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Bioaugmentation of oil and fat degradation in the laboratory

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Bioaugmentation of Oil and Fat Degradation in the Laboratory

Thesis submitted in accordance with the requirements of the University of Wales, Bangor for the degree of Doctor of Philosophy

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By

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Abstract

In the current study the ability of commercial microbial supplements to degrade fat and oil under laboratory conditions was assessed. Several of the multi-species commercial inocula investigated were capable of significantly enhancing the degradation of a variety of oils (by ca.15-60%) whilst other multi-species supplements and all of the single strained supplements tested were not.

The principle route by which the commercial inocula degraded fat/oil in the laboratory was β-oxidation. There was also evidence of mid-chain hydroxylations and oxidations and of hydrogenation on a few occasions but these pathways appeared to be of minor importance relative to β-oxidation. The microbial supplements generally hydrolysed and degraded the major fatty acids present in the oils (16:0, 18:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3) at the same rate, in contrast to environmental isolates which preferentially degraded 18:3ω3 and 18:2ω6 relative to 16:0 and 18:0 often resulting in the formation of particulate material likely to cause problems in drains, sewers and grease traps.

Some of the multi-species microbial formulations were able to grow or degrade oil over a wide range of environmental conditions, adhere to fat and/or plastic surfaces and produce digestive enzymes, indicating that they were equipped to degrade oil under field conditions. None of the supplements were capable of enhancing oil degradation in the presence of the cultured grease trap isolates, however, suggesting that the efficacy of commercial inocula in the field may be mixed.

The identities of the microorganisms involved in the degradation of oil were not fully resolved but it was clear that different groups of bacteria dominated under different environmental conditions. Efficient oil degradation was only observed when the microbial community structure was diverse and changed markedly with time, suggesting that a specific group of microoganisms was responsible for the degradation of the oil or that several microorganisms degraded the oil in a sequential manner.

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1. GENERAL INTRODUCTION

1.1. The Grease Fraction of Wastewater

1.1.1. Nature and composition of grease

Domestic wastewater is essentially a collection of discharges derived from wash-basins, bathrooms, washing machines, kitchens, lavatories, commercial activities and run-off from the land. These discharges result in a mixture that is primarily water (99%), embodying a complex blend of dissolved, colloidal, particulate organic and inorganic materials and living organisms including bacteria, viruses and protozoa (Holmes *et al.*, 1963; Metcalf & Eddy, 1991). Typical concentrations of the individual constituents found in wastewater are reported in Table 1.1.

Table 1.1. Typical composition of untreated domestic wastewater (Metcalf & Eddy, 1991).

Contaminants	(Concentration) Unit	Weak	Medium	Strong
Solids, total(TS)	$mg.l^{-1}$	350	720	1200
Dissolved total(TDS)	$mg.l^{-1}$	250	500	850
Fixed	$mg.l^{-1}$	145	300	525
Volatile	$mg.l^{-1}$	105	200	325
Suspended solids(SS)	$mg.l^{-1}$	100	220	350
Fixed	$mg.l^{-1}$	20	55	75
Volatile	$mg.l^{-1}$	80	165	275
Settleable solids	$mg.l^{-1}$	5	10	20
Biochemical oxygen demand 5-day, 20°C(BOD5, 20C)	$mg.l^{-1}$	110	220	400
Total organic carbon	$mg.l^{-1}$	80	160	290
Chemical oxygen demand	$mg.l^{-1}$	250	500	1000
Nitrogen(total as N)	$mg.l^{-1}$	20	40	85
Organic	$mg.l^{-1}$	8	15	35
Free ammonia	$mg.l^{-1}$	12	25	50
Nitrites	$mg.l^{-1}$	0	0	0
Nitrates	$mg.l^{-1}$	0	0	0
Phosphorus(total as P)	$mg.l^{-1}$	4	8	15
Organic	$mg.l^{-1}$	1	3	5
Inorganic	$mg.l^{-1}$	3	5	10
Chlorides	$mg.l^{-1}$	30	50	100
Sulphate	$mg.l^{-1}$	20	30	50
Grease	$mg.l^{-1}$	50	100	150
Total coliform	$no.100ml^{-1}$	10^6-10^7	10^7-10^8	10^7-10^9
Volatile organic compounds	$\mu g.l^{-1}$	<100	100-400	>400

The data in Table 1.1 serves only as a guide since wastewater composition is largely dependent upon the nature of the domestic and commercial inputs and may be influenced to a lesser extent by the geology and climate of the catchment area and by *in situ* processes such as sedimentation, hydrolysis, and microbial degradation (Holmes *et al.*, 1963).

Grease represents one of the most abundant organic fractions of domestic wastewater with a concentration that can be expected to average 40-100 $mg.l^{-1}$ (Loehr & Kukar, 1965). A large proportion of this grease is derived from human excreta and domestic kitchen wastes. However, elevated grease levels may arise when the wastes from commercial kitchens, food processing plants, rendering plants, tanneries, abattoirs, dairies, bakeries, laundries, oil refineries or oil mills are combined with domestic effluent (Holmes *et al.*, 1963; Loehr & Navarra, 1969).

By definition, the term 'grease' is used to denote a chemically heterogeneous group of substances having the common property of insolubility in water and solubility in solvents such as hydrocarbons, chloroform and alcohols (Gurr & Harwood, 1991). Glycerides and fatty acids constitute the major fraction of grease in wastewater although hydrocarbons, sterols and compound lipids may also be present (Kramer, 1971). Glycerides that are solid at ambient temperatures are referred to as fats, whereas those that are liquid are referred to as oils (Gurr & Harwood, 1991). Fatty acids may also found in a liquid or solid state and may be combined with an ion in the form of a sodium or calcium salt (Loehr & Roth, 1968). The nomenclature of some of the most commonly described fatty acids in this study are reported in Table 1.2

Table 1.2. Nomenclature of some of the most commonly described fatty acids in this study.

Symbol	Systematic name	Trivial name
12:0	dodecanoic	lauric
14:0	tetradecanoic	myristic
16:0	hexadecanioc	palmitic
18:0	octadecanoic	stearic
14:1ω9	tetradec-9-enoic	myristoleic
16:1ω9	hexadec-9-enoic	palmitoleic
18:1ω9	octadec-9-enoic	oleic
18:2ω6	octadeca-9c, 12c-dienoic	linoleic
18:3ω3	octadeca- 9c, 12c,15c-trienoic	α-linolenic

On entering the drains, a large proportion of grease may also be found in an emulsified state as a result of detergents and raised water temperatures. In addition, grease may become adsorbed to particles, which can either settle or remain suspended in wastewater (Loehr & Kukar, 1965).

Domestic wastewater containing the remnants of vegetable oils, animal fats and faeces has been found to have very specific fatty acid and sterol profiles. In vegetable oils, 16:0, 18:1ω9, 18:2ω6 and β-sitosterol predominate, whereas animal fats contains large proportions of 16:0, 18:0, 18:1ω9 and cholesterol (Quemeneur & Marty, 1994; Marty *et al.*, 1996). Human faeces contains 4-23% grease and the fatty acid fraction is dominated by 18:1ω9, 18:0 and 16:0. The β-stanols i.e. Coprostanol, 24-methylcoprostanol and 24-ethylcoprostanol, produced by the bacterial hydrogenation of cholesterol, campesterol and β-sitosterol in mammalian guts represent at least 50% of the faecal sterols. Anomalously low ratios of some of these sterols in domestic wastewater (coprostanol/cholesterol and 24-ethylcoprostanol/β-sitosterol) may be indicative of commercial and industrial influxes rich in animal fats (Quemeneur & Marty, 1994).

1.1.2. Problems associated with grease in wastewater

The problems associated with the grease rich fraction of wastewater are well documented in the literature. Most reports date back to the 1940's – 1960's and are based largely upon crude observations rather than quantitative data. Grease-related complications were identified in three main areas; the sewer systems, the wastewater treatment plants and the water-courses receiving the effluent. The majority of these problems could be attributed to the physical characteristics of fats and oils which include an insolubility in water and a propensity to adhere and entrap other materials such as paper, sticks and sewage solids to form deposits.

In drains and sewer lines grease can separate out from the wastewater within 10m of the source to form solid deposits. These deposits reduce the capacity of drains and sewers lines and may even result in blockages causing the sewage to back-up and/or overflow. Further along the sewer lines, in the pumping stations, grease may also interfere with float and probe level operation and clog pumps (Mahlie, 1940; Baig & Grenning, 1976).

On reaching the primary separation units of the wastewater treatment plant, grease can either rise to the surface, adhere to particles and settle, or adhere to the walls of the settlement tanks. Grease that floats to the surface and is not removed by skimming results in blocked strainers and filters whilst the grease that accumulates in the primary sludge may cause further problems in the anaerobic sludge digestors (Hanaki *et al.,* 1981). If large quantities of grease remain in the sludge at the end of the treatment, the sludge is more difficult to handle and de-waterability may be reduced to a great extent (Stoll & Gupta, 1995).

Excessive grease accumulations have also been blamed for the production of foul and rancid odours, which may result in air pollution in the vicinity of both the sewers and treatment plant. The hydrolytic and oxidative deterioration of grease results in the evolution of offensive smelling odours whilst other food waste held in anaerobic pockets of grease may be decomposed by bacteria leading to the production of hydrogen sulphide (Horsfall, 1978).

Grease that is not removed by primary wastewater treatment is conveyed to the biological units of the wastewater treatment plant where further problems may be experienced. Floating grease can reduce the permeability of the surface to oxygen preventing normal digestion processes (Mahlie, 1940). Moreover, grease coating the microorganisms interferes with oxygen transfer from the liquid to their cell interior, impeding the function of the biological digester further (Sawyer *et al.*, 1994). Experiments with activated sludge have also shown that high levels of grease in wastewater can overload sludge and thus reduce its capacity for adsorption (Heukelekian, 1943). In addition, large quantities of lipids in activated sludge encourage the growth of the filamentous actinomycete, *Nocardia amarae*, which is involved in the formation of undesirable scum and stable foam (Becker *et al.*, 1999).

Treatment plants that fail to produce the required quality of effluent may result in large quantities of grease entering rivers and seas. Grease enriched wastewater not only produces unsightly formations at outfalls and streams (Loehr & Navarra, 1969) but may also pose an increased risk for oxygen depletion in the receiving waters. Unusually large inputs of grease rich effluent may cause additional problems amongst the local wildlife. Besides the direct smothering effect, animals coated in vegetable oil can ingest the grease while cleaning themselves, leading to lipid aspiration pneumonia and dehydration as the laxative effect of the oil takes effect (Gunstone, 1994).

1.1.3. Regulations governing the discharge of grease-rich wastewater

Untreated domestic wastewater harbours numerous pathogenic microorganisms that are released from the human digestive tract. It also contains toxic compounds and decomposing organic matter that can lead to the production of offensive odours and gases (Holmes *et al.*, 1963). For these reasons, domestic wastewater is immediately removed from its sources of generation. In the United Kingdom, drains and sewers were constructed during the early nineteenth century which merely transferred the waterborne wastes from industry and homes to the rivers. The outcome was a deterioration in the condition of many rivers such that aquatic life was killed and the water became unusable for either domestic or industrial consumption (Bartlett, 1971). These problems were resolved to a degree by establishing 'sewage farms' which provided some form of treatment of the sewage before its discharge into the rivers. However, major efforts to improve the quality of surface waters did not occur until more recently. These efforts resulted in part from (1) an improved understanding of the environmental effects caused by wastewater discharges (2) a developing knowledge of the adverse long-term effects caused by specific constituents of wastewater and (3) a national concern for environmental protection (Metcalf & Eddy, 1991).

Regulations and procedures affecting wastewater discharges are diverse and subject to change. Domestic and industrial effluent must satisfy stipulated requirements (Holmes *et al.*, 1963) and in the UK, legal and administrative control over pollution is well established (Bartlett, 1971). Effluent standards are recommended by Royal Commissioners and are enforced by parliamentary acts such as the Water Resources Act 1991, Control of Pollution Act 1989, Water (prevention of pollution) Order 1998, Control of Pollution Regulation 1997, Pollution and Prevention and Control act 1999, Anti-Pollution Works Regulation, Industrial Pollution Control 1997 Order, Environmental Protection Act 1990, and Environmental Act 1995. A number of international treaties, which may influence local pollution regulations, have also been ratified by most countries. These include: The Rio convention, Convention of marine pollution by dumping of wastes and other matter 1972/1976, MARPOL 1973/1978, Protocol concerning regional co-operation in combating pollution by oil and other harmful substances, Protocol for the protection of the marine environment against pollution from land sources, United Nations convention on the law of the sea, Basel convention on the

transboundary movements of hazardous wastes and their disposal (Stephenson & Blackburn, 1997).

In the developed world, regulations specific to grease disposal have been imposed internationally and are typically based on the arbitrary limit of 100 $mg.l^{-1}$ of grease (Mendoza-Espinosa & Stephenson, 1996). Most developing countries also have extensive laws and regulations that are usually based on European or US legislation. However, regulations governing grease disposal in many developing nations are either non-existent or are not enforced (Stoll & Gupta, 1997).

To comply with the effluent standards imposed, many countries have introduced wastewater treatment technologies and stimulated research into the fundamental principles of wastewater treatment (Metcalf & Eddy, 1991). In the UK technology forcing requirements are found under the pollution and prevention and control act 1999, which stipulates that discharges from industrial or commercial installations must be treated by the 'Best available techniques'. However, despite the efforts in the developed world to reduce grease rich effluent by implementing treatment technologies, even the most rudimentary measures of containing grease, for example grease traps, are relatively uncommon in the developing world. Those grease traps that are installed generally function at decreased efficiencies due to a lack of proper operation and maintenance (Stoll & Gupta, 1997).

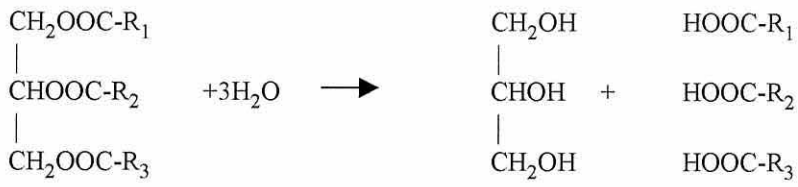
1.2. The Degradation of Grease

1.2.1. Microbial degradation of grease

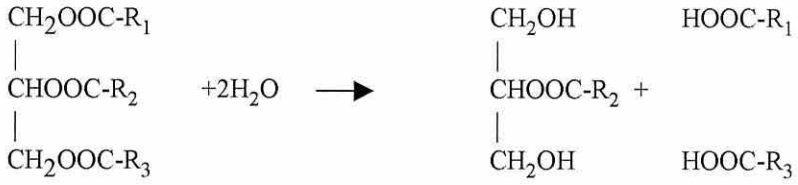
In wastewater, a significant proportion of the lipids are in the form of glycerides (Kramer, 1971) and cannot be directly assimilated by microorganisms. The first stage in the microbial degradation process is thus the hydrolysis of fats and oils to fatty acids and glycerol. This hydrolysis proceeds via the action of extracellular lipases or by lipases associated with microbial cell walls (Ratledge, 1994).

Microbial lipases have been identified which exhibit either a non-specific hydrolysis of glycerides, a specificity for an acyl substituent or a specificity for the position at which hydrolysis occurs (Figure 1.1).

(a) Non-specific lipase reaction



(b) 1,3-specific lipase reaction



(c) Acyl-group specific lipase reaction

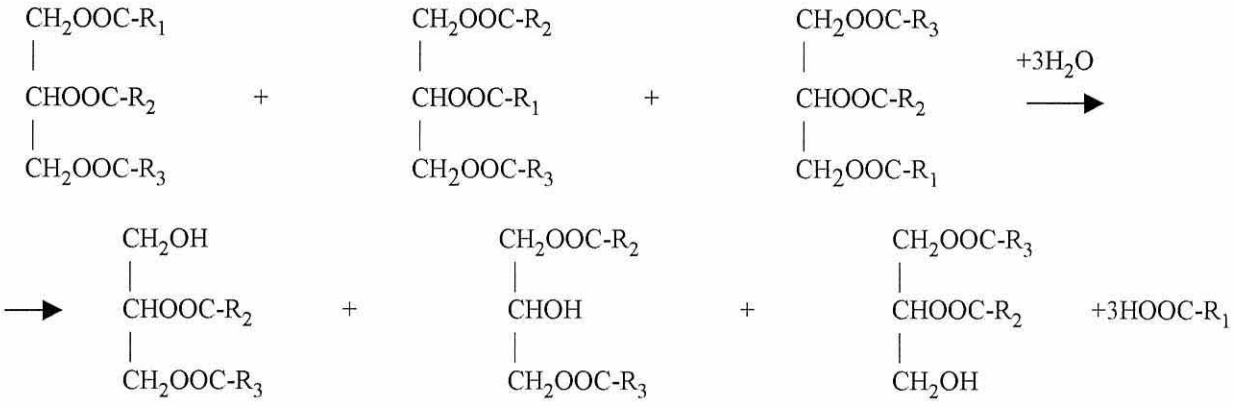


Figure 1.1. Hydrolytic reactions of lipases (Ratledge, 1994).

Lipases which exhibit no substrate or positional specificity appear to be the most commonly found. These lipases will remove fatty acyl groups from all three positions of the glyceride at more or less the same rate (Finnerty, 1989; Ratledge, 1994). The 1,3-specific lipases catalyse hydrolysis reactions at the C_1 and C_3 positions of the glyceride molecule only to yield the free fatty acids, 1,2(2,3) diglycerides and 2-monoglycerides. Although lipases that are specific towards the C_2 acyl constituents remain to be found, several microoganisms have been shown to have a low activity towards the C_2 ester bond. Some lipases have also been identified which exhibit specificity towards the fatty acid itself. The best known example is the lipase from *Geotrichum candidum*, which displays a high degree of specificity for fatty acid acyl substituents with a double bond at the 9,10-position and then only provided that there is no double bond between the $\Delta 9$ bond and carboxylic ester group (Alford *et al.*, 1964).

Microbial lipase can be produced in the absence of lipoidal material although production is generally stimulated by the inclusion of lipids such as lard and olive in the medium. The production and activity of microbial lipase is also affected by other factors such as nutrient availability, temperature, pH, and oxygen levels (Alford *et al.*, 1971; Ratledge, 1994).

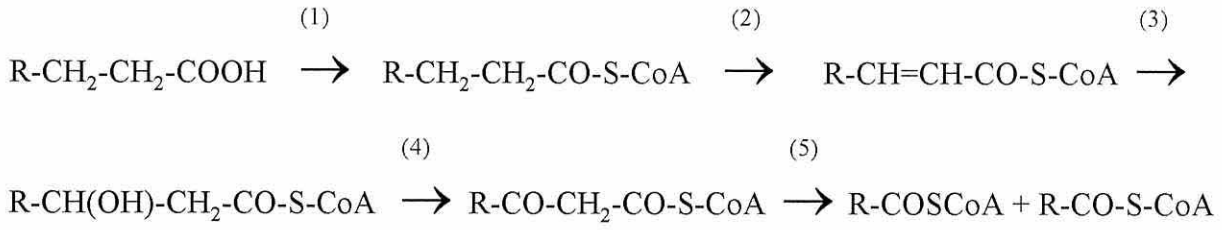
Fatty acids released during hydrolysis are transported into microbial cells by mechanisms that may involve simple diffusion processes or specific transport proteins. These fatty acids may then be metabolized to generate energy or to yield carbon intermediates for cell synthesis (Gunstone and Norris, 1983). Fatty acids that are not accommodated directly into the cell may also be elongated and/or de-saturated beforehand.

Biochemical studies have revealed that fatty acids and glycerol follow distinct oxidation pathways. Glycerol is taken up by a process of facilitated diffusion and is converted to glyceraldehyde-3-phosphate, its metabolism following that of glucose. Fatty acid oxidation, on the other hand, is representative of true fat oxidation and may proceed through a number of different oxidation pathways (Deuel, 1957).

Whilst the following oxidation reaction are discussed as separate processes, it is important to recognize that in order to complete acyl chain catabolism, it may be necessary to use several different oxidation pathways. Furthermore, some of the oxidation routes that are discussed are concerned primarily with biotransformation reactions rather than with the complete degradation of fatty acids (Koritala *et al.*, 1987).

β-oxidation

β-oxidation, the most common mechanism of fatty acid degradation, involves a cycle of reactions whereby an acetyl-CoA and an acyl-CoA containing two less carbon atoms than the original acid are generated. The latter subsequently re-enters the cycle until acetyl-CoA is the terminal product (Figure 1.2). Complete degradation of acetyl-CoA to water and carbon dioxide is achieved through the citric acid cycle (Gunstone & Norris, 1983).



(1) Fatty acid converted to its CoA ester through a reaction with ATP and coenzyme A (CoASH) in the presence of Mg^{2+} and an acyl-CoA synthetase.
(2) CoA ester is dehydrogenated to the Δ2t dehydroacyl-CoA with acyl-CoA dehydrogenase.
(3) Δ2t dehydroacyl-CoA is hydrated by a regiospecific and stereospecific reaction occurs under the influence of enoyl-CoA hydratase.
(4) Stereospecific reaction utilizing an oxidizing enzyme.
(5) Acetyl-acyl-CoA transacylase and coenzyme A effect cleavage.

Figure 1.2. β-oxidation cycle (Gunstone & Norris, 1983).

Some modification of the cycle shown in Figure 1.2 may be necessary if the fatty acid being oxidized is not fully saturated, branched or contains an odd number of carbon atoms. Unlike even numbered *n*-saturated fatty acids that are degraded by β-oxidation to acetyl-CoA, *n*-saturated acids with an odd number of carbon atoms give rise to acetyl-CoA and propionyl-CoA. Similarly, branched chained fatty acid of even chain length may eventually yield propionate, whilst the oxidation of an odd numbered branched chain acid generates a 3-methyl-branched acid with acetoacetate and 3-hydroxy-3-methylglutarate as intermediate metabolic products. Unsaturated fatty acids pose a different problem because the β-oxidation process furnishes Δ2c or Δ3c acids and additional enzymes are required to alter the configuration and position of the double bond. One such enzyme, an isomerase converts *cis*-3 fatty acid into *trans*-2 fatty acids allowing the reaction to continue in the normal way (Gunstone & Norris, 1983; Gurr & Harwood, 1991; Ratledge, 1994). It has also been suggested that the *cis* bond can be hydrogenated to yield a saturated acyl-CoA, which can be oxidized in the usual manner (Mead *et al.*, 1986).

In the absence of an external electron acceptor such as oxygen, the acetate produced by the β-oxidation does not enter the citric acid cycle, but may be converted into methane and carbon dioxide via methanogenesis (Angelidaka & Ahring, 1995). Methanogenesis may occur via two different pathways, catalysed by two different groups of microorganisms. The most common route is the acetoclastic reaction in which acetate is directly converted into methane

and carbon dioxide by acetate utilizing methanogenic bacteria. The second reaction, syntrophic acetate oxidation, is carried out by a syntrophic consortium of acetate-oxidizing organisms and hydrogen-utilizing methanogens. The acetate-oxidizing microorganisms first metabolize the acetate to produce carbon dioxide and hydrogen which are then converted in to methane by hydrogen-utilizing methanogens (Smith & Mah, 1966; Petersen & Ahring, 1991; Angelidaka & Ahring, 1995).

α-oxidation

α-oxidation involves the transformation of a fatty acid, rather than the CoA ester of a fatty acid, into a hydroxy fatty acid, which is then rapidly converted into a fatty acid containing one less carbon atom than the original acid (Yano *et al.*, 1971; Gunstone & Norris, 1983). The essential reaction is summarized below although the exact mechanism is unknown. Oxygen or hydrogen peroxide may be the oxidizing substrate with formation of hydroperoxide (R-CHOOH-COO-) as the intermediate (Ratledge, 1994).

$$R\text{-}CH_2\text{-}COO\text{-} \rightarrow R\text{-}CHOH\text{-}COO\text{-} \rightarrow R\text{-}CHO + CO_2 \rightarrow R\text{-}COO\text{-}$$

The α-oxidation pathway is widely established in plants and animals and is particularly significant if the β-oxidation pathway is blocked (Gunstone & Norris, 1983; Harwood & Russell, 1984; Mead *et al.*, 1986).

ω-oxidation

Bacteria are also capable of oxidizing the methyl end of an acyl chain using ω-hydroxylase systems to form ω-hydroxy fatty acids. Like α-oxidation, this process is commonly observed for compounds which cannot by degraded by β-oxidation (Gunstone & Norris, 1983; Mead *et al.*, 1986; Ratledge, 1994). Diterminal carboxylation proceeds via the action of an O_2-dependent ω-hydroxylase enzyme system and results in the ω-hydroxylation of fatty acids to ω-hydroxy fatty acid followed oxidation to an aldehydic fatty acid and to α, ω-dicarboxylic acid (Finnerty, 1989). Hydroxy fatty acids and similarly oxidized materials that are produced during the ω-oxidation reaction are then degraded by β-oxidation rather than experiencing further oxidation at the ω-end of the molecule. The ω-hydroxylation pathway is highlighted below:

$CH_3(CH_2)nCOOH \rightarrow HOCH_2(CH_2)nCOOH \rightarrow$

$OCH(CH_2)nCOOH \rightarrow COOH(CH_2)nCOOH$

In addition to terminal methyl group oxidation, oxidation of carbon atoms within the acyl chain can occur forming a number of hydroxy, oxo, epoxy, hydroperoxy and polyoxygenated derivatives (Ratledge, 1994).

Hydrogenation

The hydrogenation of an isolated *cis* double bond in the acyl chain of a fatty acid molecule is a reaction carried out by only a few microorganisms (Gurr & Harwood, 1991). These microorganisms are most commonly found in the rumen of animals such as cows and sheep but have also known to arise in the intestinal contents of non-ruminant animals, including humans (Eyssen & Verhulst, 1984; Verhulst *et al.*, 1986; McInerney, 1988) and in aquatic environments such as marine sediments (Rhead *et al.*, 1971; Pereira, 1999). The general pathways for biohydrogenation are illustrated in Figure 1.3

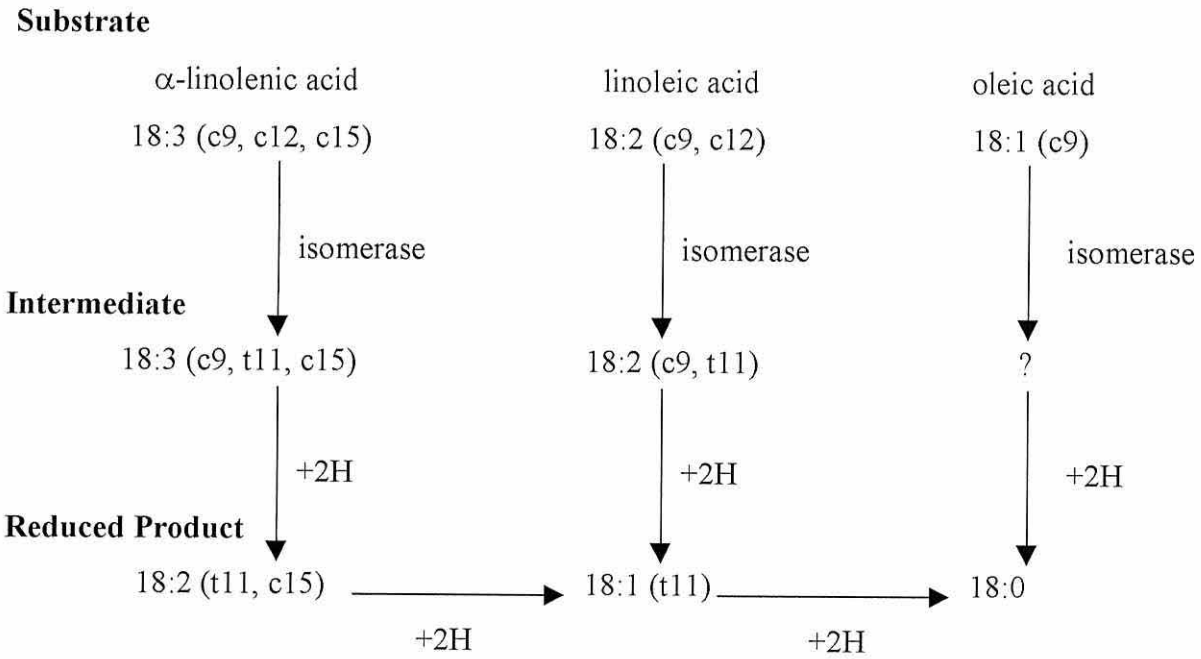


Figure 1.3. Major pathways of fatty acid degradation in rumen bacteria (Harwood & Russell, 1984).

Most biohydrogenation studies have focussed upon microorganisms isolated from the rumen (Polan *et al.*, 1964; Wilde & Dawson, 1966; White *et al.*, 1970; Kemp *et al.*, 1975; Kemp & Lander, 1983; Kemp *et al.*, 1984; Kellens *et al.*, 1986) and have shown that two distinct

groups of microorganisms are involved. The first group contains species such as *Ruminococcus albus*, *Eubacterium* and *Butyrivibrio*, that are unable to hydrogenate octadecenoic acid any further but can hydrogenate linoleic acid and α-linolenic acid to *trans*-octadec-11-enoic acid and related isomers. The second group is comprised of only three known bacteria; two strains of *Fusocillus* and an unidentified gram negative rod, which can hydrogenate many octadecenoic acids, including oleic, linoleic and *trans*-octadec-11-enoic acid to stearic acid. The products of α-linolenic hydrogenation, namely, *cis* and *trans*-octadec-15-enoic acids are not further hydrogenated even by mixed ruminal bacteria (White *et al.*, 1970; McInerney, 1988).

With the exception of *Eubacterium lentum,* biohydrogenating microorganisms that inhabit non-ruminant environments have yet to be described. *E. lentum,* isolated from the intestinal contents of animals and humans, behaves in a similar manner to the first category of ruminant organisms, transforming linoleic, α-linolenic and γ-linolenic to *trans*-octadec-ll-enoic without hydrogenating the fatty acid any further (Eyssem & Verhulst, 1984; Verhulst *et al.*, 1986). A consortium of unidentified microorganisms in marine sediments has also been shown to hydrogenate linoleic and linolenic to stearic acid. The occurrence of several 18:2 and 18:3 isomers, identical to those observed in rumen microorganisms, suggests that the biohydrogenation pathway in marine sediments is similar to that found in the rumen (Pereira, 1999).

In the case of linoleic acid (9-*cis*-12-*cis*-octadecadienoic acid), the hydrogenation reaction proceed with the action of Δ^{12}-*cis*, Δ^{11}-*trans*-isomerase to produce a conjugated diene *cis*-9, *trans*-11-octadecadienoic. This is followed by the stereospecific *cis*-addition of hydrogen atoms to yield *trans*-11-octadecenoic and finally hydrogenation of the *trans* bond to generate stearic acid. The former of the two reactions can take place under aerobic conditions and only after the fatty acids have been liberated from their ester linkages. Additional requirements for the reaction are a *cis*-9, *cis*-12 pentadiene system (Kepler *et al.*, 1970; Vernhulst *et al.*, 1986) although the isomerization of arachidonic acid, homo-γ-linolenic acid and *cis*-*cis*-nonadec-10-13-enoic acid by many *E. lentum* strains suggests that the two double bonds do not have to be strictly located at the C-9 and C-12 positions (McInerney, 1988). Attempts to identify the enzyme responsible for later reaction, reduction, or the nature of the electron donor(s) have been met with little success. The hydrogenation reaction appears to be obligatory anaerobic

(Rosenfelt & Tove, 1971) and studies have shown that the hydrogen atoms that reduce the *cis* bonds are in rapid equilibrium with water (Rosenfield & Tove, 1971; Hughs *et al.*, 1982). The stimulatory effect of viologen dyes and sodium dithionite also suggests the involvement of a biological electron carrier, such as ferredoxin (Yokoyama & Davis, 1971).

1.2.2. Oxidation of grease by oxygen

The oxidation of fats and oils by atmospheric oxygen is of great importance both in the development of rancidity and in the early stages of polymerisation (Gunstone, 1958; Gunstone & Norris, 1983; Dobarganes & Marquez-Ruiz, 1996). These processes are associated with autoxidation reaction, which proceeds via a free radical mechanism (Figure 1.4).

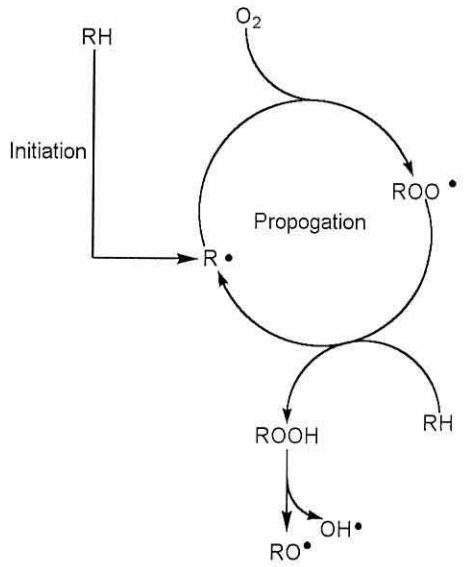


Figure 1.4. Scheme for the free radical autoxidation mechanism of an acyl group (Nawar, 1984).

The first reaction, initiation, involves the production of a very small number of radicals, for example transition metal ions or an oxygen radical by photolysis which allow the production of R• from a substrate RH (Nawar, 1984). After the formation of sufficient free radicals the reaction is propagated by the removal of hydrogen atoms at position alpha to the double bond and is proceeded by oxygen attack at these locations. The resulting peroxy radical (ROO•) in turn abstracts hydrogen from other molecules (RH) to form hydroperoxides, ROOH and R•, which react with oxygen and so on (Nawar, 1984).

The hydroperoxides formed as a result of the lipid autoxidation process may enter into numerous and complex reactions to produce a myriad of compounds with variation in molecular weight and polarity. Cleavage of the O-O bond in the hydroperoxide yields an alkoxy radical which may result in the hydroxy or keto derivative on addition or abstraction of the hydrogen atom, respectively. Alternatively, the alkoxy radical may decompose via carbon cleavage on either side of the alkoxy group to form aldehydes, hydrocarbons and acids (Nawar, 1984). The carbon alkyl radicals that are produced during autoxidation may also interact to form a combination of dimers and trimers that are joined through C-C or C-O-C linkages (Dobarganes & Marquez-Ruiz, 1996). Large numbers of these radicals can also combine to generate cross-linked structure of high molecular weight known as polymers. (Gunstone, 1958; Gere 1982; Dobarganes & Marquez-Ruiz, 1996).

The course of the autoxidation reaction is largely dependant upon the lipids composition and the conditions to which the lipid is exposed. At elevated temperatures hydroperoxide decomposition and secondary oxidations take place at extremely rapid rates resulting in the rapid decomposition of fats and oils. Similarly, the addition of certain metals or metal salts or exposure of the oil to ultraviolet light increases the rate of autoxidation whilst the addition of antioxidants, for example gallic acid and its esters, tanic acid and tannins, ascorbic acid and its esters, butylhydroxyanisole (BHA), *nor*-dihyroguaiaretic acid (NDGA) and the tocopherols has the reverse effect. Antioxidants interfere with the propagation sequence by converting propagating radicals into non-propagating species. Their effectiveness may be increased by adding compounds such as ascorbic acid, phosphoric acid and citric acid, known as synergists (Gunstone, 1958).

The effects of triglyceride structure and composition on the stability of fats and oils to oxidation has been investigated by a number of authors. Unsaturated fatty acids are known to be more susceptible to oxidation than their saturated analogs, with the rate of autoxidation increasing with the number of double bonds present in the molecule (Gunstone, 1958; Gere, 1982). Relative to their free fatty acids, esters also autoxidize more slowly (Gunstone, 1958) and those unsaturated fatty acyl group located in the 2-position of a triglyceride molecule are oxidized more slowly than those situated at the outer positions (Martin *et al.*, 1998).

Given that polymers constitute the major group of compounds generated during frying (Martin *et al.*, 1998) and that any unsaturated fats and oils released into the sewers will be

susceptible to oxidation it is postulated that polymers must be present in wastewater. Assuming that polymerization does take place, the polymers formed are likely to be similar in appearance to those observed in the lab and other aquatic environments and may exacerbate problems in sewers and wastewater treatment plant by virtue of their gelatinous, rubber-like state. Studies with pancreatic lipase have shown that the hydrolysis of triglyceride monomers were impaired by the presence of dimers and that the susceptibility of various oxidized and polymeric triglycerides to enzymatic hydrolysis was in the following order; oxidized monomers>dimers>polymer, (Marquez-Ruiz *et al.*, 1998). Thus, the polymers formed in wastewater may also be more resistant to biodegradation than the original oil and persist for longer. Indeed, the polymers formed by purely physical processes in the marine environment have been shown to be relatively non-biodegradable (Mudge *et al.*, 1994). It has also been demonstrated that polymer formation in such environments is enhanced by the presence of marine bacteria and nutrients (Mudge *et al.*, 1994) although their role in the polymerization process remains unclear.

1.2.3. The degradation of grease in wastewater

Fats, oils and fatty acids are known to be particularly susceptible to biodegradation and, in comparison to the hydrocarbon components of grease, are readily utilized by bacterial communities (McCarty *et al.*, 1972; Groenewold *et al.*, 1982). A large proportion of these lipids are degraded in biological wastewater treatment systems; namely aerated/activated sludge process and anaerobic digestion, which have been reported to achieve grease removal efficiencies as high as 74-94% (Loehr & Kukar, 1965; Loehr & Navarra, 1969; Quemeneur & Marty, 1994) and 70-92% (O'Rouke, 1968; McCarty *et al.*, 1972) respectively.

Knowledge of the fate of grease in biological wastewater treatment systems is scarce. In activated sludge plants adsorption plays a major role in the removal of grease, with hydrolysis and β-oxidation following later (Heukelekian, 1943). It has also been demonstrated that the rate limiting step in the degradation of oil by activated sludge is hydrolysis rather than adsorption or oxidation of the oil (Hsu *et al.*, 1983).

Partial biodegradation of grease by activated sludge has been known to modify effluent characteristics. Quemeneur and Marty (1994) reported that the content of 16:1ω7, 18:1ω7 and branched fatty acids increased in the particulate fraction, whilst the proportion of 18:1ω9 and

18:2ω6 decreased in the dissolved fraction. The work of Loehr and Roth (1968) also indicated that a change in effluent composition during activated sludge treatment might be expected since under aerobic conditions, the rate of fatty acid degradation decreases with increasing chain length and degree of saturation in the fatty acid molecule.

In anaerobic sludge digesters lipids are usually degraded to carbon dioxide and methane (Roy *et al.*, 1985). Triglycerides are hydrolysed to glycerol and fatty acids and the fatty acids produced by hydrolysis are then reduced and/or further degraded via the β-oxidation cycle to produce acetate (Hanaki *et al.*, 1981; Lalman & Bagley, 2001). The acetate produced via β-oxidation is finally converted to methane and carbon dioxide by methanogenesis which may proceed solely by the aceticlastic reaction (Angelidaki & Ahring, 1992) or synotrophic acetate oxidation (Roy *et al.*, 1985). Both hydrolysis (Cail *et al.*, 1986) and oxidation (O'Rouke, 1968) have been reported to be rate limiting steps in anaerobic lipid degradation studies.

As with aerobic digestion, partial anaerobic degradation of fats and oils may induce compositional changes. The effect of fatty acid size and structure on degradation rates is similar to that in an aerobic environment (Novak & Carlson, 1970) and several authors have reported that saturated or unsaturated n-2 long chain fatty acids (LCFA) intermediates may be produced from the β-oxidation of unsaturated LCFA (Novak & Carlson, 1970; Angelidaki & Ahring 1995; Lalman & Bagley, 2000, 2001).

In both aerobic and anaerobic treatment process, the fatty acids released via the hydrolysis of fats/oils can also be inhibitory to microbial growth and activity (Roy *et al.*, 1985; Angelidaki & Ahring, 1992; Becker *et al.*, 1999; Becker & Markl, 2000; Alves *et al.*, 2001). However, tolerance to long chained fatty acid toxicity may be improved by acclimatizing the microbial populations to lipids and by maintaining lower lipid concentrations (Alves *et al.*, 2001).

1.2.4. Factors that may influence microbial grease degradation in wastewater

Biosurfactants/synthetic surfactants

Given that the rate of lipid biodegradation in wastewater is often limited by mass-transfer effects (Becker & Markl, 2000) and that surfactants are known to enhance the microbial degradation of hydrocarbons (Goma, 1975; Bardi *et al.*, 2000), the efficacy of a commercial inocula may be improved by incorporating synthetic surfactants, biosurfactants or biosurfactant producing microorganisms into the product.

Biosurfactants are produced by microorganisms as metabolic products or membrane components. They are comprised of a hydrophobic and hydrophilic moiety and are capable of reducing surface interfacial tensions. The apolar, hydrophobic region of the surfactant molecule is typically a hydrocarbon chain whilst the polar, hydrophilic section is comprised of either sugars, amino acids, phosphates, alcohols or ester groups. Biosurfactants may thus be classified as glycolipids, acylpolyols, lipopeptides, fatty acids, phospholipids or neutral lipids (Van Dyke, 1991; Hommel, 1994).

Interest in biosurfactants has increased considerably over the last decade since they have potential applications in environmental protection, crude oil drilling and in the pharmaceutical and food processing industry (Vater, 1986; Mulligan *et al.*, 1989: Wei & Chu, 1998). Biosurfactants are also less toxic (Lang & Philp, 1998) and have been known to operate at extremes of temperature and pH (Makkar & Cameotra, 1998), indicating that they have the potential to augment grease control in a wide variety of environments.

Despite the aforementioned advantages, surfactants are not yet competitive with synthetic surfactants from an economic standpoint (Makkar & Cameotra, 1997). Effort has therefore been made to maximise biosurfactants yields by utilising cost-free feedstock and optimizing media conditions. A knowledge of these media conditions may also be useful if considering the use of biosurfactant-producing microorganisms in grease control.

A great variety of raw materials have been used to produce biosurfactants on an industrial scale. Traditionally, hydrocarbons have been the substrates of choice. However, water soluble

substrates such as glucose have also been used. Only a few attempts have been made to use industrial bi-products and waste materials. These include lactic whey from the dairy industry, olive oil mill effluent, molasses from the sugar cane industry and urban wastes (Makkar & Cameotra, 1997).

Yields of surfactin, one of the most effective biosurfactants known, have been optimized by supplying *Bacillus subtilis* with nitrate ions as the nitrogen source. The addition of either iron or manganese salts to the media has also been shown to enhance surfactin production in this species whilst sodium acetate and hydrocarbons inhibited production (Cooper *at al.*, 1981; Makkar & Cameotra, 1998; Wei & Chu, 1998).

Biofilm Formation

Specialist bacterial cultures, when introduced to the drains or sewer system, may not initiate grease degradation instantaneously. Instead, the selected strains may take several weeks to acclimatize (Grubbs *et al.*, 1991) and once established, may take several days to degrade the grease. Given that the retention time of water in drains and sewers is likely to be short the efficacy of a commercial microbial supplement in a wastewater environment may be influenced by the commercial supplements capacity to adhere to surfaces.

In aqueous environments, such as sewage, bacteria can occur in both the suspended or attached state. However, more recently, it has been proposed that the attached state is the predominant form and that attached cells act as reservoirs for suspended forms (Gilbert *et al.*, 1993). Advanced microscopic techniques, specifically confocal scanning laser microscopy (CSLM) has permitted the nondestructive, in-situ analysis of 'living' biofilms and thus provided detailed information on the physical architecture, chemistry and microbial components of the biofilm matrix (Costerton *et al.*, 1995). Recent studies have shown that the structure of a biofilm is complex, having cells in streamlined orientations enveloped by extracellular polymeric substances (EPS). The spatial pattern of EPS is not uniform but may contain void spaces and highly permeable water channels which serve as storage facilities for periods of carbon limitation (Costerton *et al.*, 1995; Lindsay & Von Holy, 1997). Chemical analysis of sewer biofilm material has also revealed that cell biomass is only a minor fraction of the organic matter and 70-98% of total organic carbon is extracellular. The extracellular

fraction is dominated by protein although humic substances, polysaccharides, uronic acids and DNA may also be present (Jahn & Nelsen, 1998).

Samples taken from the surfaces of trickling filters and the aerobic/anaerobic locations of wastewater treatment plants have revealed that slime and zoogleal bacteria predominant in the biofilm. Filamentous bacteria and fungi, protozoa and algae may also be present although zooglea bacteria and bacteria such as *Beggiotoa* and *Thiotrix* become more abundant as the biofilm develops. Under aerobic conditions the predominant group of bacteria tends to be cocci whilst rods appear more important anaerobically (Characklis, 1973).

The organization of bacterial cells at a surface to form a biofilm conveys many advantages to the component cells. Biofilms provide increased protection from phagocytosis (Gilbert *et al.*, 1993) and have been reported to be at least 500 times more resistant to antimicrobial agents (Costerton *et al.*, 1995). Growth prospects are also improved through the localization of nutrients and by establishing electropotential gradients across the biofilm. Additionally, the close proximity to other bacterial cells may promote genetic exchange (Bale *et al.*, 1988).

Biofilm formation is believed to be a rapid process with some bacteria requiring only 20 minutes for attachment (Lindsay & Von Holy, 1997). However, for non-motile bacteria such as *B. subtilis*, the process is likely to take longer. Initial adhesion is aided by fluid dynamic forces, which concentrate suspended organisms at the viscous boundary layer near a surface. (Gilbert *et al.*, 1993). Irreversible and reversible attachment then follows, as described by the Colloidal chemistry laws, specifically the Derjaguin-Landau and Verwy-Overbeek theory. Once the cell is close to the surface other forces such as hydrogen bonding come into play and reinforce adhesion. Finally, firm adhesion is achieved through the production of exopolymers, which behave like a cement. Exopolymers are typically fibrillar in nature and may have a polysaccharide and/or globular protein composition and are usually induced on adhesion. Adhesion triggers the expression of a sigma factor that derepresses several genes resulting in biofilm cells that are phenotypically distinct from the plankton form (Costerton *et al.*, 1995).

In order to survive and colonize other environments individual microbial cells must be detached and dispersed from the biofilm. Parts of the biofilm will continuously slough from pipework as a consequence of shear forces in turbulently flowing water. Once in the water

flow the resultant clump of cells is transported until the current is low enough to allow adhesion. Bacteria experiencing a decrease in metabolic activity through starvation have also been observed to detach from surfaces and colonize new environments in this way (Gilbert *et al.*, 1993).

1.3. Methods of Removing Grease from Wastewater

1.3.1. General methods of grease removal

Wastewater treatment plants

Domestic effluent and certain industrial and commercial wastes are transferred via the drains and sewers to wastewater treatments plants where a large proportion of the grease can be removed. Wastewater plants convert the components in raw wastewater into relatively harmless effluent for discharge to a receiving body of water. A scheme of the primary and secondary treatment process are illustrated in Figure 1.5.

The preliminary form of wastewater treatment, primary treatment essentially involves physical processes. Wastewater is first screened to separate large objects such as rags and sticks from the sewage flow whilst grinders and shredders incorporated into the screening facility macerate solids into slurry. Sand-like materials are also removed form the bulk of the wastewater and the remainder of the waste material is directed to the primary settling tanks, where the settleable and floatable solids are separated from the liquid waste by processes such as flocculation and adsorption (Holmes *et al.*, 1963).

Secondary treatment employs chemical and biological methods to remove or transform contaminants. The most widely used form of secondary treatment, the activated sludge process, involves blowing oxygen through effluent in an aeration tank. Aeration retains both high levels of bacterial activity in the tank and assists in the suspension of sludge. Microorganisms that are present convert organic material into microbial biomass and carbon dioxide. The cell material produced during this process is kept in the aeration tank until the microorganisms are past the log-phase of growth at which point the cells flocculate and settle out to form sludge (Speight, 1996).

When a high quality effluent is required tertiary treatment may be employed to remove constituents not adequately removed during secondary treatment such as nitrogen, phosphorus and other soluble organic and inorganic compounds. Advanced forms of wastewater treatment may involve chemical precipitation of phosphorus, nitrification, denitrification, ammonia stripping, break-point chlorination, filtration, carbon adsorption, ion exchange, reverse osmosis and electrodialysis (Holmes *et al.*, 1963).

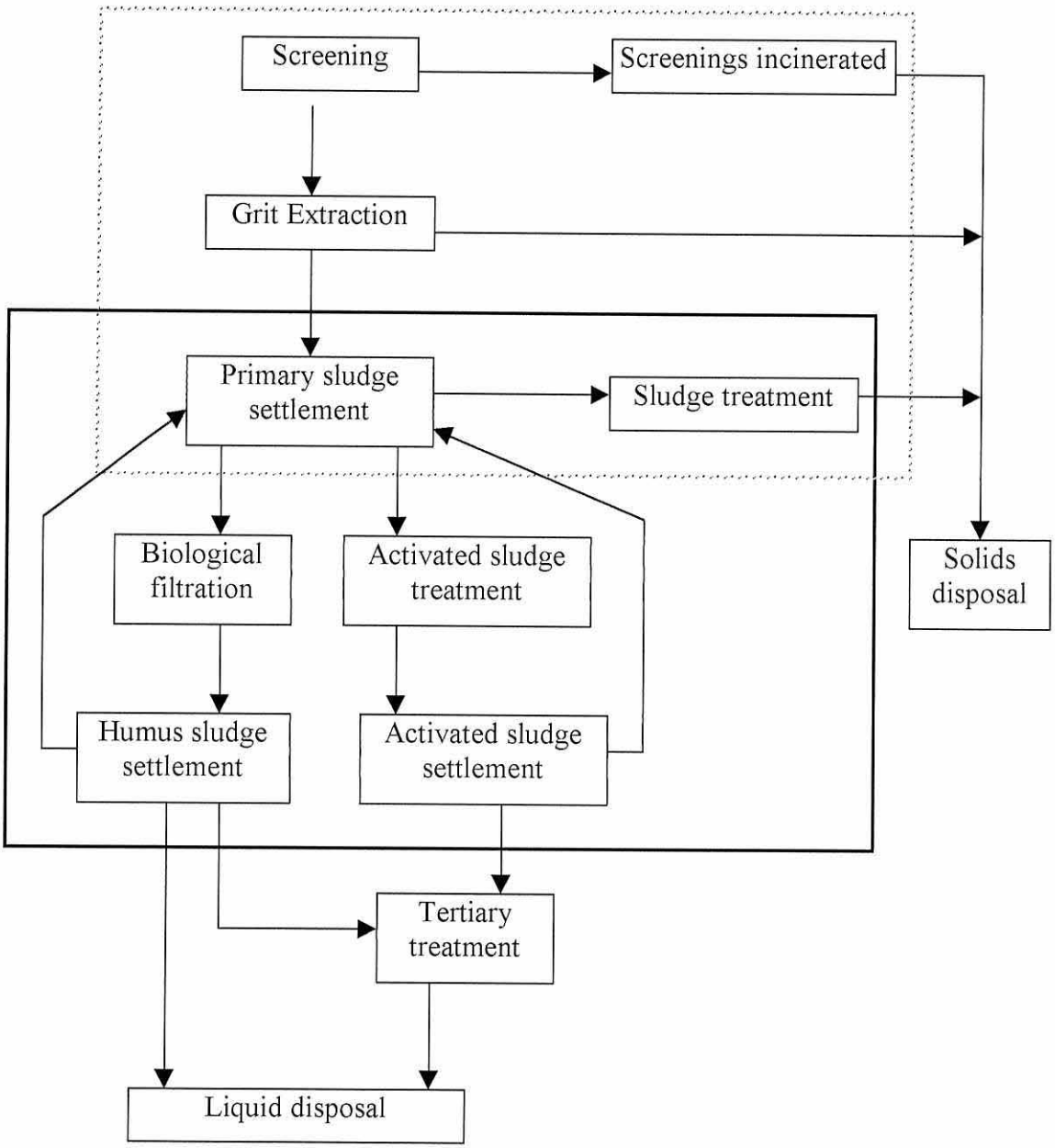


Figure 1.5. Scheme of primary (enclosed grey-dashed box) and secondary (enclosed in black box) wastewater treatment (Clark, 1997).

The treatment of sludge arising from the primary sedimentation tanks and the final settling tanks is also addressed. Common sludge management processes include thickening, stabilization, de-watering and disposal of the sludge. Sludge thickening involves gravity thickening, dissolved air flotation and centrifugation to concentrate solids and reduce volume. Stabilization may entail anaerobic or aerobic digestion, chemical oxidation, lime stabilization and thermal conditioning. Any remaining sludge is disposed of by incineration, wet oxidation, pyrolysis, composting or land filling (Holmes *et al.*, 1963).

1.3.2. Specific methods of grease removal

Wastes that arises from certain industrial and commercial activities are particular rich in grease and may require some preliminary form of treatment before discharge into the sewerage system or environment. Likewise, wastewater treatments plants that receive large quantities of grease may require additional facilities to assist in the removal of grease. These preliminary or additional treatment facilities may involve a combination of physical, chemical and biological methods of grease control:

Physical methods

The traditional method of physically limiting the amount of grease discharged into drains is to install a grease trap. Grease traps are essentially tanks of varying size and configuration that are designed to retain grease and settleable solids whilst allowing clarified water to be discharged into the sewers (Figure 1.6).

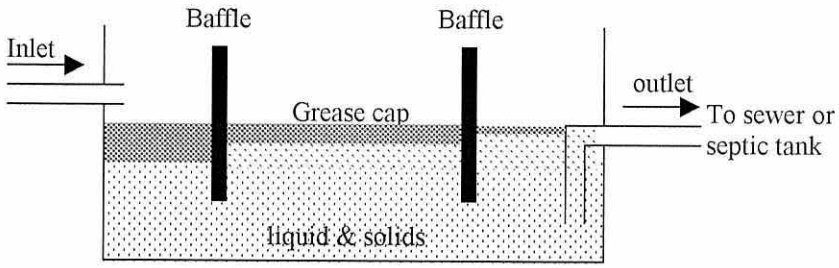


Figure 1.6. Typical configuration of a grease trap (adapted from Grubbs *et al.*, 1991)

Fats and oils that are present in the liquid waste float to the surface where they are prevented from flowing into the sewers by a series of 'baffles' covering both the inlet and outlet of the

tank (Grubbs *et al.*, 1991). Large surges of grease escaping from poorly maintained grease traps are often more detrimental than a continuous influx of grease into the sewer systems therefore grease traps must be pumped regularly to prevent accumulated fats and greases overflowing into the sewer system (Grubbs *et al.*, 1991).

The instillation of a grease trap often results in only a partial recovery of oil and grease (Steiner & Gec, 1992) and may be inadequate if the oil is emulsified. Grease removal may thus be improved by the introduction of air in the form of fine bubbles using perforated pipes or baffles, or by running screened sewage through high velocity jets placed above the surface. The bubbles of air that are released from these structure adhere to the emulsified particles of oil and grease and are carried to the surface where they can be removed by skimming (Mahlie, 1940). Once the grease has been removed from the wastewater, it is usually collected by the responsible authority and may be re-used in animal feed or human consumer products such as soap. When re-use is not feasible grease may be disposed of by land filling, incineration or anaerobic digestion (Stoll & Gupta, 1997).

Although the preferred approach from a drainage point of view is to limit the quantity of grease discharged into drains, some grease inevitably enters the sewerage system where it is likely to accumulate. One method of dealing with the deposits of grease is mechanical cleaning which involves rodding, winching and jetting. Mechanical cleaning, being a relatively expensive method, is not recommended as a long-term solution. Even when operated on an emergency basis, it may cause disruptions, potential health hazards or damage to pipework (Baig & Grenning, 1976).

Chemical methods

Since most discharges contain emulsified oil in addition to free fat and oil it may be necessary to break down the emulsion prior to the flotation step in order to get efficient separation. The most common method of de-emulsification involves the addition of acid which achieves coagulation of the oil droplets by neutralizing the droplets negative charge (Steiner & Gec, 1992). Alternatively, polyvalent cations such as Fe^{3+} and Al^{3+} and polyelectrolytes can be used to both destabilize emulsions and achieve de-emulsification (Roggatz & Klute, 1988).

Other reagents that have been proved to be very effective in the treatment of oily emulsions include chlorine and hydrogen peroxide (Mahlie, 1940; Steiner & Gec, 1992). Chlorine promotes de-emulsification by releasing the oils and fats from their protected, gelatinous, colloidal states but may also react with other constituents present in the effluent to yield potentially carcinogenic organochlorines (Clarke, 1997). Hydrogen peroxide enhances flotation by gradually decomposing in the wastewater to form additional micro-bubbles of oxygen. Unlike other chemical treatments, hydrogen peroxide, does not introduce additional salts or compounds to the water system. Any residual hydrogen peroxide will decompose to oxygen and water (Steiner & Gec 1992).

Solvents and emulsifiers may also be used in grease control but are not recommended except for emergency use or in situations where other methods of grease control are not practical. Although relatively cheap, these techniques can be hazardous to operators and result in ground water contamination when used in systems where exfiltration occurs. Emulsified grease can also create problems down stream particularly if conditions promote de-emulsification of the grease (Price, unpub.).

Biological methods of grease removal (Bioremediation)

Bioremediation is the utilization of microorganisms to enhance the removal of a pollutant from the environment. One bioremediation process, natural attenuation, relies on the fact that the environment into which a pollutant is introduced may already contain microorganisms that are capable of degrading the pollutant. In environments that do not favour microbial growth, the process of biostimulation, which involves the artificial introduction of nutrients and/or air, may improve the indigenous microbial communities' capacity to degrade the pollutant. Nutrients, specifically nitrogen and phosphorus are often employed to enhance the natural attenuation of mineral-based oils in marine environments (Brenchley, 1997). However, the impact of nutrients on the natural attenuation of grease in wastewater is not reported on, possibly because nutrient levels in wastewater are considered adequate to support microbial growth (Brock & Madigan, 1991).

Another bioremediation approach which is frequently employed in the treatment of grease-rich wastewater is bioaugmentation. Bioaugmentation is the process of adding non-indigenous microorganism to a biological system with the principle purpose of improving process

performance. By artificially increasing bacterial diversity and/or activity of the microbial population previously, untreatable pollutant (i.e. grease) may be degraded and/or depleted at a faster rate (Stephenson & Stephenson, 1992; Lange *et al.*, 1998).

Bioaugmentation, if successful, has several benefits over conventional forms of grease removal. Unlike physical or chemical methods of grease treatment which are expensive and short term solutions to grease problems, bioaugmentation can prevent further grease accumulations without large capital expenditure. This method is also environmentally acceptable since the microorganisms ultimately convert the grease to carbon dioxide and water, and there are no hazardous reagents or flammable solvents involved. (Grubbs *et al.*, 1991).

Limitations to the bioaugmentation method may include a sensitivity of the microorganisms to chemicals such as bleach and caustic, extremes of temperature and pH, and reduced oxygen levels. Microorganisms that are introduced to heavily greased sewers and drains may also dislodge excess grease from the drain lines creating further blockages and enhanced grease concentrations downstream (Baig & Grenning, 1976; Grubbs, 1983). There is also the possibility that bioaugmentation could actually decrease the 'quality' of the wastewater as measured by Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) (Grubbs, 1983) since the parameters generally used by monitoring agencies simply measure the quantity of organics being discharged into the sewer rather than the quality of discharge i.e. if it will create blockages or how treatable it is. Partial biodegradation of the grease may result in the production of shorter chained fatty acids, which are more soluble and thus amenable to biodegradation or oxidation by the COD procedure.

Conventional methods of developing commercial bioaugmentation products involve adaption, selection and mutation techniques (Figure 1.7). Microorganisms are first collected from environments that have already favoured adaption to certain substrates and conditions. Microorganisms that are capable of degrading specific substrates are then isolated and exposed to increasing quantities of the substrate they are required to degrade. This process naturally selects those microorganisms that are best able to adapt to the pollutant laden conditions of the media. The microorganisms ability to adapt to the specialized environment can then be accelerated by subjected the culture to mutagenic agents such as UV, nitrous

oxide or radiation. By increasing mutation rates, the genetic variation of the microbial population expands and ideally improves degradation rates. After strain development, the microorganisms are tested for safety, efficiency and stability and are grown-up on a commercial scale (Stephenson & Stephenson, 1992).

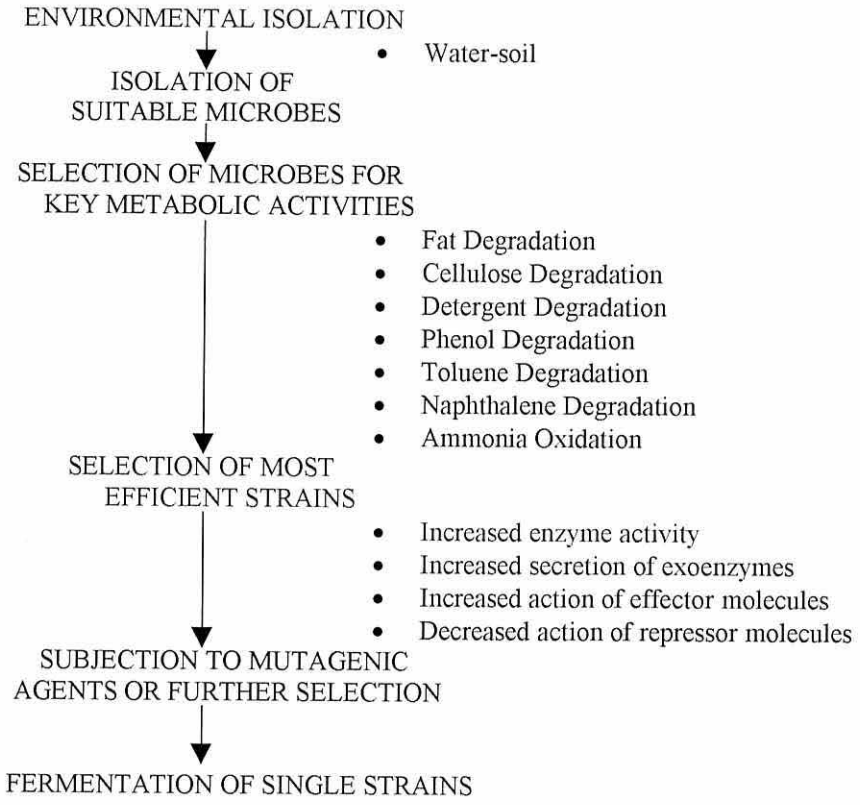


Figure 1.7. Scheme of microbial product development (Stephenson and Stephenson, 1992).

Increased control over the random procedure of mutation can be achieved by using recombinant DNA technology. Although this technique has huge potential, regulations governing the release of genetically modified microorganisms into the environment are stringent. The major concern is that microorganism could transmit genetically altered DNA to other organisms and thus change an ecosystem (Powledge, 1983).

Most commercial microbial supplements consist of mixed microbial populations including autotrophs, heterotrophs, facultative anaerobes and aerobes that are intended to serve a broad range of purposes (Hull & Kapuscinski, 1987). The final product may also incorporate wetting agents, buffers, stabilizing agents, perfumes, nutrients, emulsifiers and enzymes (Stephenson & Stephenson, 1992) and may be freeze-dried or stabilized in liquid form (Hull & Kapuscinski, 1987). A search of the internet revealed that there are a large number of

commercial bacterial preparations, that are specifically designed as the manufacturers claim, to reduce COD, BOD and grease levels in wastewater, eliminate blockages, odours and the need for other methods of grease control (Table 1.3).

Methods of application vary from the direct delivery in an undiluted form by hand to the controlled dose of the product through electro-mechanical dispensing machines (e.g. the Grease Guzzler™). Some formulations may also require pre-soaking or diluting with water to re-hydrate and/or activate the product before use. The type and quantity of bioaugmentation product used is usually determined by evaluating existing conditions and varies in accordance with system flow, organic loadings, trap and pipe size. Manufacturers generally prescribe higher initial doses of the product to hasten colonization of the desired colonies and lower subsequent doses to maintain the active populations. They also recommend that grease traps and sewers lines are mechanically cleaned of grease before dosing commences.

Table 1.3. Microbial products currently available that claim to reduce COD, BOD and grease levels in wastewater, eliminate blockages, odours and the need for other methods of grease control.

MANUFACTURER	PRODUCT
BIOCAT	WD10
BIOCHEM	250FE, TD507L,GC600L, 2006RG,
BIO-INDUSTRIES LTD	BIO-DEGREASER
BIOLOGIC	GTT
BIOTAL	HOMEBREW GD
BIO-SYSTEMS EUROPE LTD	FOGFREE, E60
CLEVELAND BIOTECH	AMINITE L1OO, L50
ENSOLVE BIOSYSTEMS INC	GREASE FEAST
HARVARD CHEMICAL RESEARCH	6036 BACTERIA PLUS
HODGE SEPARATORS LTD	OLEOBAC
INTERBIO	BIOFREE, BIOLYTE MX25, MX40
OIL CLEANING BIO-PRODUCTS	HEGTRAP
PPD LTD	GREASE BUGGA
S.F. INDUSTRIES	SF-800-GT
SYBRON	RG2006
TELAMODE	TELA-CHEM LDF
TRI SYNERGY INC	BIOLINE

Although a large number of bacteria, fungi and yeasts have been identified that are able to hydrolyse and/or grow on grease under ideal laboratory conditions (Koritala *et al.*, 1987; Tano-Debra *et al.*, 1999; Markossian *et al.*, 2000; Mihara *et al.*, 2000) attempts to

demonstrate the efficacy of commercially available bioaugmentation products have proved somewhat variable. Several authors have reported that the use of bioaugmentation products in the field have improved the quality of wastewater in terms of fat/oil concentration and/or COD and BOD (Baig & Greening, 1976; Grubbs *et al.*, 1991; Holt, 1992; Maes, 1994; Keenan & Sabelnikov, 2000) whilst lab-based studies have shown that bioaugmentation enhances fat/oil degradation in some cases (Keenan & Sabelnikov, 2000) but not in others (Salome & Bonvallot, 1994; Mendoza-Espinosa & Stephenson, 1996).

Enzyme preparations have also been shown to assist in the removal of grease from wastewater (Cail *et al.*, 1986; Gary & Sneddon, 1999). These enzymatic products are primarily composed of lipases although amylases, proteases and cellulases may also be present to assist in the degradation of grease deposits of heterogeneous nature. Unlike microbial supplements, which require only top-up doses once the wastewater is seeded, lipase treatments require large regular doses and can therefore be costly. This method of grease treatment is further limited because lipase itself is only capable of accelerating the hydrolysis reaction and is unable to accomplish complete degradation alone (Grubbs *et al.*, 1991).

1.3.3 Aims and objectives

No clear consensus on the merits of bioaugmentation has been reached. Thus, the broad aims of this work were to;

- Establish whether commercial microbial supplements were capable of enhancing the degradation of fat/oil under ideal laboratory conditions
- Determine whether the commercial microbial supplements were adapted for use in the field by examining (1) the growth/oil degrading abilities of commercial inocula under a wide variety of environmental conditions, (2) the ability of the supplements to produce protease and amylase and (3) the ability of the microbial formulations to adhere to surfaces, including that of fat/oil.
- Establish the major fat/oil degradation pathways involved in bioaugmentation and identify the rate limiting steps in the degradation process.

2. MATERIALS AND METHODS

2.1. Activation and Enumeration of Commercial Microbial Supplements

The Grease Guzzler™ (manufactured by WPL Ltd) was configured to dilute commercial microbial supplements with water (1:30) and incubate the diluted formulations at 37 °C for 5-8 hours prior to release into the drains (Figure 2.1).

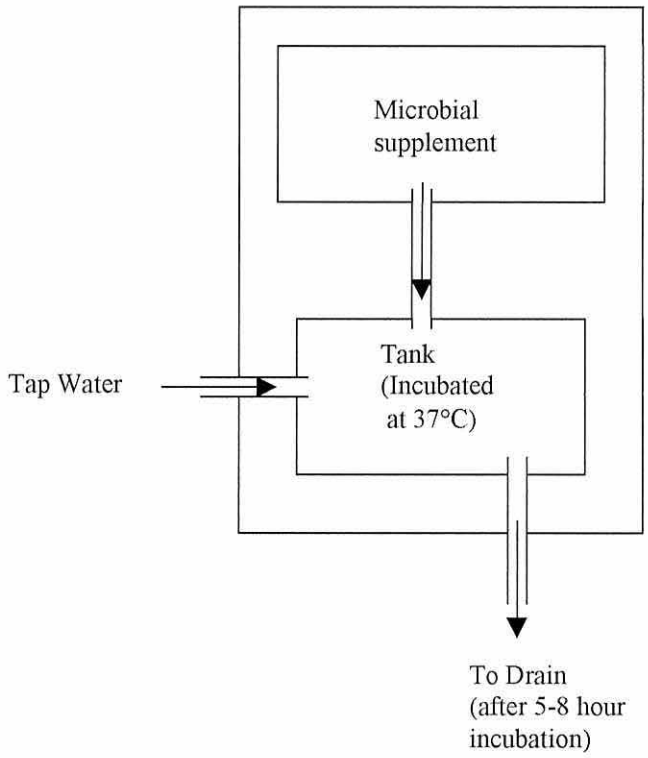


Figure 2.1. Schematic of Grease Guzzler™ operation.

This activation procedure was mimicked in the laboratory although the type of water, dilution factor and length of incubation varied between experiments. The water employed to dilute the bacterial formulations ranged from distilled to autoclaved nutrient enriched distilled water. Distilled water was favoured over tap water because the quality of distilled water was consistent and maintained to a high standard. Nutrient additions served to encourage bacterial growth, as many of the formulation investigated contained little or no nutrients. To ensure that the number of contaminating bacteria were minimized, the dilution water was autoclaved before use. The dilution factor (1:30) was also adjusted to ensure that any inhibitors present in

the product were sufficiently diluted. The dilution factors of formulations F33 and S45 (Appendix VII) were adjusted to 1:1000 and 1:10000 respectively on the recommendation of the manufacturers. A small quantity of oil may have also been added to the inocula during activation in an attempt to stimulate lipase activity and β-oxidation before addition of the inocula to the fat rich sample. Effort was made to ensure that this oil added was not transferred to samples during inoculation.

The manufacturers of the commercial bacterial supplements claimed that each product contained approximately 10^8-10^9 cfu.ml^{-1}. Bacterial numbers were verified by preparing dilutions of the activated product down to 10^{-10} using Lab M maximal recovery diluent and plating 20-50 μl of each dilution on Lab M or Oxoid nutrient agar. Plates were incubated at 30°C and bacterial growth was assessed at each dilution after 48-72 hours.

2.2. Isolation and Enumeration of Grease Trap Bacteria

Water samples (500 ml) were collected in triplicate from a local grease trap serving a kitchen catering for students in the nearby halls of residence (Ceris). Samples were removed at the end of the summer semester when the kitchens were unlikely to have been functioning at maximum capacity.

Bacterial numbers and species composition of the grease trap samples were estimated by preparing dilutions down to 10^{-8} in universal bottles using Lab M maximal recovery diluent and plating 20 or 50 μl of each dilution onto Oxoid nutrient agar. Agar plates were incubated at 30°C for 48 hours after which the number of colonies and different morphologies were counted.

Grease trap bacteria were grown by incubating 1ml of each grease trap sample in 99ml of Oxoid nutrient broth at 30°C, with shaking at 150 rpm for 24 hours. The resulting broth was preserved by mixing with an equal volume of 40% glycerol followed by freezing. Bacterial numbers and colony morphologies were examined both after incubation with the nutrient broth and after thawing of the nutrient broth/glycerol mix to ensure that the grease trap isolates had withstood the preservation process. Where appropriate the preserved inoculate was diluted to achieve a final bacterial count of approximately 4.02 x 10^5 cfu.ml^{-1} in each

treatment. A sub-sample of the preserved inoculate was also cultivated on nutrient agar to confirm that bacteria were present at the predicted levels.

2.3. Optical Density Measurements at 600nm

Bacterial numbers can also be estimated by measuring the optical density (540-620 nm) of a sample (Fehrenbach *et al.*, 1992; krist *et al.*, 1998; Vand-Walle & Shiloach, 1998) and several authors have established relationships between optical density and culturable bacterial numbers (Chorin *et al.*, 1997; Augustin *et al.*, 1999). Direct bacterial counts may also be used to overcome the potential problem that both viable and non-viable bacteria may contribute to the optical density of sample but are time consuming.

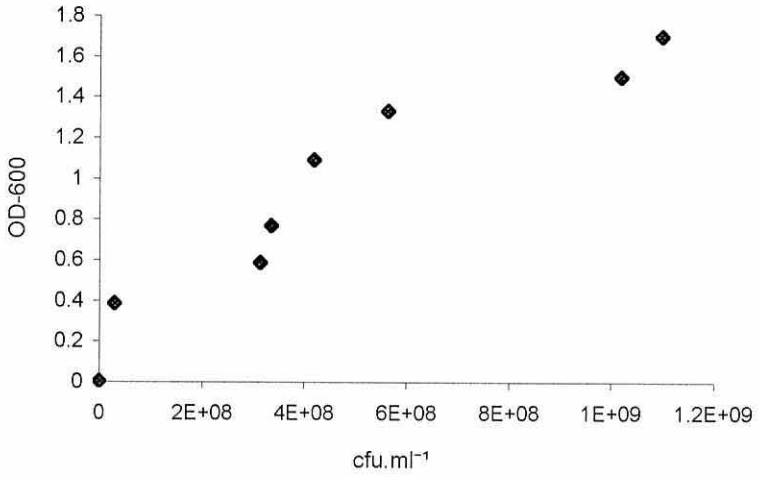


Figure 2.2. Correlation between optical density at 600nm and bacterial numbers.

To confirm that there was a relationship between the optical density (600nm) of a solution and bacterial numbers, Lab M nutrient E broth was inoculated with F33 and incubated at 30°C, 150rpm for 24 hours. Sub-samples of the inoculated nutrient broth were removed over the course of the incubation period and bacterial counts were assessed using the method outlined above. Absorbance at 600nm was measured with a spectrophotometer using sterile nutrient broth as the blank. The results of the preliminary experiment (Figure 2.2) confirmed there was significant correlation between bacterial numbers and optical density (Pearson's correlation; $r=0.942$, $P<0.001$) suggesting that the optical density measurements could be employed to estimate bacterial abundance.

Optical density measurements were used to assess the growth of the microbial supplements under a variety of conditions and to assess the ability of the microorganisms to grow on fats, oils and fatty acids as a sole carbon source at a variety of concentrations. Experiments were conducted in 25 ml vials containing 10 ml of a sterile nutrient media or synthetic sewage media (Appendix I & II) inoculated with 200 μl of a bacterial culture with an OD-600 adjusted to lie between 0.8-1.2.

The bacterial inocula used in the optical density experiments were prepared by inoculating 100 ml of lab M nutrient broth with 200 μl of the microbial supplement. After 24 hours incubation at 30°C, 130rpm, a centrifugation and rinsing procedure was adopted to ensure that all traces of organic nutrients had been eliminated from the bacterial cultures. Any limited microbial growth that may have been facilitated by residual nutrients or reserves of carbon/energy within the microbial cells was compensated for by controls. These controls were comprised of the salt solution and inoculum alone.

Physico-chemical processes such as emulsification, which may have also resulted in an increase in optical density, were taken into account by a second set of controls. These controls were identical in composition to the samples under investigation but were not inoculated. Optical density measurements were accomplished using a spectrophotometer adjusted to a wavelength of 600nm. The spectrophotometer was blanked with distilled water or in the case of the preliminary experiments, the appropriate physico-chemical control.

2.4. Measurement of Microbial Enzyme Activity

Lipase activity

Agar based methods

Tributryn agar was prepared by homogenizing 10 ml of tributryn with 1000 ml of Oxoid nutrient agar. Tween agar plates and other fat/oil plates were prepared using 2.5% of nutrient agar, 2% tween or other fat/oils and 0.01% Victoria blue B (Samad *et al.*,1989).

The ability of the various agar plates to detect lipase activity was investigated by boring circular wells (6mm diameter) into plates and filling them with 50 μl of a lipase preparation (*Candida rugosa*). Positive results were obtained in all in all cases, as indicated by a zone of clearance (tributryn agar) or colour intensification that formed around the wells containing the enzyme preparation. By varying the enzyme concentration (4, 2, 1, 0.5, 0.1, 0.05, 0.01 $mg.ml^{-1}$) it was possible to confirm that there was a significant positive correlation between the diameter of clearing/colour intensification and concentration (log_{10}) of the lipase after both 24 and 48 hours incubation on, tributryn, lard oil, soya oil, rape oil, sunflower oil, tween-20 (monolaurate), tween-40 (monopalmitate) and tween-80 (monooleate) (Pearson's correlation: $r=0.93-0.99$, $p<0.01$).

The lipolytic activities of individual microorganisms were assessed by pre-growing cultures on nutrient agar and then transferring a small portion of the bacterial colony onto the tween/tributryn plates using a sterile wire loop. To determine the overall lipolytic activity of a culture, wells (6mm in diameter) were bored into the plates and filled with 50 μl of culture. Inoculated plates were then incubated at 30°C and were examined after 24, 48 and/or 72 hours. Lipase activity on the tributryn plates and tween/fat/oil agar was indicated by a clear zone or colour intensification around each colony/well, respectively.

Quantitative, titrimetric determination of lipase

A Sigma diagnostic kit was employed to measure lipase activity in the various commercial microbial formulations. Lipase activity was quantified by measuring the amount of fatty acids generated from the hydrolysis of olive oil triglycerides. Fatty acids were determined by titrating the sample with sodium hydroxide.

Procedure

Water (2.5 ml), sigma lipase substrate (3 ml) and TRIZMA buffer (1 ml) were pipetted into several test tubes. Half of these test tubes were inoculated with 1 ml of activated product, the remaining half received only the reagents. All test tubes were capped with Nesco film, shaken for 5 seconds and placed in a 37°C incubator for 3 or 6 hours. Immediately after starting the incubation, 1ml of activated product was also pipetted into a 50 ml erlenmeyer flask and stored in a refrigerator.

After the prescribed incubation period the contents of the test tubes were poured into erlenmeyer flasks. Those test-tubes that had not been dosed with the activated product prior to incubation were emptied into the erlenmeyer flasks that contained the refrigerated activated product. Each test tube was then shaken and rinsed with 3 ml of 95% ethanol and the contents poured into their respective flasks. Finally, thymolphthalein indicator solution was added to each flask (4 drops if the incubation period was 6 hours, 6 drops if the incubation was 3 hours) and each flask was titrated against 0.05N NaOH until the emulsion turned from white to a slight but definite blue.

Calculation

All samples and the corresponding controls were tested in triplicate hence it was possible to determine whether the volume of sodium hydroxide used in the controls was significantly different to the volume used in the test samples.

Volume of NaOH (ml) needed to neutralize fatty acids formed =
volume of NaOH (ml) used for test - volume of NaOH (ml) used for blank

Sigma-Tietz units.ml^{-1}= volume of 0.05N NaOH (ml) needed to neutralize fatty acid)

Amylase activity

Amylase activity was detected using agar prepared from 2 g of starch, 2 g of nutrient agar and 100 ml of distilled water. Once the agar had solidified, wells (6mm in diameter) were bored into the plates and filled with 50 µl of the microbial culture. After 24 or 48 hours incubation, a few drops of iodine were added to the surface of the agar and a zone of clearing was indicative of amylase activity.

Protease activity

Protease activity was detected using milk agar. Milk agar was prepared by combining two different media after autoclaving. One media was comprised of 4 g of skimmed milk and 60 ml of water whilst the other was comprised of 3 g of agar and 130 ml of water. Once the agar had solidified, wells were bored into the agar and 50 µl of microbial culture were added. The

agar plates were incubated for 24 or 48 hours and protease activity was indicated by a zone of clearing.

2.5. Assessing the Density of Bacteria Attached to Surfaces

Glass coverslips (2cm x 2cm) were cleaned using hot chromic acid and attached to the base of glass trays (30cm x 30cm) using a silicone-based gasket sealent (Rhodorseal). Each tray contained roughly 9 coverslips, which were immersed in 500 ml of distilled water or nutrient solution (appendix) inoculated with activated bacterial supplements. Over a period of approximately 3-6 weeks, glass cover slips were regularly removed using forceps, rinsed with distilled water and fixed in 2.5% glutaraldehyde for 1 hour. After fixing, samples were dehydrated using a sequential alcohol series (30 minutes at 30%, 50%, 70% and 100% ethanol) and mounted on stainless steel stubs once the alcohol had evaporated. Graphite was used to maintain electrical conductivity between the stub and coverslip and the uppermost surface of the coverslip was coated in gold using a gold spluttering device. Samples were then viewed using a scanning electron microscope (Leo S120, scanning at 10000eV).

In later experiments, some of the glass coverslips and ABS plastic were coated with lard. Since the high, localised temperatures generated during electron microscopy would melt any lard present an alternative method for examining the adhering bacteria was devised. This alternative procedure involved staining the microorganisms adhering to the slides with DAPI (4,6-Diamidino-2-phenylindole) at 50 $\mu g.ml^{-1}$ for 10-20 minutes and then viewing the slides under a UV-light microscopy (Nikton optiphot HB-1010AF). The number of microorganisms adhering to the slides was then assessed using a graticule (16 x 36 μm) in 10-25 random fields of view.

2.6. Chemical Oxygen Demand Determinations

Background

The Chemical Oxygen Demand (COD) determination provides a measure of the oxygen equivalent of the organic material present in a sample that is susceptible to oxidation by a strong chemical oxidant. The preferred oxidant is dichromate due to its superior oxidising ability, applicability to a wide variety of samples and ease of manipulation.

Oxidation reaction

$Cr_2O_7^{2-} + 8H^+ \rightarrow 2Cr^{3+} + 4H_2O + 3O_2$

The ferrous iron/dichromate titration

$6Fe^{2+} + Cr_2O_7^{2-} + 14H^+ \rightarrow 6Fe^{3+} + 2Cr^{3+} + 7H_2O$

The dichromate-reflux technique, although more effective than the permanganate method, does have some limitations and interferences. Straight-chain aliphatic compounds, aromatic hydrocarbons, and pyridine are not oxidized to any appreciable extent, whilst nitrite and reduced inorganic species such as ferrous iron, sulfide and manganous manganese interfere with the procedure. Some of the interferences, for example nitrite, may be eliminated by the addition of sulfamic acid, and straight-chain aliphatic compounds may be oxidized more effectively when silver sulphate is present as a catalyst. However, silver sulphate also reacts with chloride, bromide and iodide to produce precipitates that are oxidized only partially by the procedure. These difficulties may be overcome, though not completely, by complexing the halides with mercuric sulphate before the refluxing procedure.

Chloride interference reaction

$6Cl^- + Cr_2O_7^{2-} + 14H^+ \rightarrow 3Cl_2 + 2Cr^{3+} + 7H_2O$

Experimental design

The ability of the microbial supplements to degrade both oil and other organic material was assessed using COD determinations. Experiments were generally conducted in 250 ml flask which were stoppered with cotton wool to encourage oxic conditions and reduce the risk of contamination. The final volume for all treatments was 100 ml, 90 ml of which was nutrient solution and where appropriate, 10 ml of activated microbial supplement. Soya oil, when required, was added at concentrations of 0.1-1% (v/v) and if necessary was mechanically emulsified using a blender or chemically emulsified using 400 µl of an emulsifier provided by Organica.

Procedure

A sub-sample (10 ml) was measured into a round bottomed flask followed by 1 ml of 20% (w/v) mercuric sulphate, 5 ml of 0.020833M potassium dichromate, and 15 ml of 1% (w/v) silver sulphate in concentrated sulphuric acid (d_{20} 1.84). After the contents of the flask were swirled and connected to a condenser, the flask and its contents were placed on a heating mantle to boil gently. The source of heat was removed after 2 hours and allowed to cool for approximately 10 minutes. After 10 minutes, 25 ml of water was added via the condenser to rinse any remaining dichromate from the condenser. The flask was then disconnected from the condenser and cooled to room temperature in running water.

Residual dichromate was determined titrimetrically using a standard solution of ferrous ammonium sulphate (0.025 M) and 1-2 drops of 1:10 phenanthroline complex (Ferroin). The ferrous ammonium sulphate was standardized each day before use against 0.020833 M potassium dichromate.

To standardize the ferrous ammonium sulphate, 5 ml of 0.020833M potassium dichromate solution was diluted with water to approximately 60 ml and 15 ml of sulphuric acid (d_{20} 1.84) was added carefully. Once cooled, 1-2 drops of 'Ferroin' indicator were added and the solution was titrated against the ferrous ammonium sulphate solution.

The molarity (M) of the ferrous ammonium sulphate solution is given by

$$M = (0.020833 \times 30)/V \text{ or } M = 5/(8V)$$

where V is the volume (in ml) of ferrous ammonium sulphate solution titrated.

It was also necessary to determine a blank value by replacing the 10 ml sample with an equivalent quantity of water. The blank value was the mean of three determinations and if any value differed by more than ±0.5 ml from the mean value it was rejected and a new mean recalculated from the acceptable blank values.

Calculation of COD results

$COD = 800\ M\ (V_B - V_S)\ mg.l^{-1}$

V_B = average volume of ferrous ammonium sulphate (ml) used in titrating the blanks
V_S = average volume of ferrous ammonium sulphate (ml) used in titrating the sample
M = molarity of standard ferrous ammonium sulphate solution

In most cases it was necessary to pre-dilute a sample, therefore, the calculation was modified to take the dilution factor into account.

The technique and quality of the reagents were evaluated using a standard solution of glucose (0.4686 $g.l^{-1}$) which theoretically yields a COD of 500 $mg.l^{-1}$. With reagent grade glucose, the coefficient of variation was ±0.81% of the mean, which was calculated to be 100.75% of the theoretical oxygen demand (503.76 +/-4.06 $mg.l^{-1}$).

2.7. Oxygen Consumption Measurements

Preparation and collection of samples

Sub-samples were removed from some of the mechanically prepared vegetable oil emulsions employed in the COD experiments and were diluted with distilled water that had been equilibrated at 20°C. Incubation bottles ranging between 50-60 ml were filled by siphoning and any air bubbles were displaced before the glass stoppers were replaced. An initial oxygen determination was performed on one sample and a replicate sample was placed in a 20°C incubator for a further 24 hours before the next oxygen determination was carried out.

Standardization of thiosulphate

Thiosulphate is not a primary analytical standard and consequently for accurate work it was necessary to standardize the thiosulphate using a standard solution of potassium iodate. Each of the two reagents, sulphuric acid (0.5 ml) and alkaline iodide (0.5 ml) were added to about 25 ml of distilled water in a conical flask. After the additions were made, 5ml of potassium iodate standard (0.0007M) was added and the solution was covered and allowed to stand for 10 minutes to allow the reaction to go to completion. Finally, the whole contents of the

conical flask were titrated with thiosulphate and the molarity of the thiosulphate (M) was calculated as followed:

$M = (6 \times V_2 X M_1)/V_1$

Where:

V_1 = volume of the thiosulphate added (ml)

V_2 = volume of iodate added (ml)

M_1 = molarity of the iodate standard

Fixing and titration of samples

Manganese sulphate solution (0.5 ml) followed by alkali-iodide reagent (0.5 ml) were carefully added to the incubation bottle below the surface of the liquid. The incubation bottle was stoppered with care to exclude air bubbles and the contents were mixed by inverting the bottle several times. After the precipitate had settled leaving a clear supernatant, the sample was shaken again and then left to stand until at least 30 ml of supernatant had cleared.

When settling had produced sufficient supernatant the stopper was carefully removed and 1 ml of concentrated H_2SO_4 was added by allowing the acid to run down the neck of the bottle. After re-stoppering the contents were mixed by gentle inversion until dissolution was complete. Once the iodine was uniformly distributed throughout the bottle 3 x 10 ml sub-samples were removed and titrated with 0.002M thiosulphate solution until a pale straw colour was obtained. 1-2 ml of starch solution were then added and the titration was continued until the first disappearance of the blue colour.

Calculation of oxygen concentration and consumption

Oxygen concentration ($\mu moles.l^{-1}$) $= A \times M \times V \times 10^6 / ((B \times (V-1) \times 4)$

Where:

V = volume of the sample bottle (ml)

A = volume of thiosulphate added (ml)

B = volume of sample titrated (ml)

M = molarity of thiosulphate used

Oxygen consumption ($\mu moles.l^{-1}$) $= ((D_1-D_2)-(B_1-B_2)) / P$

Where:

D_1 = oxygen concentration of diluted sample immediately after preparation ($\mu moles.l^{-1}$)

D_2 = oxygen concentration of diluted sample after 24 hours incubation at 20°C ($\mu moles.l^{-1}$)

B_1 = oxygen concentration of distilled water control before incubation ($\mu moles.l^{-1}$)

B_2 =oxygen concentration of distilled water control after incubation ($\mu moles.l^{-1}$)

P = decimal volumetric fraction of sample used

2.8. Gravimetric Analysis of Fat/Oil Degradation

Experimental design

The degradative abilities of the microbial supplements were estimated by incubating the activated commercial bacterial supplements with lard or vegetable oil for a designated period of time and then extracting the fat/oil into an organic solvent to determine the mass remaining. Experiments were conducted in either 25 ml 'universal' bottles or 250 ml conical flasks which were stoppered with cotton wool to encourage oxic conditions and reduce the risk of contamination. Fats and oils, which were purchased from the local supermarket were added to achieve a final concentration of ca. 0.5-10%(v/v) in samples. The lard emulsion was prepared using ca.10g of lard (melted), 195ml of hot tap water and 5ml of surfactant (Fairy liquid) and was diluted 10 fold in the samples. Final volumes for all treatments were generally 10 ml and 100 ml in the case of the universal bottles and 250 ml conical flasks respectively. 9 or 90 ml of the total volume was either water or nutrient solution and the volume of activated product added to the universal bottles and conical flasks was typically 1 ml and 10 ml respectively. Controls were prepared in the same manner but received the equivalent dose of a water/nutrient solution instead of the activated inoculum. The flasks/universal bottles and their content were often autoclaved prior to inoculation to ensure that samples were free from contaminating microorganisms. All samples were incubated at a room temperature (20-25°C) or 30°C and may have been shaken at 130-150 rpm.

Extraction of lipids from samples

Lipids were extracted from samples in separating funnels using 15 ml or 40 ml of chloroform or dichloromethane for 10 ml or 100 ml samples, respectively. Any oil that remained in the universal bottles or conical flasks was rinsed out with the appropriate solvent before addition

of the solvent to the separating funnel. The contents of the separating funnel were shaken vigorously after which the mixture was allowed to settle and the organic phase transferred to a florentine flask. Some samples were centrifuged at 2000-5000 rpm for 2-10 minutes and a few drops of saturated sodium chloride solution or ethanol may have also been added to assist separation of the two phases if emulsification had occurred. The entire extraction procedure was repeated 4 times and after the second extraction, 10% HCl was added, when required, to acidify the aqueous phase to pH 2-3.

To eliminate contaminating water from the lipid samples, the dried sample collected in the florentine flask was re-dissolved in a few ml of solvent and transferred to a vial containing anhydrous sodium sulphate and was then filtered through Whatman number 4 or 6 filter paper into a pre-weighed vial. The solvent was again evaporated under oxygen free nitrogen at 40°C to allow the mass of extracted oil to be calculated.

2.9. Adsorption Chromatography

Principles of adsorption chromatography

Adsorption chromatography is a technique that is frequently employed to separate large quantities of lipids by selective elution from a solid support. Compounds that are bound to a solid adsorbent by a combination of polar, ionic and Van der Waals forces are separated according to the relative polarities of the individual lipid groups. By eluting the column with increasingly polar solvents it is possible to separate the lipid mixture into increasingly polar classes of compounds. For example, a mixture of free fatty acids and glycerides treated with increasingly polar solvents would be eluted in the following order; triglycerides, diglycerides, monoglycerides and free fatty acids.

Separation of free fatty acids from the mono, di and tri-glycerides was achieved by modifying a recognized adsorption chromatography technique (Kates, 1986). The procedure outlined in Kates (1986) was suitable for separating large lipid mixtures (120 -180mg) composed of hydrocarbons, steryl esters, methyl esters, cholesterol, in addition to fatty acids, mono, di and triglycerides. Modification of the technique was necessary to deal both with the smaller

quantities of lipid used in the degradation experiments (<120mg on occasions) and the restricted number of lipid classes.

The ability of the adsorption chromotography method to separate the glycerides from free fatty acids was assessed using a lipid standard containing roughly equal proportions of a free fatty acid and a mono, di and tri-glyceride (heptacosanoic acid (27:0), 1-monomyristoyl-rac glycerol (14:0), dipalmitin (16:0) and trinonadecanoin (19:0), respectively). After the eluted fractions were derivatized using 14 % boron trifluoride in methanol, the lipid classes present in each fraction were identified and quantified using gas chromatography.

Method

Hydrated florisil (4% or 7%) was prepared using 10 g of 60-100 mesh florisil activated at 130°C for 16 hours with 0.4 or 0.7 ml of distilled water and shaking in a stoppered flask overnight. The hydrated florisil was slurried in hexane and poured into a 0.5 x 30 cm chromatography tube to achieve a column length of ca. 10 cm. The lipid sample (2-10 mg) was applied in 2-5 ml of hexane and the column was eluted with the sequence of solvents given in table at a flow rate of ca. 1.5-2 $ml.min^{-1}$.

Glyceride fraction	=	Hexane/ethyl ether and/or ethyl ether/methanol
Fatty acid fraction	=	Ethyl ether/acetic acid

The preliminary investigations (Table 2.1. method number 1-3) were concerned exclusively with the purity of the glyceride and fatty acid fractions therefore the yield of each lipid class was not calculated. These experiments established that complete separation of the glyceride and fatty acid fractions had not been achieved. In subsequent assays solvent volumes, solvent ratios and adsorbent moisture content were progressively adjusted in an attempt to improve glyceride yields without compromising the purity of either the glyceride or fatty acid fractions.

Table 2.1. Yields (%) of the mono, di, tri-glycerides and free fatty acid eluted from the florisil packed columns using various volumes/ratios of organic solvents (methods 4-9 used 4% hydrated florisil, 1-3 used 7% hydrated florisil) (*-not measured, ND-not detected).

Method Number	Solvents used	Total Volume	Ratio	Yield (%) of each lipid class Glycerides			Fatty acid
				14:0	16:0	19:0	27:0
1	Ethyl ether/methanol	35	98:2	*	*	*	*
	Ethyl ether/acetic acid	7	93:7	*	*	*	*
2	Ethyl ether/methanol	70	98:2	*	*	*	*
	Ethyl ether/acetic acid	20	93:7	*	*	*	*
3	Ethyl ether/methanol	157	92:8	*	*	*	*
	Ethyl ether/acetic acid	20	93:7	*	*	*	*
4	Ethyl ether/methanol	100	97:3	77.5	100.9	104.1	ND
	Ethyl ether/acetic acid	20	93:7	1.8	5.6	0.0	88.0
5	Ethyl ether/methanol	150	97:3	92.8	104.0	105.1	0.6
	Ethyl ether/acetic acid	20	93:7	0.3	0.7	0.6	34.4
6	Ethyl ether/methanol	150	95:5	110.4	108.6	115.5	0.63
	Ethyl ether/acetic acid	20	93:7	0.2	0.2	0.4	71.3
7	Ethyl ether/methanol	155	96:4	116.7	113.6	110.5	2.0
	Ethyl ether/acetic acid	20	93:7	5.2	9.7	9.7	114.0
8	Ethyl ether/hexane	50	50:50				
	Ethyl ether/methanol	150	95:5	76.5	71.1	86.8	6.3
	Ethyl ether/acetic acid	20	93:7	0.52	1.0	0.4	73.2
9	Ethyl ether/hexane	50	50:50				
	Ethyl ether/methanol	200	95:5	69.3	79.2	76.3	ND
	Ethyl ether/acetic acid	20	93:7	0.7	ND	ND	63.0

Method 9 achieved complete separation of the glyceride and fatty acid fraction and was repeated in triplicate to ensure that the results were reproducible (Table 2.2). Despite contamination of the glyceride fraction with small quantities of the mono and diglycerides method 9 was adopted as standard

Table 2.2. Yield (%) of each lipid class eluted from the columns using the volumes/ratios of organic solvents described in method 9.

	(Glyceride fraction)		(Fatty acid fraction)	
	Mean	SD	Mean	SD
Monacylglycerol (14:0)	68.96	1.06	0.61	0.11
Diacylglycerol (16:0)	77.27	1.82	0.16	0.27
Tiacylglycerol (19:0)	74.14	2.32	0.00	0.00
Fatty acid (27:0)	0.00	0.00	68.54	5.80

2.10. Gas Chromatography/Mass Spectroscopy

Principles of GC/MS

Gas chromatography accomplishes the separation of compounds by partitioning solutes between the moving (mobile) gas phases and a stationary phase bonded to a solid support. Samples are introduced into a stream of heated carrier gas where they are volatized and swept through the chromatographic column. Solutes in the sample are retarded according to their interactions with active groups co-valently bonded to the solid support and are eluted sequentially before entering a detector attached to the column exit. The detector converts the concentration of the corresponding component into electrical signals, which are amplified and registered as chromatographic peaks (Nyberg, 1986). The principle components of a gas chromatograph are illustrated in Figure 2.3.

One of the most widely used method of detection in the GC-analysis of lipids is mass spectrometry. A mass spectrometer, performs three basic tasks: (1) creates gaseous ion fragments from a sample, (2) sorts ions according to mass and (3) measures the relative abundance of ion fragments of each mass. Viewed in its simplest form, a mass spectrometer consists of a sample inlet system, ion source, ion acceleration and mass ion analyser, ion-collection system, data handling system and vacuum system (Evershed, 1992).

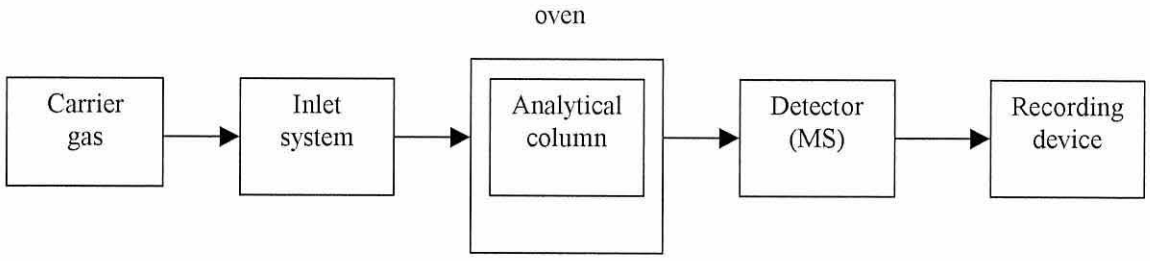


Figure 2.3. Principle components of a gas chromatograph.

In principle, molecules are bombarded with electrons to form positively charged ions that fragment and rearrange in a number of different ways to generate a variety of smaller neutral or charged species. The resultant species are then accelerated by an electrical potential and pass through a magnetic or electrostatic field where they deviate from their initial straight line of flight according to their mass to charge ratio.

The overall result of an electron impact on a molecule is illustrated by a series of peaks corresponding to the ratio of mass to charge (m/z) and the relative abundance of the ions produced (mass spectrum). The peak of highest abundance (100%) is known as the base peak and the ion derived from the charged parent molecule is referred to as the molecular ion (M+). Molecules tend to fragment in very predictable ways, for example cleavage tends to occur at weak bonds or adjacent to specific functional groups. Consequently, it is possible to infer molecular structure by analysis of the fragmentation pattern in the mass spectrum of an unknown (Christie, 1989).

The combination of gas chromatography with the mass spectrometer provides one of the most sensitive and specific means of analysing and identifying organic compounds in complex mixtures. Mass spectra are recorded continuously and in many cases, complete structure assignments may be made by consideration of mass spectra and GC retention alone. The availability of computer databases assembled from the mass spectra of many commonly occurring lipids also provides a very convenient means of interpretation. However, the results of many such computerized library searches may be misleading and must be assessed in conjunction with retention times and elution orders (Evershed, 1992).

Derivatization of samples

Fatty acids are derivatized both to increase volatility and to improve analysis by enhancing chromatographic behaviour and detectability. The derivatization method followed was that of methylation, which involves the substitution of a hydrogen atom from the carboxyl end of the fatty acid by a methyl group to form a methyl ester. The reaction is catalysed by a boron trifluoride-methanol reagent and is based upon the work by Morisson and Smith (1964)

Lipid samples were dissolved in chloroform to achieve a final concentration of 1-2 $mg.ml^{-1}$ and were transferred to a 5 ml teflon lined screw cap vial with 4 ml of boron trifluoride methanol complex in a nitrogen free atmosphere. After heating the vial and contents to 100°C for 1 hour, 3 ml of water and 2 x 6 ml of pentane was used to clean and separate the fatty acid methyl esters (FAME's). The pentane was evaporated under an oxygen free nitrogen flow and the fatty acids were finally dissolved in hexane.

Operation of the GCMS

In the GC-Ms analysis of fatty acids, due consideration must be given to the operating conditions. The GC-MS operating conditions used in this work are summarised in Table 2.3.

Table 2.3. Summary of GC/MS operating conditions used in this study.

Operating conditions	Fisons MD 800	
Column	**Capillary**	**Capillary**
Phase	BPX-70	RTX2330
Type	Bonded phase	cross bonded phase
Film thickness(μm)	0.25	0.2
Length (m)	60	60
I.D. (mm)	0.32	0.25
Carrier gas	helium	helium
Flow rate (ml min^{-1})	2	2
Sample injection		
Volume size (μl)	1	1
Injector	on-column	on-column
Oven temperature	Isothermal at 80°C for 2 minutes, 40°C.min^{-1} to 160°C, 0.5°C.min^{-1} to 170°C, 10°C min^{-1} to 250°C and isothermal at 250°C for 10 minutes	
Detector	Electron ionisation	Electron ionisation
Temperature(°C)	230	230
Ion source (eV)	70	70
Mass scan range (m/z)	45-400	45-400

Fatty acids quantification

A calibration was performed to establish the relationship between the compound to be analysed (FAME's) and the response from the instrument (peak area). The instrument was calibrated using the principle components of soya, sunflower, lard and rape oil (16:0, 18:0, 18:1ω9, 18:2ω6, 18:3ω3) over the concentration range in which the samples were expected to fall. Standard solutions of each fatty acid at the concentrations of 0.1, 1.0, 3.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 40.0, 60.0, 80.0, and 100 μg.ml^{-1} or concentrations of 0.2, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 12.0 and 16.0 μg.ml^{-1} were prepared in hexane and injected in triplicate.

Regression analysis established that the relationship between FAME concentration and peak area (as derived from the base ion) was linear. Using equation 2.1 and the parameters obtained from the linear regression (Appendix VI.), the concentration of FAME's in each sample was calculated.

$$\text{Fatty acid Concentration } (\mu g.ml^{-1}) = (\text{peak area} - \text{intercept}) / \text{slope} \qquad 2.1.$$

Experimental errors associated with the injection step and changes in detector sensitivity were often compensated for by the presence of an internal standard (23:0), which was added to the standards at a fixed concentration (50 $\mu g.ml^{-1}$). In this case, the peak area of each FAME was divided by the area of an internal standard (IS) and was multiplied by the concentration of the internal standard as shown in equation 2.2. The new variable named 'response' then replaced 'peak area' in equation 2.1.

$$\text{Response } (\mu g.ml^{-1}) = (\text{area} / \text{area IS}) \times \text{IS concentration} \qquad 2.2.$$

In order to establish how the response of the GC-MS changed during the analysis of some samples one standard of intermediate fatty acid concentration was run in triplicate every 10 samples (Figure 2.4).

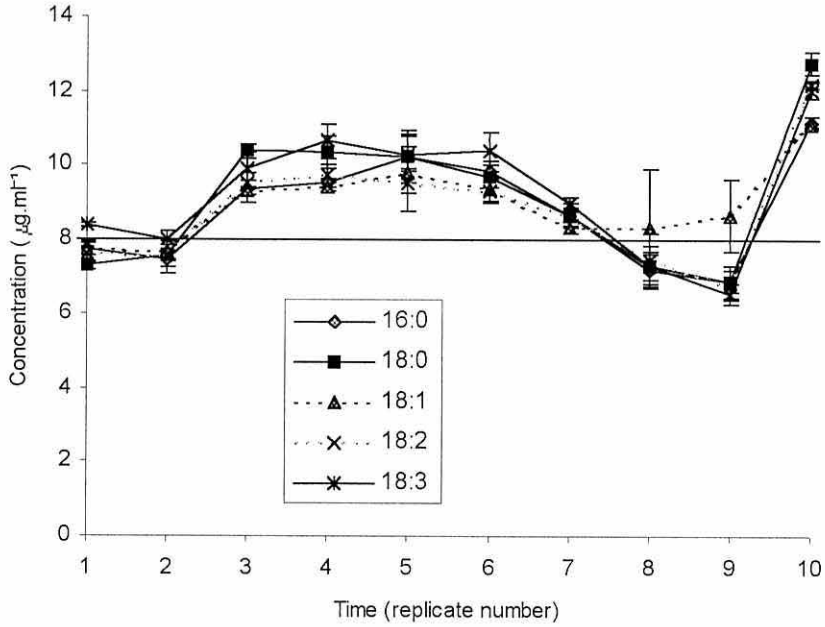


Figure 2.4. Change in the apparent concentration (mean ± SD) of 16:0, 18:0, 18:1, 18:2 and 18:3 with time in a standard containing 8$\mu g.ml^{-1}$ of each fatty acid.

It was observed that the sensitivity of the GC-MS fluctuated markedly with time (from ca. 7 to 13 $\mu g.ml^{-1}$) but within a given replicate sample the response of the instrument to individual fatty acids was similar. As indicated by Figure 2.3. and the coefficients of variation outline in Table 2.4, the absolute concentration data was considerably less reliable than the percentage

composition data. Fatty acid data in this study was therefore expressed in terms of percentage composition rather than concentration.

Table 2.4. Apparent concentration ($\mu g.ml^{-1}$) and proportion (mean± SD & CV) of 16:0, 18:0, 18:1, 18:2 and 18:3 in a standard containing $8\mu g.ml^{-1}$ of each fatty acid run on 32 separate occasions.

	Concentration			% composition		
	Mean	**SD**	**CV**	**Mean**	**SD**	**CV**
16.0	8.81	1.45	16.51	19.58	0.53	2.70
18.0	9.11	1.89	20.77	20.14	0.90	4.49
18.1	8.97	1.05	11.67	20.11	1.74	8.63
18.2	8.85	1.58	17.88	19.64	0.48	2.42
18.3	9.26	1.71	18.44	20.52	1.00	4.89

Quantification of fatty acids that were not used in the calibration standards

Some of the fatty acids that were detected in the samples were not used in the calibration due to the unavailability of the standards. Since some of these fatty acids were quantitatively important, their concentrations were calculated from an average of the closest FAME's present in the calibration. Any saturates present were quantified using the average regression of the closest saturate standards present. Similarly, monunsaturates, diunsaturates and polyunsaturates were calculated from the regression of the 18:1ω9, 18:2ω6 and 18:3ω3, respectively. The results obtained from those FAME's excluded from the calibration were interpreted with care since the regression coefficients were not the same for each FAME. 'New' fatty acids were defined as those fatty acids not detected originally in the fats and oils Only 'new' fatty acids which constituted greater than 1% of the total were reported in the results section.

2.11. Measurement of 'Particulate' Production

'Particulate' material was defined as the opaque yellow/white lumps of partially solidified material generated when soya oil was incubated with nutrients under non-sterile conditions. Quantification of the 'particulate' material was first achieved by filtering the contents of the flasks through pre-weighed Whatman No.4 paper filters. Given that paper filters may have

been adsorbing oil an alternative method of separating the particulate material from both the aqueous and oil phase was devised. Centrifugation at 2000 and 4500 rpm for 5-10 minutes failed to separate a mixture of water, oil and particulate material. However, nylon mesh (600μm) was capable of retaining most of the particulate material whilst allowing the bulk of the oil to pass through. The appearance of particulate material collected by nylon mesh filter after drying at 40°C for 18 hours is shown in Figure 2.5.

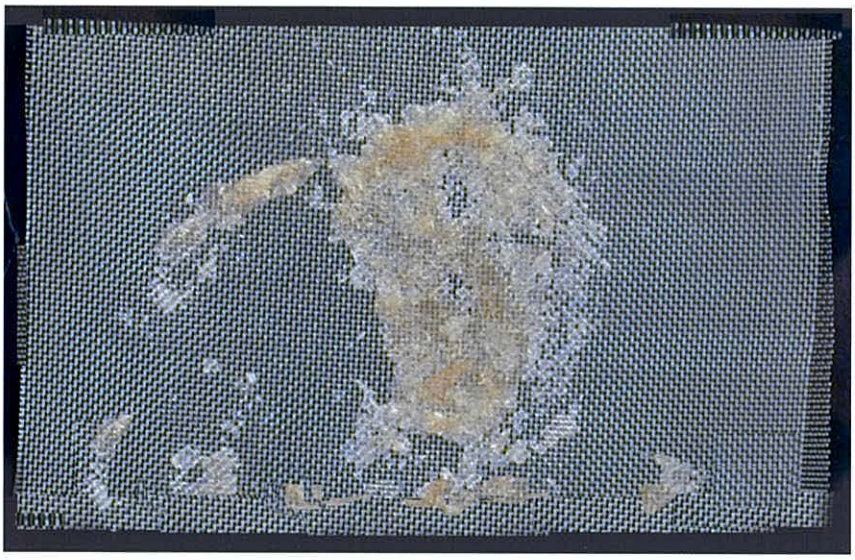


Figure 2.5. Appearance of particulate material collected by nylon mesh filter after drying at 40°C for 18 hours.

The relatively non-absorbent nature of the nylon mesh was demonstrated by passing 20 ml of water and 0.5 ml of vegetable oil through pre-weighed paper (Whatman No.4) and nylon filters (600 um). After laying the filters upright on adsorbent paper and drying at 40°C for 18 hours the mass of oil that had been retained by the filters was determined (Table 2.5).

Table 2.5. Mass of oil absorbed (mean+/-SD) by paper and nylon filters (ca. 96cm^2) after filtering a mixture of oil (20 ml) and water (0.5ml).

	Mass of oil absorbed (g)
Paper	0.3093 ± 0.0222
Nylon	0.0239 ± 0.0158

A two-sample t-test was performed and revealed that the nylon mesh filter absorbed significantly less oil than the paper filter. (T=18.12, P=<0.001) (Data normally distributed, no significant heterogeneity of variance, F=1.97, P=0.673). Consequently, 600μm nylon mesh

filters (ca.12cm x 8cm) were employed to quantify particulate production in the majority of experiments.

2.12. Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis

Principles of T-RFLP

T-RFLP is a quantitative molecular technique that was developed by Liu *et al.* (1997) for the rapid analysis of microbial community diversity in various environments. The technique involved the amplification of a selected region of bacterial genes encoding 16S RNA using the Polymerase Chain Reaction (PCR) and two primers fluorescently labeled at the 5' end. The PCR products were then digested using restriction enzymes with 4-base pair recognition sites and the fluorescently labeled terminal restriction fragments (T-RF's) were measured using an automated DNA sequencer.

Procedures

DNA extraction

A pellet of bacterial biomass was first suspended in 300 μl of lysis buffer (100 mM Tris-HCl, 100 mM EDTA, 0.75M sucrose and 1.6%, w/v CTAB). 36 μl of lysozyme (0.1mg.ml^{-1}) and 36 μl of achromopeptidase (0.02 mg.ml^{-1}) were then added and the suspension was incubated at 37°C for 30 minutes. After the addition of 41 μl of sodium dodecyl sulphate (10%,w/v) and 4 μl of proteinase K (10 mg.ml^{-1}), the samples were incubated for a further 2 hours at 37°C. During this incubation period each tube was gently inverted several times every 30 minutes to ensure the samples were well mixed. 68 ul of NaCl (5M) was then added to the mixture and samples were incubated at 65°C for 20°C. Once the mixture had been allowed to cool, samples were mixed with 500 μl of phenol:chloroform and centrifuged for 10 minutes at 10000 rpm. After centrifugation, the upper aqueous phase was removed and transferred to another tube. The samples was then mixed with 400 μl of chloroform:isoamyl alcohol and centrifuged for a further 5 minutes at 10000 rpm after which the upper phase was removed and transferred to another tube. The DNA was recovered by adding an equal volume of isopropanol and incubating the sample for 1 hour. Samples were then centrifuged at 13000 rpm for 10 min and the pellet was washed with 70% ethanol, dried and resuspended in 50 μl

of water. Additional purification was achieved using Wizard DNA purification columns (Promega).

PCR Conditions

Reaction mixtures for PCR contained 5 μl of DNA (of varying concentrations) 66.5 μl of water, 10 μl of Mg free Buffer (Promega), 6 μl of $MgCl_2$ (25 mM), 8 μl of deoxynucleotide triposphate (10 mM), 0.5 μl of Taq polymerase (5 units.μl^{-1}). The primers used 8f-Hex (5'→3':AGAGTTTGATCCTGGCTCAG) and 926r-TET, (5'→3':CCGTCAATTCCTTTR AGTTT) (2μl each of 10 *p*M.μl^{-1}) were labeled at the 5' end with the phosphoramidite dyes, 5-hexachlorofluorescein and 5-tetrachlorofluorescein (Liu *et al.*, 1997). Amplification of the DNA was performed with a PCR machine (Techne Genius) using the following programme: 1-3min hot start at 94°C, followed by 35 cycles consisting of denaturation (30secs at 94°C), annealing (45secs at 56°C) and extension (2mins at 72°C) and a final extension at 72°C for 3 minutes. PCR products were verified by electrophoresis of 5μl aliquots of PCR mixtures in 1.0% agarose (w/v) in 100ml of TBE buffer (0.045M Tris-Borate, 0.001M EDTA) with 7.5μl of ethidium bromide (10mg.ml^{-1}) as illustrated in Figure 2.6.

Restriction enzyme digestion

Fluorescently labeled PCR products (80-300 μl) were purified by using Wizard PCR purification columns (Promega). Aliquots (5 μl) of the purified PCR products were then separately digested with 5 units of *Hha*I, *Rsa*I and *Msp*I at 37°C for 2 hours. Enzyme digests were verified by electrophoresis of 5μl aliquots of PCR mixtures in 1.0% agarose (w/v) in 1 x TBE buffer, as illustrated in Figure 2.7.

DNA sequencing

The precise lengths of the terminal restriction fragments from the amplified 16S rDNA products were determined by electrophoresis using an automated sequencer. The DNA sequencing work was carried out by an independent commercial laboratory (Advanced Biotechnology Center, Charing Cross Trust Hospital, London) using an ABI sequencer capable of detecting T-RF's of 50-500 base pairs in length. T-RF's <400 base pairs in length were accurately sized by ±1 base pair whilst T-RF's >400 base pairs in length were accurately sized by ± 2 base pairs.

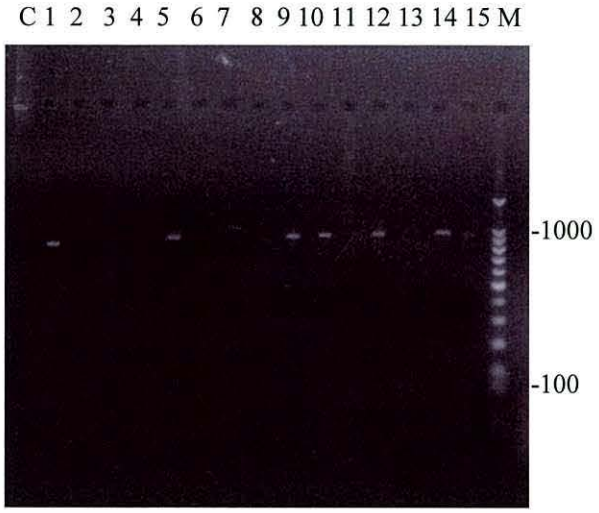


Figure 2.6. PCR amplified 16s rDNA from pF33 incubated under various environmental conditions (Lanes 1-15). M=100 base pair marker, C=control .

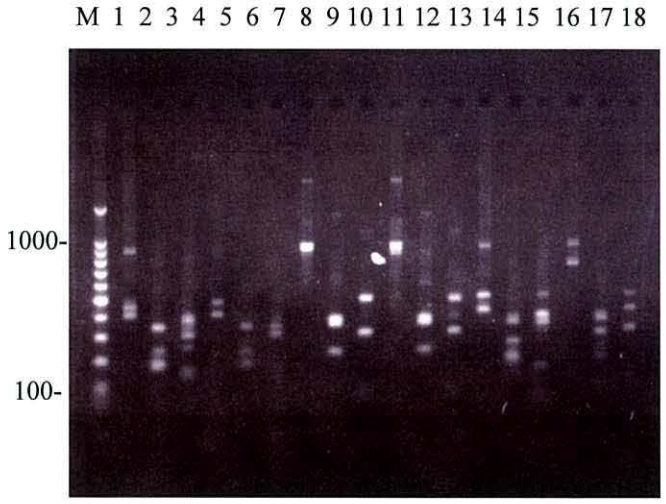


Figure 2.7. *Hha* I, *Msp* I and *Rsa* I digests of PCR amplified 16s rDNA from pF33 incubated under various environmental conditions (Lanes 1-18). M=100 base pair marker.

3. EFFECT OF COMMERCIAL INOCULA ON THE PHYSICAL AND CHEMICAL CHARACTERISTICS OF FAT/OIL

3.1. Introduction

Numerous microorganisms are capable of degrading fat/oil under ideal laboratory conditions and may thus be potential candidates for bioaugmentation products. Koritala *et al.* (1987) assessed the ability of over 100 different strains of microorganisms to degrade/hydrolyse soya oil and reported that fungi were the most effective. Similarly, Mihara *et al.* (2000) examined the oil degrading capacity of two microorganisms isolated from soil and reported that combined inocula achieved greater levels of oil degradation than the single strains alone. The work of Tano- Debrah *et al.* (1999) led the isolation of a multi-strain bacterial inocula that was able to degrade a range of oils under a variety of conditions. These workers further determined that degradation rates were strongly influenced by medium composition, temperature, and pH. Markossian *et al.* (2000) identified a thermophyllic bacterium capable of growing on a variety of oils and fatty acids at high temperatures, indicating that bioaugmentation products may have the potential to operate over a wide range of environmental conditions.

Despite these studies indicating that a large number of microorganisms are capable of degrading fat/oil under laboratory conditions, results of experiments assessing the efficacy of commercially available bioaugmentation products are rather mixed. Baig and Greening (1976) conducted a telephone survey of users of bioaugmentation products for the treatment of grease containing wastes on a wide variety of scales and reported improvements in terms of BOD and odour reduction and reduced build up of grease. Another similarly vague article highlighted the apparent success of bioaugmentation at a chocolate factory when several inches of solid chocolate were removed from grease traps (Anon, 1980). Grubbs *et al.* (1991) conducted a more thorough assessment of the bioaugmentation process and showed a reduction in free fatty acid and triglyceride concentration in a grease trap effluent from around 2500 ppm to 900-1800 ppm and around 1200 ppm to 400-700 ppm respectively following bioaugmentation. Bioaugmentation also prevented the formation of a grease cap

and induced changes in the fatty acid composition of the grease, with a reduction in the relative proportion of palmitic acid from approximately 50 to 40% coupled with slight increases in the relative proportions of myristic and lauric acids. In a similarly designed study, Holt (1992) compared the COD and BOD of a grease trap effluent prior to and after bioaugmentation and showed a 15.4% drop in average COD (1862 to 1423 $mg.l^{-1}$) coupled with a 26% drop in BOD. Interestingly, the average grease concentration in the effluent actually increased from 152 to 209 $mg.l^{-1}$. Thus, it is unclear what was responsible for the reduction in COD and BOD. The work of Keenan and Sabelnikov (2000) demonstrated at both laboratory and field scales that bioaugmentation reduced the fat/oil content of bakery wastewater to within the discharge regulatory requirements. Mixed cultures were also reported to outperform single strained cultures for fat/oil degradation. Maes (1994) tested 40 products in full scale treatment plants and found they did enhance grease degradation. In one of the treatment plant investigated, the proportion of grease removed increased from 40 to 75% following bioaugmentation. In contrast, Salome and Bonvallot (1994) examined five bioadditives for grease removal and found that none significantly degraded olive oil. Mendoza-Espinosa and Stephenson (1996), studying laboratory scale batch reactors, also found little difference in the ability of natural activated sludge bacteria and commercial additives to acclimatise biological processes to removing grease.

Although commercial microbial supplements have been known to enhance fat/oil degradation in some studies, the degradation pathways involved have not be examined in any detail. It is assumed that the first stage in the microbial degradation process is the hydrolysis of fats and oils to fatty acids and glycerol. This hydrolysis is known to proceed via the action of extracellular lipases or by lipases associated with microbial cell walls that are released into solution with cell fragments as the cell ages (Kramer, 1971; Ratledge, 1994).

Whilst it is considered to be the exception rather than rule to find microorganisms that are devoid of lipase activity (Ratledge, 1994), hydrolysis has been reported to be the rate limiting step in some degradation studies (Hsu *et al.*, 1983; Cail *et al.*, 1986) implying that either the microorganisms used in such investigations were either unable to produce sufficient quantities of lipase or that the lipase produced was unable to hydrolyse the fat/oil. It has also been shown that microbial lipases can either attack the triglyceride randomly or may exhibit positional or substrate specificity. Lipases of the first category, which exhibit no substrate or

positional specificity appear to be the most common and have been isolated from microorganisms such as *Staphylococcus aureus*, *Staphylococcus hyicus*, *Corynebacterium acnes*. *Pseudomonas species* kwi56 and *Chromobacterium viscosum*. Lipases of the second category, which attack the R_1 & R_3 ester groups without hydrolysing the R_2 position to any appreciable extent have been isolated from *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas alcaligines*, amongst other microorganisms (Kramer, 1971; Jaegar *et al.*, 1994). The final group of lipases, which exhibit a pronounced fatty acid preference appear to have been found only in yeasts and fungi. Lipase from *Geotrichum candidum* displays a high degree of specificity for fatty acid acyl substituents with a double bond at the 9,10-position and then only provided that there is no double bond between the Δ9 bond and carboxylic ester group (Alford *et al.*, 1964). This lipase also hydrolyses fatty acyl groups with *cis*-double bonds more rapidly than those fatty acids possessing *trans*-double bonds (Kramer, 1971; Gurr & Harwood, 1991). Other lipases that display a preference for fatty acids of particular chain length include the lipases secreted from microorganisms such as *B. subtilis 168*, *Aeromonas hydrophila*, *Propionibacterium acnes*, *P. aeruginosa*, *P. aeruginosa EF2*, *P. alcaligines*, *P. species* ATCC 21808 and *Pseudomonas fluorescens* SIKWI which preferentially hydrolyse fatty acids with chain lengths of, 8, 6-8, 4-6, 18, 12-18, 8-10, 6-8 carbon atoms respectively (Thomson *et al.*, 1999).

Fatty acids that are released during hydrolysis may be transported into microbial cells by mechanisms that may involve simple diffusion process or specific transport proteins. They can either be modified by elongation and/or desaturation to ensure that the functional lipids of the microbial cell are maintained or may be metabolized to yield energy or carbon intermediates for cell growth (Ratledge, 1994).

β-oxidation is the major route by which fatty acid are oxidized (Ratledge, 1994). Several environmental isolates, including those used in commercial inocula, have been shown to reduce the total quantity of fat/oil present (Koritala *et al.*, 1987; Mendoza-Espinosa & Stephenson, 1996; Funtikova *et al.*, 1999; Tano-Debrah *et al.*, 1999; Markossian *et al.*, 2000; Mihara *et al.*, 2000) implying that β-oxidation must have taken place. According to Chappe *et al.* (1994) the microbial activity of grease bioaugmentation products is often limited to hydrolysis, which suggests that β-oxidation may be a rate limiting step in the degradation of fats and oils. Other workers have also shown that β-oxidation, rather than hydrolysis, is the

rate limiting step in the microbial oil degradation process (Becker & Markl, 2000) and suggested that the free fatty acids released from hydrolysis accumulate and thus inhibit the growth of lipid-degrading microorganisms.

Work with anaerobic batch scale reactors has further demonstrated that saturated or unsaturated long n-2 chain fatty acids (LCFA) intermediates are produced from the β-oxidation of unsaturated LCFA but no intermediates are produced from the β-oxidation of saturated LCFA (Novak & Carlson, 1970; Angelidaki & Ahring 1995; Lalman & Bagley, 2000, 2001). In such studies, Palmitic and mystiric acid have been reported to be the primary products of oleic acid degradation whilst oleic, palmitoleic, palmitic, mystiric, lauric, hexanoic acid and acetic acid have been detected during linoleic degradation. Sediment studies have also shown that a large proportion of the lipids are mineralised during diagenesis and that oleic acid can yield shorter chained saturated intermediates (Rhead *et al.*, 1971; Gaskell *et al.*, 1976) whilst palmitic acid cannot (Sun *et al.*, 1997).

The detection of saturated intermediates in the above mentioned degradation studies implied that hydrogenation of a double bond was also taking place. Earlier workers proposed that the β-oxidation of unsaturated LCFA, such as linoleic acid can proceed only after the double bonds were saturated (Novak & Carlson, 1970). However, stearic acid has never been observed as a product of either oleic acid or linoleic acid anaerobic degradation. Lalman and Bagley (2001) detected palmitoleic acid and oleic acid during linoleic acid degradation and thus concluded that LCFA need not be completely saturated prior to β-oxidation.

Microorganisms have also been known to oxidize the terminal methyl group of a fatty acid molecule via ω-oxidation to produce ω-hydroxy fatty acids, ω-aldehydic fatty acids or a ω, α-dicarboxylic fatty acid (Finnerty, 1989; Ratledge 1994). One example of fatty acid ω-oxidation is the oxidation oleic acid and elaidic acid to the corresponding unsaturated dicarboxylic acids 9-*cis*1,18-octadecenedioic acid and 9-*trans*1,18-octadecendioic acid, respectively by the yeast *Candida tropicalis* (Yi & Rehm, 1988a,b).

In addition to terminal methyl group oxidation, microorganisms can also oxidize carbon atoms within the acyl chain to form a number of hydroxy, oxo, epoxy, hydroperoxy and polyoxygenated derivatives. The strongest evidence of there being a sub terminal hydroxylase

system comes from work with *Bacillus megaterium*. Fulco and co-workers demonstrated that enzyme preparations from *B.megaterium* hydroxylate free fatty acids to isomeric mixtures of ω-1, ω-2 and ω-3 monohydroxy fatty acids (Miura & Fulco, 1974, 1975). The same enzyme system was also shown to catalyse double bond epoxidation of palmitoleic acid and a variety of other monounsaturated acids (Narhi & Fulco, 1982, 1986). Many of the mid-chained oxidations reviewed were complex involving a combination of bond migration, hydration, dehydrogenation, and hydroxylation at more than one position. Oleic acid, for example, may experience a number of different microbial modifications to yield 12-hydroxyoleic acid (Soda, 1987), 10-hydroxyoleic acid, 10-hydroxyoctadecanoic acid, and 10-oxo-octadecanoic acid (El-Sharkawy *et al.*, 1992).

α-oxidation is another route by which fatty acids may be oxidized. In the few bacterial species that have been studied, however, it is apparent that the decarboxylation process, if present at all, represents an oxidation pathway of minor importance. The occurrence of small quantities of 2-hydroxy 12:0 and 2-hydroxy 14:0 in a number of Gram negative bacteria suggests, but does not prove, the existence of this oxidation route in bacteria (Wilkinson, 1988; Finnerty, 1989; Ratledge, 1994).

The work of Hita *et al.* (1996) indicated that several combination of metabolic pathways, may be involved in the degradation of a fat/oil. Hitu and co-workers reported that tristerin in soil that was not mineralized to carbon dioxide, was converted into simple esters, di-, keto and hydroxy acids. It was proposed that these metabolic bi-products may have been derived from one or several of the following reactions; bioesterification, alcoholysis, β-oxidation, ω-oxidation and autoxidation.

The main objective of the work outlined in the following chapter was to determine whether commercial microbial supplements were capable of modifying the physical and chemical characteristics of fat and oil under laboratory conditions. The physico-chemical changes to the fat/oil were quantified by a variety of techniques including: COD measurements, gravimetric analysis, lipase activity measurements and GC-MS analysis.

3.2. Results

3.2.1. Lipolytic activities of commercial inocula

The Sigma lipase diagnostic kit was used to determine the lipase activities (Table 3.1) of various activated microbial supplements (Appendix VII).

Table 3.1. Lipase activities (Sigma Tietz units.ml^{-1}) of activated microbial formulations (All formulation activated in water unless otherwise stated) * activated in Organica nutrient media. Also shown are the average volumes of NaOH (ml) required to titrate the free fatty acid present in the test and blank samples and the results of two sample t-tests comparing the volume of NaOH used in the test and blank samples.

Sample	Vol of NaOH used (ml)		Lipase activity (Sigma-Tietz units.ml^{-1}	Test Statistic	
	Test	Blank		T	P
P80	6.36 ± 0.02	6.30 ± 0.05	0.06	-2.16	0.10
GTL	2.91 ± 0.08	2.87 ± 0.01	0.05	-0.80	0.48
G40	7.60 ± 0.08	7.48 ± 0.06	0.12	-2.04	0.11
G40 (added oil)	7.27 ± 0.06	7.23 ± 0.04	0.10	-0.87	0.43
F33 (aerated)*	2.97 ± 0.04	2.93 ± 0.01	0.03	-1.03	0.36
F33*	2.97 ± 0.01	2.94 ± 0.04	0.04	-1.11	0.33

The lipase activities of the activated microbial supplements were low and variable (0.03-0.12 Sigma-Tietz units.ml^{-1}). Aeration and the addition of oil during the activation procedure also had little effect, lowering lipase activity by 0.01 and 0.02 Sigma-Tietz units.ml^{-1}, respectively.

To determine whether the volume of NaOH used in the test sample was significantly greater than that of the blank, a series of two samples t-tests were performed (Table 3.1). (Residuals were normally distributed and there was no significant heterogeneity of variance (F=0.008-12.00, P=0.07-0.698)) The results of the two samples t-test suggest that volume of NaOH used in the control sample was not significantly different to that used in the test sample i.e. lipase was not detected in any of the samples.

The lipase production/activity of another supplement, S45, was also measured during activation in a nutrient media containing emulsified oil. Optical density measurements were also carried out in an effort to determine whether lipase activity was related to bacterial density (Figure 3.1).

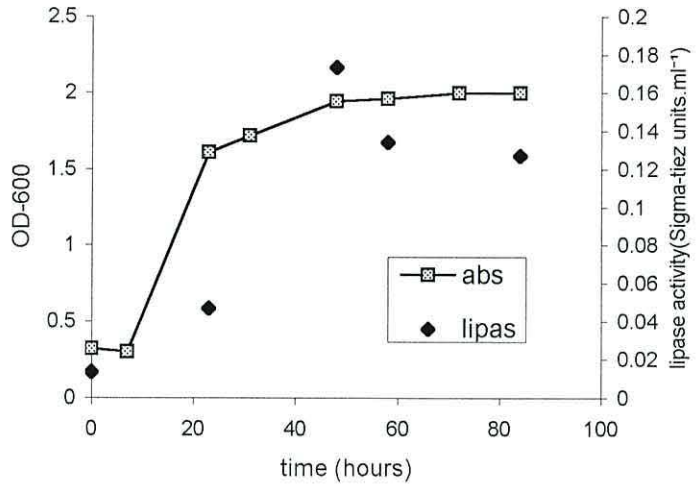


Figure 3.1. Relationship between lipase activity and OD-600 (a measure of bacterial density) with time for lab M nutrient broth containing mechanically emulsified soya oil (0.01%) inoculated with S45(0.01%v/v) incubated at 30°C with shaking at 150rpm.

The optical density of the sample increased markedly over the first 7-23 hours of the incubation period (by a factor of 6.7) and continued to increased over the following 25 hours of the experiment but at a progressively diminishing rate (increased by a factor of 1.2 between 23 and 84 hours). The lipase activity of the sample also increased appreciably during the first 48 hours of the experiment (by a factor of ca. 13.) and declined to a value that was greater than that measured at time 0 over the remainder of the incubation period.

3.2.2.Gravimetric analysis of fat/oil degradation

Degradation of lard present at a concentration of 0.5% in 10ml samples

The effect of G40 and oil activated G40 on the degradation of chemically emulsified lard suspended in distilled water is shown in Figure 3.2. After 1 days incubation, the quantity of lard extracted from the inoculated samples was more than twice that extracted from the control and the addition of oil during activation appeared to have little effect. In contrast, after a 6 day incubation period, inoculation with G40 appeared to have reduced the quantity of lard (by ca. 30%) whereas the oil activated supplement resulted in an increase in the quantity of lard relative to the control. Interestingly, both the control samples and samples inoculated with oil activated G40 experienced an apparent increase in oil levels from day 1 to 6.

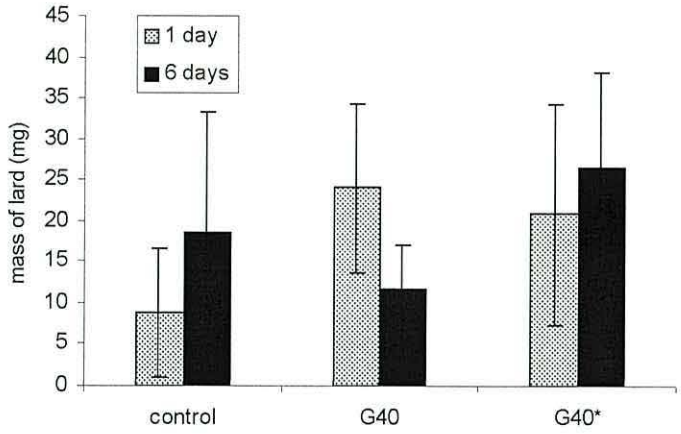


Figure 3.2. The effect of G40 and oil activated G40* on the degradation of chemically emulsified lard (ca. 50mg) suspended in 10ml of distilled water (non sterilized). Data shows the mass (mean ± SD) of lard remaining in samples after 1 and 6 days incubation at room temperature, without shaking.

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=1.815, P=0.874) and a two-factor ANOVA analysis established that there was no interaction between factors (F=1.53, 0.260) and that the extent of lard degradation was not significantly influenced by G40, oil activated G40 (F=1.10, P=0.366) or incubation time (F=0.05, P=0.836).

In the previous experiment, samples were prepared from a 'stock' mixture of lard, distilled water and washing-up liquid. Given that it was difficult to maintain a homogenous lard emulsion and remove representative sub-samples, a 'stock' solution of emulsified lard was no longer used. Instead, 50 μl of melted lard was dispensed into a sample containing 10 ml of distilled water. The effect of G40 on the degradation of lard was then monitored as a function of time (Figure 3.3).

Lard levels in the control sample fluctuated marginally with time (25.4-29.8mg) whilst those in the inoculated sample fluctuated over the first 4 days of the experiment (26.8-30.0mg) and declined by approximately 50% thereafter.

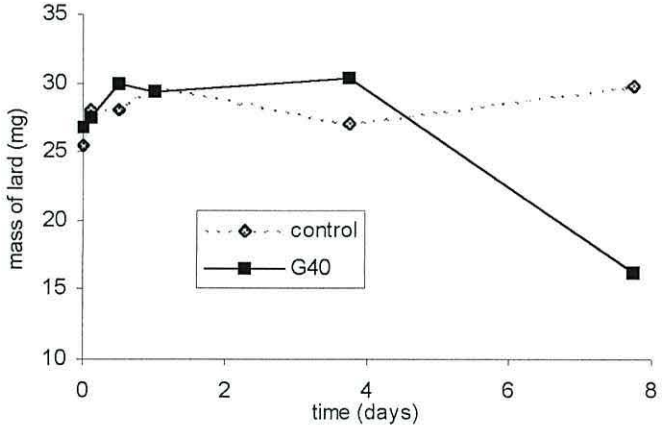


Figure 3.3. Effect of G40 on the degradation of lard (50μl) suspended in 10ml of distilled water (non sterilized). Data shows the mass of lard extracted from samples incubated at room temperature without shaking.

Degradation of vegetable oil present at a concentration of 0.5-1% in 10ml samples

The effect of G40 on the degradation of sunflower oil was investigated since lard tended to solidify in the tip of the pipette. Conditions were identical to those used in the previous experiments except that the 50μl of lard were replaced with 50μl of sunflower oil (Figure 3.4).

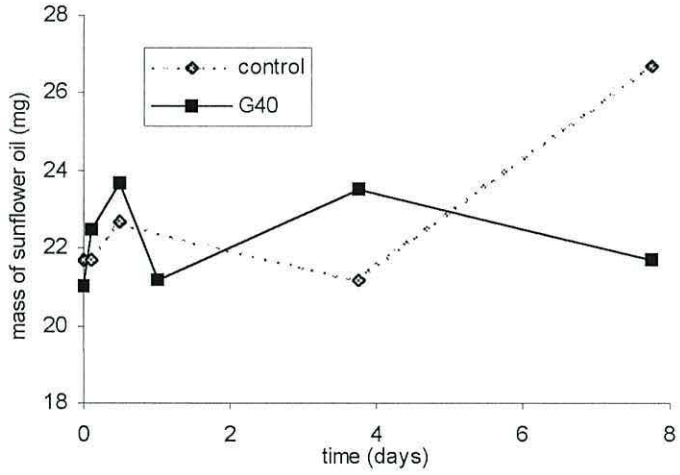


Figure 3.4. Effect of G40 on the degradation of sunflower oil (50μl) suspended in distilled water (10ml) (non sterilized). Data shows mass of lard extracted from samples incubated at room temperature without shaking.

As with the lard samples, the mass of sunflower oil fluctuated considerably with time (21.0-26.7mg). G40 did not appear to effect the mass of sunflower oil extracted from the samples and there was no trend in the mass of extracted sunflower oil and incubation time.

The volume of sunflower oil used was increased from 50 to 100 μl in an attempt to reduce the errors associated with extracting and pipetting small volumes of oil. Degradation was assessed after 5 days incubation and the effect of adding oil during activation was also investigated (Table 3.2).

Table 3.2. Effect of G40 and oil activated G40* on the degradation of sunflower oil (100 μl) suspended in 10ml of distilled water (non sterilized). Data shows the mass (mg) of sunflower oil (mean ± SD) remaining in samples incubated for 5 days at room temperature without shaking.

Treatment	Mass of sunflower oil
G40	90.61 ± 1.35
G40*	104.21 ± 12.62
Control	97.38 ± 3.32

Samples incubated with G40 experienced only fractionally more degradation than the controls (7%) whereas the oil-activated supplement appeared to have the reverse effect. In comparison to the earlier degradation experiment with chemically emulsified lard, the variability between replicate samples was reduced considerably (coefficients of variation; 43.5-89.4% and 1.5-12.1%, respectively).

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=6.681, P=0.035) and a one-way ANOVA confirmed that G40 or oil activated G40, failed to enhance the degradation of the oil (F=2.42, P=0.170).

Further attempts to minimize experimental errors in subsequent investigations addressed the problem of distributing oil by pipette. Errors that were introduced by oil adhering to both the interior and exterior of the pipette were eliminated by weighing vials before and after pipetting to calculate the exact amount oil transferred by the pipette. The proportion of oil degraded or lost during the extraction procedure was then calculated using equation 3.1.

$$\frac{\text{(initial mass of oil (g)- mass of extracted oil(g))}}{\text{initial mass of oil (g)}} \times 100 \qquad 3.1$$

The effect of G40, oil activated G40 and a chemical surfactant (Fairy Liquid) on the degradation of oil was assessed (Figure 3.5).

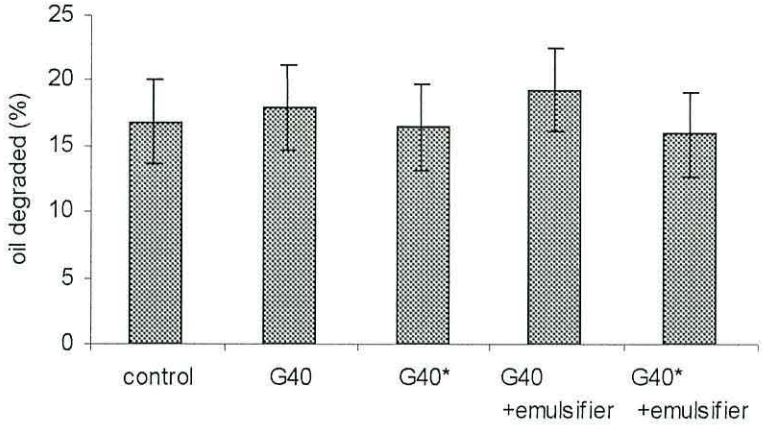


Figure 3.5. Effect of G40, oil activated G40* and a chemical surfactant (0.5% v/v) on the mean degradation (± SD) of sunflower oil (100μl) suspended in 10ml of distilled water (non sterilized), incubated at room temperature without shaking for 6 days.

The addition of surfactants, G40 or oil activated G40 appeared to have very little impact on the degradation of oil (Figure 3.5). The average extent of degradation in samples treated with surfactant and/or a commercial microbial was within 1% of the controls and there was no obvious relationship between the extent of degradation and the presence of surfactant and/or microbial products.

The residuals were normally distributed with no significant heterogeneity of variance (Bartlett's χ^2=1.528, P=0.882) and a one-way ANOVA established that neither G40 nor oil activated G40, in the presence or absence of a surfactant, significant influenced the extent of oil degradation (F=0.31, P=0.867).

A similar experiment was conducted using two new supplements (P80 and GTL) and a chemical surfactant/emulsifier known as EC-200 (Figure 3.6). As in the previous experiment, the presence of P80, GTL and the chemical surfactant had little impact on the degradation of sunflower oil with the average extent of oil degradation varying between 8.1 and 9.9%.

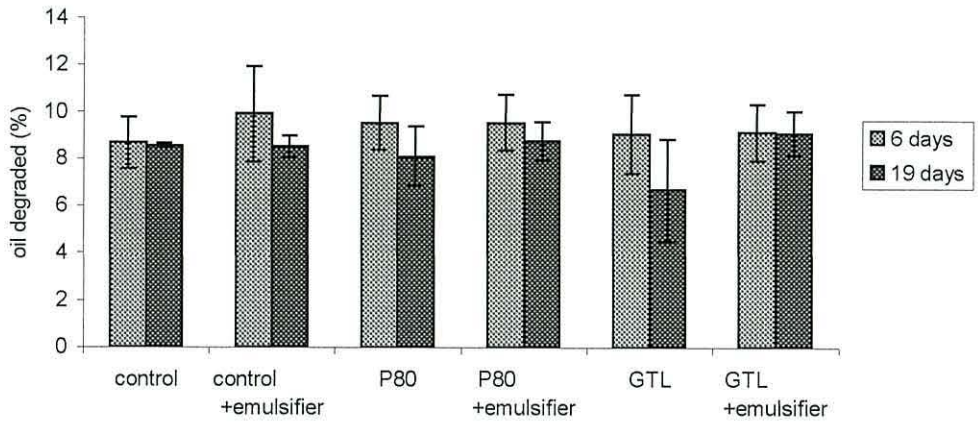


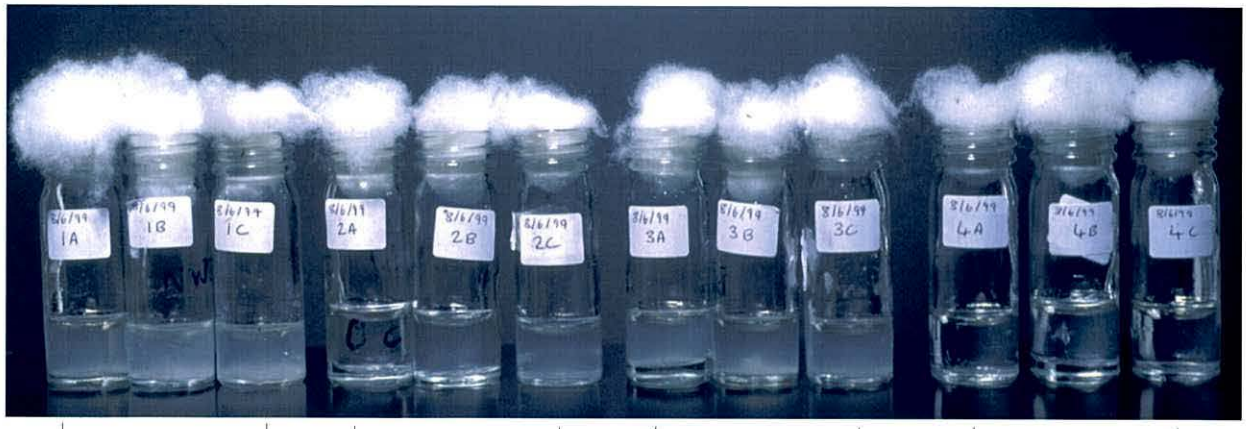
Figure 3.6. Effect of P80, GTL and EC200 (0.5% v/v) on the mean degradation (± SD) of sunflower oil (100μl) suspended in 10ml of distilled water (non sterilized) incubated at room temperature without shaking for 6-19 days .

The residuals were normally distributed with no significant heterogeneity of variance (Bartlett's χ^2=5.891, P=0.881). A two-factor ANOVA analysis confirmed that there was no interaction between factors (F=0.66, P=0.657) and that the addition of the surfactant and/or P80/GTL to the samples did not significantly influence the extent of oil degradation (F=0.88, P=0.512). Those samples that were incubated for 6 days, however, experienced, on average, a significantly greater extent of oil degradation than those samples incubated for 19 days (F=5.24, P=0.031).

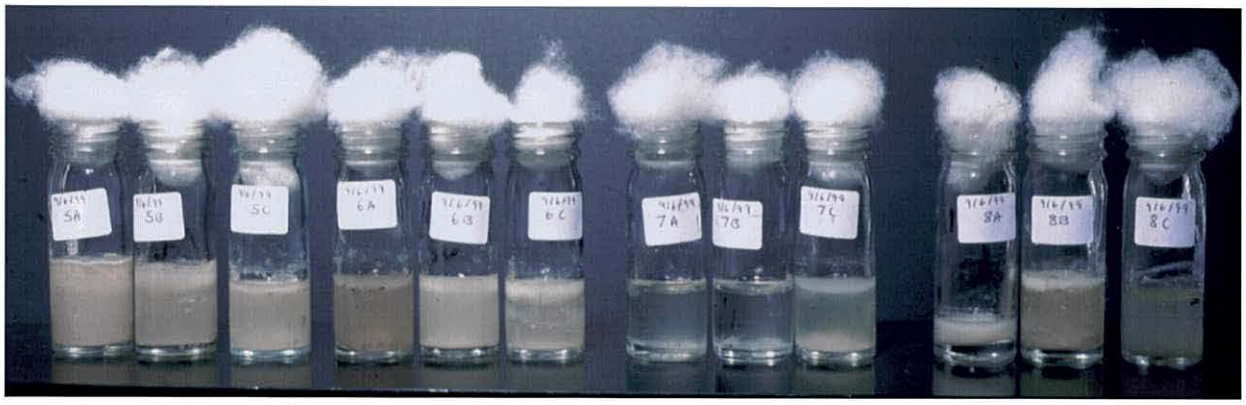
Degradation of vegetable oil present at a concentration of 10% in 10ml samples

In an effort to enhance oil degradation rates samples were incubated at 30°C with shaking at 140rpm. Nutrients, lipase and a surfactant (EC200), were also added to the samples in various combinations to establish whether the oil degradation process was limited by nutrient availability, hydrolysis or the bioavailability of the oil. The concentration of sunflower oil used was further increased (to 10%v/v) in an attempt to minimize the errors associated with extracting and weighing small quantities of oil (Table 3.3).

Prior to extracting the sample the appearance of the oil was noted. In the presence of nutrients the appearance of the oil had changed quite dramatically (Figure 3.7). The oil, which was originally a clear, pale yellow viscous liquid, had developed into large sticky, white-yellow, opaque lumps resembling chewing gum.



P80 + Lipase | P80 | GTL + Lipase | GTL



Lipase + Nutrients | Nutrients only | P80 + Lipase + Nutrients | P80 + Nutrients

GTL + Lipase + Nutrients | GTL + Nutrients | GTL + Lipase + Nutrients + Surfactant | P80 + Lipase + Nutrients + Surfactant

Figure 3.7. Appearance of sunflower oil after incubation with various combinations of P80, GTL, lipase surfactant and nutrients at 30°C, shaken at 140rpm for 21 days.

Table 3.3. Effect of P80, GTL, lipase (0.5mg.ml^{-1}) and surfactant (0.5% v/v) on the mean degradation (± SD) of sunflower oil (1ml) suspended in 10ml of distilled water or nutrient media 1 (non sterilized) incubated at 30°C, shaken at 140rpm for 21 days. * not tested

	Oil degraded (%)	
Treatment	Water	Nutrients
P80 + lipase	10.27 ± 1.12	22.50 ± 12.63
P80	8.79 ± 0.70	61.40 ± 33.40
GTL + lipase	11.33 ± 2.25	20.82 ± 15.44
GTL	9.08 ±1.48	23.06 ± 10.97
Lipase	*	45.60 ± 24.90
Control	*	56.00 ± 26.70
GTL + lipase + surfactant	*	11.67 ± 8.69
P80 + lipase + surfactant	*	29.60 ± 19.50

The effect of microbial supplementation on the degradation of oil was unclear (Table 3.3). In the presence of nutrients only, P80 marginally enhanced the degradation of oil relative to the control (by a factor of 1.1) whereas GTL had the reverse effect (reduced extent of degradation by ca. 50%). The effect of lipase additions was similarly variable. In the absence of nutrients, lipase additions marginally enhanced the extent of oil degradation whilst in the presence of nutrients, the reverse trend was observed (extent of degradation reduced by >50% in those samples inoculated with P80). The addition of surfactants to inoculated samples containing both lipase and nutrients was also inconsistent. Supplementation with the surfactant increased the extent of oil degradation in samples inoculated with P80 but not with GTL. The addition of nutrients to samples inoculated with either P80 or GTL increased the extent of oil degradation by a factor of ca. 2.5 to 7. However, when exogenous lipase was present the effect of the nutrients on oil degradation was less dramatic (increased extent of degradation by a factor of ca. 1.8-2.2).

After transformation ($\log_{10}$), the residuals were normally distributed with no significant heterogeneity of variance (Bartlett's χ^2=14.872, P=0.188). A one-way ANOVA indicated that there were significant differences between some of the treatments (F=5.26, P<0.001) and a series of Bonferoni's selected pairwise comparisons (Table 3.4) determined that the addition of nutrients significantly enhanced the degradation of oil in samples inoculated with P80 but that oil degradation was not influenced significantly by the addition of surfactants, lipase, GTL and P80.

Table 3.4. Bonferoni's selected pairwise comparisons of the mean difference (log_{10}%) of sunflower oil degraded in samples treated with a combination of nutrients (nutrient media 1), emulsifiers, lipase and microbial supplements (GTL & P80) incubated at 30°C, shaken at 140rpm for 21 days. 95% confidence interval of any difference = 0.5985 *significant differences

Pairwise comparison	Difference in means
Effect of surfactant	
(GTL+lipase+nutrients) & (GTL+lipase+nutrients+surfactant)	0.2567
(P80+lipase+nutrients) & (P80+lipase+nutrients+surfactants)	-0.1090
Effect of lipase	
(GTL) & (GTL+lipase)	0.0943
(P80) & (P80 +Lipase)	0.0668
(GTL+nutrients) & (GTL+nutrients+lipase)	-0.1027
(P80+nutrients) & (P80+nutrients+lipase)	-0.4339
(nutients only) & (lipase + nutrients)	0.0941
Effect of nutrients	
(P80) & (P80+nutrients)	-0.7950*
(GTL) & (GTL+nutrients)	-0.3786
(P80+lipase) & (P80+lipase+nutrients)	-0.2943
(GTL+lipase) & (GTL+lipase+nutrients)	-0.1816
Effect of microbial supplement	
(P80+nutrients) & (nutrients only)	0.0238
(GTL+nutrients) & (nutrients only)	-0.3813
(P8O+lipase+nutrients) & (lipase+nutrients)	-0.3160
(GTL+lipase+nutrients) & (lipase+nutrients)	-0.3899

To determine whether the change in appearance of the oil due to the activity of contaminating bacteria rather than physico-chemical process the experiment was repeated using media and glassware that were sterilized prior to inoculation. The extraction procedure was also modified to ensure that the rubber-like material was completely extracted by the solvent (Table 3.5).

In comparison to the previous experiment, the change in the appearance of sunflower oil was not quite as dramatic. The extent of oil degradation was also considerably lower with mean oil losses ranging from 5 and 13% (average oil loss in previous experiment; 10-60%). Furthermore, there was no obvious relationship between the extent of oil degradation and presence of lipase, surfactants and/or microbial supplement.

Table 3.5. Effect of P80, GTL, lipase (0.5mgml^{-1}) and surfactant (0.5% v/v) on the mean degradation (± SD) of sunflower oil (1ml) suspended in 10ml of distilled water or nutrient media 1 (sterilized) incubated at 30°C, shaken at 140rpm for 14 days. * not tested

	Oil degraded (%)	
Treatment	**Water**	**Nutrients**
P80 + lipase	9.71 ± 2.41	10.73 ± 0.38
P80	4.91 ± 5.26	9.87 ± 3.91
GTL + lipase	12.08 ± 6.72	10.01 ± 5.70
GTL	10.34 ± 3.23	10.53 ± 1.70
Lipase	*	7.43 ± 2.62
Control	*	12.53 ± 2.45
GTL + lipase + surfactant	*	6.04 ± 4.80
P80 + lipase + surfactant	*	7.52 ± 2.77

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=12.354, P=0.338) and a one-way ANOVA confirmed that the extent of oil degradation was not significantly altered by the addition of exogenous lipase, surfactant, nutrients and microbial supplements to samples (F=1.08, P=0.418).

Further attempts were made to determine if commercial microbial products were capable of reducing oil/fat concentrations since the previous experiments had demonstrated that they were not. A new microbial supplement was employed (liquid F33) and the nutrient solution used was recommended and supplied by the manufacturers of the microbial supplement. Some samples were also aerated and/or received one or several doses of the microbial product over the 3 week incubation period (Figure 3.8).

The extent of oil loss in samples that were inoculated with F33 was within 1% of the controls and there was no obvious relationship between the extent of oil degradation and aeration (Figure 3.8).

The residuals were normally distributed and there was no significant heterogeneity of variance (Bartlett's χ^2=1.665, P=0.893). A two-factor ANOVA established that there was no interaction between factors (F=0.65, 0.541) and that inoculation with F33 (F=1.10, P=0.366) or aeration (F=0.05, P=0.836) did not influence the extent of oil degradation.

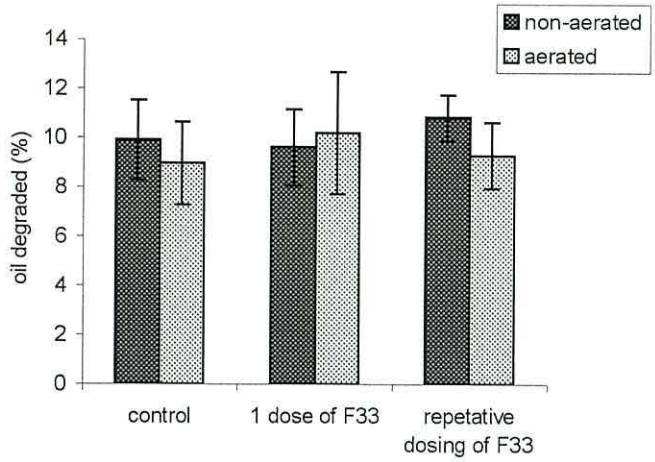


Figure 3.8. Effect of aeration and repetitive dosing with F33 on the mean degradation (±SD) of soya oil (1ml) suspended in 10ml of Organica nutrient media (media sterilized prior to inoculation) incubated at 30°C for 21 days.

Oil was also pre-treated with lipase to determine, whether hydrolysis was the rate limiting step in the degradation process. Furthermore two different oils were investigated (soya and sunflower) to establish if the performance of the microbial supplement was affected by the nature of the vegetable oil used (Figure 3.9).

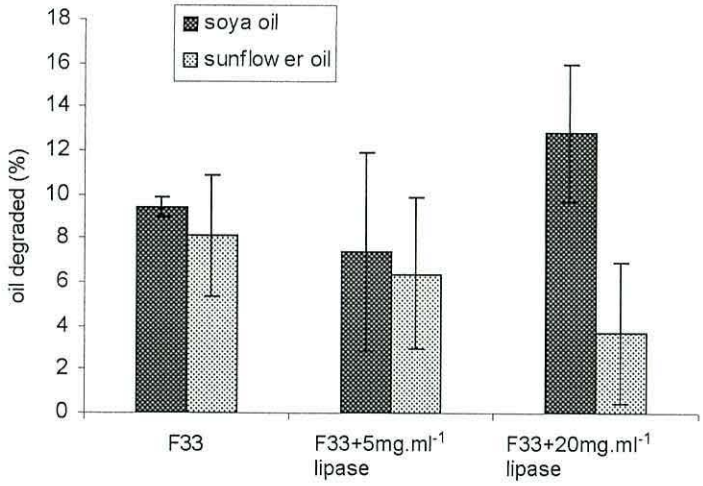


Figure 3.9. Effect of lipase on the mean (± SD) degradation of soya and sunflower oil (1ml) suspended in 10ml of Organica nutrient media inoculated with F33. (Media sterilized prior to inoculation, samples incubated at 30°C for 20 days)

Pre-treatment of the oil with 5 $mg.l^{-1}$ of lipase appeared to marginally reduce the degradation of both soya and sunflower oil relative to the controls (F33 only). At elevated lipase concentrations (20 $mg.l^{-1}$), however, the effect of the lipase on oil degradation was unclear. Soya oil samples experienced an apparent increase in degradation whilst sunflower oil

samples experienced the reverse effect. In all treatments, soya oil also experienced a marginally greater extent of degradation than sunflower oil.

The residuals were normally distributed and there was no significant heterogeneity of variance (Bartlett's χ^2=5.559, P=0.352). A two-factor ANOVA indicated that there was no interaction between factors (F=3.17, P= 0.079), that pre-treatment with lipase did not significantly influence the extent of oil degradation (F=0.56, P=0.584) but that soya oil, on average, experienced significantly greater degradation than sunflower oil (F=3.17, P=0.026).

Degradation of vegetable oil present at a concentration of 0.5-1% in 100/200ml samples

Oil and yeast/glucose concentrations were reduced from 10% to 0.5-1%, v/v and 3.0 $g.l^{-1}$ to 0.3 $g.l^{-1}$, respectively to ensure that the high oil and nutrient concentrations used were not inhibiting the microbial degradation of the oil. Rather than reducing the volume of oil being used in the experiment (which would compromise earlier efforts to minimise experimental errors) the volume of nutrient media used was increased from 10 to 100/200 ml.

The ability of F33 to degrade mechanically emulsified oil was investigated and measured as a function of time (Figure 3.10).

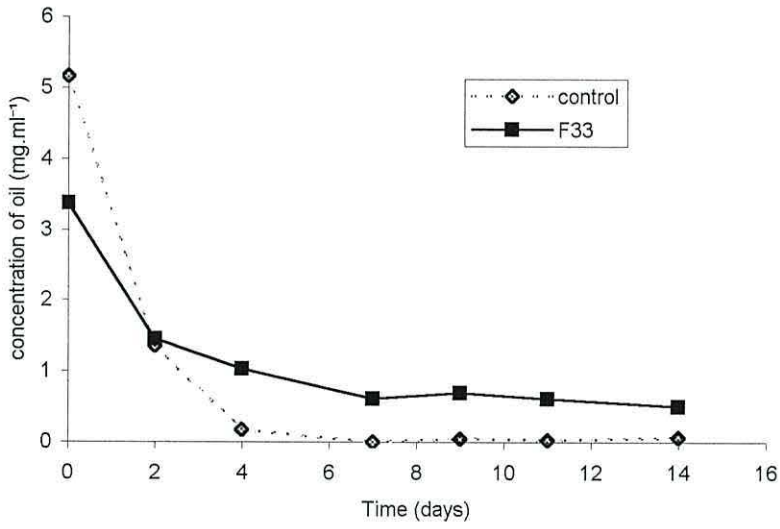


Figure 3.10. Effect of F33 on the degradation of mechanically emulsified soya oil (0.6% v/v oil) suspended in 100ml of nutrient media 2 (non sterilized). Data shows the change in oil concentration with time in samples incubated at 30°C, with shaking at 150rpm.

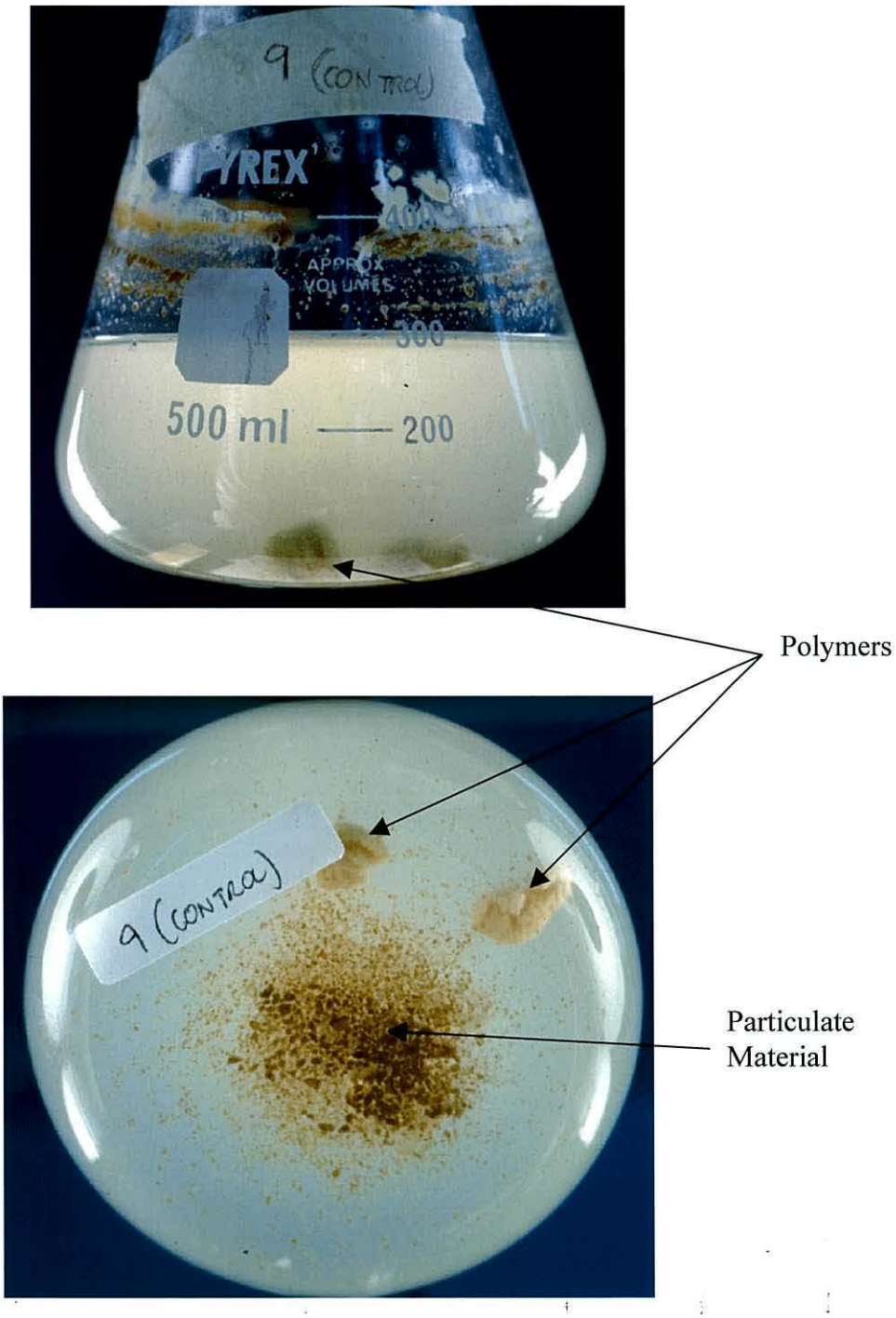


Figure 3.11. Appearance of polymers and particulate material that formed in sample of mechanically emulsified soya oil (0.6%v/v oil) suspended in 100ml of nutrient media 2 (non sterilized) incubated for 4 days at 30°C, with shaking at 150rpm.

The concentration of oil in both the control (contaminants only) and inoculated sample (contaminants+F33) declined dramatically over the first 4 days of the experiment (by 96.7 and 69.4%, respectively) and fluctuated at a low level over the remaining 11 days. On all sampling occasions, except day 0 and 2 of the experiment, the concentration of oil in the inoculated sample was greater than in the control sample (by a factor of 6.1-20.3) which implied that F33 was inhibiting the degradation of oil.

The appearance of both the inoculated and control sample also varied with time (Figure 3.11). Small, yellow lumps appeared in both the control and inoculated sample and a large white lump of rubber-like material was observed to form in the control between day 2 and 4 of the experiment. The small yellow lumps were known as particulate material (see section 3.2.4 for further details) whilst the large white lumps of rubber like material were thought to be polymerized oil. These polymers and particulate material may have prevented the removal of representative sub-samples and may thus account for the apparently greater extent of oil degradation in the control.

The effect of nutrient media 2 and S45 (which contained the same microbial population as F33) on the degradation of oil was also investigated (Table 3.6).

Table 3.6. Effect of S45 and nutrient media 2 on the mean degradation (± SD) of soya oil (1ml) suspended in 100ml of distilled water/nutrient media (non sterilized) incubated for 16 days at 25°C with agitation on a regular basis.

Treatment	Oil degraded (%)
S45 + nutrients	41.97 ± 6.36
Control + nutrients	53.70 ± 21.10
S45 + water	15.69 ± 1.39
Control + water	16.48 ± 4.12

In both the inoculated and control samples, the extent of oil degradation in the nutrient enriched samples was considerably greater than in non-nutrient enriched samples (by a factor of 2.7-3.3). Inoculation with S45 had little effect on the degradation of oil in distilled water but reduced oil degradation in the nutrient enriched samples markedly (by 21.8%).

The residuals of the transformed data (log_{10}) were normally distributed and displayed no significant heterogeneity of variance (Bartlett's χ^2=3.257, P=0.354). A two-factor ANOVA indicated that there was no significant interaction between factors (F=0.39, P=0.552), that the addition of S45 did not significantly effect oil degradation (F=0.73, P=0.418) but that nutrient additions significantly influenced the extent of oil degradation (F=58.73, P=<0.005).

According to the manufacturers, the concentration of preservative in S45 was considerably higher than in F33 and may have been inhibiting the growth of the bacteria. S45 was therefore diluted after activation in attempt to reduce the concentration of the inhibitor and improve oil degradation rates (Table 3.7).

Table 3.7. Effect of S45 concentration on the mean degradation (± SD) of soya oil (1ml) suspended in 100ml of nutrient media 2 (sterilized prior to inoculation) incubated for 19 days at 30°C

S45 concentration (%v/v)	oil degraded (%)
10	19.30 ± 1.55
1	19.39 ± 4.55
0.1	17.68 ± 4.37
0.01	22.13 ± 6.25
0	16.54 ± 2.00

The extent of degradation in samples inoculated with S45 was marginally greater than that of the controls (by a factor of 1.1-1.3). The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett't χ^2=3.78, P=0.436) and a one-way ANOVA confirmed that oil degradation was not significantly enhanced by S45 at any concentration (F=0.78, P=0.560).

On recommendation of the manufacturers, the nutrient solution used was changed to peptone (0.9 $g.l^{-1}$) and the dilution factor during activation was changed from 1:30 to 1:10000. Some samples were also inoculated with neat S45 (not activated) to achieve a final S45 concentration of ca. 0.008% (Table 3.8).

Table 3.8. Effect of S45 concentration on the mean degradation (±SD) of soya oil (1ml) suspended in 100ml of $0.9g.l^{-1}$ peptone media (sterilized prior to inoculation) incubated for 30°C for 19 days. Also shown are the bacterial counts taken on day 5 of the experiment.

S45 concentration (%v/v)	oil degraded (%)	bacterial counts ($cfu.ml^{-1}$)
Neat	14.27 ± 1.93	2.20×10^7
10	22.17 ± 7.75	2.48×10^6
1	16.62 ± 2.65	6.80×10^6
0.1	15.69 ± 2.07	4.00×10^2
0	16.98 ± 1.59	0.00×10^0

Non-activated S45 and low concentrations of activated S45 (<10%) marginally reduced the extent of oil degradation (by 2.1-16.0% relative to controls) whilst a concentration (10%) of activated S45 enhanced the extent of oil degradation (by a factor of 1.3, relative to the controls). Bacteria were present in all treatments although numbers were appreciably lower at an S45 concentration of 0.1%.

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=6.612, P=0.158) and a one-way ANOVA indicated that the extent of oil loss in the samples was not significantly affected by inoculation with S45 at any concentrations. (F=1.73, P=0.219).

Many of the previous experiments were conducted using media and glassware that were not sterilized prior to inoculation to simulate the use of the microbial formulations in the field. The following experiment was designed to assess the oil degrading abilities of F33 and the 'contaminating' bacteria by using both sterile and non-sterile media and glassware prior to inoculation with S45 (Table 3.9).

Both F33 and the contaminating bacteria induced appreciable oil degradation relative to the sterile control (by a factor 1.7-1.8). The ability of the contaminants to degrade oil was marginally greater than that of F33 (extent of oil degradation within ±2.19%) and combining the two microbial communities resulted in less oil degradation than samples inoculated with F33 or contaminants alone.

Table 3.9a. Effect of F33 and the contaminants on the mean degradation (±SD) of soya oil (1ml) suspended in 200ml of nutrient media 2 incubated for 21 days at 30°C with agitation on a regular basis.

Treatment	Oil degraded (%)
F33 alone	35.08 ± 9.35
Control (sterile)	20.76 ± 1.91
F33 + Contaminants	25.27 ± 0.61
Contaminants alone	37.27 ± 4.30

Table 3.9b. Sheffe's pairwise comparisons of the mean difference ($\log_{10}$%) (± 95% confidence intervals) of oil degraded. *significant difference

Pairwise comparison	Difference in means ± 95 CI
F33 alone & Control (sterile)	0.2192 ± 0.2072*
F33+contaminants & contaminants alone	-0.1667 ± 0.2317
F33 alone & F33+contaminants	0.1326 ± 0.2317
Control (sterile) & contaminants alone	-0.2533 ± 0.2072*
F33+contaminants & control (sterile)	0.0866 ± 0.2317
Contaminants alone & F33 alone	-0.0341± 0.2072

The residuals of the transformed data ($\log_{10}$) were normally distributed (Bartlett's χ^2=6.20, P=0.102), there was no significant heterogeneity of variance and a one-way ANOVA revealed that there were significant differences between treatments (F=8.21, P=0.011). Sheffe's pairwise comparisons (Table 3.9) revealed that F33 alone was capable of significantly enhancing oil degradation relative to the sterile control but was not capable of significantly enhancing oil degradation in the presence of the contaminants. The contaminating bacteria were also capable of significantly enhancing the degradation of oil. However, inoculation with F33 did not significantly effect their ability to degrade oil.

To further simulate conditions in the field, the ability of F33 and S45 to degrade oil in the presence of microorganisms isolated from a grease trap was investigated (Table 3.10).

Oil degradation took place in all samples inoculated with the grease trap community (2.1-2.7 orders of magnitude greater than in the controls). The ability of the grease trap isolates to degrade oil was reduced considerably by supplementing the samples with F33 but only marginally reduced by supplementing the samples with S45.

Table 3.10a. Effect of S45 and F33 on the mean degradation (±SD) of soya oil (1ml) suspended in nutrient media 2 (100ml) by microorganisms isolated from community. (Samples were sterilized prior to inoculation and were incubated for 18 days at 30°C with occasional agitation)

Treatment	oil degraded (%)
F33+Grease trap isolates	32.84 ± 10.98
S45+Greasetrap isolates	39.46 ± 8.83
Grease trap isolated only	42.14 ± 1.55
Control (Sterile)	15.40 ± 6.33

Table 3.10b. Bonferoni's pairwise comparisons of the mean difference (%) of soya oil degraded in samples inoculated with various combinations of S45, F33 and the grease trap isolates (GI). 95% confidence interval of any difference is 21.27%. *significant differences

Pairwise comparison	Difference in means
F33+GI & GI	-9.31
S45+GI & GI	-2.69
GI & Sterile	26.75*
F33+GI & Sterile	17.44
S45+GI & Sterile	24.33*

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=4.442, P=0.218) and a one-way ANOVA confirmed that there were significant differences between treatments (F=7.20, P=0.012). Bonferoni's pairwise comparisons (Table 3.10) confirmed that the oil degrading ability of the grease trap isolates was not significantly different to that of the grease trap isolates supplemented with S45 and F33. However, grease trap isolates alone or supplemented with S45 significantly enhanced the degradation of oil relative to control but grease trap isolates supplemented with F33 did not.

3.2.3 Assessment of oil degradation using COD determinations

Effect of F33 on the degradation of nutrient media supplemented with oil

The effect of F33 on the COD of nutrient media supplemented with mechanically emulsified soya oil is depicted in Figure 3.12.

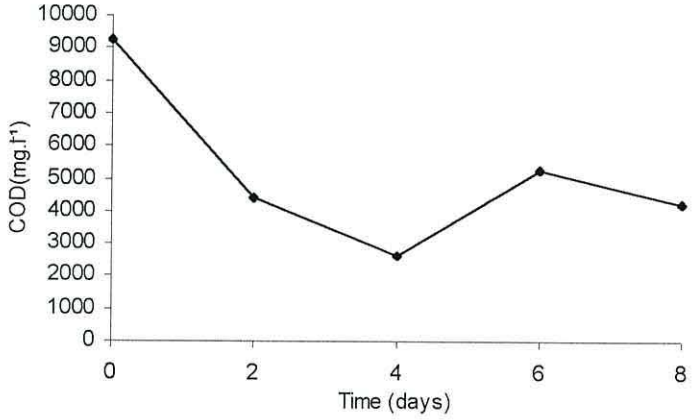


Figure 3.12. Change in COD with time for non-sterilized nutrient media 1 (100ml) containing mechanically emulsified soya oil (0.5ml) inoculated with F33 incubated at 30°C, shaken at 150 rpm.

The COD of the sample varied considerably with time. From day 0 to day 4 of the experiment the COD of the sample declined markedly (by approximately 70%), after which the COD fluctuated below that measured at the start.

The experiment was repeated and additional samples were also prepared to assess whether the method of oil emulsification affected the performance of F33 (Table 3.11) and to determine how the pH and 24-hour oxygen consumption rate varied between the different treatments and with time (Figures 3.13 & 3.14).

Table 3.11. Initial and final mean COD ($mg.l^{-1}$) (± SD) of non sterilized nutrient media 1 (100ml) supplemented with mechanically emulsified soya oil (0.5ml), chemically emulsified soya oil (0.5ml of oil + 0.4ml of emulsifier EC200) or non-emulsified soya oil (0.5ml) inoculated with F33. Samples were incubated for 4 days at 30°C with shaking at 150rpm.

Treatment	Initial COD	Final COD
Mechanically emulsified oil	8004 ± 405	8703 ± 812
Chemically emulsified oil	14498 ± 170	14458 ± 183
Non-emulsified oil	11863 ± 416	10901 ± 519

As indicated by the initial COD values of the samples, the method of emulsion preparation affected the COD appreciably. Between day 0 and 4 of the experiment, however, the COD changes in all treatments was inconstant and relatively small (within ± 9% of the initial COD values).

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=9.145, P=0.103) and a two-factor ANOVA indicated that there was no interaction between the factors (F=1.49, P=0.264) and that the COD of the samples did not significantly change over the course of the incubation period (F=0.07, P=0.801).

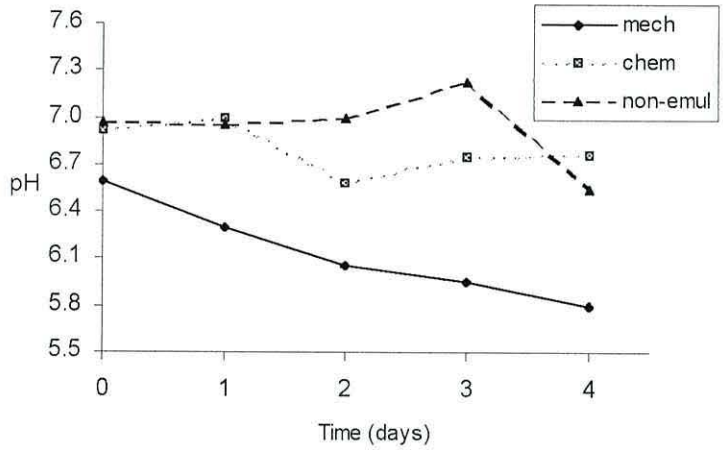


Figure 3.13. Change in pH with time for non sterilized nutrient media 1 (100ml) supplemented with 0.5 ml of mechanically emulsified soya oil (mech), 0.5ml of soya oil + 0.4ml of emulsifier EC200 (chem) or 0.5 ml of non-emulsified soya oil (non-emul) inoculated with F33. Samples incubated for 4 days at 30°C shaken at 150rpm.

Prior to incubation, the pH of the samples ranged between 6.6 (mechanical emulsion) and 7.0 (no emulsion). During the experiment, the pH of the chemically emulsified and non-emulsified samples fluctuated, whilst the pH of the mechanically emulsified sample declined progressively with time. For all samples, the pH at day 4 of the incubation was below that at time zero (Figure 3.13) .

The change in oxygen consumption with time in the chemically emulsified, non emulsified and mechanically emulsified oil samples is shown in Figure 3.14. At time 0, oxygen consumption in the mechanically emulsified sample was considerably lower than in the chemically and non-emulsified samples. During the experiment, oxygen consumption either progressively declined (by ca. 37% and 18% in the chemically emulsified and non-emulsified samples, respectively) or fluctuated widely (mechanically emulsified sample). The decline in oxygen consumption was greater in the chemically emulsified sample than in the non-emulsified sample although the decline in oxygen consumption occurred over the last 2 days of the experiment rather than over the entire length of the incubation period. Despite the wide

difference in oxygen consumption at the start of the experiment, oxygen consumption in all samples was similar and within 16336-21731 μmoles.l^{-1} at the end of the experiment.

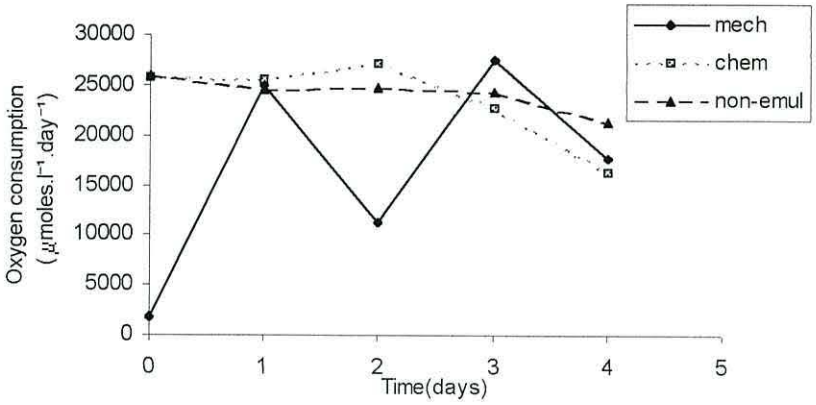


Figure 3.14. Change in oxygen consumption with time for non sterilized nutrient media 1 (100ml) supplemented with 0.5ml of mechanically emulsified soya oil (mech), 0.5ml of soya oil + 0.4ml of emulsifier EC200 (chem) or 0.5ml of non-emulsified soya oil (non-emul) inoculated with F33. Samples incubated for 4 days at 30°C shaken at 150rpm.

Bacterial counts on day 4 of the incubation (mechanically emulsified oil = 9.2 x 10^5 cfu.ml^{-1}, chemically emulsified oil = 4.08 x 10^8 cfu.ml^{-1}, non-emulsified oil = 4.02 x 10^8 cfu.ml^{-1}) confirmed that bacteria were present in relatively high numbers.

The effect of F33 on both mechanically and chemically emulsified oil samples was investigated further. Oil concentrations in the samples were reduced from 0.5% to 0.1% and the incubation period was extended to 15 days. Additional samples were also prepared to assess the performance of F33 in the absence of oil (Figure 3.15). The starting COD of the samples varied considerably and were greatest in those samples prepared with the chemical emulsifier. Over the course of the experiment all four treatments experienced a reduction in COD although the decline was most dramatic in samples that were prepared without the chemical emulsifier (COD reduced by 70.6%, 75.9%, 28.0% and 24.5% in samples containing mechanically emulsified oil, no oil, chemically emulsified oil and chemical emulsifier only, respectively). In the absence of the chemical emulsifier, the reduction in COD was most marked during the first 5 days of the experiment and the COD continued to decline at a progressively reduced rate over the remainder of the experiment. In comparison, the COD levels of samples prepared with a chemically emulsifier fluctuated slightly and the decline in COD appeared to take place in stages (between day 0-3, 4-7, 11-15). Given that the COD of the mechanically emulsified oil sample declined by more than 3000 mg.l^{-1} and that the

organic nutrients contributed ca. 3000 mg.l^{-1} to the initial COD of the sample, it was assumed that degradation of the oil, in addition to that of the nutrient media had taken place.

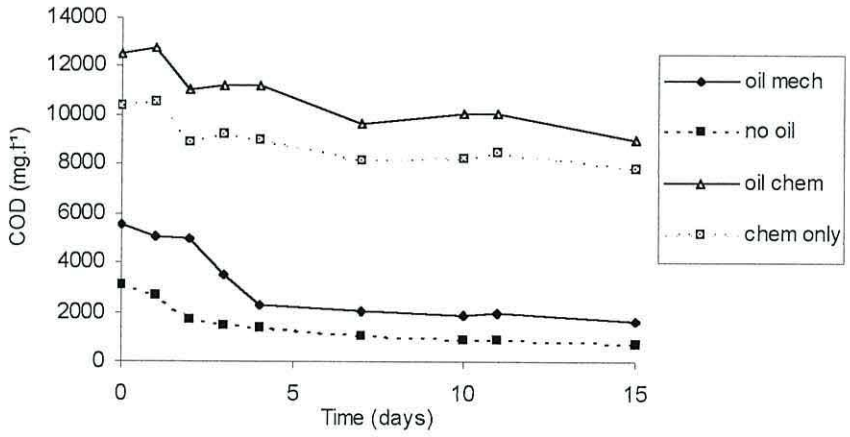


Figure 3.15. Change in COD with time in samples prepared with 100ml of nutrient media 1 inoculated with F33 (no oil) or supplemented with either 0.1ml of mechanically emulsified soya oil (oil mech), 0.1ml of soya and 0.4ml of chemical emulsifier (EC200) (oil chem) or 0.4ml of chemical emulsifier (chem only). Samples were not sterilized prior to inoculation and were incubated at 30°C with shaking at 150rpm.

The pH profiles of the four treatments were very similar (Figure 3.16). All four treatments experienced an initial drop in pH during the first 24 hours of the experiment followed by a rise in pH over the remaining 14 days. pH increased most steeply between days 1 and 4, after which the pH continued to rise but at a diminished rate. The reduction in pH during the first 24 hours of the experiment was most dramatic in the mechanically emulsified sample.

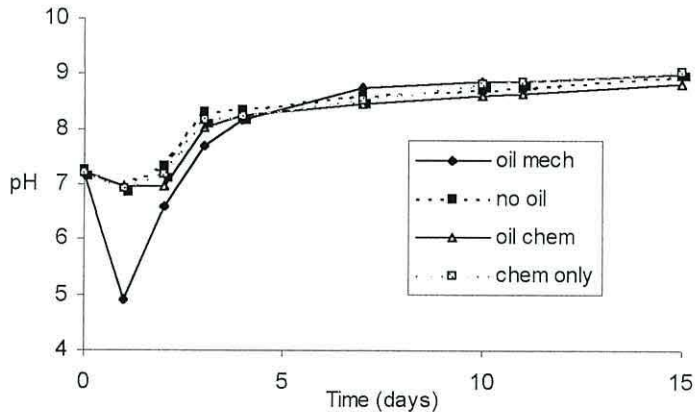


Figure 3.16. Change in pH with time in samples prepared with 100ml of nutrient media 1 inoculated with F33 (no oil), supplemented with either 0.1ml of mechanically emulsified soya oil (oil mech), 0.1ml of soya and 0.4ml of chemical emulsifier (EC200) (oil chem) or 0.4ml of chemical emulsifier (chem only). Samples were not sterilized prior to inoculation and were incubated at 30°C with shaking at 150rpm.

The oxygen consumption profiles of the chemically emulsified and oil free samples were very similar (Figure 3.17). All three samples displayed a lag in oxygen consumption at time 0, followed by a steep rise in oxygen consumption (20000 μmoles.l^{-1}) over the following 24 hours of the experiment. After the initial rise in oxygen consumption, oxygen consumption levels declined to 1000-2000 μmoles.l^{-1} for the duration of the experiment. The mechanically emulsified sample, in contrast, displayed high oxygen consumption at the start of the experiment and a less rapid decline over the following 6 days. There was a significant relationship between COD and oxygen consumption in the mechanically emulsified oil sample (r=0.979, P<0.01) but not in any of the other samples (r=0.487-0.595, P=0.213-0.327).

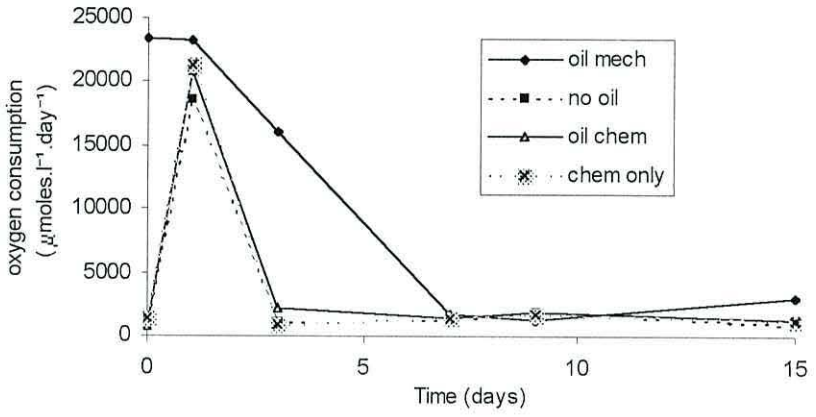


Figure 3.17. Change in oxygen consumption with time in samples prepared with 100ml of nutrient media 1 inoculated with F33 (no oil), supplemented with either 0.1ml of mechanically emulsified soya oil (oil mech), 0.1ml of soya and 0.4ml of chemical emulsifier (EC200) (oil chem) or 0.4ml of chemical emulsifier (chem only). Samples were not sterilized prior to inoculation and were incubated at 30°C with shaking at 150rpm.

Bacterial numbers were measured on two occasions and confirmed that bacteria were present in large numbers in all 4 treatments (Table 3.12).

Table 3.12. Number of bacteria (cfu.ml^{-1}) in samples prepared with 100ml of nutrient media 1 inoculated with F33 (no oil), supplemented with either 0.1ml of mechanically emulsified soya oil (mechanically emulsified), 0.1ml of soya and 0.4ml of chemical emulsifier (EC200) (Chemically emulsified) or 0.4ml of chemical emulsifier (chemical emulsifier only). Samples were not sterilized prior to inoculation and were incubated at 30°C with shaking at 150rpm for 1 or 7 days.

Treatment	Day1	Day7
Mechanically emulsified	1.44×10^8	1.66×10^9
No oil	2.32×10^8	7.40×10^8
Chemically emulsified	1.86×10^8	9.40×10^8
Chemical emulsifier only	1.32×10^8	1.02×10^9

There was also a significant relationship between oxygen consumption and bacterial numbers on day 7 (r=0.990, P=0.01) but not on day 1 (r=-0.870, P=0.130) of the experiment.

The effect of F33 on the COD, pH and oxygen consumption of the chemically emulsified oil sample was also investigated using a higher concentration of oil (1%) and an extended incubation period (40 days) (Figure 3.18).

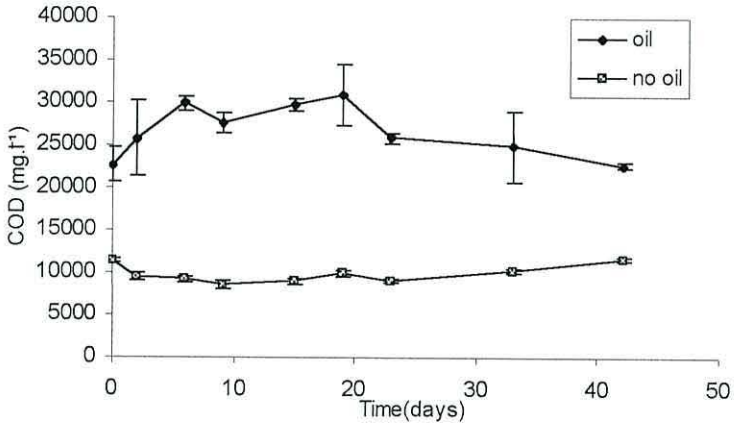


Figure 3.18. Change in COD with time in nutrient media 1 (100ml) inoculated with F33 and supplemented with 1ml of mechanically emulsified soya oil. Samples were not sterilized prior to inoculation and were incubated at 30°C with shaking at 150rpm.

Over the course of the experiment, COD levels in the oil enriched and oil free sample fluctuated between 22666-30876 $mg.l^{-1}$ and 8583-11734 $mg.l^{-1}$ respectively. In both treatments, the final COD was marginally greater than the initial COD (increase of 0.3% and 2.5%, respectively) indicating that little degradation of the oil or nutrient media had taken place.

The change in pH with time for the oil-enriched and oil free samples is depicted in Figure 3.19. During the first 6-9 days of the incubation the pH in both samples increased dramatically (from pH 7.2 to 8.6- 8.9). After the initial rise in pH, values progressively declined and/or fluctuated, but were consistently above those measured at day 0. pH in the oil-free sample were also consistently higher than those of the oil-enriched sample although the difference in pH was less marked at the end of the incubation.

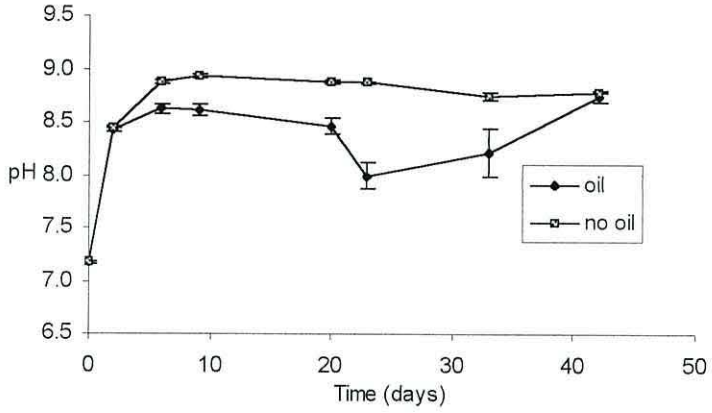


Figure 3.19. Change in pH with time in nutrient media 1 (100ml) inoculated with F33 and supplemented with 1ml of mechanically emulsified soya oil. Samples were not sterilized prior to inoculation and were incubated at 30°C with shaking at 150rpm.

Oxygen consumption measurements for the oil-enriched and oil-free sample are illustrated in Figure 3.20.

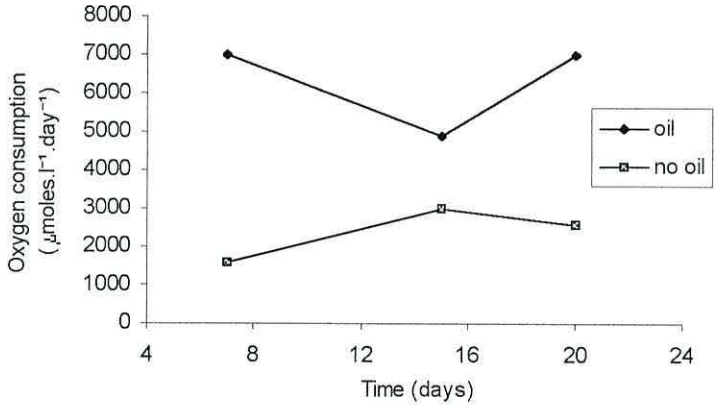


Figure 3.20. Change in oxygen consumption with time in nutrient media 1 (100ml) inoculated with F33 and supplemented with 1ml of mechanically emulsified soya oil. Samples were not sterilized prior to inoculation and were incubated at 30°C with shaking at 150rpm.

Oxygen consumption in both the oil-free and oil-enriched samples fluctuated over the course of the experiment, with values ranging from 1570 $\mu moles.l^{-1}$ (oil free sample, day 7) to 7000 $\mu moles.l^{-1}$ (oil enriched samples, day 7 & 20). On all sampling occasions, oxygen consumption in the oil-enriched samples was greater than in the oil-free samples.

Plate assays on day 7 indicated that bacteria were present in high numbers (2.1×10^8 $cfu.ml^{-1}$ and 5.4×10^9 $cfu.ml^{-1}$ in non-enriched and oil-enriched cultures respectively).

To establish whether F33 was solely responsible for the COD reductions previously observed in the mechanically and chemically emulsified samples, controls were prepared to take into account any COD changes which were the result of physico-chemical processes and/or microbial contamination (Table 3.13). The concentration of yeast/glucose in the nutrient solution was also reduced by a factor of 10, since the degradation of organic nutrients may have been masking and/or inhibiting the degradation of the oil

Table 3.13 Effect of F33 on the COD (mgl^{-1}) of nutrient media 2 (200ml) containing mechanically emulsified soya oil (0.3 or 0.6% v/v). Data shows the mean (± SD) starting COD, (COD_0), COD after 7 (COD_7) and 13 days incubation (COD_{13}) and the mean difference in COD (COD_{0-7} and COD_{7-13}). Samples incubated for 9-13 days at 30°C with shaking at 140rpm. * sterilized prior to inoculation

Treatment		COD_0	COD_7	COD_{13}	Difference in COD COD_{0-7}	COD_{7-13}
0.6% oil	**F33**	6792 ± 78	4953 ± 613	3851 ± 1098	1839 ± 676	1102 ± 529
	Control	5776 ± 293	5455 ± 2214	2284 ± 944	321 ± 1936	3171 ±1628
0.3% oil*	**F33**	4795 ± 205	4219 ± 720	-	576 ± 906	-
	Control	4327 ± 322	2272 ± 758	-	2055 ± 842	-

Prior to the COD measurement, it was noted that lumps of white/yellow material had collected at the bottom of the flask and were also adhering to the sides of the flasks. This material was dislodged and sometimes re-homogenized using a blender (in the case of the 0.3% emulsion) to facilitate the removal of representative sub-samples.

The COD of all treatments declined with time. At low oil concentrations (0.3%), the COD of the control sample declined more rapidly than the COD of the inoculated sample resulting in an overall COD reduction of 47.5% and 12.0 % respectively. By increasing the concentration of oil in the sample to 0.6% and performing the experiment under non-sterile conditions, this trend in degradation was reversed over the first 7 days incubation (COD of control and inoculated sample declined by 5.6% and 27.1% respectively). After 6 days incubation, however, the COD of the control samples decreased more rapidly than that of the inoculated samples, resulting in an overall reduction in COD of 60.5% and 43.3 %, respectively. These results implied that the contaminating bacteria may have been responsible for the reductions in COD observed and that F33 was reducing degradation rates.

The residuals were normally distributed and there was no significant heterogeneity of variance (Bartlett's χ^2=1.159-3.437, P=0.136-0.926). A two-sample t-test indicated that an oil concentration of 0.3%, F33 had no significant impact on the COD_{0-7} (F=-2.07, P=0.11). Likewise, a one-way ANOVA determined that at an oil concentration of 0.6%, F33 had no significant effect on the COD_{0-7} and COD_{7-13} (F=2.47, P=0.136).

Bacterial counts confirmed that at an oil concentration of 0.6%, contaminants were present at high numbers in the control samples (Table 3.14). At oil concentration of 0.3%, however, there were very few bacteria present in the control, suggesting that physico-chemical processes were also responsible for the reductions in COD.

Table 3.14. Number of bacteria (mean ± SD) in nutrient media 2 (200ml) containing mechanically emulsified soya oil (0.3 or 0.6%v/v) incubated for 9-13 days at 30°C with shaking at 140rpm. *sterilized prior to inoculation

Treatment	Bacterial numbers ($cfu.ml^{-1}$)
F33 (0.3%)*	$3.32 \times 10^9 \pm 4.25 \times 10^8$
Control (0.3%)*	50.00 ± 50.00
F33 (0.6%)	$4.40 \times 10^9 \pm 1.00 \times 10^8$
Control (0.6%)	$3.20 \times 10^9 \pm 1.43 \times 10^9$

Effect of the hydrolysis reaction on COD levels

To determine whether some of the COD fluctuations observed in previous experiments were due to the hydrolysis of triglycerides to free fatty acids, COD measurements were carried out after oil was incubated with lipase (Table 3.15).

Incubation with 20 $mg.l^{-1}$ of lipase resulted in COD levels that were marginally smaller than the controls (decrease of 3.1%), whilst incubation with 40 $mg.l^{-1}$ of lipase resulted in COD levels which were greater than the controls (increase of 15.3%).

The residuals were normally distributed and there was no significant heterogeneity of variance (Bartlett's χ^2=1.671, P=0.643). A two-factor ANOVA indicated that there was significant interaction between factors (F=7.82, P=0.023), but that pre-treatment with lipase did not significantly impact the COD of the oil (F<0.01, P=0.952). Bonferoni's selected

pairwise comparisons (Table 3.15) also confirmed that pre-treatment with lipase did not significantly impact the COD of the oil.

Table 3.15a. Effect of lipase pre-treatment on the COD ($mg.l^{-1}$, mean ± SD) of nutrient media 2 (100ml) containing 1ml of soya oil and 0.4ml of chemically emulsifier EC200 (* oil was not incubated with lipase for 24-48 hours, instead lipase was added immediately before COD measurement was carried out)

Treatment	20 $mg.l^{-1}$	40 $mg.l^{-1}$
lipase	30188 ± 1473	31198 ± 714
control*	28452 ± 1178	32792 ± 596

Table 3.15b. Bonferoni's selected pairwise comparisons of the mean difference in COD ($mg.l^{-1}$). 95% confidence interval of any difference is 2362 $mg.l^{-1}$.* significant difference

Pairwise Comparisons	Difference in means
20 $mg.l^{-1}$ lipase & 20 $mg.l^{-1}$ control	1736
40 $mg.l^{-1}$Lipase & 40 $mg.l^{-1}$ control	-1660

The effect of organic nutrients on the degradation of oil

In previous experiments, nutrient additions were shown to have a significant effect on oil degradation rates. An experiment was therefore designed to establish whether oil degradation could take place in the absence of yeast/glucose and whether the yeast/glucose concentration had any effect on the extent of oil degradation (Figure 3.21).

The COD of all treatments varied considerably with time (Figure 3.21). With the exception of treatments 7 and 8 (high oil concentrations, low-intermediate yeast/glucose concentrations) the majority of samples experienced a marked reduction in COD during the first 7 days of the experiment, after which the COD continued to decline but at a diminished rate.

The decline in COD appeared to most rapid at elevated yeast/glucose concentrations and at intermediate-high oil concentrations. At an oil concentrations of 0.6%, there was evidence of oil degradation at high yeast/glucose concentrations only and the overall decline in COD was 9.7%, 0% and 61.6% at yeast/glucose concentrations of 0, 0.03 and 0.3$g.l^{-1}$ respectively. To determine whether the effect of the yeast/glucose concentration was significant, a series of Pearson's correlations were performed. These correlations confirmed that the relationship

between COD and time was significant at a yeast/glucose concentration of 0.3 $g.l^{-1}$ (r=-0.89, P<0.05) but not at a yeast/glucose concentration of 0.03 $g.l^{-1}$ (r = -0.50, P=0.095) or 0.00 $g.l^{-1}$ (r=-0.34, P=0.279).

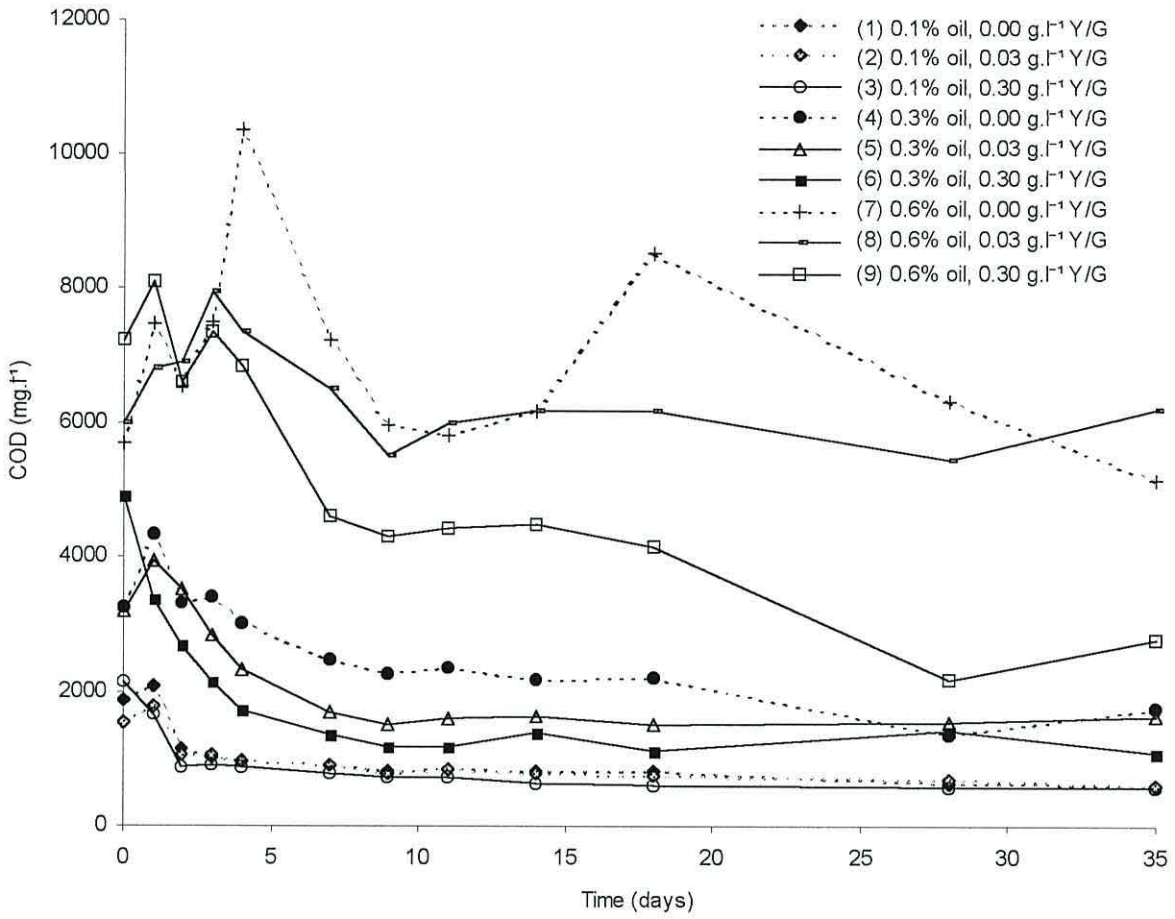


Figure 3.21. Change in COD with time for a range of non-sterilized nutrient solutions (0.00-0.30g/l^{-1} yeast/glucose 2:1 (Y/G)) supplemented with oil (0.1-0.6%v/v) inoculated with F33 and incubated at 30°C with shaking at 150rpm.

At lower oil concentrations, the decline in COD with time was significant at all yeast/glucose concentrations (Pearson's correlation, r=-0.607-0.837, P=0.001-0.036). Oil degradation appeared to be taking place in all samples and the overall COD decline in treatment 1, 2 ,3, 4, 5 and 6 was 68.3%, 61.1%, 73.3%, 46.3%, 48.9% and 77.8%, respectively.

To determine whether the yeast/glucose concentration was significantly affecting the rate at which the COD declined, regression analysis was performed over the linear portions of the data series and the gradients of the different treatments were compared. After transformation (log_{10}) the residuals were normally distributed, there was no significant heterogeneity of

variance (Bartlett's χ^2=0.664-1.051, P=0.591-0.718) and there was linear relationship between COD and time over days 2-35, (oil concentration of 0.1%) and days 1-7 (oil concentration of 0.3%) A two-factor ANOVA determined that yeast/glucose concentration significantly affected the rate at which the COD declined at an oil concentration of 0.3% over days 1-7 of the experiments (F=8.32,P<0.05) but not at an oil concentration of 0.1% over days 2-35 of the experiment (F=0.20, P=0.823). These results implied that ratio of nutrients to oil is important for oil degradation at intermediate/high oil concentrations but not at low oil concentrations.

The appearance of all the emulsified oil samples also varied with time. Small white-yellow lumps were observed to form in all treatments over the course of the experiment (Figure 3.23) which made the removal of a representative sub-samples difficult. The small white-yellow lumps were referred to as particulate material and are discussed in section 3.2.4.

In addition to the COD determination, the change in pH with time for the various nutrient solutions was assessed (Figure 3.22).

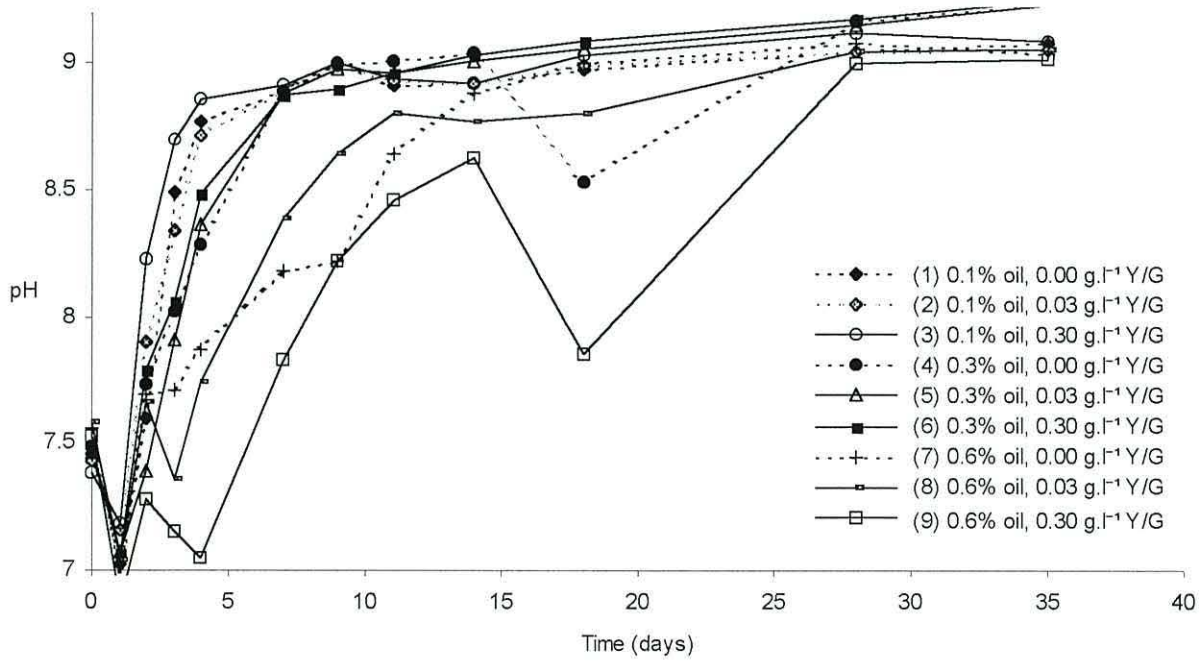
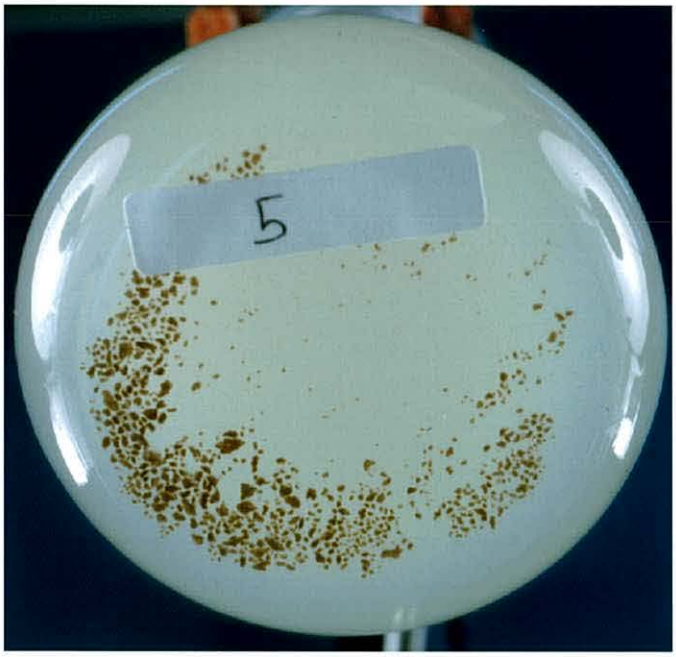


Figure 3.22. Change in pH with time for a range of non-sterilized nutrient solutions (0.00-0.30g/l^{-1} yeast/glucose 2:1 (Y/G)) supplemented with oil (0.1-0.6%v/v) inoculated with F33 and incubated at 30°C with shaking at 150rpm.

Treatment 7



Treatment 5

Figure 3.23. Appearance of particulate material that was observed to form in non sterilized nutrient solutions supplemented with oil (treatment 7; 0.6%v/v oil, 0.03 $g.l^{-1}$ yeast/glucose, treatment 5; 0.3%v/v oil, 0.00 $g.l^{-1}$ yeast/glucose) after 4 days incubation at 30C, with shaking at 150rpm.

After an initial decline in pH (ca. pH 7.5 to pH 7.0) over the first 24 hours of experiment, the pH of all treatments increased markedly between days 1 and 9 and continued to increase over the remainder of the experiment at a much slower rate. The pH was generally lower at elevated oil concentrations (0.6%) and anomalously low pH values were measured in treatments 9 and 4 on day 18 of the experiment.

Bacterial counts and oxygen consumption measurements were also performed (Table 3.16). Bacteria were present at high numbers in all treatments and the oxygen consumption measurements also confirmed that the bacteria were active in the majority of the treatments. There was a significant correlation between oxygen consumption and COD levels on day 14 and 18 of the experiment (day 14, r=0.758, P=0.018; day 18, r=0.785, P=0.012) but no significant correlation between oxygen consumption and bacterial numbers (r=0.288, P=0.453).

Table 3.16. Number of bacteria (cfu.ml^{-1}) and oxygen consumption (μmoles.l^{-1}day^{-1}) in a range of non-sterilized nutrient solutions (0.00-0.30g/l^{-1} yeast/glucose 2:1 (Y/G)) supplemented with oil (0.1-0.6%v/v) inoculated with F33 and incubated at 30°C with shaking at 150rpm for 17 days.

Treatment	Bacterial counts	oxygen consumption
0.1% oil, 0.00 g.l^{-1} Y/G	1.28 x 10^9	912
0.1% oil, 0.03 g.l^{-1} Y/G	1.21 x 10^9	0
0.1% oil, 0.30 g.l^{-1} Y/G	6.70 x 10^8	0
0.3% oil, 0.00 g.l^{-1} Y/G	1.74 x 10^9	3364
0.3% oil, 0.03 g.l^{-1} Y/G	1.39 x 10^9	804
0.3% oil, 0.30 g.l^{-1} Y/G	1.33 x 10^9	851
0.6% oil, 0.00 g.l^{-1} Y/G	1.40 x 10^9	6036
0.6% oil, 0.03 g.l^{-1} Y/G	8.75 x 10^8	1840
0.6% oil, 0.30 g.l^{-1} Y/G	9.90 x 10^8	5392

The effect of glucose and inorganic nitrogen on the degradation of oil

The effect of glucose and inorganic nitrogen on the degradation of oil was also investigated to determine whether inorganic forms of nitrogen have a similar effect to organic forms of nitrogen (Figure 3.24).

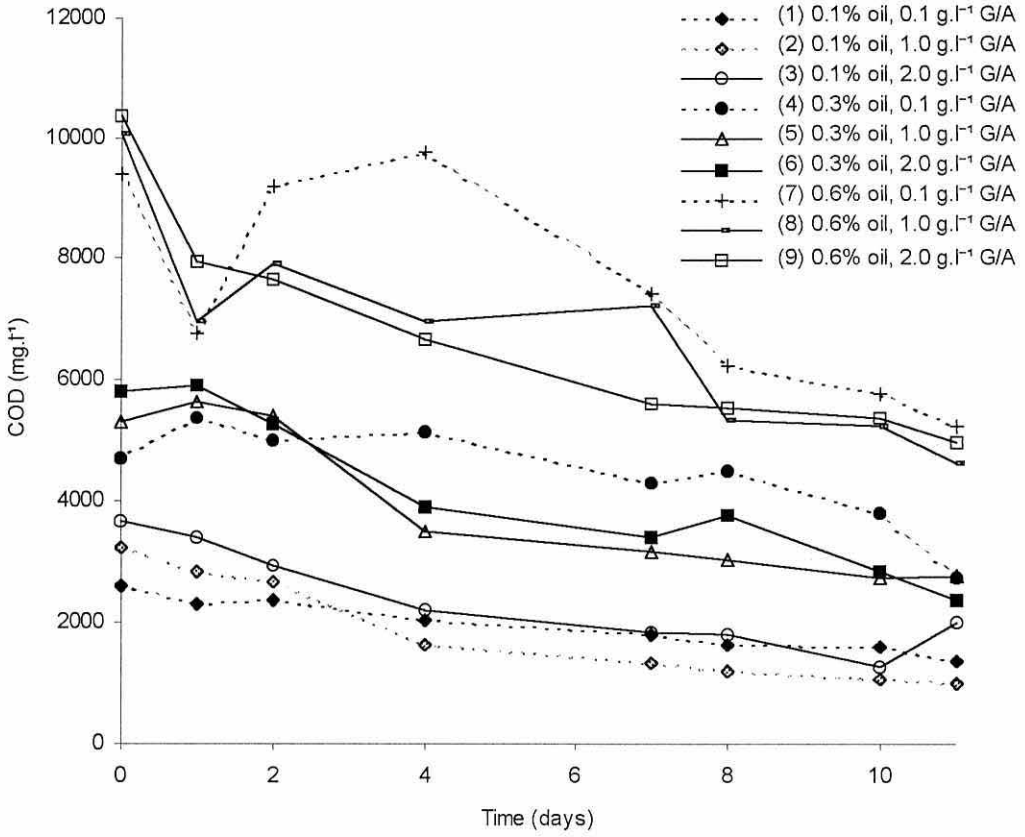


Figure 3.24. Change in COD with time for a range of non-sterilized nutrient solutions (0.1-2.0g/l^{-1} glucose/ammonium nitrate 2:1 (G/A)) supplemented with oil (0.1-0.6% v/v) inoculated with F33 and incubated at 30°C with shaking at 150rpm.

Over the course of the experiment, the COD of all treatments declined by 41.8% to 69.4%. At elevated oil concentrations and low-intermediate $C_6H_{12}O_6/NH_4NO_3$ concentrations (treatment 7 and 8), the COD also fluctuated considerably. There did not appear to be a strong relationship between the $C_6H_{12}O_6/NH_4NO_3$ concentration and rate at which the COD declined with time. Nor was there a relationship between oil concentration and the reduction in COD.

Figure 3.25 illustrates the change in pH with time at various oil and glucose/ammonium nitrate concentrations. Between day 0 and day 4 of the experiment, the pH of all samples also declined. This reduction in pH was extremely rapid at high/intermediate $C_6H_{12}O_6/NH_4NO_3$ concentrations (ca pH 7.5 to 4.0) but less marked at low $C_6H_{12}O_6/NH_4NO_3$ concentrations (ca. pH 7.5 to 6.7). After 4 days, the pH of the extreme $C_6H_{12}O_6/NH_4NO_3$ concentrations (0.1 and 2.0g.l^{-1}) continued to decline at a diminished rate, whilst the pH levels of intermediate $C_6H_{12}O_6/NH_4NO_3$ concentration progressively increased. The pH at low $C_6H_{12}O_6/NH_4NO_3$ concentrations were consistently higher than those of a high/intermediate $C_6H_{12}O_6/NH_4NO_3$

concentration for all treatments and the final pH values were below those measured at the start.

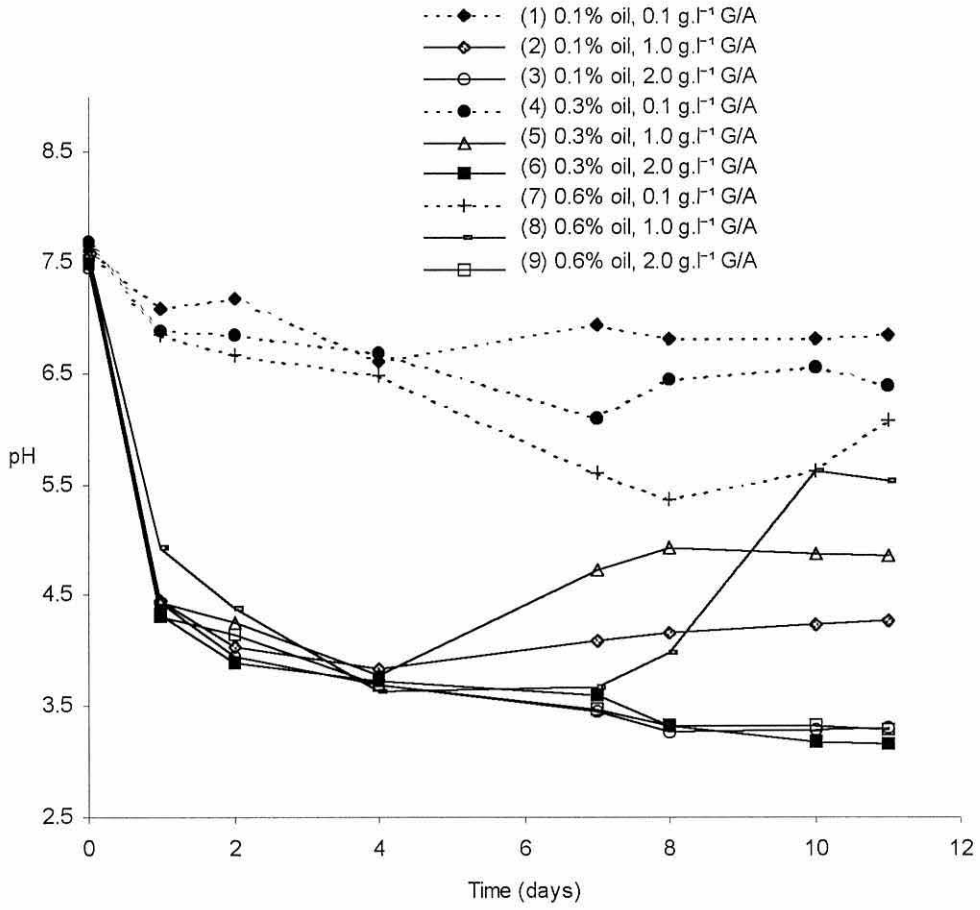


Figure 3.25. Change in pH with time for a range of non-sterilized nutrient solutions (0.1-2.0g/l^{-1} glucose/ammonium nitrate 2:1 (G/A)) supplemented with oil (0.1-0.6% v/v) inoculated with F33 and incubated at 30°C with shaking at 150rpm

3.2.4. Production of particulate material

Preliminary observations

'Particulate' material was first observed in the mechanically emulsified oil samples prior to the solvent extraction of the oil and the COD determinations (Figures 3.11 & 3.23). In these preliminary experiments, the quantity of particulate material was determined using paper rather than nylon filters. The quantity of suspended and adhering particulate material in a mechanically emulsified oil sample is reported in Table 3.17.

Table 3.17. Effect of F33 on the quantity of suspended and adhering particulate material (mg, mean ± SD) produced in non-sterilized nutrient media 2 (135ml) containing mechanically emulsified soya oil (0.6%v/v) incubated for 14 days at 30°C with shaking 150rpm.

Treatment	Suspended material	Adhering material
F33	42.90 ± 26.30	4.10 ± 2.93
Control	219.10 ± 106.90	121.70 ± 84.80

The residuals for the suspended and $\log_{10}$ transformed adhering data were normally distributed and there was no significant heterogeneity of variance (F=0.514-16.53, P=0.114-0.474). Analysis of the data using two-sample t-tests confirmed that F33 significantly reduced the quantity of both suspended and adhering particulate material (T=2.77-4.73, P=0.009-0.05)

Effect of F33 and S45 on the adherence of particulate material to glass and plastic

The effect of F33 and S45 on the adherence of particulate material to glass and plastic is shown in Table 3.18.

Table 3.18. Effect of F33 on the quantity of particulate material (mg, mean ± SD) adhering to the sides of glass and plastic vial containing nutrient media 2 (10-25ml) and soya oil (0.1-0.25ml) incubated at 30°C with occasional shaking for 19 (I) or 25 (II) days.([a] = sample volume of 25ml [b] =sample volume of 10ml)

	Treatment	Plastic vials[a]	Glass vials[b]
(I)	F33	28.30 ± 17.10	4.85 ± 1.80
	Control	44.17 ± 9.21	10.38 ± 1.60
(II)	F33	18.60 ± 3.80	5.1 ± 1.90
	S45	85.10 ± 87.90	47.7 ± 33.8
	Control	11.75 ± 3.50	8.6 ± 3.50

On the first occasion (I), F33 appreciably reduced the quantity of particulate material adhering to the sides of both the plastic and glass vials by 35.9% and 52.9%, respectively.

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2 =1.323-3.461, P=0.199-0.766) and two two-sample test, confirmed that the

effect of F33 was significant in the case of glass (T=-5.69, P<0.001) but not plastic (T=-2.00, P=0.074).

Contrary to the earlier work, the second experiment (II) showed, that both F33 and S45 increased the quantity of particulate material adhering to the plastic, relative to the control (by a factor of 1.6 and 7.2, respectively). The effect of S45 on the adherence of particulate material to glass, was similar to that with plastic (increasing adherence by factor of 5.5, relative to the control). However, the effect of F33 was consistent with that observed in the previous experiment (A) and thus the reverse of that observed with plastic (quantity of adhering material reduced by approximately 40.7%, relative to control).

The residuals for both the glass and plastic data were continuously distributed and there was significant heterogeneity of variance (Levene's statistic=5.108-6.294, P=0.010-0.020). Two Mood's median tests indicated that there were significant differences between treatments in the case of glass (χ^2=12.00, P=0.010) but not plastic (χ^2-=4.00, P=0.135).

Effect of microbial community combination on the production of particulate material

The total quantity of particulate material produced by the different combinations of bacterial communities is illustrated in Table 3.19. The effect of F33 and S45 on the production of particulate material was first assessed in the presence of the contaminants. Relative to the control (contaminants only), both S45 and F33 appreciably reduced the production of particulate material by 31.9 % and 86.3% respectively.

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=0.208, P=0.901) and a one-way ANOVA confirmed that there were significant differences between treatments (F=20.44, P=0.002). Tukey's pairwise comparisons (Table 3.19) indicated that samples inoculated with F33 produced significantly less particulate material than samples containing contaminants only or contaminants combined with S45.

Table 3.19a. Effect of grease trap isolates (GI), F33, S45 and contaminants on the production of particulate material (mg, mean ± SD) in nutrient media 2 (100ml-200ml) containing soya oil (1% v/v, non-emulsified) incubated at 30°C for 10 days (I), 18 days (II) and 20 days (III)

	Treatment	Particulate production
(I)	F33 +contaminants	45.8 ± 49.4
	S45 +contaminants	227.0 ± 66.6
	Contaminants only	333.3 ± 49.2
(II)	Contaminants only	336.8 ± 33.3
	Contaminants+GI	346.4 ± 38.3
	Contaminants+S45+GI	285.55 ± 17.8
	Contaminants+F33+GI	241.8 ± 21.3
(III)	S45 only	487.0 ± 209.0
	F33 only	absent
	GI only	649.1 ±18.5
	Contaminants only	576.2 ±78.6

Table 3.19b. Tukey's pairwise comparisons of the mean difference (mg) of particulate material produced in samples inoculated with S45, F33 and contaminants. 95% confidence interval of any difference =139.5 *significant differences

Pairwise comparison	Difference in means
S45+contaminants & F33 + contaminants	181.2*
S45+ contaminants & contaminants only	-106.0
F33+contaminants & contaminants only	-287.3*

Table 3.19c. Tukey's pairwise comparisons of the mean difference (mg) of particulate material produced in samples inoculated with S45, F33, grease trap isolates (GI) and contaminants. 95% confidence interval of any difference =139.5 *significant differences

Pairwise comparison	Difference in means
S45+contaminants+GI & contaminants + GI	60.9
F33+contaminants+GI & contaminants +GI*	104.6
F33+contaminants+GI & s45+contaminants+GI	43.7
Contaminants+GI & contaminants only	-9.6

In the presence of the grease trap isolates and contaminants the effect of S45 and F33 on particulate production was similar. Once again, particulate production was reduced by inoculating samples with S45 and F33, (by 17.56% & 30.2%, respectively). However, relative to samples containing the contaminants only, the reduction in particulate production was less

marked. Grease trap isolates also appeared to have very little effect on the ability of the contaminants to generate particulate material.

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=1.217, P=0.749) and a one-way ANOVA confirmed that there were significant differences between the various treatments (F=8.46, P=0.007). Bonferoni's pairwise comparisons (Table 3.19) indicated that F33 significantly reduced the production of particulate material relative to the control (i.e. contaminants+GI).

With the exception of F33, all of the microbial communities investigated generated particulate material. The quantity of particulate material produced ranged from 487-689 mg. However, a one-way ANOVA indicated that the quantity of particulate material produced by the contaminants, grease trap isolates and S45 was not significantly different (F=1.18, P=0.371) (Residuals approximately normally distributed, no significant heterogeneity of variance; Levene's statistic=1.882, P=0.232).

Effect of autoclaving on the ability of F33 to reduce particulate production

To determine whether the ability of F33 to reduce the production of particulate material was the result of biological or physico-chemical processes, the effect of autoclaved F33 on the production of particulate material was investigated (Table 3.20). Inoculation of the samples with both autoclaved and non-autoclaved F33 reduced the quantity of particulate material produced by approximately 50%. Plate counts also confirmed that the autoclaving process successfully destroyed the microorganism present in F33.

After transforming the data ($\log_{10}$), the residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=1.727, P=0.422) and a one-way ANOVA established that there were significant differences between treatments (F=26.72, P=0.010). Tukey's pairwise comparisons (Table 3.20) indicated that there were no significant differences between F33 and autoclaved F33, although both treatments produced significantly less particulate material than the controls. These results indicated that either a non-biological component of F33 was inhibiting the production of particulate material or that a heat tolerant substance produced by the F33 microorganisms during activation was responsible for the inhibition.

Table 3.20a. Effect of autoclaved and non-autoclaved F33 on the production of particulate material (mg, mean ± SD) in non-sterilized nutrient media 2 (100ml) containing soya oil (1ml), incubated at 30°C for 22 days. (Autoclaving of F33 took place after activation)

Treatment	Particulate production
Autoclaved F33	123.7 ± 19.9
F33	125.9 ± 10.5
Control	321.9 ± 79.6

Table 3.20b. Tukey's pairwise comparisons of the mean difference of particulate material produced (log_{10} mg) 95% confidence interval of any difference = 189.1 *significant difference.

Pairwise comparison	Difference in Means
Autoclaved F33 & F33	-20.7
Control & autoclaved F33	379.9*
Control & F33	400.1*

3.2.5. Assessment of fat/oil degradation using column chromatography and/or GC-MS

Previous experiments (section 3.2.2 & 3.2.3) had demonstrated that most microbial supplements were unable to enhance the degradation of fat/oil. Gas chromatography-mass spectrometry was therefore used to determine whether the microbial supplements were capable of altering the relative proportions of the major fatty acids in the fat/oil rather than completely mineralising the oil.

Effect of G40 on the fatty acid composition of lard and sunflower oil

The relative proportions of the various fatty acids in the glyceride fraction of lard fluctuated by ca. 3-4% with time and inoculation with G40 did not alter the relative proportion of any fatty acid greatly (Figure 3.26).

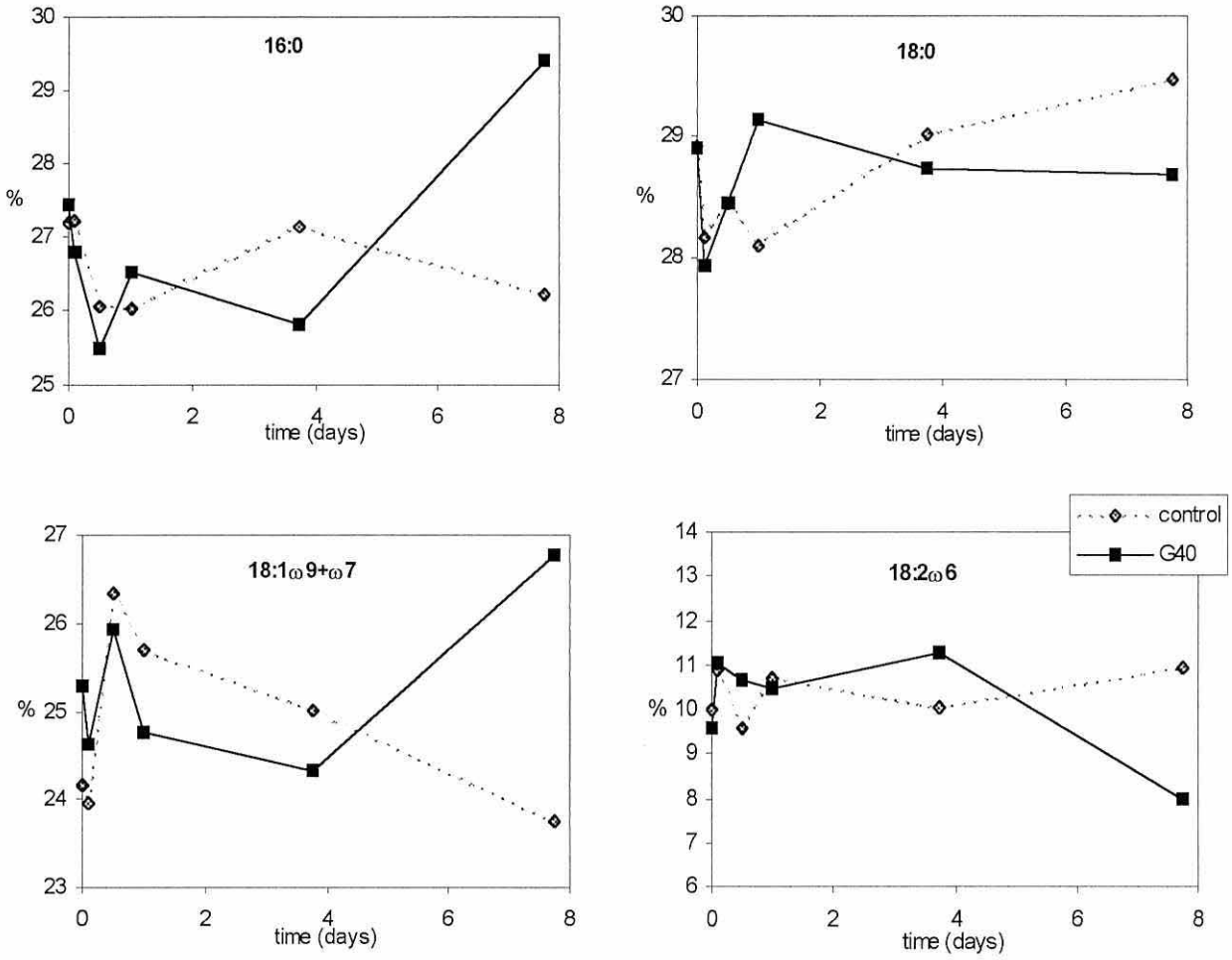


Figure 3.26. Effect of G40 on the relative proportions of the major fatty acids in the glyceride fraction of lard (50μl) suspended in 10ml of distilled water (non-sterilized) incubated at room temperature without shaking.

In comparison to the glyceride fraction of lard, the relative proportions of the fatty acids in the free fatty acid fraction of lard (Figure 3.27) fluctuated marginally more extensively with time (by ca. 6-9%) and inoculation with G40, did not alter the relative proportion of the fatty acids greatly.

The fatty acids classified as 'other' were present in lard oil at time 0 (14:0, 15;0, 17:0, 16:1ω?'s, 18:1ω? and 18:3ω3). These fatty acids constituted 8.8-14% of the lard at time 0 and were observed to fluctuate by ca. 5% over the course of the experiment. No 'new' fatty acids were detected in any of the samples.

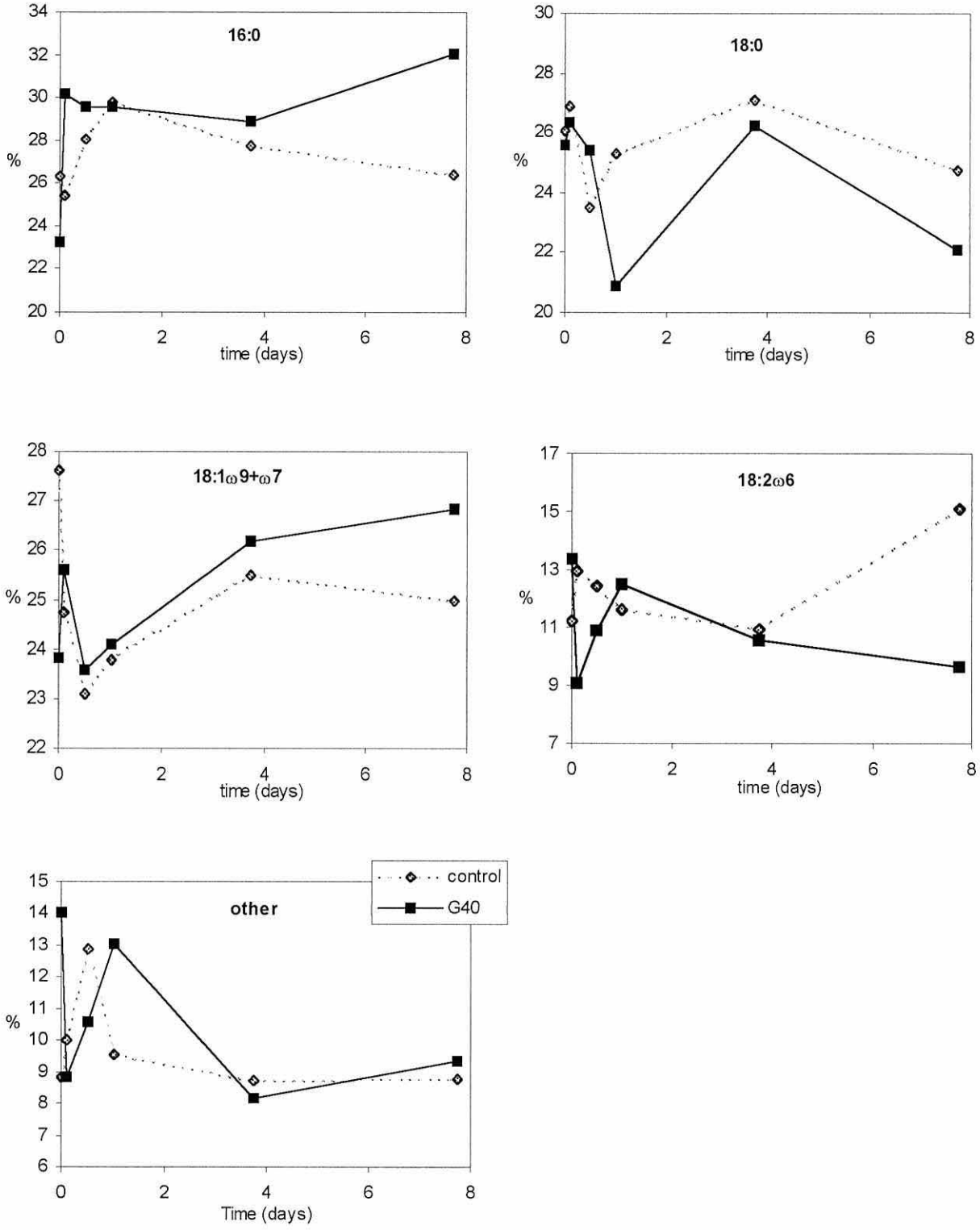


Figure 3.27. Effect of G40 on the relative proportions of the major fatty acids in the free fatty acid fraction of lard (50μl) suspended in 10ml of distilled water (non-sterilized) incubated at room temperature without shaking.

As was observed with lard, the relative proportions of the fatty acids in the glyceride fraction of sunflower oil (Figure 3.28) fluctuated within narrow limits over the course of the experiment (by ca.2-5%) and inoculation with G40, did not alter the relative proportion of the fatty acids greatly.

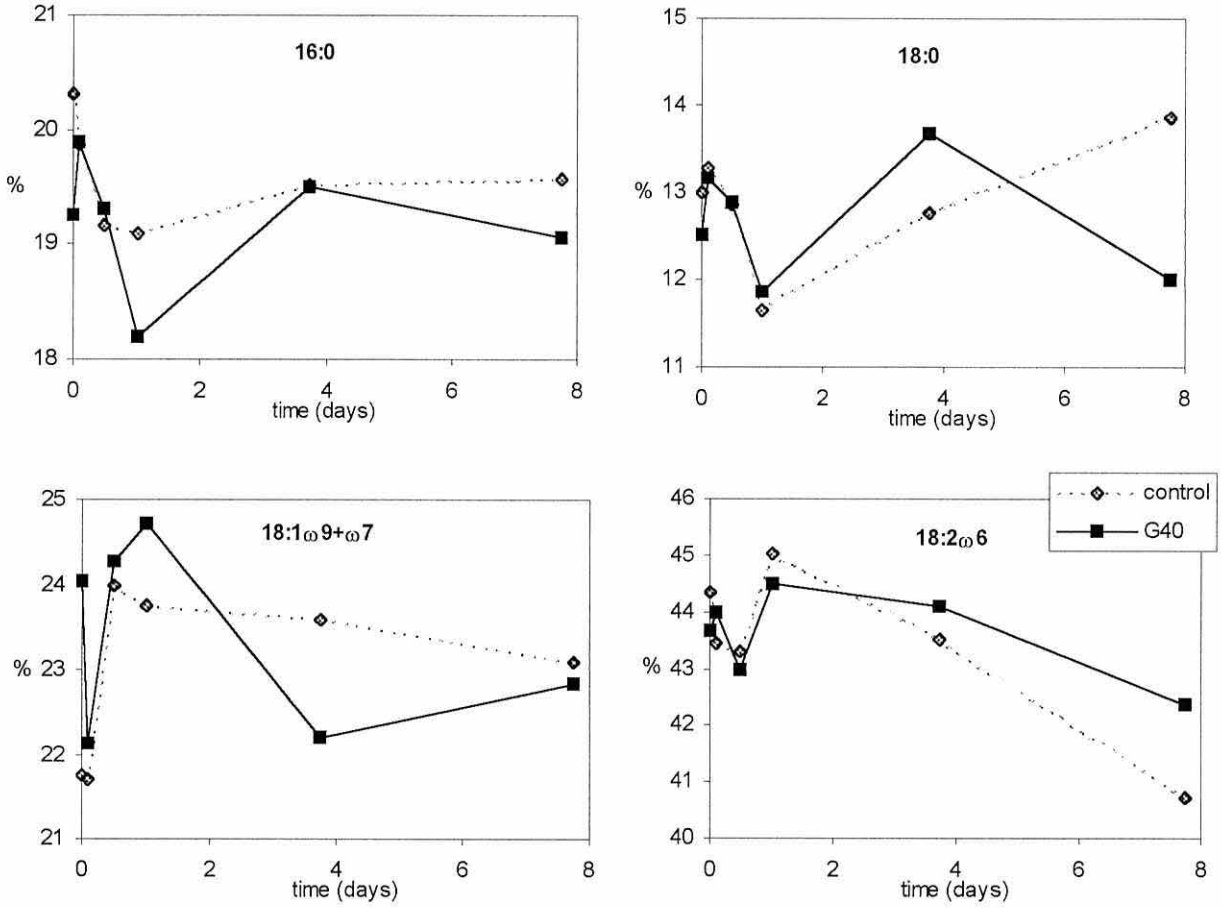


Figure 3.28. Effect of G40 on the relative proportions of the major fatty acids in the glyceride fraction of sunflower oil (50μl) suspended in 10ml of distilled water (non-sterilized) incubated at room temperature without shaking.

In the free fatty acid fraction of sunflower oil (Figure 3.29), however, the relative proportions of the major fatty acids fluctuated extensively with time (by ca. 7-27%) and inoculation with G40 appeared to have a considerable impact on the relative proportions of the fatty acids at the start of the experiment, but very little effect after day 1. At time 0, the inoculated sample was enriched in 16:0 and deficient in 18:1ω7+9 and 18:2ω6 relative to the control, which implied that there was a problem with the method or contamination of the sample rather than the selective degradation of certain fatty acids. The minor fatty acids ('other'; 14:0, 15;0,

17:0, 16:1ω?'s, 18:1ω? and 18:3ω3) also represented a variable proportion of the total at the start of the experiment (2.8-10.8%) and fluctuated thereafter. No 'new' fatty acids were detected in any of the samples.

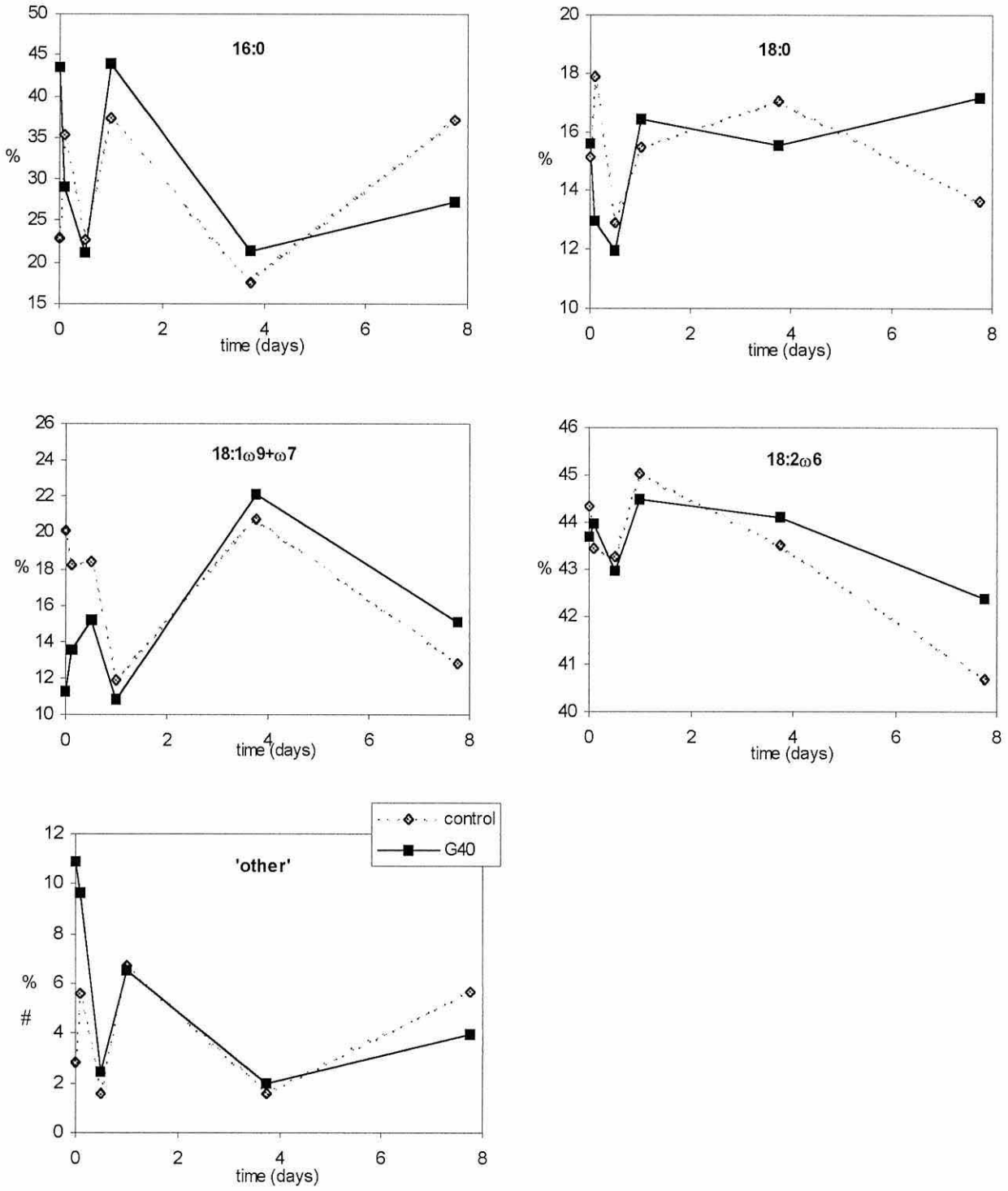


Figure 3.29. Effect of G40 on the relative proportions of the major fatty acids in the free fatty acid fraction of sunflower oil (50μl) suspended in 10ml of distilled water (non-sterilized) incubated at room temperature without shaking.

Effect of F33 on the fatty acid composition of soya oil

The composition of the glyceride fraction of soya oil varied considerably over the course of the experiment (Figure 3.30). In the inoculated sample, the relative proportions of 18:3ω3 and 18:2ω6 generally declined with time from ca. 6 to 1% and 52 to 20% respectively, whilst the relative proportions of 16:0 and 18:0, 18:1ω9 increased by a factor of 1.9-3.8 overall. Similar trends were observed in the control sample, although the change in the percentage composition of 18:1ω9 with time was the reverse of that observed in the inoculated sample after day 4 of the experiment.

In the inoculated free fatty acid fraction of soya oil (Figure 3.31), the relative proportions of 18:3ω3, 18:2ω6 fluctuated considerably but generally declined with time from ca. 3.2 to 0.3% and from 2.7 to 10% respectively whilst the relative proportion of 16:0 fluctuated with an overall increase from ca. 20 to 33%. The proportion of 18:1ω 9, in contrast, declined markedly (by ca. 17%) during the first 4 days of the experiment and subsequently increased by the same degree over the remaining 10 days of the incubation period . Similar trends were observed in the control sample. However, the changes were more marked and the proportion of 18:1ω 9 declined over the course of the experiment.

The fatty acids classified as 'other' include the minor fatty acid constituents that were present in the free fatty acid fraction of the original oil at time 0 and several 'new' fatty acids that were not detected in the original oil. In both the control and inoculated sample, the relative proportion of these 'other' fatty acids increased markedly between day 0 and day 4-8 and appeared to gradually decline thereafter. New fatty acids that were detected in the inoculated and control sample are reported in Table 3.21 . These new fatty acids constituted 1-6% of the total.

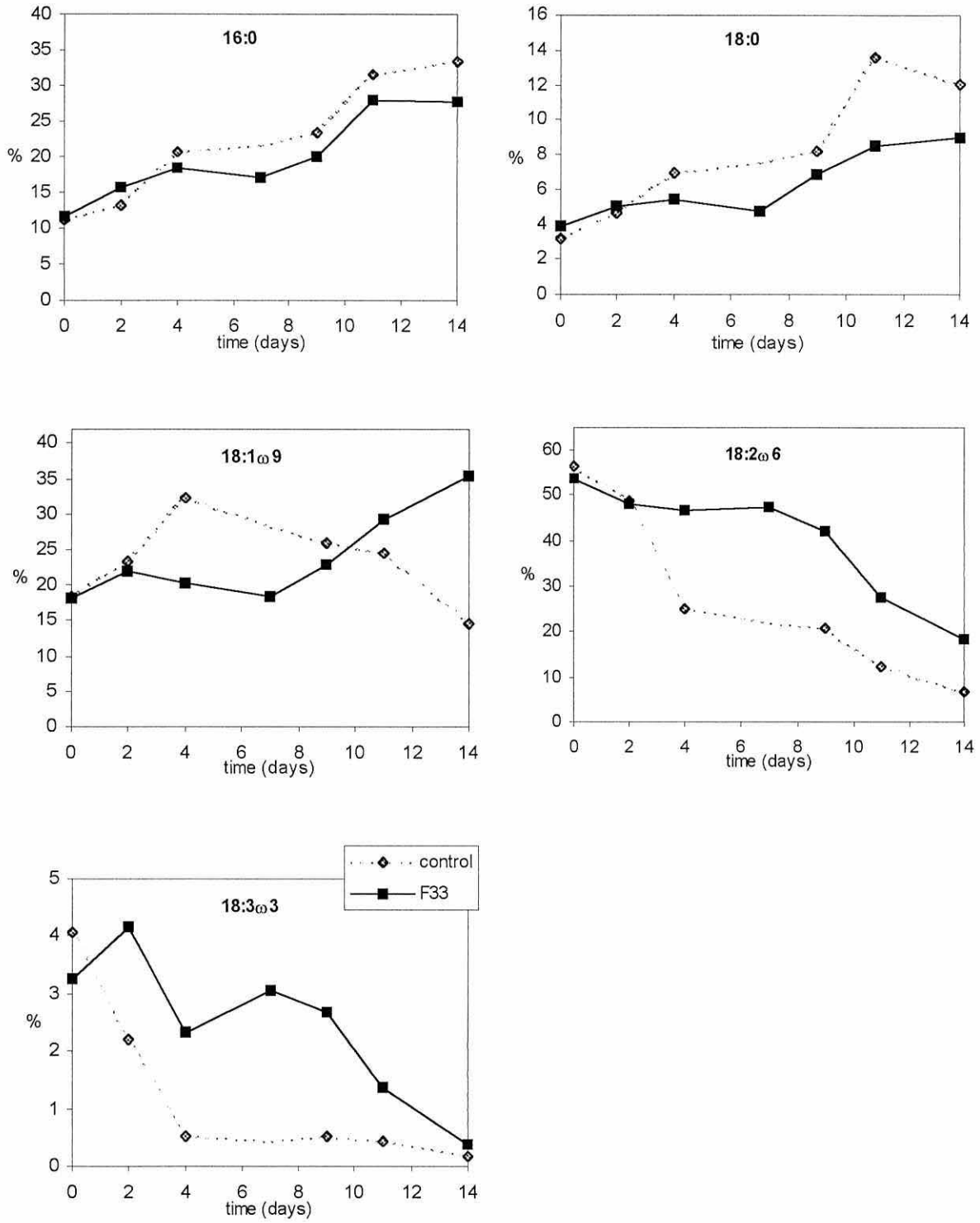


Figure 3.30. Effect of F33 on the relative proportions of the major fatty acids in the glyceride fraction of soya oil mechanically emulsified (0.6% v/v oil) in 100ml of nutrient media 2 (non sterilized) incubated at 30°C, with shaking at 150rpm.

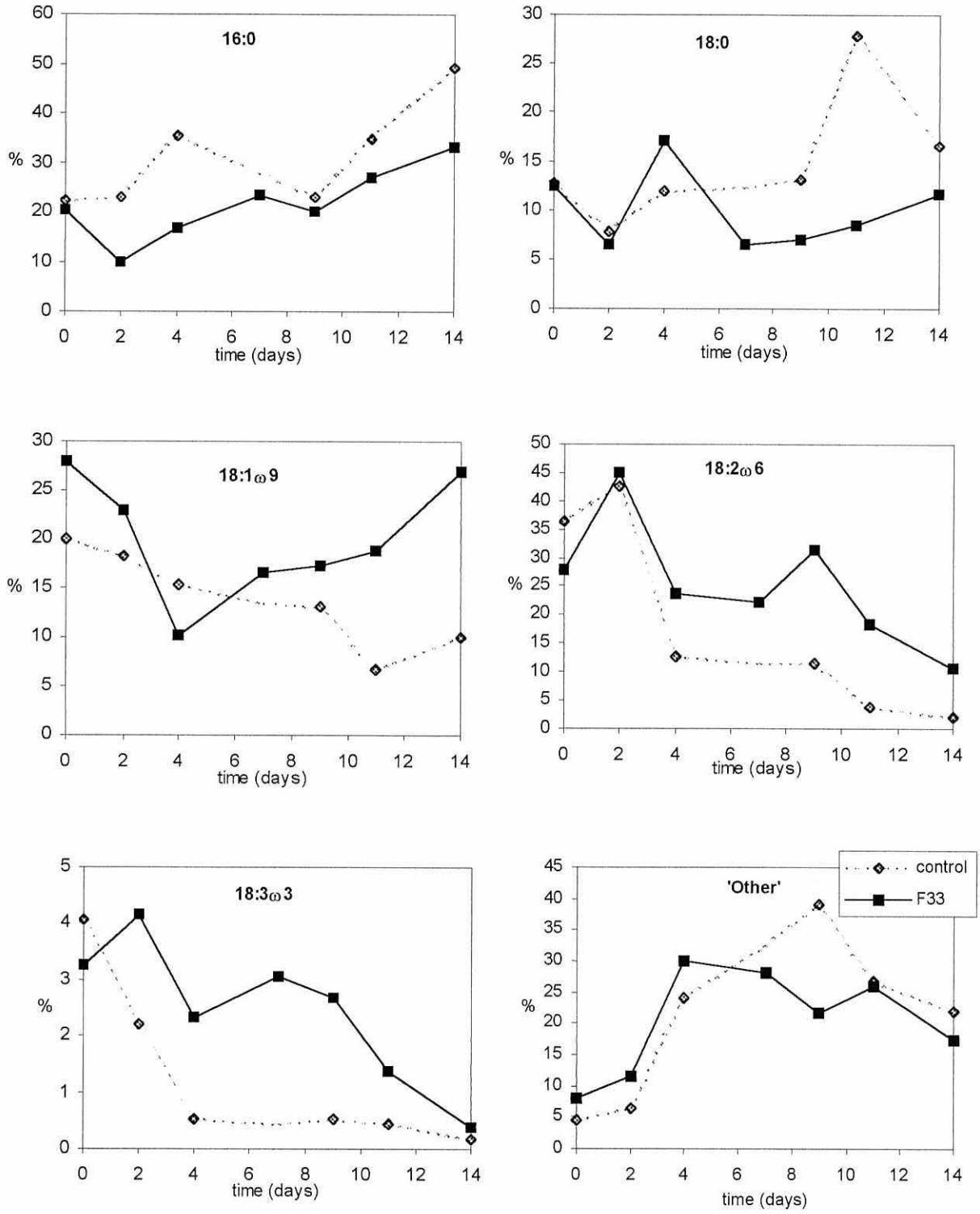


Figure 3.31. Effect of F33 on the relative proportions of the major fatty acids in the free fatty acid fraction of soya oil mechanically emulsified (0.6% v/v oil) in 100ml of nutrient media 2 (non-sterilized) incubated at 30°C, with shaking at 150rpm.

Table 3.21. New fatty acids detected in the free fatty acid fraction of soya oil mechanically emulsified (0.6%v/v oil) in 100ml of nutrient media 2 (non sterilized) incubated at 30°C, with shaking at 150rpm (retention times of partially identified new fatty acids are also reported)

New fatty acid	F33	Control
saturated(110)	✗	✓
16:1ω? (314)	✓	✓
monunsaturated(257)	✗	✓
monunsaturated(301)	✗	✓
monunsaturated(332)	✓	✗
monunsaturated(450)	✗	✓
monunsaturated(535)	✓	✗
monunsaturated(578)	✓	✓
14:2ω?(170)	✓	✗
diunsaturated(712)	✓	✗
18:3ω?(765)	✗	✓
18:3ω?(793)	✗	✓
polyunsaturated(895)	✓	✗
nonanoic acid 9-oxo	✓	✗
10-hydroxy-octadecanoic acid	✗	✓
3-OH (284)	✓	✗
3-OH (710)	✓	✗

Effect of F33 and the contaminants on the fatty acid composition of soya oil

Previous work (Table 3.9) had shown that the combined oil degrading ability of F33 and the contaminating bacteria was appreciably lower than that of the two microbial communities alone. In an attempt to provide an explanation for the diminished oil degrading ability of the combined microbial community the fatty acid compositions of the partially degraded oil samples and the control were investigated.

Figure 3.32. illustrates the percentages of the main fatty acids present in the glyceride fraction of soya oil incubated with different microbial communities. The composition of oil incubated with F33 alone did not differ greatly from that of the sterile controls. However, oil that was incubated with the contaminating bacteria was characterised by high proportions of 16:0, 18:0 and 18:1ω9 and low proportion of 18:2ω6 and 18:3ω3 relative to the controls. The addition of F33 to the contaminants did not affect the ability of the contaminants to alter the composition of the glyceride fraction greatly although the variability between replicate samples increased considerably.

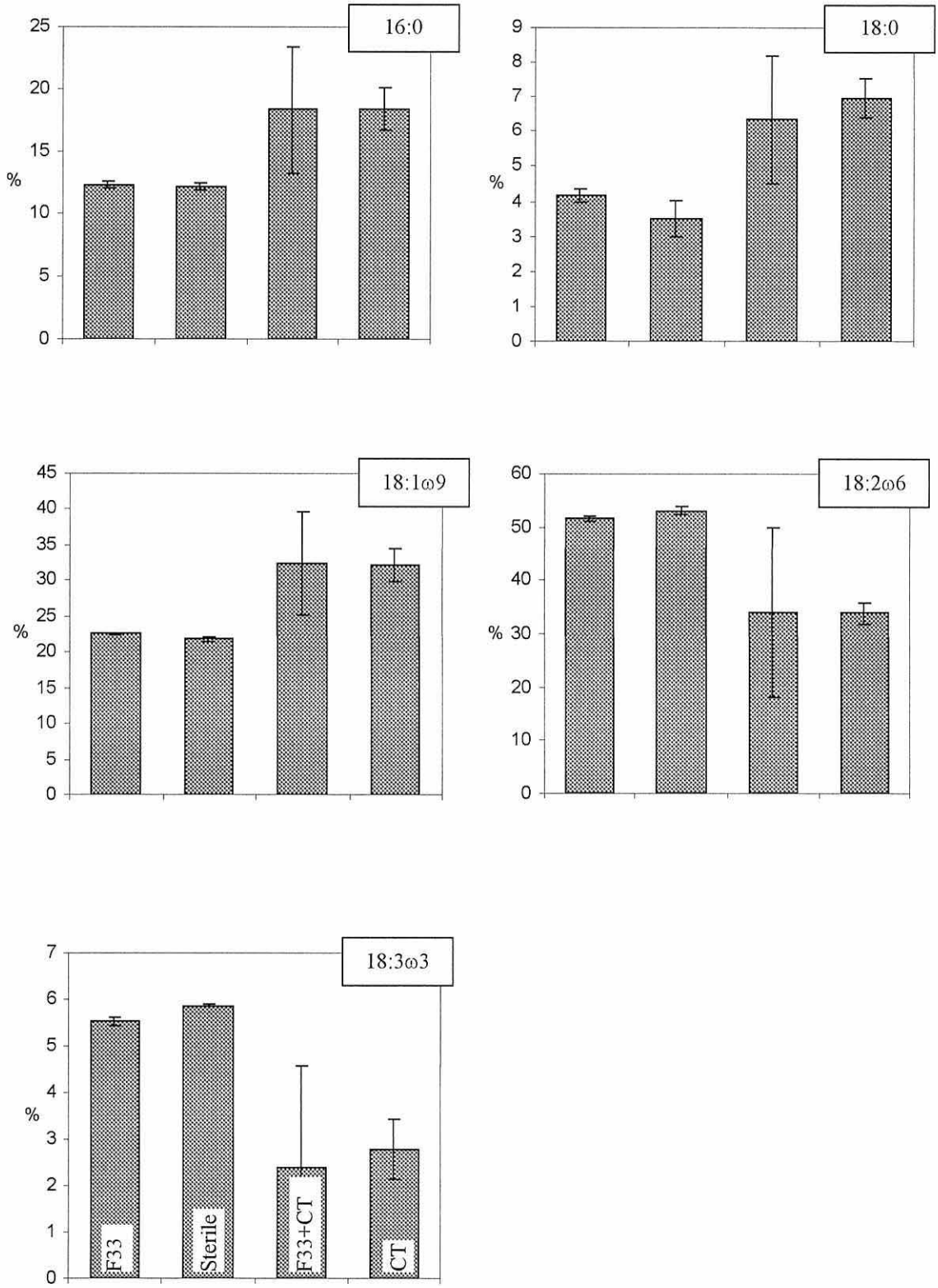


Figure 3.32. Effect of F33 and the contaminants (CT) on the relative proportions (%, mean ± SD) of the major fatty acids in the glyceride fraction of soya oil (1ml) suspended in nutrient media 2 (200ml) incubated for 21 days at 30°C with agitation on a regular basis.

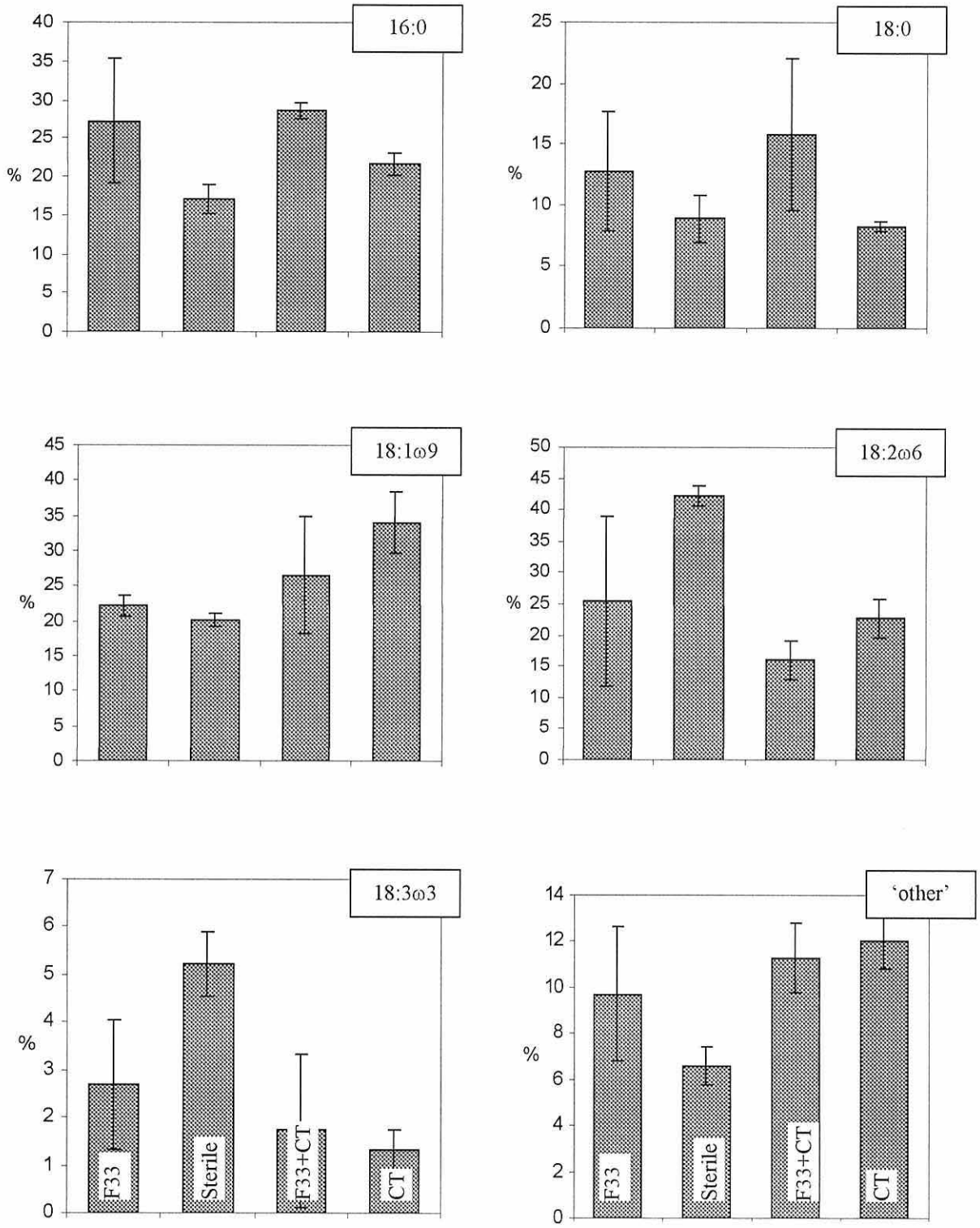


Figure 3.33. Effect of F33 and the contaminants (CT) on the relative proportions (%, mean ± SD) of the major fatty acids in the free fatty acid fraction of soya oil (1ml) suspended in nutrient media 2 (200ml) incubated for 21 days at 30°C with agitation on a regular basis.

In the free fatty acid fraction of soya oil (Figure 3.33), incubation with F33 and/or the contaminants resulted in a reduction in the proportion of 18:2ω6 and 18:3ω3 coupled with an increase in the proportion of 16:0, 18:0 , 18:1ω9 and minor fatty acid constituents, relative to the control. Although the overall effect of the microbial communities on the composition of the free fatty acid fraction was similar, the relative proportions of the major fatty acids changed by varying degrees indicating that the different microbial communities or combinations of microbial communities degraded fatty acids at different rates and/or followed different degradation pathways. All samples inoculated with F33 (in presence or absence of contaminants) were enriched in 16:0 and 18:0 relative to both the control and contaminants alone. Incubation with the contaminants alone also enhanced the proportion of 16:0 relative to the control but did not alter the proportion of 18:0 in the sample. Unlike F33 alone, the contaminants, both in the presence and absence of F33, resulted in an increase in the proportion of 18:1ω9 relative to the control.

No 'new' fatty acids that represented greater than 1% of the total were detected.

Principle component analysis of the standardized data was used to determine whether there was any relationship between the microbial communities present and the fatty acid composition of the glyceride and free fatty acid fraction of soya oil (Figures 3.34 & 3.35). In the glyceride fraction of soya oil, principle components 1 and 2 accounted for 75.6% and 8.2% of the variability, respectively. Several fatty acids had a similar influence on principle component 1 whilst 14:0 and 9-oxo-nonanoic acid (keto) appeared to have the greatest influence on principle component 2 (Appendix III). In terms of the major fatty acids, principle component 1 represented high proportions of 16:0, 18:0 and 18:1ω9, together with low proportions of 18:2ω6 and 18:3ω3. Principle component 2, on the other hand represented a high proportion of 14:0 and a low proportion of 9-oxo nonanoic acid (keto). In the free fatty fraction of oil, principle components 1 and 2 accounted for 35.0% and 28.5%. As in the glyceride fraction, several fatty acids contributed to principle component 1 whilst 18:1ω9, 18:1ω7 and 18:3ω3 appeared to have the greatest influence on principle component 2 (Appendix III). Principle component 1 again represented a high proportion of 16:0 and 18:0 together with a low proportion of 18:2ω6, 18:3ω3 and 18:1ω9. Principle component 2, in contrast, represented a low proportion of 18:1ω9 and 18:1ω7 and a high proportion of 18:3ω3.

Figures 3.34. and 3.35. illustrate score plots of principle components 1 and 2 for both the glyceride and free fatty acids fraction of soya oil.

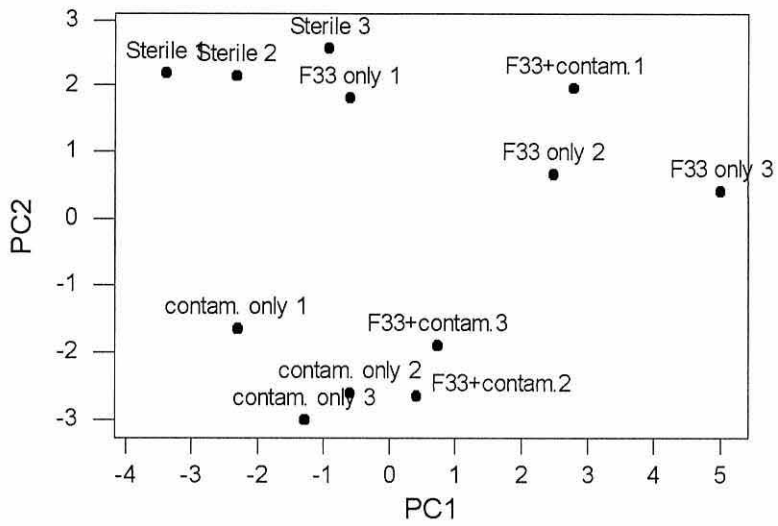


Figure 3.34. Score plot of principle components 1 and 2 for the relative proportion of all fatty acids in the free fatty acid fraction of soya oil (1ml) suspended in nutrient media 2 (200ml) incubated under sterile conditions or in the presence of F33 and the contaminants (contam.) at 30°C with agitation on a regular basis for 21 days

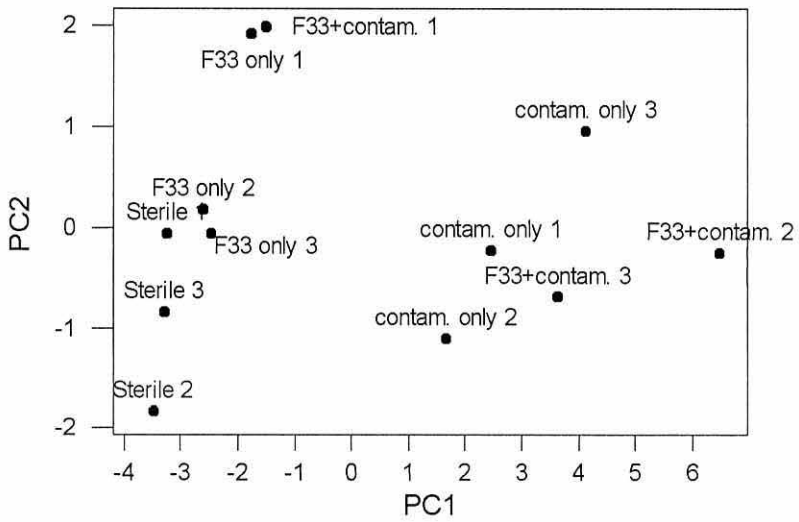


Figure 3.35. Score plot of principle components 1 and 2 for the relative proportion of all fatty acids in the glyceride fraction of soya oil (1ml) suspended in nutrient media 2 (200ml) incubated under sterile conditions or in the presence of F33 and the contaminants (contam.) at 30°C with agitation on a regular basis for 21 days

In both score plots the replicate samples were separated in to two groups on the basis of whether particulate material was produced, rather than on which microbial communities were present. Particulate producers were separated from non particulate producers by principle components 1 and 2 in the glyceride and free fatty acid fractions, respectively. In the

glyceride fraction, replicate samples that were observed to form particulate material scored positively on principle component 1whilst in the free fatty acid fraction replicate samples that were observed to form particulate material scored negatively on principle component 2

Particulate material was produced only when the contaminants were present therefore it would appear that the contaminants alone were responsible for the production of particulate material and that F33 had some ability to inhibit the production of this particulate material. F33 inhibited the production of particulate material in one replicate sample only (F33+contam.1) indicating that the effect of F33 was somewhat variable. (For further information on the impact of F33 on particulate production see section 3.2.4).

In addition to principle component analysis, the data for each major fatty acid was analysed separately. After the appropriate transformation ($\log_{10}$ or arcsine) the residuals for fatty acids in both the glyceride and free fatty acid fraction of soya oil were normally or approximately normally distributed, with no significant heterogeneity of variance (Levene's statistic=1.366-2.539, P=0.13-0.279, Bartlett's χ^2=3.075-7.223, P=0.065-0.380). For all fatty acids, except 18:0 in the glyceride fraction of soya, a series of one-way ANOVA's confirmed that there were significant differences between the various sets of samples tested (Table 3.22).

Table 3.22. F and P values for the series of one-way ANOVA's applied to compare the relative proportions of 16:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3 in the glyceride and free fatty acid fraction of soya oil (1ml) suspended in nutrient media 2 (200ml) incubated with F33 and the contaminants at 30°C with agitation on a regular basis for 21 days.

	Glyceride fraction		**Free fatty acids fraction**	
	F	**P**	**F**	**P**
16:0	5.08	0.029	4.58	0.038
18:0	8.33	0.008	2.47	0.137
18:1ω9	6.99	0.013	4.74	0.035
18:2ω6	5.32	0.026	8.23	0.041
18:3ω3	7.68	0.010	7.22	0.012

Tukey's pairwise comparisons (Table 3.23) indicated that both the contaminants only and contaminants combined with F33, resulted in a significantly greater proportion of 18:0 and 18:1ω9 and significantly smaller proportion of 18:3ω3 relative to the sterile control in the glyceride fraction of soya oil. The contaminants alone also resulted in significantly more 18:0

than F33 alone and when combined with F33 resulted in significantly less 18:3ω3 than F33 alone. In the free fatty acid fraction of soya oil, both the contaminants alone and combined with F33 resulted in a significantly greater proportion of 16:0 (contaminants+F33 only) and a significantly smaller proportion of 18:2ω6 and 18:3ω3 than the sterile control.

Table 3.23. Tukey's pairwise comparisons of the mean difference ± 95% confidence interval of the proportion of 16:0, 18:0, 18ω:9, 18:2ω6 and 18:3ω3 (%, $\log_{10}$ or arcsine) in the glyceride (G) and free fatty acid fraction (F) of soya oil incubated under sterile conditions (strle) or in the presence of F33 and the contaminants (ctm).

		16:0	18:0	18:1ω9	18:2ω6	18:3ω3
F33 only & Strle	**G**	0.078 ±7.102	0.673 ±2.624	0.647 ±10.000	-1.465 ±20.925	-0.345 ±2.956
	F	0.106 ±0.116	- -	0.040 ±0.204	-16.683 ±18.755	-2.517 ±2.952
Ctm. only & Strle	**G**	6.193 ±7.102	3.462* ±2.624	10.304* ±10.000	-19.205 ±20.925	-3.091* ±2.956
	F	0.048 ±0.116	- -	0.224* ±0.204	-19.405* ±18.75	-3.902* ±2.952
Ctm.+F33 & Strle	**G**	6.127 ±7.102	2.859* ±2.624	10.537* ±10.000	-19.110 ±20.925	-3.474* ±2.956
	F	0.119* ±0.116	- -	0.104 ±0.204	-26.095* ±18.755	-3.490* ±2.952
F33 only & ctm.only	**G**	-6.116 ±7.102	-2.789* ±2.624	-9.658 ±10.000	17.740 ±20.925	-2.746 ±2.956
	F	0.058 ±0.116	- -	-0.185 ±0.204	2.720 ±18.755	-1.385 ±2.952
F33 only & ctm.+F33	**G**	-6.049 ±7.102	-2.186 ±2.624	-9.891 ±10.000	17.645 ±20.925	3.129* ±2.956
	F	-0.026 ±0.116	- -	-0.064 ±0.204	9.410 ±18.755	0.973 ±2.952
Ctm.only & ctm+F33	**G**	0.133 ±7.102	0.604 ±2.624	-0.233 ±10.000	-0.190 ±20.925	0.383 ±2.956
	F	-0.071 ±0.116	- -	0.120 ±0.204	6.690 ±18.755	-0.412 ±2.952

Effect of F33 and S45 on the fatty acid composition of soya oil incubated with microorganisms isolated from a grease trap

The ability of the grease trap isolates to degrade oil was impaired by inoculating the samples with F33 and to a lesser extent, with S45. The fatty acid composition of these partially degraded lipid samples were also investigated but due to time restraints the glyceride and free fatty acids fractions were not examined individually.

The relative proportions of the major fatty acids in the control samples and samples inoculated with the various microbial communities are shown in Figure 3.36. All microbial communities or combinations of microbial communities resulted in an increase in the proportion of minor fatty acids (other), 16:0, 18:0 and 18:1ω9 coupled with a decrease in the proportion of 18:2ω6 and 18:3ω3, relative to the sterile control. The degree to which the microbial communities altered the fatty acid composition of the oil was similar. However, the combined effects of the grease trap isolates and F33 were more variable than those of the individual microbial communities or grease trap isolates combined with S45.

No 'new' fatty acids that constituted greater than 1% of the total were present in the inoculated sample.

Principle component analysis of the standardized data was used to determine whether there was any relationship between the microbial communities present and the fatty acid composition of the soya oil (Figure 3.37). Principle components 1 and 2 accounted for 62.8% and 16.5% of the variability, respectively. Several fatty acids had a similar influence on principle component 1 whilst numerous minor 'new' fatty acids (15:0, 18:2(1046), 18:2(1052), polyunsaturated(1328) and polyunsaturated(1342)) contributed to principle component 2 (Appendix III).

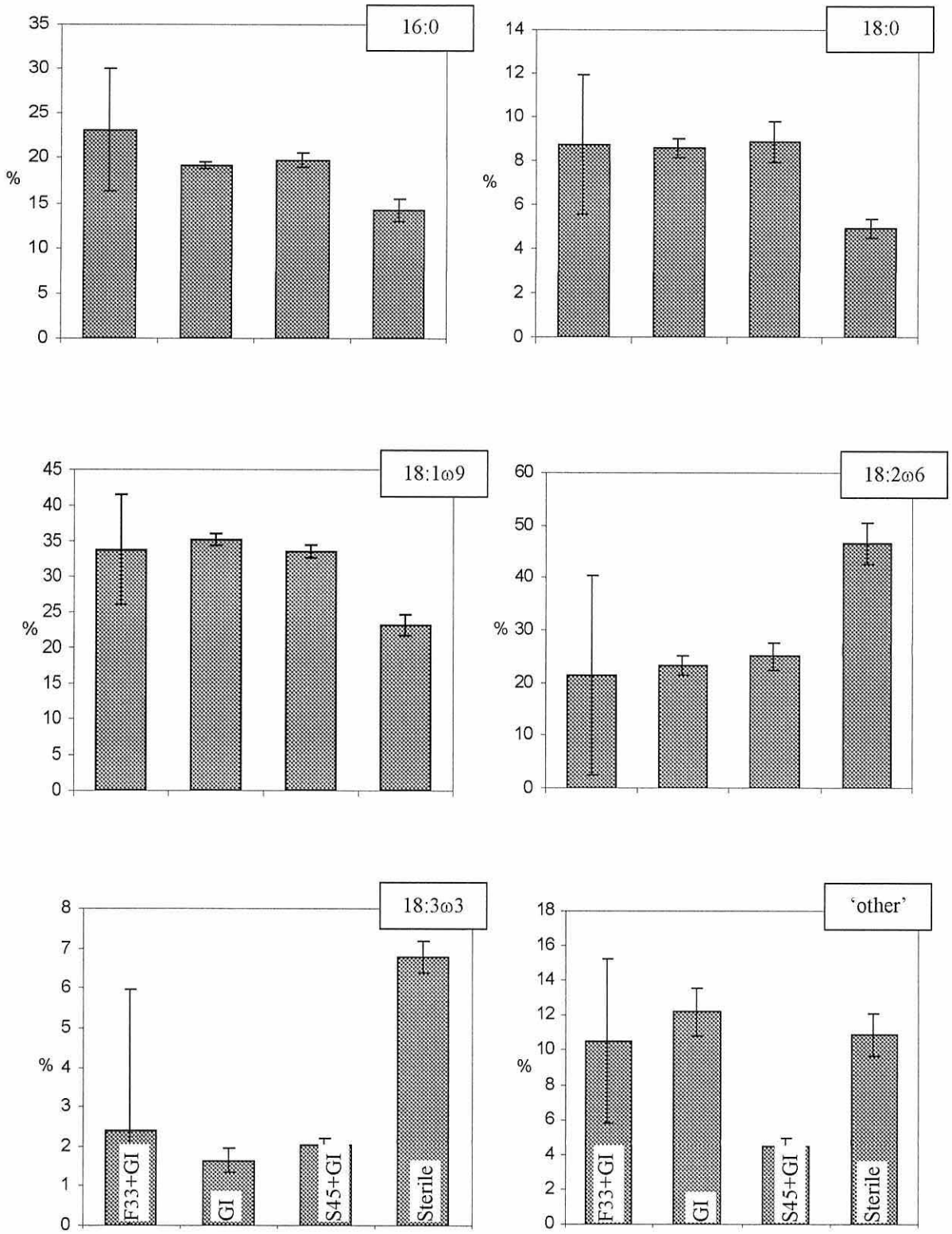


Figure 3.36. Effect of S45 and F33 on the relative proportions of the major fatty acids (mean ± SD) in soya oil (1ml) suspended in nutrient media 2 (100ml) inoculated with microorganisms isolated a grease trap (GI). (Samples were sterilized prior to inoculation and were incubated for 18 days at 30°C with occasional agitation)

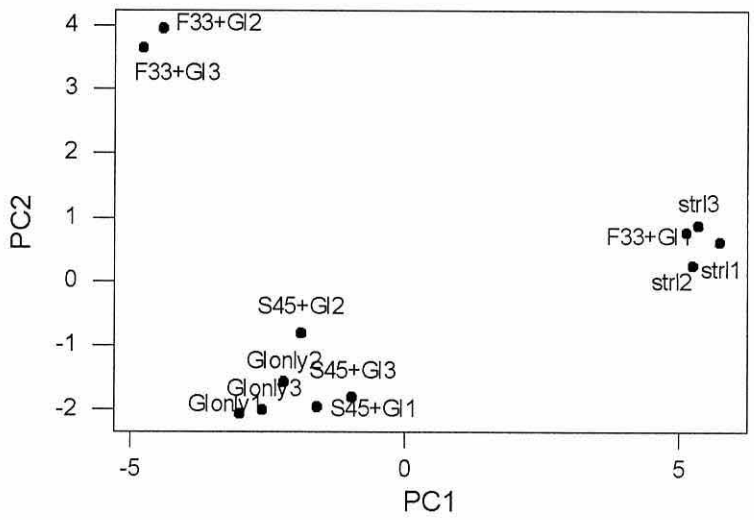


Figure 3.37. Score plot of principle components 1 and 2 for the relative proportion of all fatty acids in soya oil (1ml) suspended in nutrient media 2 (100ml) incubated under sterile conditions (strl) or with various combinations of the grease trap isolates (GI), F33 and S45 for 18 days at 30°C with occasional agitation.

The score plot of principle components 1 and 2 for soya oil is shown in Figure 3.37. Principle component 1 separated the replicate samples on the basis of whether oil degradation had occurred or not. Those replicate samples that scored positively on principle component 1 (i.e. sterile control samples 1-3 and F33+GI 1) experienced low levels of oil degradation. Samples that experienced relatively high levels of oil degradation were also separated into two groups by principle component 2. These findings implied that the inhibitory effect of F33 on oil degradation was inconsistent and that when oil degradation does take place in the presence of F33, the pathway of oil degradation is different from that induced by S45 and the grease trap isolates.

In addition to principle component analysis, the data for the major fatty acids was analysed separately (Table 3.24). The residuals for all fatty acids were approximately normally distributed and there was no significant heterogeneity of variance (Levene's statistic=0.682-1.064, P=0.417-0.588). A series of one-way ANOVA's were performed and revealed that there were significant differences in the relative proportions of 18:1ω9, 18:2ω6 and 18:3ω3 only. Tukey's pairwise comparisons (Table 3.24) confirmed that all microbial communities significantly altered the proportion of at least one fatty acid in soya oil. The grease trap isolates alone or combined with F33 resulted in a significantly greater proportion of 18:1ω9 relative to the sterile control whilst the grease trap isolates alone or combined with S45 also gave rise to a significantly smaller proportion of 18:3ω3 in comparison to the sterile control.

Table 3.24a. F and P values for the series of one-way ANOVA's applied to compare the relative proportions of 16:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3 in soya oil (1ml) suspended in nutrient media 2 (100ml) incubated with various combinations of the grease trap isolates, F33 and S45 for 18 days at 30°C with occasional agitation)

	F	P
16:0	3.26	0.081
18:0	3.83	0.057
18:1ω9	5.83	0.021
18:2ω6	4.27	0.045
18:3ω3	5.40	0.025

Table 3.24b Tukey's pairwise comparisons of the mean difference ± 95% confidence interval of the proportion of 18:1 ω:9, 18:2ω6 and 18:3ω3 in soya oil incubated under sterile conditions or with various combinations of the grease trap isolates (GI), F33 and S45.

	18:1ω9	18:2ω6	18:3ω3
F33+GI & GI only	-1.42 ± 10.40	-1.90 ± 25.67	0.78 ± 4.69
F33+GI & S45+GI	0.25 ± 10.40	-3.55 ± 25.67	0.38 ± 4.69
F33+GI & sterile	10.59 ± 10.40*	-25.05 ± 25.67	-4.38 ± 4.69
GI only & S45+GI	1.67 ± 10.40	-1.65 ± 25.67	-0.40 ± 4.69
GI only & sterile	12.01 ± 10.40*	-23.16 ± 25.67	-10.32 ± 4.69*
S45+GI & sterile	10.35 ± 10.40	-21.50 ± 25.67	-4.76 ± 4.69*

Fatty acid composition of particulate material

The fatty acid composition of the particulate material discussed in section 3.2.4. was also investigated. Figure 3.38. illustrates the relative proportions of the major fatty acids present in both the original oil and the two forms of particulate material. The fatty acid compositions of the suspended and adhering particulate material did not differ greatly. However, both forms of particulate material contained a greater proportion of the minor fatty acids (other), 16:0, 18:0 and 18:1ω7+9 and a lower proportion of 18:2ω6 and 18:3ω3, relative to the original oil. A 'new' fatty acid that constituted 1-2.5% of the particulate material was identified as 9-oxo nonanoic acid.

The residuals for all fatty acids ($\log_{10}$ transformed in case of 18:0) were normally distributed with no significant heterogeneity of variance (Bartlett's χ^2=0.779-3.218, P=0.2-0.677). A series of one-way ANOVA's were performed (Table 3.25) and confirmed that there were significant between some of samples for all fatty acids tested. Tukey's pairwise comparisons (Table 3.25) revealed that, in terms of the major fatty acids, the composition of suspended

particulate material was not significantly different from that of adhering particulate material. However, both forms of particulate material contained a significantly greater proportion of 16:0 , 18:0 and 18:1ω9+7 and significantly smaller proportion of 18:2ω6 and 18:3ω3, relative to the original oil.

Table 3.25a. F and P values for the series of one-way ANOVA's applied to compare the relative proportions of 16:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3 in pure soya oil and suspended and adhering particulate material produced in non-sterilized nutrient media 2 (135ml) containing mechanically emulsified soya oil (0.6% v/v) incubated for 14 days at 30°C with shaking 150rpm.

	F	P
16:0	49.15	<0.001
18:0	31.16	0.001
18:1ω7+9	57.18	<0.001
18:2ω6	95.35	<0.001
18:3ω3	489	<0.001

Table 3.25b. Tukey's pairwise comparisons of the mean difference ± 95% confidence interval of the proportion of 16:0, 18:0, 18:1ω9+7, 18:2ω6 and 18:3ω3 in pure soya oil and adhering (adh.) and suspended (sus.) particulate material (P)

	16:0	18:0	18:1ω9+7	18:2ω6	18:3ω3
soya oil & sus. P	-28.301* ±9.241	-0.381* ±0.170	-20.567* ±8.124	58.33* ±14.785	3.515* ±0.395
soya oil & adh. P	-22.381* ±9.241	-0.378* ±0.170	-27.129* ±8.124	56.855* ±14.785	3.454* ±0.395
sus. & adh. P	5.920 ±9.241	0.003 ±0.170	-6.562 ±8.124	-1.475 ±14.785	0.061 ±0.395

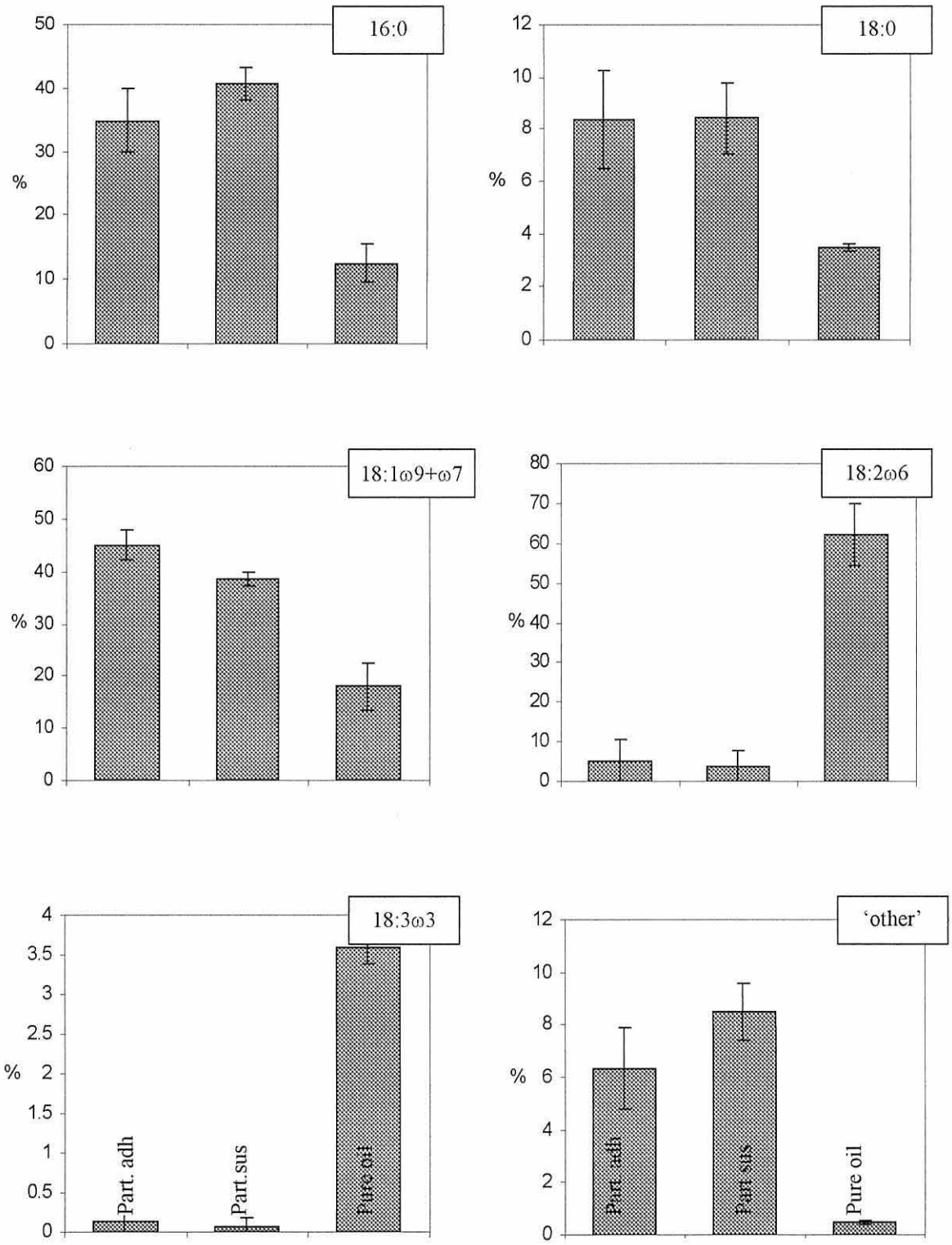


Figure 3.38. Composition (%) of pure soya oil and suspended (part.sus) and adhering particulate (part.adh) produced in non-sterilized nutrient media 2 (135ml) containing mechanically emulsified soya oil (0.6% v/v) incubated for 14 days at 30°C with shaking 150rpm.

Effect of frying on the fatty acid composition of vegetable oil

Given that most oil is fried before it is discharged into the drains and sewers, the impact of frying on the fatty acid composition of rape oil was also assessed (Table 3.26).

Table 3.26. Effect of frying on the relative proportions (%, mean ± SD) of 16:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3 in pure rape oil (oil fried in the absence of a food source at 180°C for 15 minutes with 20 minutes for cooling on 14 separate occasions). Also shown are the two sample t-tests applied to compare the relative proportions of 16:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3 in pure and fried rape oil. (Significant level; P=0.01)

	Pure	Fried	T	P
16:0	5.12 ± 0.16	5.73 ± 0.66	1.54	0.199
18:1ω9	51.00 ± 1.34	46.65 ± 4.71	-1.54	0.199
18:1ω7	6.14 ± 1.55	11.30 ± 1.76	3.80	0.019
18:2ω6	21.20 ± 0.10	18.83 ± 1.79	-2.29	0.150
18:3ω3	10.11 ± 0.45	10.24 ± 1.65	0.13	0.902
other	6.42 ± 0.24	7.25 ± 0.82	-	-

The frying process appeared to have little impact on the relative proportions of the minor fatty acids (other), 16:0, 18:2ω6 and 18:3ω3 in rape oil, but did result in an appreciable reduction in proportion of 18:1ω9, together with an increase the proportions of 18:1ω7. A 'New' fatty acid (10-OH) that constituted 1.23 ± 1.25% of the fried oil was also detected.

The residuals were normally distributed and with the exception of 18:2ω6, (Bartlett's χ^2=359.186, P=0.006) there was no significant heterogeneity of variance (Bartlett's χ^2=1.291-17.874, P=0.106-0.150). A series of two sample t-tests (Table 3.26) determined that the frying process did not significantly change the relative proportions of the major fatty acids.

Effect of autoclaving on the fatty acid composition of soya oil.

The effect of autoclaving on the fatty acid composition of soya was also investigated since several oil samples were autoclaved prior to inoculation with the microbial supplements (Table 3.27). The autoclaving process had little impact on the relative proportions of fatty acids in the glyceride fraction of soya oil (effect was within +/-5.5%) but did induce larger compositional changes in the free fatty acid fraction of soya oil (by as much as 45.2%). The major compositional changes observed included a reduction in the proportion of the minor fatty acids (other), 16:0 and 18:3ω3 and an increase in the proportion of 18:0, 18:1ω9 and

18:2ω6. For each 'free' fatty acid, the standard error was also observed to increase considerably after autoclaving (3.87-18.92% to 15.85-84.73%) reflecting the large variability between replicate samples.

There were no 'new' fatty acids detected in the autoclaved samples.

Table 3.27a. Effect of autoclaving (121°C, 15 PSI for 20 minutes) on the relative proportions (%, mean ± SD) of 16:0, 18:0 18:1ω9, 18:2ω6 and 18:3ω3 in the glyceride and free fatty acid fraction of soya oil.

	Glyceride fraction		Free fatty acids fraction	
	Non-autoclaved	**Autoclaved**	**Non-autoclaved**	**Autoclaved**
16:0	2.14 ± 0.71	12.73 ± 0.84	25.34 ± 0.98	20.69 ± 3.28
18:0	4.40 ± 0.28	4.16 ± 0.38	14.22 ± 2.69	18.29 ± 10.68
18:1ω9	26.72 ± 1.21	27.04 ± 0.93	16.61 ± 1.01	18.20 ± 6.72
18:2ω6	47.99 ± 2.43	46.92 ± 2.17	27.56 ± 3.28	33.93 ± 7.56
18:3ω3	5.75 ± 0.55	5.95 ± 0.68	12.19 ± 1.76	6.68 ± 5.66
'other'	3.01 ± 0.15	3.20 ± 0.58	4.09 ± 2.54	2.22 ± 1.93

Table 3.27b. two sample t-tests applied to compare the relative proportions of 16:0, 18:0 18:1ω9, 18:2ω6 and 18:3ω3 in the free fatty acid and glyceride fraction of pure and autoclaved soya oil. (Significant level; P=0.01)

	Glyceride Fraction		Free Fatty acid fraction	
	T	**P**	**T**	**P**
16:0	0.92	0.410	-2.35	0.078
18:0	-0.86	0.438	0.64	0.557
18:1ω9	0.37	0.730	0.41	0.724
18:2ω6	-0.57	0.600	1.34	0.252
18:3ω3	0.39	0.713	-1.61	0.182

The residuals of the fatty acids in both the glyceride and free fatty acid fraction of soya oil were normally distributed with no heterogeneity of variance (Bartlett's χ^2=0.596-15.777, P=0.119-0.887), except in the case of 18:1ω9 in free fatty acid fraction (Bartlett's χ^2=44.75, P=0.044). A series of two sample t-tests (Table 3.27) indicated that the autoclaving process did not significantly alter the relative proportion of fatty acids in either the glyceride or free fatty acid fraction of oil.

3.3. Discussion

A variety of techniques were employed to quantify the effects of commercial inocula on the physico-chemical characteristics of fat/oils. The ability of the supplements to produce lipase and thus initiate the first stage in the lipid degradation process, hydrolysis, was investigated by means of a Sigma-diagnostic kit. The diagnostic kit indicated that all microbial supplements assayed were unable to produce a measurable quantity of lipase after the activation procedure. These results were unexpected as it is considered to be more unusual to find a microorganism that is unable to produce lipase than one that does (Ratledge 1994). Furthermore, F33 significantly enhanced the degradation of oil in a later experiment and must therefore have been capable of producing lipase and hydrolysing the oil.

The microbial supplements may have failed to produce a measurable quantity of lipase due to unfavourable experimental conditions. In many cases, the activation media was devoid of fat/oil and triglycerides which are known to stimulate lipase production (Ota *et al.*, 1968). The nutrients present in the activation media may also have been insufficient, particularly when the only supply of nutrients was from the microbial supplement itself (i.e. G40 and GTL). A more likely explanation for the failure of Sigma-diagnostic kit to detect lipase, however, was that the diagnostic kit was not sensitive to the levels of lipase produced by the bacteria. This was not unexpected given that the sigma-diagnostic kit was designed for assessing pancreatis and other clinical conditions.

Despite the apparent insensitivity of sigma diagnostic kit to microbial lipase, there was evidence to suggest that there was a positive relationship between the lipase activity and microbial growth. These results contradict the work of Alford and Pearce (1963), however, who found that the quantity of lipase or rate of lipase production was not a function of cell growth or concentration.

The ability of the microbial supplements to mineralise fat/oil was assessed gravimetrically and by COD determinations. Gravimetric analysis of the fat/oil degradation process indicated that only one multi-strained product was capable of significantly enhancing oil degradation (ca.15% of oil degraded). None of the other multi-strained products; (GTL and S45) or single strained microbial product (G40 and P80), investigated had any significant effect on fat/oil degradation.

The failure of the commercial inocula to significantly enhance the degradation of oil except on one occasion was unexpected given that single or multi-strained microbial or fungal inocula have been shown to improve oil degradation rates under laboratory conditions (Koritala *et al.*, 1987; Funtikova *et al.*, 1999; Tano-Debrah *et al.*, 1999; Mihara *et al.*, 2000) and have achieved fat/oil degradation efficiencies as high as 95%.

Whilst the work of the above mentioned authors cannot be compared directly to this study due to differences in experimental set-up (i.e. nutrient media, oil concentration, incubation temperature), it was known that these workers had encouraged microbial growth on oil through the provision of nutrients and the shaking of samples during incubation. Several experiments in this study, however, were design to mimic conditions in the sewers or grease trap therefore an essential requirement for the microbial oil degradation process may not have been met. F33 was incubated without shaking, which may explain why this supplement did not perform as well as the other inocula reported in the literature. Similarly, G40 was not shaken and the only source of nutrients in the media was the molasses in the activated supplement.

In the case of P80, GTL and S45, all the requirements for oil degradation, (i.e. nutrients and shaking), were met on at least one occasion. Attempts was also made to encourage these microbial supplements to degrade oil by adding surfactant and lipase and by adjusting the nutrient media and concentration of microbial supplement used. However, none of these microbial supplements were able to degrade oil under any of the conditions imposed. One reason why P80 and GTL may not have been capable of enhancing the degradation of oil was because extremely high oil concentrations were used (10%v/v). Whilst high oil concentrations have not presented a problem in other studies (Tano-Debrah *et al.*, 1999), they may have been detrimental to bacterial growth in this work by raising the carbon to nitrogen ratio of the sample to a high level (Lancelot & Billin, 1985) or by impeding the transfer of oxygen into the liquid below. Oil that was present at high concentrations may have also been poorly dispersed in the media as shaking appeared to have little effect. Some samples with high oil concentration were aerated but not shaken during incubation, which implies that poor dispersal of the oil in the media rather than oxygen limitation was the problem. If the availability of oxygen in the sample was limited, however, only microbial supplements comprised of both aerobes and anaerobes (i.e. F33, GTL and S45) would continue to degrade

oil. Generally, organic compounds degrade more rapidly under aerobic conditions (Bourquin, 1984) However, work with sediments has shown that the degradation of organic matter in anaerobic environments can proceed at a similar or more rapid rate to that in aerobic environments (Westrich & Berner, 1984; Henrichs & Reeburgh, 1987).

Another factor that may have contributed to poor oil degradation rates was the presence of contaminating bacteria in the samples. The effect of contaminants and grease trap bacteria on the performance of F33 was investigated in some detail. It was shown that F33 significantly enhanced the degradation of oil in sterile media but not under non-sterile conditions (i.e. in the presence of contaminants or grease trap bacteria). The interaction between commercial microbial supplements and microbial communities indigenous to a wastewater environment has never been demonstrated before. However, several authors have postulated that competition with other microorganisms is one reason why inocula fail to do in the field what they can do successfully in the axenic culture (Horsfall, 1979; Goldstein *et al.*, 1985; Stephenson & Stephenson, 1992).

The commercial microbial supplements may have been unable to compete with the environmental isolates for several reasons; (1) the environmental isolates may have been better adapted to the conditions found in the sample e.g. nutrient availability and oil levels. (2) the environmental isolates may have contained predators such as myxobacteria and cellular slime moulds which are able to feed on the microorganisms (Alexander, 1984). (3) the environmental isolates may secrete materials (e.g. bacteriocin) which are inhibitory to the growth of the microbial supplement (Parret & De Mot, 2002).

Unlike the majority of commercial microbial supplements investigated, the contaminants and grease trap isolates significantly enhanced the degradation of oil (ca. 17-27% of oil degraded). Their ability to degrade oil was appreciably reduced in the presence of F33, however, implying that the use of some microbial supplements in the field could be detrimental to grease control. The non-biological components of F33 (e.g. inhibitors, preservatives, perfumes, thickners, colourants) or the F33 microorganisms themselves may have inhibited the growth of the environmental isolates.

It may also be argued that some of the practical difficulties experienced during gravimetric analysis of fat/oil content may have masked the true extent of oil degradation in certain experiments and thus led to false conclusions. Emulsification of the solvent and aqueous phase during extraction was a frequently encountered problem and may have interfered with the ability of the solvent to extract the oil. Furthermore, the microbial supplements may have either contained or produced emulsifiers over the incubation period and thus increased the susceptibility of the solvent and aqueous phase to emulsification. Another problem encountered was particulate production and/or polymerisation of the oil which prevented representative sub-sampling from taking place in one experiment. The particulate material/polymer may have also been less extractable than the original oil due to the reduction in lipid surface area/volume ratio and a change in polarity.

Despite the fact that the gravimetric determinations of fat/oil content indicated that F33 significantly enhanced oil degradation, it was never established using the COD measurements, that F33 was capable of significantly enhancing the degradation of oil. It was observed, nonetheless, that the COD of samples inoculated with F33 either fluctuated or declined with time indicating that degradation of the oil/nutrient media was sometimes taking place. On several occasion, it was also shown that the reduction in COD was a result of oil degradation rather than the degradation of glucose or yeast, which were also present in the media.

The failure of F33 to significantly reduce the COD of the oil/nutrient media relative to a control, suggests that contaminating bacteria and/or physico-chemical process may have been responsible for many of the COD reductions observed or that the COD measurements did not accurately depict the degradative changes taking place. Indeed, it has been proposed by Grubbs (1983) that bioaugmentation could actually decrease the 'quality' of wastewater as measured by COD and BOD, since partial breakdown of oil results in shorter chained fatty acids which are more soluble and thus amenable to oxidation by the COD procedure (or microbial oxidation in case of BOD). The relative susceptibility of triglycerides and free fatty acids to oxidation by the COD procedure was not reported in the literature. However, in this study it was shown that increasing the relative proportion of free fatty acids in the sample via incubation with lipase did not significantly alter the COD.

The production of short chained fatty acids during oil degradation may also explain why the COD levels of some sample fluctuated or remained approximately constant whilst bacterial counts and oxygen consumption measurements indicated that bacteria were present and active. Another explanation for some of the unexpected COD changes observed was that it impossible to maintain a homogenous media and remove representative sub-samples due to the formation of 'particulate' material .

COD changes were generally assessed in mechanically or chemically emulsified oil/nutrient media. Oil degradation appeared to take place in both mechanically and chemically emulsified samples. However, chemical emulsification did not generate a completely homogenous media and the chemical emulsifier used contributed considerably to the COD. The effect of emulsification on degradation rates was investigated but since there was no degradation in either the non-emulsified oil sample (free-floating oil) or the mechanically/chemically emulsified oil samples, results were inconclusive.

The COD measurements also highlighted the importance of alternative carbon and/or organic nitrogen sources on the degradation of oil. Intermediate-high concentrations of yeast extract/glucose were essential for the degradation of intermediate-high concentrations of oil but had no significant effect on the degradation of oil present at low concentrations. It was impossible to establish whether it was the glucose or yeast extract enhancing oil degradation in this work. However, yeast extract was likely to be the most important substrate since a change in glucose/ammonium nitrate concentration appeared to have no effect on oil degradation rates. Furthermore, Tano-Debrah *et al.* (1999) demonstrated that oil degradation was enhanced by an organic form of nitrogen but not by glucose. This may relate to microbial lipase production/activity which is generally stimulated by organic sources of nitrogen (Alford & Pierce, 1963; Al-Saleh & Zahran, 1999) but may be enhanced (Elsawah *et al.*, 1995; El-Shafei & Rezkallah, 1997; Al-Saleh & Zahran, 1999; Lopes *et al.*, 1999) or inhibited (Alford & Pierce, 1963; Tsuzuki *et al.*, 1999) by glucose.

Yeast extract was thought to have more of an impact at intermediate-high oil concentrations than at low concentrations because the C/N ratios of these samples were raised and thus the nitrogen requirements of the bacteria were greater. It would appear that the ratio of organic nitrogen to carbon was more important than the ratio of mineral nitrogen to carbon in the

media since an excessive quantity of inorganic nitrogen was present in the media (KNO_3) and bacteria are known to take up mineral nitrogen from the media if organic nitrogen is not present in a sufficient amount (Lancelot & Billen, 1985). The availability of organic nitrogen in the media may have an important role in lipase production given that lipase production in some microorganisms is stimulated by organic sources of nitrogen but not by mineral nitrogen (Alford & Pierce, 1963).

Samples incubated with F33 experienced large changes in pH as the COD of the sample changed and the oil was degraded. Some samples experienced either a decrease or increase in the pH over the course of experiment, whilst others experienced an initial reduction in pH, followed by an increase in the pH over the remainder of the incubation period. Unlike the work of Tano-Debrah *et al.* (1999), the pH changes could not be related to the to the initial pH of the media and the final pH of the media was rarely in the neutral range (6.5-7.5).

Major metabolic processes may account for some of the pH changes that took place in the nutrient/oil media. Reductions in pH may arise from the production of fatty acids during the hydrolysis of oil (Desnuelle *et al.*, 1955) and/or the production of H^+ from the nitrification of ammonium ions (when present). An increase in pH, on the other hand, may have been due to the uptake of free fatty acids from the media and/or the release of the weak base; ammonia, during the ammonification of organic nitrogenous material. Nitrification may also explain why samples of high/intermediate glucose/ammonium nitrate concentrations experienced more dramatic reductions in pH than samples of low glucose/ammonium nitrate concentrations.

The oxygen consumption measurements, which were performed in conjunction with the pH and COD measurements indicated that the biodegradation of organic material was taking place in all of the samples investigated. The relationship between oxygen consumption and bacterial numbers/COD levels was unclear, indicating that oxygen consumption was not necessarily governed solely by the quantity of organic material present or number of bacteria but was more likely to be influenced by both.

As mentioned above, oil was sometimes observed to develop into an opaque, semi-solid sticky material, referred to as particulate material. Initially, this particulate material was

considered to be polymerised oil, as oil in other microbial degradation studies had been observed to solidified via polymerisation. Furthermore, GC-MS analysis revealed that particulate material, like the polymers examined by Mudge *et al.* (1994), was rich in 16:0 and 18:1ω9 relative to the original oil. It was later realized, however, that particulate material may not have simply been polymerized oil. Unlike the polymers observed in Mudge and co-workers degradation experiments, particulate material developed only in the presence of certain groups of microorganisms (i.e. S45, contaminants and the grease trap isolates) and was not of a 'used chewing gum-like' appearance, except on one occasion. Particulate production was therefore more likely to be a consequence of microbial activity than polymerisation, which is essentially an autoxidation process. Microbial degradation alone could account for the solidification of the oil since the preferential degradation of fatty acids with lower melting points (i.e. 18:2ω6 and 18:3ω3) would result in lipoidal material that was enriched in fatty acids with higher melting points (i.e. 18:1ω9, 16:0 and 18:0).

It was postulated that solidification of the oil by bacteria would exacerbate the problems in drains and sewers, as solid material was more likely to create blockages than a liquid. Particulate material was also fairly sticky in nature and was therefore more likely to adhere to the sides of the drains and sewers than the original oil. One of the microbial formulations investigated (F33), however, did not generate particulate material during incubation with the oil and, on at least one occasion, significantly reduced the quantity of particulate material produced by the contaminants and grease trap isolates. The same microbial supplement also demonstrated a significantly ability to reduce the quantity of particulate material adhering to glass on some occasions, but not plastic. These results implied that F33 may reduce the likelihood of blockages in drains and sewers by reducing the ability of the indigenous microbial community to solidify the oil. Given that autoclaving had no significant effect on the ability of F33 to reduce the production of particulate material, it was postulated that either the F33 microorganisms were not responsible for inhibiting particulate production or that a material produced by the F33 microorganisms prior to autoclaving was exerting an inhibitory effect. If the F33 microorganisms were not responsible for the reduction in particulate production, one of the non-biological components of F33 (e.g. preservative, thickener, perfume) must have reduced the growth or activity of the contaminants and grease trap bacteria and thus hindered their ability to produce particulate material.

Although F33 significantly enhanced the degradation of oil on one occasion, GC-MS analysis of oil indicated that none of the microbial supplements investigated (F33 and G40) significantly altered the composition of the glyceride or free fatty acid fraction of the oil relative to sterile control. Only the contaminants and grease trap isolates investigated (both in the presence and absence of F33 and S45), were observed to induced significant compositional changes.

Compositional changes were evident in both the glyceride fraction and free fatty acid fraction of the oil or the two fractions of the oil combined. In the case of glyceride fraction of the oil, the contaminants (alone and combined with F33), resulted in a decline in the relative proportions of 18:2ω6 and 18:3ω3, an increase in the proportion of 16:0, 18:0, and an increase or decrease in the proportion of 18:1ω9. These results implied that 18:2ω6 and 18:3ω3 were being hydrolysed more rapidly than 16:0 and 18:0 and reflected either the specificity of the lipase towards 18:2ω6 and 18:3ω3 or a specificity of the lipase towards the positions at which the majority of 18:2ω6 and 18:3ω3 fatty acids were situated in the triglyceride molecule. Although there are some fungal lipases which catalyse the hydrolysis of fatty acyl substituents with a double bond at the 9,10 position (Alford *et al.*, 1964; Ratledge, 1994) it was unlikely that this particular lipase was responsible for the changes observed since 18:1ω9 would have been hydrolysed at a similar rate to 18:2ω6 and 18:3ω3. A lipase specific towards the position at which the majority of 18:2ω6 and 18:3ω3 fatty acids were situated in the triglyceride molecule was also improbable given that unsaturated fatty acids tend to be situated in the central position of a triglyceride (Gurr & Harwood, 1991) and no microbial lipases specific for this central position have been reported on before.

In contrast to the contaminants, F33 alone was capable of mineralizing the oil but did not significantly alter the relative proportion of fatty acids in the glyceride fraction which suggests that the lipase produced by the F33 microorganisms was non-specific. G40, on the other hand, was not capable of mineralizing the oil, therefore the absence of any compositional changes in the glyceride fraction of oil was probably due to G40's inability to degrade oil.

The composition changes observed in the free fatty acid fraction of the oil and also in the glyceride and free fatty acid fraction combined, were similar to those observed in the

glyceride fraction alone. The contaminants and grease trap isolates (alone and combined with F33/S45) induced a decline in the proportion of 18:2ω6 and 18:3ω3, an increase in the proportion of 16:0 and 18:0 and an increase or decrease in the proportion of 18:1ω9. These results imply that the contaminants were degrading 18:2ω6 and 18:3ω3 more readily than 18:0 and 16:0 and that 18:1ω9 was degraded at a similar or faster rate than 18:0 and 16:0. This is in accordance with the results of several other studies which have shown that unsaturated fatty acids are more reactive than saturated fatty acids of a similar chain length (Parker & Leo 1965; Loehr & Roth, 1968; Malaney & Gerhold, 1969; Farrington & Quinn 1971; Sun & Wakeham 1994). Several theories have been proposed to account for the rate differential between saturated and unsaturated fatty acids. Loehr and Roth (1968) suggested that because saturated fatty acids are less soluble than unsaturated fatty acids of a similar size they are less accessible to microbial cell and are thus degraded at a slower rate. Work with heart mitochondria, by contrast, indicates that the long chain acyl-CoA synthetases are more active towards the unsaturated fatty acids (Gurr and Harwood, 1991) suggesting that the β-oxidation may be controlled by biochemical rather than physico-chemical effects.

The rise in the proportion of 16:0, 18:0 and 18:1ω9 in the free fatty acid fraction of the oil, reflects not only the preferential degradation of 18:2ω6 and 18:3ω3 but may also imply that 16:0, 18:0 and 18:1ω9 were produced from the hydrogenation and partial β-oxidation of 18:3ω3 and 18:2ω6. The production of these fatty acids from 18:2ω6 and 18:3ω3 has been observed in other studies (Novak & Carlson, 1970; Angelidaki & Ahring 1995; Pereira, 1999; Lalman & Bagley, 2000, 2001) and is thought to indicate that either the microorganisms capacity to process intermediates has been saturated or that other microorganisms are required in the media to degrade the intermediates produced (Lalman & Bagley, 2000, 2001). In the case of F33 alone, the free fatty acid fraction was not significantly different to that of the controls, indicating that there was no preferential degradation of fatty acids and that the F33 microorganisms were better able to process the intermediates produced.

It might also be argued that the compositional changes observed in both the glyceride and free fatty acid fraction of soya oil were the result of autoxidation. Unsaturated fatty acids and their esters react with molecular oxygen more readily than their saturated counterparts (Gunstone 1958) and some autoxidation processes such as polymerization, result in the loss of a double bond in the acyl group of a triglyceride molecule to form a more saturated acyl group (Mudge

et al., 1994; Dobarganes & Marquez-Ruiz, 1996). If polymerization was responsible for the compositional changes observed in this study, however, the polymers analysed must have been of similar polarity to the glycerides or free fatty acids otherwise they would not have been extracted and eluted along with these two fractions of the oil. Furthermore, the derivatization agent (BF_3-MeOH) must have been capable of cleaving the inter- and intra-molecular bonds formed in the polymers.

Oil that was incubated with the contaminants and grease trap isolates also experienced an increase in the proportion of 'other' fatty acids. This increase may have reflected a reduction in the proportion of some of the major fatty acids or the production of new fatty acids. The 'new' 18:2ω? and 18:3ω? isomers detected had longer retention times than 18:2ω6 and 18:3w3 indicating that conjugated tri/diene systems may have been present (Pereira, 1999). Conjugated 18:3ω3 and 18:2ω6 isomers are produced by rumen microorganisms during the first stage in the hydrogenation process; isomerisation (Kepler *et al.*, 1970, Verhulst *et al.*, 1986, McInerney, 1988) implying that some of the 18:1ω9, 16:0 and 18:0 in this study may have been produced via the hydrogenation of 18:2ω6 and 18:3ω3. The F33 microorganisms appeared to be limiting the grease trap isolates ability to produce 18:2ω? and 18:3ω? isomers and may thus have been reducing the capacity of the grease trap isolates to hydrogenate 18:2ω6 and 18:3ω3. This may explain why F33 was sometimes able to inhibit the production of particulate material, which is rich in saturated fatty acids relative to the raw oil.

Shorter chained unsaturated fatty acids (16:1ω? and 14:2ω?) were also detected a few samples indicating that β-oxidation of 18:1ω9, 18:2ω6 or 18:3ω3 was taking place. The failure to detect β-oxidation intermediates in other samples was not unexpected, however, since the reaction occurs in the cell (Lalman & Bagley, 2000). In addition to the β-oxidation intermediates, other new fatty acids (3-hydroxy fatty acids, 10-hydroxy fatty acid and 9-oxo nonanoic acid) were detected in the oil. It was not known whether these fatty acids were the result of autoxidation processes or bioconversion activity. Hydroxy fatty acids have been known to arise from the autoxidation of oils (Nawer, 1984; Dobarganes & Marquez-Ruiz, 1996), which may explain why 10-OH was detected in fried oil. However, there have also been several reports of microorganisms converting oleic acid to 10-hydroxyocatadecanoic (El-Sharkawy *et al*, 1992; Koritala & Bagby, 1992; Lanser *et al*, 1992) and 3-OH fatty acids

are known to arise as intermediates in fatty acid biosynthesis and are also characteristic of the lipopolysaccharide found in the cell envelope in Gram-negative bacteria (Schweizer, 1989). There are no reports of microorganisms synthesising 9-oxo nonanoic acid from other fatty acids, therefore the most likely source of 9-oxo nonanoic acid is autoxidation. This assumption is further supported by the fact that the carboxylic acid derivative of 9-oxo nonanoic acid; nonanedioic acid (azaleic acid), has been detected during the autoxidation of methyl oleate (Gunstone, 1958). Autoxidation processes are thought to prevail during frying or when oil is heated (Gere, 1982; Nawer, 1984; Dobarganes & Marquez-Ruiz, 1996; Martin *et al.*, 1998) However, neither frying nor autoclaving had any significant impact on the relative proportions of the major fatty acids in oil.

4. FACTORS AFFECTING THE EFFICACY OF COMMERCIAL INOCULA

4.1. Introduction

Several factors may influence a commercial microbial supplement's capacity to modify the physico-chemical characteristics of fat/oil.

Wastewater characteristics vary both spatially and temporarily (Holmes *et al.*, 1963; Metcalf & Eddy, 1991), thus a commercial inocula must remain viable over a range of temperatures, pH's, oxygen and nutrient levels if it is to be effective.

In order to initiate the degradation of fat/oil, a microbial supplement must also be capable of producing lipase and numerous factors have been described which influence the production and activity of microbial lipases. Alford and Pierce (1963) established that the addition of an organic source of nitrogen to the growth media stimulated lipase production in *Pseudomonas fragi* whilst inorganic nitrogen sources had no affect. Various workers have demonstrated that the addition of glucose to the growth media improved lipase production in some microorganisms, including those of the genus *Bacillus* and *Lactobacillus* (Alford & Pierce, 1963; Elsawah *et al.*, 1995; El-Shafei & Rezkallah, 1997; Al-Saleh & Zahran, 1999; Lopes *et al.*, 1999) but reduced or inhibited lipase production and activity in others (Alford & Pierce, 1963; Tsuzuki *et al.*, 1999). Marek and Bednarski (1996), reported that the activity of intra- and extracellular lipases increased with increasing lipid concentrations. However, the work of Obradors *et al.* (1993) implied that excessive lipids levels in the growth medium may be cytotoxic. Earlier workers also demonstrated that unsaturated triglycerides were good inducers of lipase production whilst the saturated triglcyerides and fatty acids were not (Ota *et al.*, 1968). In addition, several authors have shown that the addition of Ca^{2+} ions to the growth media enhanced the activity of *Bacillus*, *Lactobacillus* and *Pseudomonas* sp. lipase (Smith & Alford, 1966; Lesuisse *et al.*, 1993; Gobbetti *et al.*, 1996) but that other metal ions such as Hg^{2+} exerted an inhibitory effect. Helisto and Korpela (1998) established that detergents such as Tween-20, Trition X-100, polyvinyl alcohol and linear alkylbenzene sulfonate inhibited the activity of eukaryotic lipases but that *Bacillus* sp. lipase was tolerant to high concentrations of these detergents.

Aside from nutrients, metal salts and detergents, other media conditions such as pH, temperature and oxygen availability have been known to influence the production and activity of lipase. The effect of temperature and pH on lipase production and activity is difficult to generalize upon. Optimum temperatures and pH's for lipase production/activity are strain dependent and thus highly variable. In some microorganisms, the production of lipase is unrelated to the temperature of the media (Alford *et al.*, 1971) whilst in others lipase production is temperature dependent (Lopes *et al.*, 1999). The effect of dissolved oxygen concentration and aeration on the production of lipase is similarly variable. Several authors have reported that lipase production, particularly in *Bacillus* sp. is enhanced by stirring, shaking and/or aerating cultures (Ota *et al.*, 1968; Savitha & Ratledge, 1992; El-Shafei *et al.*, 1997). However, the production of lipase in other bacilli species has not been linked to specific aeration requirements (El-Hoseiny, 1986). Furthermore, there is evidence of anaerobic lipase activity in rumen studies and of vigorous aeration reducing lipase production and its accumulation (Alford *et al.*, 1971). In some bacteria, lipase production also depends more extensively on the rate of oxygen transfer than on dissolved oxygen concentrations (Chen *et al.*, 1999).

Given that wastewater conditions vary widely and that the lipolytic activities of different microorganisms under similar conditions are rather variable, wastewater might be expected to enhance or inhibit the production/activity of microbial lipases.

Once hydrolysis of the fat/oil has taken place, the fatty acids must be transferred from the lipoidal phase to the microbial cell (Ratledge, 1994). It has been reported that the mass transfer situation may be improved either through the addition of surfactants (Sekelsky & Shreve, 1999), saponifiers (Lefebve *et al.*, 1998) or by using thermophilic lipid degrading microorganisms and raising the temperature of the media (Becker *et al.*, 1999). However, the impact of surfactants on the performance of commercial supplements has not been reported on before.

Some microorganisms are also capable of producing their own surfactants, which may assist in the uptake of fatty acids. *Bacillus subtilis* is known to produce surfactin, one of the most effective biosurfactants known (Cooper *et al.*, 1981). Maximum surfactant production in this

species has been achieved when nitrate ions provided the nitrogen source. Yields have also been improved by the addition of either iron or manganese salts to the media. Sodium acetate and hydrocarbons, on the other hand, have been known to inhibit biosurfactant production in this Bacillus species (Cooper *et al.*, 1981; Makkar & Cameotra, 1998; Wei & Chu, 1998).

The extent and rate at which fatty acids are degraded by microorganisms also depends upon the degree of saturation, chain length and solubility of the fatty acid. Degradation studies have shown that amongst the various soluble fatty acids classes unsaturated fatty acids are degraded more rapidly than saturated fatty acid (Parker & Leo, 1965; Loehr & Roth, 1968; Malaney & Gerhold, 1969; Farrington & Quinn, 1971; Sun & Wakeman 1994). Furthermore, within the saturated fatty acids, short chain lengths have higher rates of degradation than longer chained ones (Loehr & Roth, 1968). In wastewater, insoluble fatty acid calcium salts often predominate therefore the rate of fatty acid degradation is governed by substrate surface area rather than fatty acid structure (Loehr & Roth, 1968).

If the uptake and oxidation of fatty acids is not particularly rapid the fatty acids released by hydrolysis may accumulate and inhibit microbial activity (Becker & Markyl, 2000). Numerous authors have reported that fatty acids are inhibitory to the growth of bacteria (Smith & Alford, 1966; Ferdinandus & Clark, 1969; Steffen & Calvin, 1971; Galbraith *et al.*, 1971; Galbraith & Miller, 1973abc; Butcher *et al.*, 1976) and the common feature apparent from these studies was that gram positive bacteria were more sensitive to the antibacterial effects of long chained fatty acids than gram negative bacteria. Given that several commercial inocula are comprised largely of gram positive bacteria (Appendix VII), commercial inocula may be particularly susceptible to this problem.

Fatty acid toxicity is also influenced by the degree of saturation, solubility and chain length of the fatty acids. Galbraith *et al.* (1971) determined that fatty acid toxicity became more pronounced as the compounds became more unsaturated and that the antibacterial activity displayed by saturated fatty acids above C_6 is in the order of $C_8 < C_{10} < C_{12} \geq C_{14} > C_{16} \geq C_{18}$. These workers further established that the addition of cholesterol, Ca^{2+}, Mg^{2+}, Fe^{2+} and Sn^{2+} reversed the inhibition of fatty acid sodium salts by forming insoluble salts and increasing interfacial tension. The mechanism of long chained fatty acid toxicity is thought to relate to

the adsorption of surface active fatty acids to the microbial cell wall which affects its transport and/or protective functions (Galbraith & Miller, 1973 abc).

Given that microbial fat/oil degradation is not instantaneous and that commercial microbial supplements may require a period of acclimatisation before grease degradation can commence (Stephenson & Stephenson, 1992), the efficacy of a microbial inocula in dynamic environments such as drains sewers and grease traps, may depend to some degree on the ability of the microorganisms to adhere to surfaces.

Several factors have been described which effect biofilm formation in aqueous environments. Rogers *et al.* (1994) and Niquette *et al.* (2000) reported that the characteristics of the material composing the pipes used in drinking water distribution systems greatly affect cell attachment and growth. In general, biofilms are encouraged to develop on materials that are able to supply nutrients, as is the case with latex. Plastic surfaces also tend to leach metal ions at non-toxic levels and may furnish cations essential for enzyme function. Materials that are known to encourage cell attachment and biofilm growth in wastewater are found in biological treatment systems, such as biological aerated filters, packed bed reactors and trickling filters. The biofilm support media used in these systems generally have large surface areas and are comprised either of expanded polystyrene, porous stone, expanded shale, high voidage plastic media, granulated activated carbon or brown coal (Pujol *et al.*, 1992; Ros *et al.*, 1992).

Surface roughness is another major cause of enhanced colonisation (Gilbert *et al.*, 1993), although it does not appear to govern the total amount of slime formed (Characklis, 1973). Rough materials encourage bacterial growth by providing attachments sites that are protected from shear forces (Gilbert *et al.*, 1993). Some rough materials, for example corroded iron, may also offer protection against chemical oxidants such as chlorine (Niquette *et al.*, 2000).

The characteristics of the aqueous media may also influence the extent of biofilm formation. Heukelekian and co-workers reported that the nature and availability of organic nutrients had a considerable impact on biofilm growth (Heukelekian & Crosby, 1955; Heukelekian, 1956). These workers observed that dense, readily adhering slimes were characteristic of low concentrated nutrient solutions while abundant slimes which readily slough were more

representative of liquids high in oxidizable material. They also noted that slime growth in sewage was enhanced in the presence of glucose and/or peptone.

Velocity and shear forces have also been known to affect biofilm growth. High velocity flows tend to impede initial biofim colonisation, but enhance growth once the biofilm is established. Those films that are grown at higher velocities also adhere to surfaces more firmly (Characklis, 1973).

The impact of temperature on biofilm formation is unclear and several temperature optimums for slime growth have been reported. According to Farrel and Rose (1967) the optimum temperature for polysaccharide synthesis is often lower than that for growth of the microorganisms, which could account for the observed temperature optimums for slime growth being somewhat lower than the optimum temperatures for suspended bacterial growth.

The effect of pH on slime growth is similarly contradictory. Reid and Assenzo (1960) reported that biofilm growth is optimal when pH is maintained near neutrality. However, Lindsay *et al.* (2000) demonstrated that the attachment of *B. subtilis* 115, *B. pumilis* 122 and *B. lichenformis* 137 to stainless steel was enhanced at pH 4 and 10 relative to pH 7.

The main objectives of the work outlined in the following chapter were to:

- Establish whether commercial inocula were capable of growth under a variety of environmental conditions.
- Determine how fat/oil composition affected lipase production/activity and microbial growth.
- Establish whether the commercial inocula were capable of adhering to surfaces, including that of fat/oil.

4.2. Results

4.2.1. Growth of commercial inocula under a variety of environmental conditions

To determine whether the commercial inocula had the potential to operate in a wastewater environment, the ability of selected microbial supplements to grow in wastewater over a wide range of environmental conditions was investigated.

Growth of formulation 1-7 on synthetic sewage media

The growth of the 7 different formulations on synthetic sewage media 1 at 30°C is shown in Table 4.1.

Table 4.1a. Optical densities at 600nm (mean ± SD) (a measure of microbial growth) of synthetic sewage media 1 inoculated with microbial formulations 1-7 incubated for 3 days at 30°C with shaking at 130rpm.

Formulation	OD-600
1	0.113 ± 0.016
2	0.122 ± 0.014
3	0.076 ± 0.006
4	0.120 ± 0.020
5	0.092 ± 0.009
6	0.148 ± 0.004
7	0.132 ± 0.008

Table 4.1b. Tukey's pairwise comparisons of the mean difference (column-row) of OD-600 in synthetic sewage media 1 inoculated with microbial formulations 1-7. 95% confidence interval of any difference = 0.034. *significant differences

	1	2	3	4	5	6
2	-0.010	-	-	-	-	-
3	0.037*	0.047*	-	-	-	-
4	-0.007	0.003	-0.044*	-	-	-
5	0.021	0.030	-0.016	0.028	-	-
6	-0.035*	-0.025	-0.072*	-0.028	-0.056*	-
7	-0.019	-0.010	-0.056*	-0.012	-0.040*	0.016

The low positive optical densities (OD-600; 0.076-0.148) implied that all 7 microbial communities were capable of only poor growth on synthetic sewage.

The residuals of the data for each formulation were approximately normally distributed and a one-sample sign test established that the bacterial densities of all formulations were greater than zero (T=10.56-67.56, P=0-0.009) indicating that significant microbial growth had occurred in synthetic sewage media 1. The residuals of the combined data set were also normally distributed with no significant heterogeneity of variance (Bartlett's χ^2=5.866, P=0.438) and a one-way ANOVA confirmed that there were significant differences between the bacterial densities of the formulations (F=12.14, P<0.001). Tukey's pairwise comparisons (Table 4.1) indicated that optical densities of samples inoculated with formulation 3 were significantly lower than those inoculated with formulations 1, 4, 6 and 7. The optical densities of samples inoculated with formulation 5 were also significantly lower than those inoculated with formulation 6 and 7 and formulation 6 resulted in a significantly greater optical densities than formulation 1.

In the following experiments the growth of only formulations 1, 6 and 7 was examined due to time restraints. Formulations 1, 6 and 7 were selected for comparison since they had demonstrated different capacities for growth on synthetic sewage media and lipase activity (section 4.2.2). (formulations 6; good growth and lipase activity, formulation 1; poor growth and lipase activity, formulation 7; good growth and poor lipase activity)

Effect of nutrients on the growth of formulations 1, 6 and 7

Growth of the microbial supplements on synthetic sewage media was relatively low and therefore the effect of various organic carbon/nitrogen supplements on microbial growth was investigated (Figure 4.1). Peptone, hydrolysed starch and glucose were selected for comparison since similar nutrients might be expected to arise in effluent derived from kitchens.

The response of the microbial formulations to changes in nutrient composition was similar. Of the nutrient supplements investigated, only peptone and glucose appreciably enhanced the optical density (by a factor of 2.8-3.5). Elevated peptone concentrations resulted in the greatest optical densities and there appeared to be a strong positive relationship between the

concentration of peptone used and bacterial growth. The optical densities of the glucose supplemented samples were greater than those of samples containing no additional nutrients (synthetic sewage salt media 2) indicating that an organic form of nitrogen was not essential for microbial growth. However, the optical densities of samples supplemented with 0.5g.l^{-1} and 0.25 g.l^{-1} peptone were in excess of those supplemented with glucose (0.5 g.l^{-1}) which strongly suggested that an organic supply of nitrogen was more favourable towards growth.

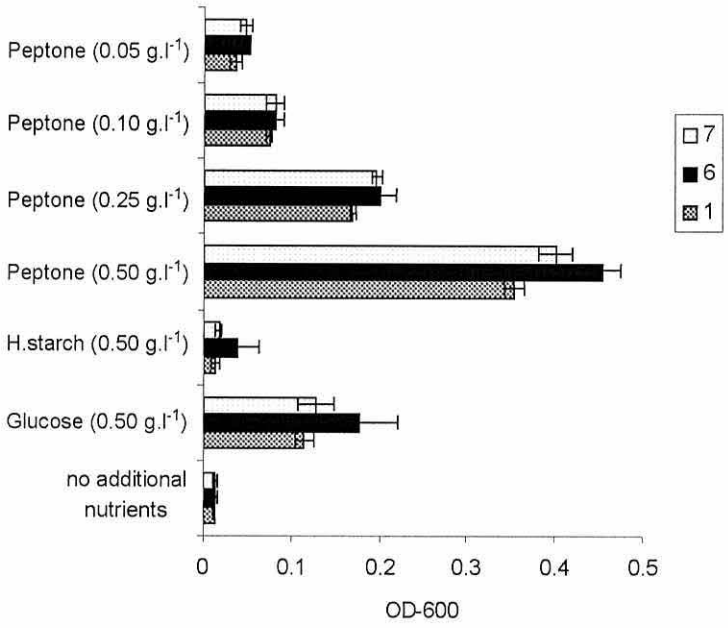


Figure 4.1. Optical density at 600nm (mean ± SD) (a measure of microbial growth) of synthetic sewage media 2 supplemented with either peptone (0.05-0.50g.l^{-1}), glucose (0.50 g.l^{-1}) or hydrolysed starch (H.starch) (0.50 g.l^{-1}) inoculated with formulations 1, 6, or 7 and incubated for 6 days at 30°C with shaking at 130rpm.

The residuals of the transformed data ($\log_{10}$) were approximately normally distributed and there was no significant heterogeneity of variance (Levene's statistic=0.660, P=0.841). A two-factor ANOVA established that was significant interaction between factors (F=2.05, P=0.044) and that the optical density of the sample was significantly affect by both the nutrient supplement (F=343.64, P<0.001) and microbial formulation used (F=15.01, P<0.001). Bonferoni's selected pairwise comparisons (Table 4.2) indicated that all nutrient supplements except hydrolysed starch inoculated with formulations 1 and 7 and peptone (0.05 g.1^{-1}) inoculated with 1 and 6, significantly enhanced the optical densities of the samples relative to the control (no additional nutrients). 0.5 g.1^{-1} of glucose also resulted in optical densities that were significantly lower than 0.5 g.1^{-1} peptone but not of 0.25 g.1^{-1}, for all formulations. Further pairwise comparisons revealed that there were no significant differences

between the optical densities of the formulations in media supplemented with peptone (0.5 $g.l^{-1}$). A series of Pearson's correlations also confirmed that there was a significant relationships between the concentration of peptone used and optical density of the sample (formulation 1; r=1.00, P<0.001, formulation 6; r=1.00, P<0.001, formulation 7; r=1.00, P<0.001).

Table 4.2. Bonferoni's selected pairwise comparisons of the mean difference of OD-600 (log_{10}) in synthetic sewage media 2 supplemented with either peptone (0.05-0.50$g.l^{-1}$), glucose (0.50 $g.l^{-1}$) or hydrolysed starch (H.starch) (0.50 $g.l^{-1}$) inoculated with formulations 1,6, or 7 and incubated for 6 days at 30°C with shaking at 130rpm. 95% confidence interval of any difference = 0.235. *significant differences

Pairwise Comparison	Difference in means
Formulation 1; (Glucose) & no additional nutrients	0.948*
Formulation 1; (H. starch) & no nutrients	-0.016
Formulation 1; (Peptone, 0.50 $g.l^{-1}$) & no additional nutrients	1.437*
Formulation 1; (Peptone, 0.25 $g.l^{-1}$) & no additional nutrients	1.118*
Formulation 1; (Peptone, 0.10 $g.l^{-1}$) & no additional nutrients	0.763*
Formulation 1; (Peptone, 0.05 $g.l^{-1}$) & no additional nutrients	0.445
Formulation 6; (Glucose) & no additional nutrients	1.101*
Formulation 6; (H starch) & no additional nutrients	0.373*
Formulation 6; (Peptone, 0.50 $g.l^{-1}$) & no additional nutrients	1.151*
Formulation 6; (Peptone, 0.25 $g.l^{-1}$) & no additional nutrients	1.574*
Formulation 6; (Peptone, 0.10 $g.l^{-1}$) & no additional nutrients	0.776*
Formulation 6; (Peptone, 0.05 $g.l^{-1}$) & no additional nutrients	0.570
Formulation 7; (Glucose) & no additional nutrients	0.969*
Formulation 7; (H starch) & no additional nutrients	0.094
Formulation 7; (Peptone, 0.50 $g.l^{-1}$) & no additional nutrients	1.470*
Formulation 7; (Peptone, 0.25 $g.l^{-1}$) & no additional nutrients	1.161*
Formulation 7; (Peptone, 0.10 $g.l^{-1}$) & no additional nutrients	0.778*
Formulation 7; (Peptone, 0.05 $g.l^{-1}$) & no additional nutrients	0.545*
Formulation 1; (Glucose) & (peptone 0.50 $g.l^{-1}$)	-0.488*
Formulation 6; (Glucose) & (peptone 0.50 $g.l^{-1}$)	-0.413*
Formulation 7 (Glucose) & (peptone 0.50 $g.l^{-1}$)	-0.501*
Formulation 1; (Glucose) & (peptone 0.25 $g.l^{-1}$)	-0.170
Formulation 6; (Glucose) & (peptone 0.25 $g.l^{-1}$)	-0.056
Formulation 7; (Glucose) & (peptone 0.25 $g.l^{-1}$)	-0.192
Peptone 0.5 $g.l^{-1}$; formulation 1 & formulation 6	-0.110
Peptone 0.5 $g.l^{-1}$; formulation 1 & formulation 7	-0.056
Peptone 0.5 $g.l^{-1}$; formulation 6 & formulation 7	0.055

After determining that synthetic sewage media 2 supplemented with peptone ($0.5g.l^{-1}$) (synthetic sewage media 3) stimulated the greatest level of microbial growth, synthetic sewage media 3 was used to investigate the growth of the microorganisms under a variety of environmental conditions and in the presence of potential inhibitors.

Effect of starting pH on the growth of formulations 1, 6 and 7

The growth of the microorganisms in peptone supplemented synthetic sewage media at various starting pH's is shown in Figure 4.2. In general, the growth of the formulations was lower at extremes of pH (i.e. pH 3.1 and 11.2) than at intermediate pH's (i.e. pH 5.6-9.0) and strongly acidic environments (i.e. pH of 3.1) resulted in lower optical densities than strongly alkaline environments (i.e. pH of 11.2). Although the responses of the formulations to changes in pH were similar, the optical densities of samples inoculated with formulation 6 were marginally greater than those inoculated with the other formulations at higher pH's

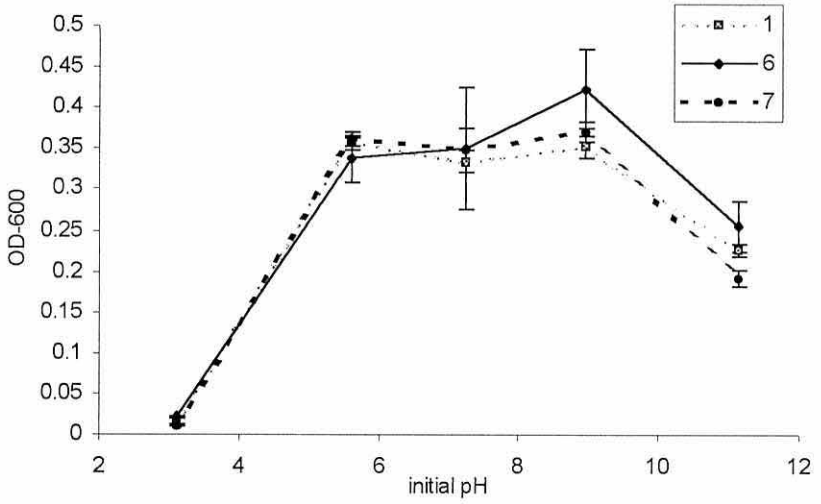


Figure 4.2. Effect of pH on the optical density at 600nm (mean ± SD) (a measure of microbial growth) of synthetic sewage media 3 inoculated with formulations 1, 6 or 7 incubated for 2 days at 30°C with shaking at 130rpm.

The residuals of the transformed data ($\log_{10}$) were approximately normally distributed, with no significant heterogeneity of variance (Levene's statistic=0.613, P=0.833). A two-factor ANOVA revealed that there was significant interaction between factors (F=11.6, P<0.001), and that varying the pH (F=2426.24, P<0.001) and inoculum source (F=26.06, P<0.001) significantly affected the optical density of the sample. Bonferoni's pairwise comparisons (Table 4.3) indicated that the optical density of formulation 6 was significantly greater than

that of formulation 7 at pH 11.2 and significantly greater than that of formulation 1 and 7 at pH 3.1

Table 4.3. Bonferoni's selected pairwise comparisons of the mean differences of the OD-600 (log_{10}) in synthetic sewage media 3, inoculated with formulations 1, and 7, incubated at different starting pH's for 2 days at 30°C with shaking at 130rpm. 95% confidence interval of any difference = 0.097. *significant differences

	pH3.1	pH5.6	pH7.2	pH9.0	pH11.2
1&6	-0.238*	0.027	-0.016	-0.078	-0.050
1&7	0.091	-0.002	-0.017	-0.023	0.070
6&7	0.329*	-0.028	-0.001	0.055	0.121*

The majority of inoculated samples also experienced a change in pH over the incubation period (Figure 4.3). Changes in pH appeared to be unrelated to the microbial supplement used and with the exception of those samples that had an initial pH of 3.1, the final pH of the inoculated samples was within 8.3 to 9.3. In general, inoculated samples that had an initial pH of 5.6 and 7.2 experienced an overall increase in pH whilst the pH of those samples that had an initial pH of 9.0 and 11.2 decreased. Compared with the corresponding inoculated sample, non-inoculated samples (physico-chemical controls) that had an initial pH of 9.0 and 11.2, experienced similar or marginally larger pH changes, which implied that the pH changes were the result of physico-chemical process. On the contrary, non-inoculated samples (physico-chemical controls) that had an initial pH of 5.6 and 7.2 experienced only a small change in pH, indicating that the microorganisms were responsible for the observed changes in pH.

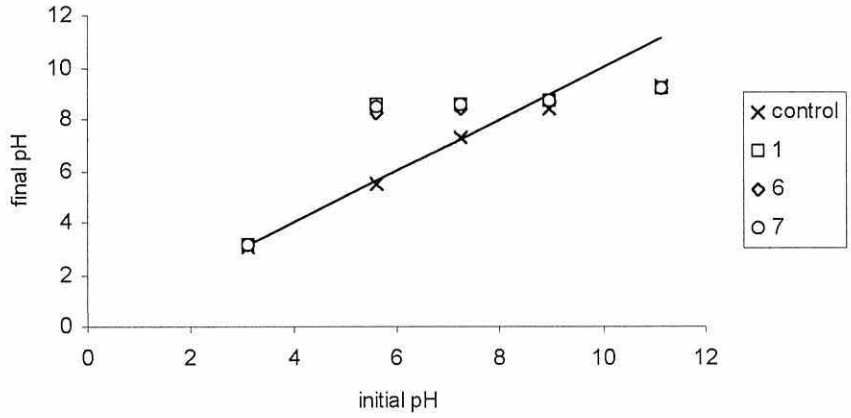


Figure 4.3. Average initial and final pH of synthetic sewage media 3 inoculated with formulations 1, 6 or 7 incubated for 2 days at 30°C with shaking at 130rpm (straight line represents no change).

Effect of temperature on the growth of formulations 1, 6 and 7

The effect of incubation temperature on the growth of formulations 1, 6 and 7 is shown in Figure 4.4.

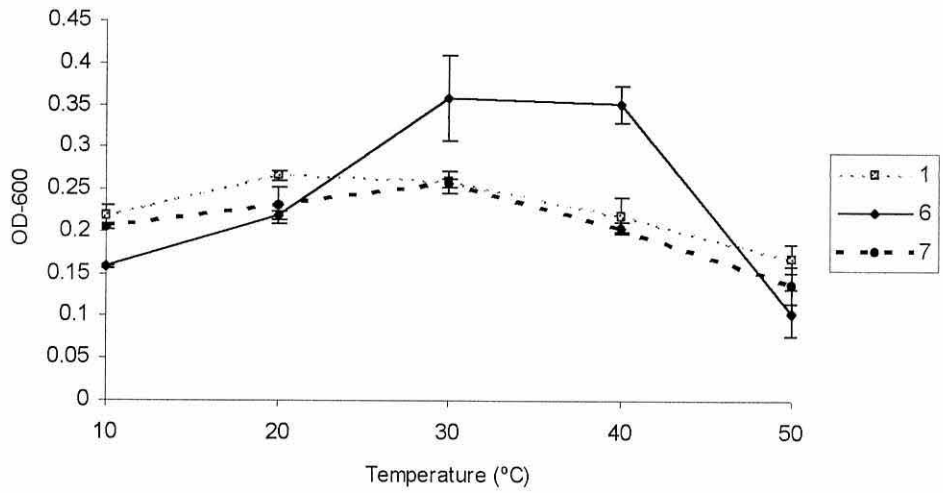


Figure 4.4. The effect of temperature on the optical density at 600nm (mean ± SD) (a measure of microbial growth) of synthetic sewage media 3 inoculated with formulations 1, 6 or 7, incubated for 6 days with intermittent shaking.

The optical densities of the formulations were greater at intermediate temperatures (20-40°C) than at extremes of temperatures (10°C and 50°C) and increasing the temperature above the optimum for growth (20°C in case of formulation 1 and 30°C in case of formulation 6 and 7) appeared to reduce the density of bacteria more markedly than lowering the temperature. At extremes of temperature and 20°C the optical densities of formulations 1 and 7 were marginally greater than those of formulation 6 whilst at 30°C and 40°C the optical densities of formulation 6 were considerably greater than those of formulation 1 and 7.

The residuals of the data were approximately normally distributed and there was no significant heterogeneity of variance (Levene's statistic=0.704, P=0.753). A two-factor ANOVA determined that there was significant interaction between factors (F=21.60, P<0.001) and that both temperature (F=83.74, P<0.001) and inoculum source (F=9.73, P=0.001) had a significant effect on optical density. Bonferoni's pairwise comparisons (Table 4.4) confirmed that at a temperature of 30°C and 40°C the optical densities of formulation 6 were significantly greater than those of formulation 1 and 7. At extremes of temperature

(10°C and 50°C), however, the optical density of formulation 6 was significantly lower than that of formulation 1.

Table 4.4. Bonferoni's pairwise comparisons of the mean differences of OD-600 in synthetic sewage media 3 inoculated with formulations 1, 6 or 7, incubated for 6 days at different incubation temperatures with intermittent shaking. 95% confidence interval of any difference = 0.052. *significant differences

	10°C	20°C	30°C	40°C	50°C
1&6	0.061*	0.046	-0.099*	-0.132*	0.064*
1&7	0.013	0.035	0.003	0.016	0.031
6&7	-0.048	-0.011	0.102*	0.148*	-0.032

Effect of oxygen availability on the growth of formulations 1, 6 and 7

The effect of oxygen availability on the growth of formulations 1, 6 and 7 is depicted in Figure 4.5. By limiting the availability of air in the samples the optical densities of all three formulations were appreciably reduced (by 37.7-62.3%). The optical densities of all three formulations were similar in the presence of little or no air, (0.14-0.16) but under oxic conditions, the optical densities of formulation 6 were considerably greater than those of the other formulations (by a factor of 1.64-1.72).

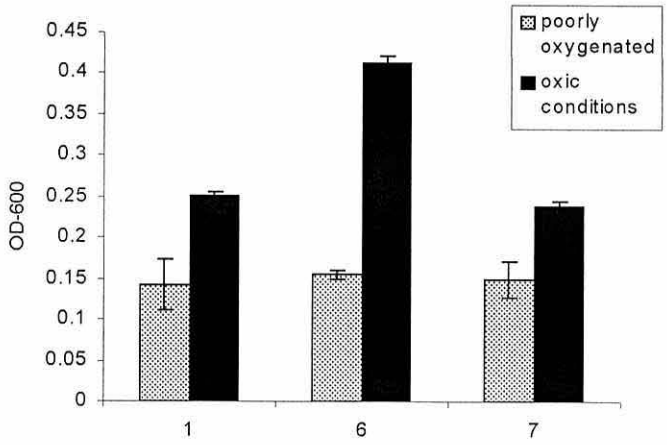


Figure 4.5. The effect of oxygen availability on the optical density at 600nm (mean ± SD) (a measure of microbial growth) of synthetic sewage media 3 inoculated with formulations 1, 6 or 7 incubated for 6 days at 30°C with intermittent shaking.

The residuals of the data were normally distributed and there was no significant heterogeneity of variance (Bartlett's χ^2=9.05, P=0.107). A two-factor ANOVA confirmed that there was significant interaction between factors (F=44.27, P<0.001) and that the level of oxygenation (F=368.36, P<0.001) and inoculum source (F=54.88, P<0.001) had a significant effect on

optical density. Bonferoni's pairwise comparisons (Table 4.5) confirmed that by limiting the availability of oxygen in the samples, the optical densities of all formulations were reduced. Further pairwise comparisons indicated that the optical density of formulation 6 was significantly greater than that of formulation 1 and 7 under aerated conditions. However, there were no significant differences in the optical densities of the microbial formulations in the presence of little or no air.

Table 4.5. Bonferoni's pairwise comparisons of the mean difference of OD-600 in synthetic sewage media 3 inoculated with formulations 1, 6 or 7 incubated for 6 days at 30°C with intermittent shaking. 95% confidence interval of any difference = 0.046 *significant differences

Pairwise comparison	Difference in means
Formulation 1 poorly oxygenated & oxic	-0.109*
Formulation 6 poorly oxygenated & oxic	-0.257*
Formulation 7 poorly oxygenated & oxic	-0.090*
Poorly oxygenated formulation 1 & formulation 6	-0.013
Poorly oxygenated formulation 6 & formulation 7	0.006
Poorly oxygenated formulation 1 & formulation 7	0.007
Oxic formulation 1 & formulation 6	0.161*
Oxic formulation 6 & formulation 7	0.172*
Oxic formulation 1 & formulation 7	0.012

Effect of bleach on the growth of formulations 1, 6 and 7

The effect of different bleach concentrations on the growth of formulations 1, 6 and 7 is shown in Figure 4.6.

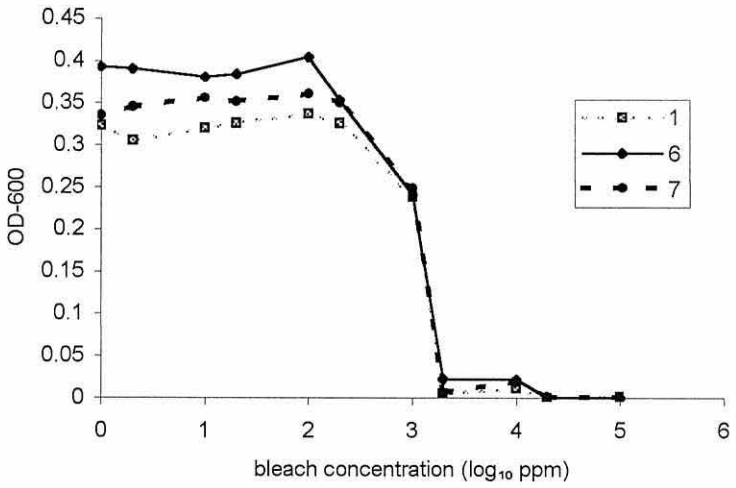


Figure 4.6. The effect of bleach concentration (co-operative brand) on the optical density at 600nm (mean ± SD) (a measure of microbial growth) of synthetic sewage media 3 inoculated with formulations 1,6 or 7 incubated for 3 days, at 30°C with shaking at 130 rpm.

The optical densities of all three formulations were affected markedly by changes of beach concentration. Optical densities increased marginally or fluctuated as the concentration of bleach was raised from 0 to 2.0 $\log_{10}$ ppm and increasing the bleach concentration from 2.0-3.33 $\log_{10}$ ppm drastically reduced the optical density of all formulations by 94.6-98.5%.

Effect of emulsifiers/surfactants on the growth of formulations 1, 6 and 7

The effect of emulsifiers/surfactant concentrations on the optical densities of the formulations is illustrated in Figure 4.7. The optical densities of all three formulations generally declined with increasing concentrations of domestic emulsifier/surfactant and the reduction in optical density was most marked in formulation 6 over a concentration range of 0-2 $\log_{10}$ ppm. The effect of increasing the commercial surfactant concentration was similar to that of the domestic surfactant over a concentration range of 0-2 $\log_{10}$ ppm. However, increasing the concentration of commercial surfactant above 2 $\log_{10}$ ppm resulted in fluctuating and/or increasing optical densities.

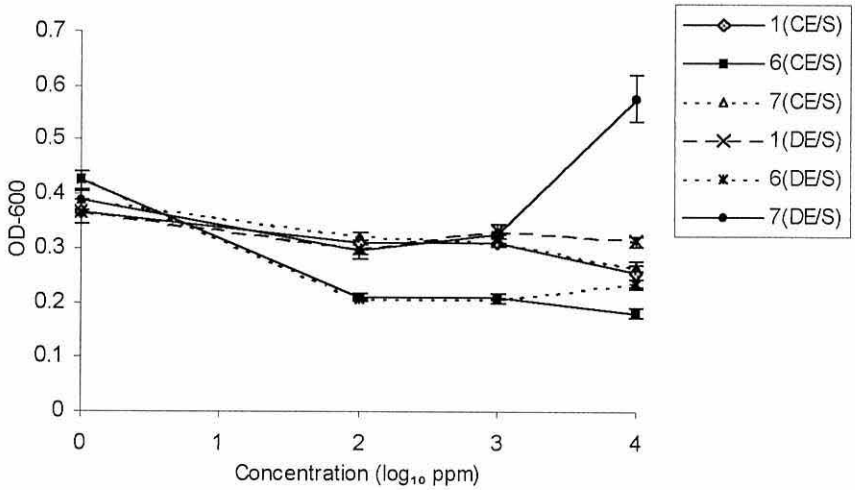


Figure 4.7. Effect of commercial and domestic emulsifiers/surfactants (CE/S and DE/S) on the optical density at 600nm (mean ± SD)(a measure of microbial growth) of synthetic sewage media 3 inoculated with formulations 1, 6 and 7 incubated for 3 days at 30°C with shaking at 130rpm.

The residuals of the transformed data ($\log_{10}$) were normally distributed and there was no significant heterogeneity of variance (Bartlett's χ^2=27.994, P=0.11). A two-factor ANOVA confirmed that there was significant interaction between factors (F=54.39, P<0.001) and that both surfactant/emulsifiers (F=181.06, P<0.001) and the inoculum source (F=571.10, P<0.001) had a significant effect on the optical density of the sample.

Bonferoni's pairwise comparisons (Table 4.6) determined that the optical densities of formulation 6 were significantly lower than those of formulation 1 and 7 at surfactant/emulsifier concentrations of 2, 3 and 4 $\log_{10}$ ppm. Furthermore, the optical density of formulation 1 was not significantly different from that of formulation 7 at all surfactant/emulsifier concentrations except at 4 $\log_{10}$ ppm.

Table 4.6. Bonferoni's pairwise comparisons of the mean difference of OD-600 ($\log_{10}$) in synthetic sewage media 3 supplemented with commercial or domestic surfactants/emulsifier (CS/E or DS/E) inoculated with formulations 1, 6 and 7 incubated for 3 days at 30°C with shaking at 130rpm. 95% confidence interval of any difference = 0.046. *significant differences

	2 $\log_{10}$ ppm	3 $\log_{10}$ ppm	4 $\log_{10}$ ppm
CS/E			
1&6	0.170*	0.172*	0.146*
1&7	-0.016	-0.002	-0.019
6&7	-0.186*	-0.174*	-0.164*
DS/E			
1&6	0.162*	0.211*	0.127*
1&7	0.009	0.096	-0.265*
6&7	-0.153*	-0.202*	-0.392*

4.2.2. Enzyme activity levels in commercial inocula

4.2.2.1. Ability of formulations 1-7 to hydrolyse variety of lipids

Non-cultured formulations

A variety of different lipid substrates were used to assess the lipolytic activities of formulations 1, 2, 3, 4, 5, 6 and 7 (Figure 4.8). After 24 hours incubation, active lipase was detected in only formulations 2 and 6, on all of substrates investigated (except formulation 2 on tween-80). Formulation 2 resulted in greater zones of clearing/colour intensification than formulation 6 on lard, sunflower, rapeseed, soya oil (by a factor of 1.4-1.6) and tween-40 (by a factor of 2.6) but not on tween-20 and tributryn.

The residuals for all substrates were normally distributed and with the exception of the sunflower oil data (F=46.848, P=0.042) displayed no significant heterogeneity of variance

(F/Bartlett's χ^2=0.312-11.651, P=0.158-0.728). A series of two sample t-tests (Table 4.7) revealed that formulations 2 and 6 did not result in zones of colour intensification that were significantly different in diameter on tween-20 and tween-40. However, formulation 2 resulted in a significantly greater zone of colour intensification than formulation 6 on lard and rapeseed whilst formulation 6 resulted in a significantly greater zone of clearing than formulation 2 on tributryn. The soya oil data was not analysed due to the lack of variability between replicate samples.

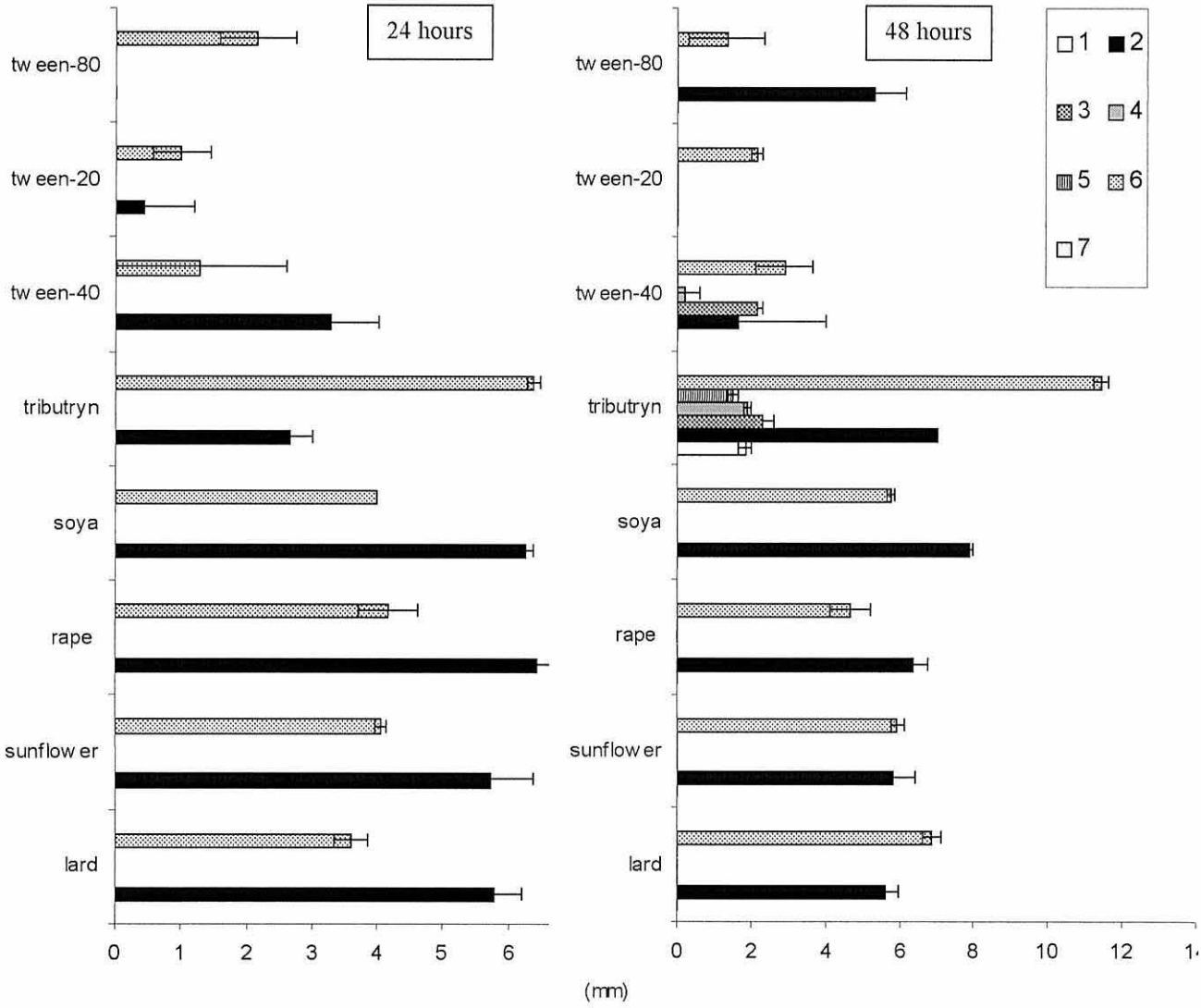


Figure 4.8. Diameters of zones of colour intensification/clearing (mm, mean ± SD) of non-cultured microbial formulations 1-7 on various lipid substrates after 24 and 48 hours incubation.

After a further 24 hours incubation lipase was detected in formulations 1, 3, 4 and 5 on tributryn (formulations 1, 3, 4, and 5) and tween-40 (formulations 3 and 4). However, formulations 2 and 6 generated greater zones of colour intensification/clearing than

formulations 1, 3, 4 and 5 on tributryn but not on tween-40 (formulation 3 >formulation 2). Only formulations 2 and 6 were capable of hydrolysing on lard, soya, sunflower oil, rapeseed, tween-80 and tween-40 after 48 hours incubation.

Table 4.7. Summary of the T and P values for various two-sample t-tests applied to compare the diameters of zones of colour intensification/clearing for the non-cultured formulations 2 and 6 on a variety of lipid substrates after 24 hours incubation. (Significant level; P=0.008)

Treatment	T	P
Lard	7.60	0.002
Rapeseed	5.36	0.006
Sunflower	4.24	0.051
Tributryn	-18.46	<0.001
Tween-40	2.25	0.088
Tween-20	-1.13	0.322

Over the later half of the incubation period (between 24 and 48 hours incubation) the diameter of the zone of colour intensification/clearing generally increased on all substrates inoculated with formulation 6 (except tween-80) whilst formulation 2 resulted in a zone of colour intensification/clearing that increased in diameter on only soya, tributryn and tween-80. The changes in the zones of colour intensification/clearing were such that formulation 6 resulted in a greater zone of colour intensification than formulation 2 on all substrates except rapeseed, soya and tween-80 after 48 hours.

The residuals for the lard, sunflower, rapeseed, soya and tween-80, tween-40 and tributryn data (excluding formulation 2) were normally distributed and displayed no significant heterogeneity of variance (F/Bartlett's χ^2=0.067-9.271, P=0.126-0.939), except in the case of tween-40 (Bartlett's χ^2=9.319, P=0.025; Levene's statistic=4.313, P=0.051).

The one-way ANOVA, two sample t-tests and Mood's median test (Table 4.8) confirmed that formulation 2 resulted in a significantly greater zone of colour intensification than formulations 6 on soya oil and tween-80 whilst formulation 6 generated a significantly greater zone of colour intensification than formulation 2 on lard. These tests further indicated that the supplements (2 and 6 in case of sunflower and rapeseed; 2, 3, 4 and 6 in case of tween-40) did not result in zones of colour intensification that were significantly different on sunflower oil, rapeseed oil and tween-40.

Table 4.8a. Summary of T, F,χ^2, and P values for two sample t-tests, Moods median tests and one-way ANOVA applied to compare the diameters of zones of colour intensification/clearing for the non-cultured formulations on various lipid substrates after 48 hours incubation. (significant level in case of 2 sample t-tests; P=0.01)

Treatment	Test statistic	P
Soya	T=27.11	<0.001
Lard	T=-5.13	0.007
Rapeseed	T=4.31	0.012
Sunflower	T=-0.30	0.777
Tween 80	T=5.28	0.006
Tributryn	F=1406.76	<0.001
Tween 40	χ^2=3.61	0.307

Table 4.8b. Tukey's pairwise comparisons of the mean difference (column-row) of diameters of zones of clearing (mm) for the non-cultured formulations on tributryn after 48 hours incubation. 95% confidence interval for any difference = 0.52. *significant difference

	1	3	4	5
3	-0.50	-	-	-
4	-0.05	0.45	-	-
5	-0.33	0.83*	0.39	-
6	-9.61*	-9.11*	-9.56*	-9.85*

Tukey's pairwise comparisons (Table 4.8) also indicated that formulation 6 resulted in a significantly greater zone of clearing than formulation 1, 3, 4 and 5 on tributryn and that formulation 3 generated a significantly greater zone of clearing than formulation 5.

In summary, the lipolytic activities of formulation 1, 3, 4 ,5 and 7 were lower than those of formulations 2 and 6 on most substrates. After 48 hours incubation formulations 1, 3, 4, 5 and 7 were only capable of hydrolysing simple triglycerides (tributryn) or in the case of formulation 3 and 4 hydrolysing synthetic esters comprised of 16:0 whilst formulations 2 and 6 were capable of hydrolysing all the natural fat/oils investigated, simple triglycerides and some synthetic esters.

Cultured formulations

Given that lipase production/activity may have been influenced by the initial bacterial densities of the formulations (OD-600; 0.104-1.999) the experiment was repeated after the formulations were grown, washed and diluted to achieve similar final bacterial densities (OD-

600; 0.599-0.757) (Figure 4.9). As in the previous experiment, lipase was evident in only formulations 2 and 6 on rapeseed, lard, soya and sunflower oil after 24 hours incubation. The zone of colour intensification was also greater with formulation 2 than formulation 6 (by a factor of 2.97-3.87) although the colour of the intensification zone was much paler. All the formulations produced lipase on tributryn and with the exception of formulation 1 and 7, produced active lipase on tween-20, 40 and 80. Formulations 2 and/or 6 resulted in greater zones of colour intensification/clearing than the other formulations on tributryn, tween-40, tween-20 and tween-80.

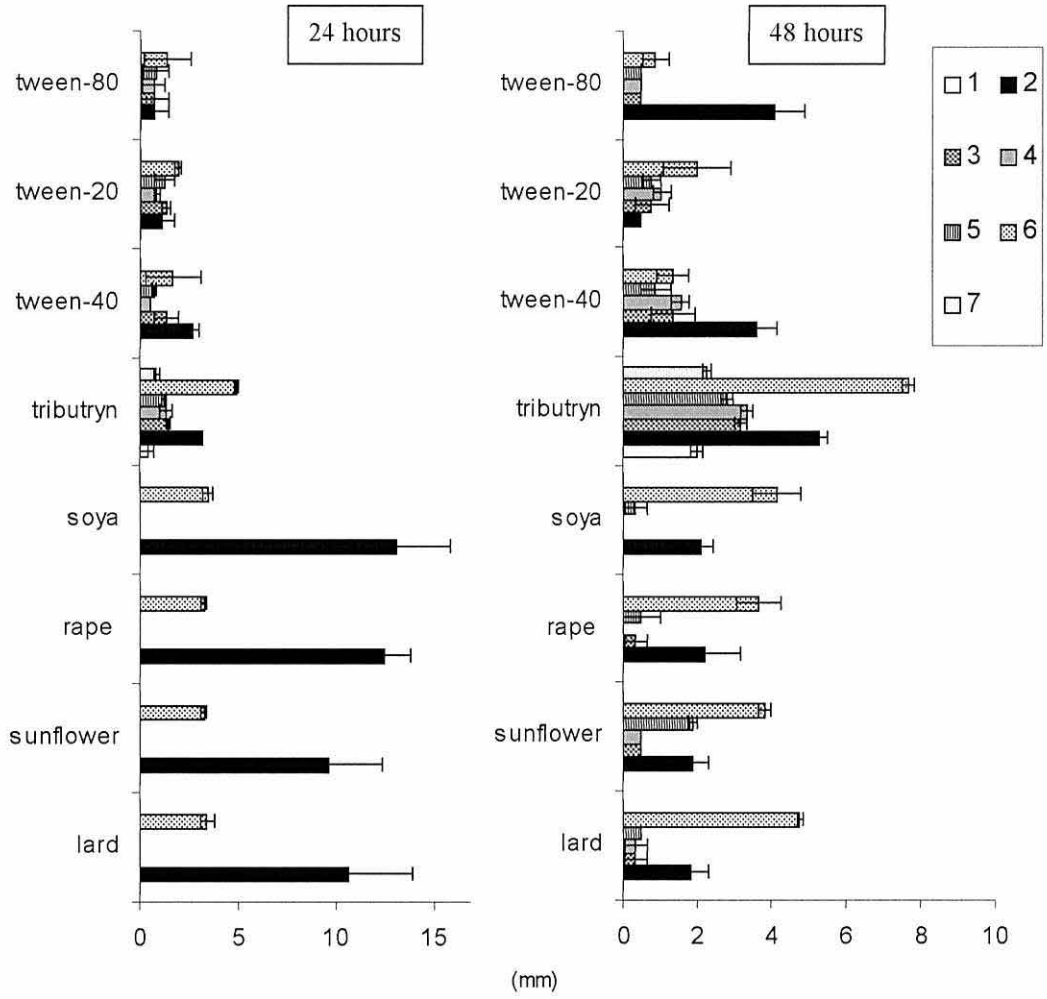


Figure 4.9. Diameters of zones of colour intensification/clearing (mm, mean ± SD) of cultured microbial formulations 1-7 on various lipid substrates after 24 and 48 hours incubation.

The residuals for each substrate were normally distributed with significant heterogeneity of variance in the case of rapeseed, soya, lard and sunflower oil (F=50.128-212.63, P=0.009-0.039) but not in the case of tributryn (excluding the data for formulation 2), tween-20, tween-40 and tween-80 (Bartlett's χ^2=1.013-5.703, P=0.226-0.908)

The two sample t-tests and one-way ANOVAs (Table 4.9) indicated that there were no significant differences in the diameters of the zones of colour intensification on soya, lard, sunflower oil, tween-20, tween-80 and tween-40. However, formulation 2 resulted in a significantly greater zone of colour intensification than formulation 6 on rapeseed oil. Tukey's pairwise comparisons (Table 4.9) revealed that formulation 6 generated greater zones of clearing formulations 1,3,4, 5 and 7 on tributryn and that formulations 3, 4 and 5 resulted in significantly greater zones of clearing than formulation 1.

Table 4.9a. Summary of T, F, and P values for two sample t-tests, and one-way ANOVA's applied to compare diameters of zones of colour intensification/clearing for the cultured formulations on various lipid substrates after 24 hours incubation.(in case of two-sample t-test significant level; P=0.01)

Treatment	Test statistic	P
Rapeseed	T=11.73	0.007
Soya	T=6.18	0.025
Lard	T=3.90	0.060
Sunflower	T=3.94	0.059
Tributryn	F=180.9	<0.001
Tween-20	F=2.95	0.075
Tween-80	F=0.36	0.835
Tween-40	F=3.84	0.076

Table 4.9b. Tukey's pairwise comparisons of the mean difference (column-row) of the diameter of zone of clearing (mm) for the cultured formulations on tributryn after 24 hours incubation. 95% confidence interval of any difference = 0.571.*significant difference

	1	3	4	5	6
3	-1.05*	-	-	-	-
4	-0.94*	-0.11	-	-	-
5	-0.83*	-0.22	0.11	-	-
6	-4.50*	-3.44*	-3.55*	-3.66*	-
7	-0.44	0.61*	0.50	0.39	4.05*

After 48 hours incubation, there was evidence of low levels of lipase activity in formulations 1, 3, 4, and 5 on lard, sunflower, rapeseed and soya oil (except formulation 4 on rapeseed oil and formulation 3 on soya oil). These formulations resulted in zones of colour intensification that were smaller in diameter than those generated by formulation 2, despite the fact that between 24 and 48 hours formulation 2 resulted in a zone of colour intensification which

appeared to decline in diameter on lard, soya, rapeseed and sunflower (diameter of colour intensification reduced by 80.3-84.0%). In contrast to formulation 2, formulation 6 resulted in a zone of colour intensification which increased in diameter on lard, soya, rapeseed and sunflower over the incubation period (diameter of colour intensification increased by a factor of 1.14-1.41). The zone of clearing for all formulations increased on tributryn over the incubation period (diameter of colour intensification increased by a factor of 1.57-5.13) and as with the 24 hour incubation period formulation 6 and 2 resulted in greater zones of clearing on tributryn than the other formulations. All formulations resulted in zones of colour intensification on tween-40, tween-80 and tween-20 which altered by differing degrees over the 24-48 hour incubation. The changes in the zones of colour intensification were such, that after 48 hours incubation formulation 2 and/or 6 resulted in greater zones of colour intensification/clearing than the other formulations.

The residuals for all substrates were normally distributed with no significant heterogeneity of variance (F/Bartlett's χ^2=1.228-5.562, P=0.189-0.953). The statistical tests (Table 4.10) confirmed that there were significant differences between the diameters of the zone of colour intensification/clearing for all substrates except tween-80. (Those treatments which exhibited no variability were removed from the analysis)

Tukey pairwise comparisons (Table 4.10) indicated that formulation 6 resulted in a significantly greater zone of colour intensification/clearing than all other formulations on all substrates, except tween-40 and tween-80. Formulation 2 also generated a significantly greater zone of colour intensification/clearing than all other formulations on soya, lard, rapeseed oil, tributryn (except formulation 6) and tween-40. Formulations 3, 4 and 5 also resulted in significantly greater zones of clearing than formulations 1 and 7 on tributryn and formulation 4 generated a significantly greater zone of clearing than formulation 5 on tributryn.

In conclusion, the lipolytic activities of cultured formulations 2 and/or 6 were significantly greater than those of the other formulations on all lipid substrates. There was evidence of lipase activity in cultured formulations 1, 3, 4, 5 and 7 but not in the equivalent non-cultured formulations on tributryn/tweens after 24 hours and soya/rapeseed/sunflower/lard, after 48 hours, implying that growth and/or a change in bacterial density was appreciably affecting the

abilities of some formulations (1, 3, 4, 5, 7) to hydrolyse simple triglycerides and synthetic esters.

Table 4.10a. Summary of F, and P values for two sample t-tests, and an ANOVA applied to compare diameters of zones of colour intensification/clearing for the cultured formulations on various lipid substrates after 48 hours incubation.

Treatment	Test statistic	P
Lard	F=123.82	<0.001
Sunflower	F=53.75	<0.001
Rapeseed	F=18.79	0.001
Soya	F=51.20	<0.001
Tributryn	F=402.17	<0.001
Tween-80	T=6.26	0.003
Tween-40	F=15.94	<0.001
Tween-20	F=3.32	0.078

Table 4.10b. Results of Tukey's pairwise comparisons of the mean difference ± 95% confidence interval of the diameters of the zones of colour intensification/clearing (mm) of the cultured formulations on a variety of substrates after 48 hours incubation

	Tween-40	Tributryn	Soya	Sunflower	Lard	Rapeseed
1&2	-	-3.28±0.48*	-	-	-	-
1&3	-	-1.17±0.48*	-	-	-	-
1&4	-	-1.33±0.48*	-	-	-	-
1&5	-	-0.83±0.48*	-	-	-	-
1&6	-	-5.67±0.48*	-	-	-	-
1&7	-	-0.28±0.48	-	-	-	-
2&3	2.28±1.25*	2.11±0.48*	-	-	1.497±0.85	1.89±1.65*
2&4	2.06±1.25*	1.94±0.48*	-	-	1.497±0.85*	-
2&5	2.72±1.25*	2.44±0.48*	1.78±1.16*	0.00±0.67	-	1.72±1.65*
2&6	2.28±1.25*	-2.39±0.48*	-2.06±1.16*	-1.95±0.67*	-2.95±0.85*	1.44±1.65
2&7	-	3.00±0.48*	-	-	-	-
3&4	-0.22±1.25	-0.17±0.48	-	-	0.00±0.85	-
3&5	0.45±1.25	0.33±0.48	-	-	-	0.17±1.65
3&6	0.00±1.25	-4.50±0.48*	-	-	-4.44±0.85*	-3.33±1.65*
3&7	-	0.89±0.48*	-	-	-	-
4&5	0.67±1.25	0.50±0.48*	-	-	-	-
4&6	0.22±1.25	-4.33±0.48*	-	-	-4.44±0.85*	-
4&7	-	1.06±0.48*	-	-	-	-
5&6	-0.45±1.25	-4.33±0.48*	-3.83±1.16*	-1.95±0.67*	-	-3.17±1.65*
5&7	-	0.56±0.48*	-	-	-	-
6&7	-	5.39±0.48*	-	-	-	-

4.2.2.2. Lipolytic activities of S45 microorganisms grown at different dilutions

S45 may have been unable to degrade oil in some of the earlier experiments (section 3.2.2) because the inhibitor and/or antibiotic producing bacteria were not adequately diluted. The ability of the S45 microorganisms to grow at various dilutions was investigated by plating serial dilutions of S45 on lab M nutrient agar (Table 4.11).

The number of bacteria in the undiluted sample of activated S45 (10^0) was $1.51 \times 10^3 \pm 5.66 \times 10^2$ cfu.ml^{-1}. A dilution factor greater than 10^4 was expected to reduce the number of S45 microorganisms to zero. However, dilution of the activated S45 by a factor of 10^4-10^{10} resulted in bacterial numbers that were greater than predicted (i.e. greater than zero). These results imply that the inhibitor and/or antibiotic producing microorganisms present in S45 may have been suppressing the growth of certain groups of bacteria at low dilutions.

Table 4.11. Number of bacteria in S45 (cfu.ml^{-1}, mean ± SD) at each dilution in after 72 hours incubation at 30°C on lab M nutrient agar.

Dilution	cfu.ml^{-1}
10^0	$1.51 \times 10^3 \pm 5.66 \times 10^2$
10^{-1}	$1.81 \times 10^3 \pm 2.01 \times 10^3$
10^{-2}	$1.53 \times 10^2 \pm 2.16 \times 10^2$
10^{-3}	$3.33 \times 10^1 \pm 3.06 \times 10^1$
10^{-4}	$0.00 \times 10^0 \pm 0.00 \times 10^0$
10^{-5}	$1.07 \times 10^2 \pm 1.33 \times 10^2$
10^{-6}	$4.00 \times 10^1 \pm 5.29 \times 10^1$
10^{-7}	$6.80 \times 10^2 \pm 1.14 \times 10^4$
10^{-8}	$2.00 \times 10^1 \pm 3.46 \times 10^1$
10^{-9}	$4.67 \times 10^1 \pm 4.16 \times 10^1$
10^{-10}	$6.67 \times 10^1 \pm 1.12 \times 10^2$

To determine whether the S45 microorganisms were capable of producing lipase, several of the S45 colonies grown on the lab M nutrient agar at different dilutions were transferred to tributryn agar. After 24, 48 and 72 hours incubation the tributryn plates were examined for evidence of lipase activity (Table 4.12).

Table 4.12. Number of colonies in S45 that produced no, low or high levels of lipase at various dilutions and incubation times (dilutions were re-classified as low (10^0-10^{-2}), mid (10^{-3}-10^{-6}) and hi (10^{-7}-10^{-10}).

	no lipase	low lipase	high lipase
low dilution			
24 hrs	38	28	5
48 hrs	23	26	22
72 hrs	15	22	34
mid dilution			
24 hrs	2	9	4
48 hrs	0	3	12
72 hrs	0	1	14
hi dilutions			
24 hrs	6	11	4
48 hrs	4	2	15
72 hrs	1	4	16

A series of chi-squared tests of independence were performed (Table 4.13). Major contributors to the chi-squared value were successively removed until the differences between the classes were no longer significant.

Table 4.13. Chi-squared statistic, P-values and degrees of freedom (DF) for the lipase activities of S45 microorganisms produced at different dilutions and incubation times. (LD=low dilution, MD=mid dilution, HD=high dilution after 24, 48, 72 hours)

Groups compared	χ^2	P	DF
LD(24,48,72), MD(24,48,72), HD(24,48,72)	102.84	<0.001	16
LD(48,72), MD (24,48,72), HD(24,48,72)	54.73	<0.001	14
LD(48,72), MD (24,48,72), HD(48,72)	41.70	<0.001	12
LD(48,72), MD (48,72), HD(48,72)	30.45	<0.001	10
LD(72), MD (48,72), HD(48,72)	20.342	0.009	8
MD (48,72), HD(48,72)	8.88	0.181	6
LD(24,48,72), MD(24), HD(24)	39.83	<0.001	8
LD(48,72), MD(24), HD(24)	12.06	0.060	6

Three distinct categories of lipase producers were identified (Group 1- mid/high dilution 48/72 hours, Group 2- low dilution 48/72 hours, mid/high dilution 24 hours, Group 3- low dilution 24 hours) and a final chi-squared test was performed to confirm that lipase activity was dependent on the three resultant classes (χ^2=87.28, P<0.001, DF=4).

The % distribution of lipase activities in each group was also calculated and is shown in Figure 4.10.

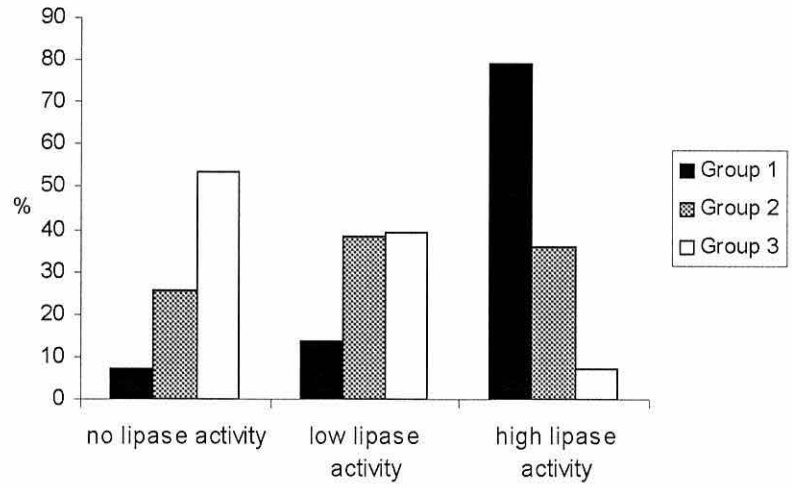


Figure 4.10. Percentage distribution of lipase activity in each dilution/time class of the S45 microorganisms (Group 1- mid/high dilution 48/72 hours, Group 2- low dilution 48/72 hours, mid/high dilution 24 hours, Group 3- low dilution 24 hours).

The lipase activities of the microorganisms appeared to depend largely upon the dilution at which they were grown and incubation time. After 24 hour incubation, microorganisms from mid/high dilutions (group 2) displayed an almost equal proportions of high (36%) and low (38%) lipase activities whilst those from the low dilutions (group 3) exhibited no (54%) or low (26%) levels of lipase activity. In general, the proportion of high lipase producers in each dilution increased as the incubation period was extended from 24 to 48/72 hours. Those microorganisms from the mid/high dilutions of S45 (group 1) incubated for 48/72 hours exhibited high levels of lipase activity (79 %) whilst microorganisms from low dilutions exhibited lipase activity that was equivalent to those from mid/high dilutions after 24 hours incubation (group 2).

4.2.2.3. Ability of formulations 1-7 to produce protease

Protease activity was investigated in both cultured and non cultured formulations (Table 4.14). As with the lipase activity measurements cultured formulations were grown washed and diluted to achieve similar starting bacterial densities.

Table 4.14a. Diameter of zone of clearing, (mm) for cultured and non-cultured microbial formulations on milk agar after 24 and 48 hours.

	Non-cultured formulations		Cultured formulations	
Formulation	**24hours**	**48hours**	**24 hours**	**48 hours**
1	ND	ND	ND	0.50 ± 0.00
2	8.22 ± 0.54	15.06 ± 0.68	5.83 ± 0.60	10.00 ± 0.93
3	3.06 ± 0.42	9.00 ± 0.33	6.11 ± 0.10	12.50 ± 0.50
4	ND	ND	5.66 ± 1.26	14.00 ± 0.58
5	ND	ND	ND	0.50 ± 0.00
6	8.67 ± 0.44	14.28 ± 0.51	8.61 ± 0.345	14.28 ± 0.92
7	ND	ND	ND	0.50 ± 0.00

Table 4.14b. Tukey's pairwise comparisons of the mean difference of the diameters of zones of clearing (mm) on milk agar for the non-cultured formulations. 95% confidence interval of any difference = 0.77. *significant differences

Pairwise comparison	**Difference in means**
Formulation 2&3	5.61*
Formulation 3&6	-5.44*
Formulation 2&6	-0.16

Table 4.14c. Results of Bonferoni's pairwise comparisons of the mean difference of the diameters of zones of clearing (mm) on milk agar for the cultured formulations after 24 and 48 hours incubation. 95% confidence interval of any difference = 2.01. *significant difference

Pairwise comparison	**24 hours**	**48 hours**
Formulation 2&3	-0.28	-2.50*
Formulation 2&4	0.17	-4.00*
Formulation 2&6	-2.78*	-4.28*
Formulation 3&4	0.45	-1.50
Formulation 3&6	-2.50*	-1.78
Formulation 4&6	-2.95	-0.27

The diameter of the zone of clearing varied considerably between formulations and with incubation time. In the non-cultured formulations, protease activity was evident only in formulations 2, 3 and 6. Formulations 2 and 6 resulted in a greater zone of clearing than formulation 3 after both 24 and 48 hours incubation (by a factor of approximately 2.8 and 1.7 respectively) and increasing the length of the incubation period increased the zone of clearing for formulations 2, 3 and 6 by a factor of 1.6-2.9.

The residuals were normally distributed and there was no significant heterogeneity of variance (Bartlett's χ=0.993, P=0.963). A two-factor ANOVA established that there was no significant interaction between factors (F=2.45, P=0.128) and that the effects of the formulation used (F=247.97, P<0.001) and incubation period were significant (F=685.93, P<0.001). Tukey's pairwise comparisons (Table 4.14) confirmed that formulation 2 and 6 resulted in a significantly greater zone of clearing and thus protease activity than formulation 3 after both 24 and 48 hours incubation.

In contrast to the non-cultured formulations, protease activity was not restricted to cultured formulations 2, 3 and 6 implying that the protease activities of some formulations were appreciably affected by growth and/or adjusting the bacterial densities of the formulations. After 24 hours incubation, cultured formulation 4 exhibited protease activity, along with formulation 2, 3 and 6 and cultured formulation 6 resulted in a greater zone of clearing than formulation 2, 3 and 4 (by 29.0-34.1%). Extending the incubation period for a further 24 hours also resulted in extremely low but detectable levels of protease in formulations 1, 5 and 7 and an increase in the zones of clearing for the other formulations (by a factor of 1.6-2.5). The relative changes in the zones of clearing were such that after 48 hours formulations 4 and 6 generated greater zones of clearing than formulations 2 and 3 (by a factor of 1.1-1.4).

The residuals were normally distributed and displayed no significant heterogeneity of variance (Bartlett's χ=8.877, P=0.262). (Low lipase producers were excluded from the analysis due to a lack of variance in the data). A two-factor ANOVA determined that there was significant interaction between factors (F=8.29, P=0.001) and that the effect of incubation time (F=414.87, P<0.001) and formulation used (F=23.39, P<0.001) was significant. Bonferoni's pairwise comparisons (Table 4.14) established that formulation 6 resulted in a significantly greater zone of clearing (and thus protease activity) than formulations 2, 3 and 4 after 24 hours incubations and that formulations 3, 4 and 6 generated significantly greater zones of clearing (and thus protease activity) than formulation 2 after 48 hours incubation.

4.2.2.4. Ability of formulations 1-7 to produce amylase

As with lipase and protease activity tests, amylase activity was assessed in both the cultured and non-cultured formulations. There was evidence of amylase activity in cultured and non-cultured formulation 2 only but it was unclear whether amylase activity in this formulation

was influenced by the length of the incubation period or by growth and adjusting the bacterial densities.

4.2.3. Ability of commercial inocula to grow on various lipids

4.2.3.1. Effect of lipid concentration on microbial growth

A series of experiments were performed to examine the effect of lipid concentration on the growth of several microbial supplements and occasionally the grease trap isolates.

Mixed vegetable oil

The optical density of nutrient media 4 inoculated with pF33 over a range of emulsified mixed vegetable oil concentrations is shown in Table 4.15. Negative optical densities occurred at all oil concentrations suggesting that either bacterial growth did not occur or that the emulsified oil was degraded over the incubation period. There did not appear to be a relationship between the magnitude of the absorbance value and the concentration of oil used.

Table 4.15. Relationship between the optical density at 600nm and mechanically emulsified oil concentration (rapeseed, soya & sunflower oil combined) in nutrient media 4, inoculated with pF33, incubated at 30°C, with shaking at 130rpm for 7 days.

Oil concentration (%,v/v)	OD-600
0.05	-0.1803
0.01	-0.0123
0.005	-0.0443

A similar experiment was conducted using non-emulsified mixed vegetable oil over a range of concentrations (Figure 4.11). The optical densities of the non-emulsified vegetable oil samples were positive indicating that both the pF33 and grease trap isolates were capable of growth using the mixed vegetable oil as the sole carbon source. The density of bacteria appeared to fluctuate or increase as the concentration of oil was raised from 0.5 to 5%, and progressively decline to a value that was lower than that measured at 0.5%. The density of pF33 bacteria was generally greater than that of the grease trap isolates, except at oil levels of 1 and 10 %

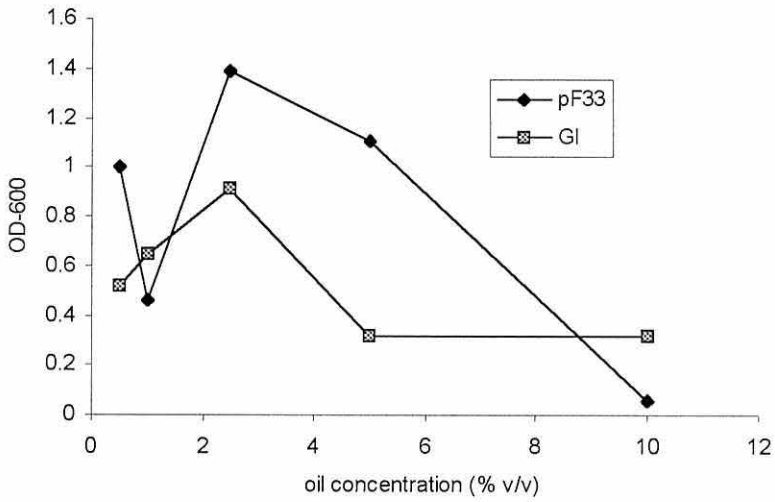


Figure 4.11. Relationship between that optical density at 600nm (a measure of microbial growth) and non-emulsified oil concentration in nutrient media 4 inoculated with pF33 and grease trap isolates (GI), incubated at 30°C with shaking at 150 rpm for 3 days.

Mixed tween

The relationship between mixed tween (40, 60, & 80) concentration and the optical density of nutrient media 4 inoculated with pF33 is depicted in Figure 4.12 .

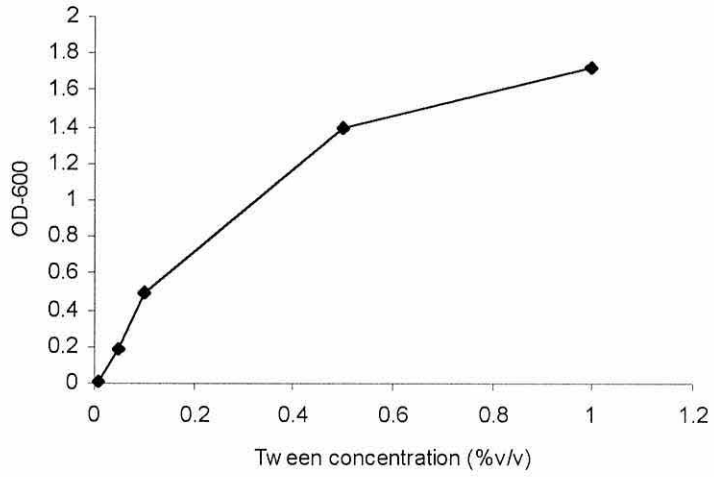


Figure 4.12. Relationship between the optical density at 600nm (a measure of bacterial density) and concentration of mixed tween in nutrient media 4, inoculated with pF33, incubated at 30°C, with shaking at 150 rpm for 5 days.

Optical densities were positive at all tween concentrations, suggesting that the pF33 isolates were capable of growth using tweens as the sole carbon source. A Pearson's correlation confirmed that there was a significant relationship between tween concentration and optical

density (r=0.952, P=0.013). The effect of tween on microbial growth was not investigated any further since tweens were not expected to arise in wastewater.

Fatty acids

The relationship between palmitic acid concentration and the optical density of nutrient media 4 inoculated with either pF33 or the grease trap isolates is shown in Figure 4.13. Positive optical densities were measured in most of the samples suggesting that the pF33 and grease trap isolates were capable of growth using palmitic acid as the sole carbon source. Although the relationship between optical density and palmitic acid concentration was not very clear, increasing the concentration of palmitic acid from 0.02-0.2 % (grease trap isolates) and 0.1-0.5% (pF33), enhanced the growth of the isolates by a factor of 8.6 and 11.6, respectively.

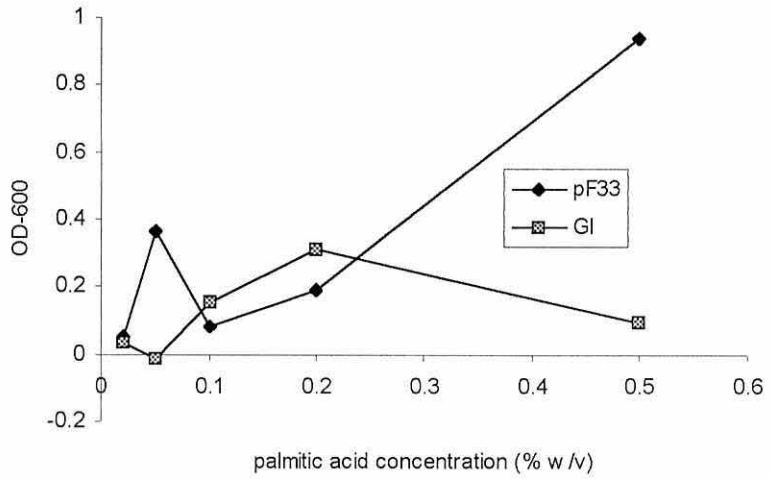


Figure 4.13. Relationship between the optical density at 600nm (a measure of microbial growth) and palmitic acid concentration in nutrient media 4 inoculated with pF33 and grease trap isolates, incubated at 30°C, with shaking at 150 rpm for 5 days)

Unlike samples containing palmitic acid, a large number of samples containing oleic acid were found to have negative optical densities (Figure 4.14). Optical densities were generally positive at low oleic levels (<2%) and negative at high levels (>2%) suggesting that the microorganisms were only capable of growth when oleic acid was present at low concentrations or that high concentrations of oleic acid were inhibitory. At low oleic acid concentrations, the density of the F33 isolates was also greater than that of the grease trap isolates.

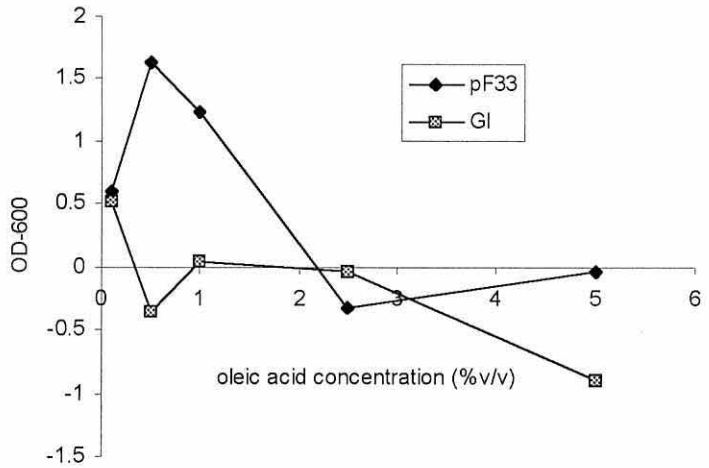


Figure 4.14. Relationship between the optical density at 600nm (a measure of microbial growth) and oleic acid concentration in nutrient media 4 samples inoculated with pF33 and grease trap isolates (GI) incubated at 30°C with shaking at 150 rpm for 5 days.

In comparison to the samples containing palmitic and oleic acid, even fewer of the samples containing linoleic acid were found to have positive optical densities (Figure 4.15). Optical densities generally fluctuated between -0.023 and -0.073, indicating that both F33 and the grease trap isolates were incapable of growth using linoleic acid as the sole carbon source or that linoleic acid was inhibitory to their growth. A positive optical density was measured when linoleic was present at a concentration of 0.1%, indicating that pF33 may have been capable of utilising linoleic acid as the sole carbon source only at very low concentrations.

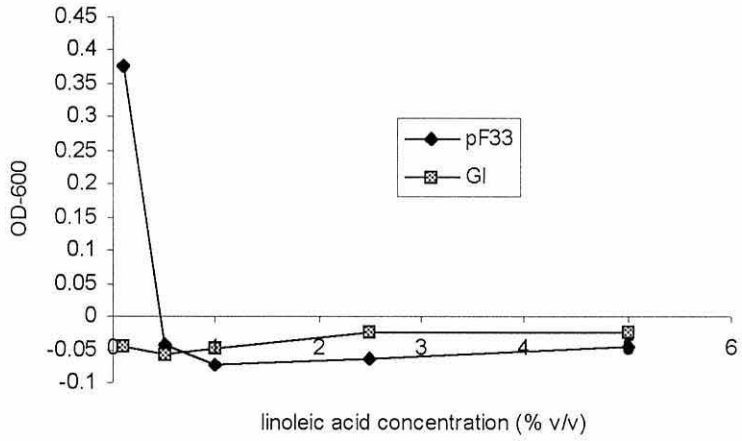


Figure 4.15. Relationship between optical density at 600nm (a measure of microbial growth) and concentration of linoleic acid in nutrient media 4 inoculated with pF33 and grease trap isolates (GI), incubated at 30°C, with shaking at 150 rpm for 14 days.

Fatty acid sodium salts

The effect of fatty acid sodium salt concentration on the growth of the microbial supplements was also investigated since wastewater contains fatty acids both in an uncombined and salt form (Figures 4.16-4.18). The optical densities of samples containing sodium oleate and sodium palmitate samples were either negative or very low and positive indicating that either the fatty acid salts were being degraded or that the salts were inhibitory to microbial growth (N.B.-like emulsified oil, solutions prepared from sodium palmitate and sodium oleate are optically dense). The optical densities of the sodium linoleate samples (which were not initially optical dense) were low and positive indicating that growth at all linoleate concentrations was relatively poor. There was also a positive relationship between microbial growth and the concentration of sodium linoleate.

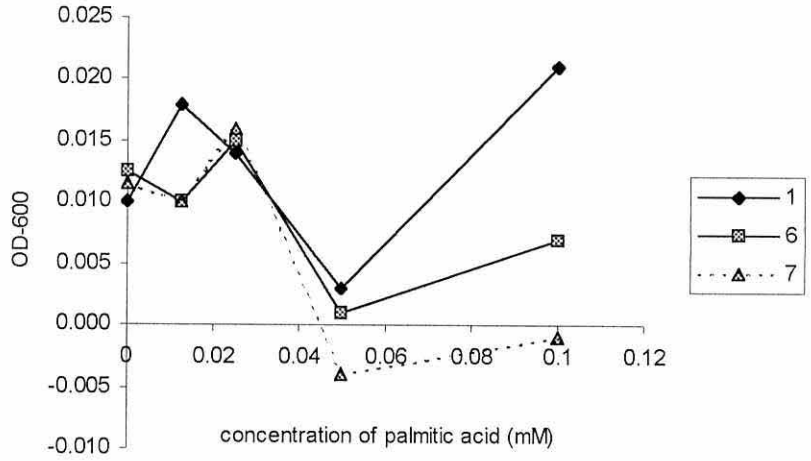


Figure 4.16. Relationship between optical density at 600nm and concentration of sodium palmitate in synthetic sewage media 2 inoculated with formulations 1, 6 and 7 incubated at 30°C with shaking at 150rpm, for 22 days.

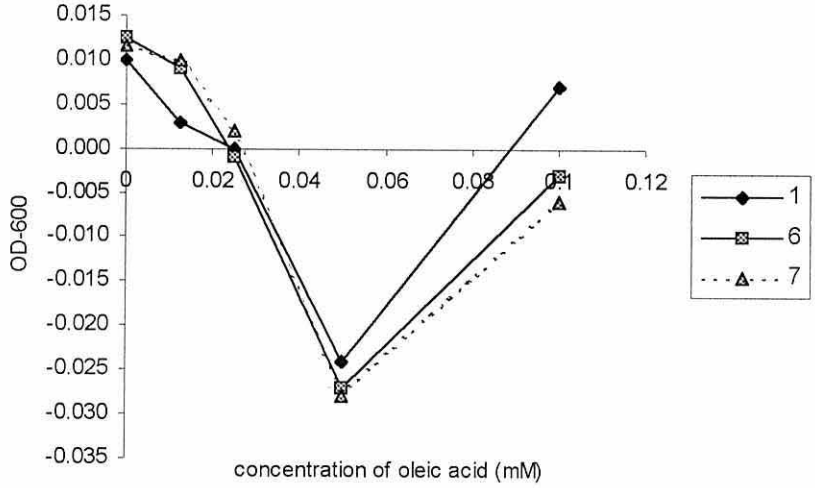


Figure 4.17. Relationship between optical density at 600nm and concentration of sodium oleate in synthetic sewage media 2, inoculated with formulations 1, 6 or 7 incubated at 30°C with shaking at 150rpm for 22 days.

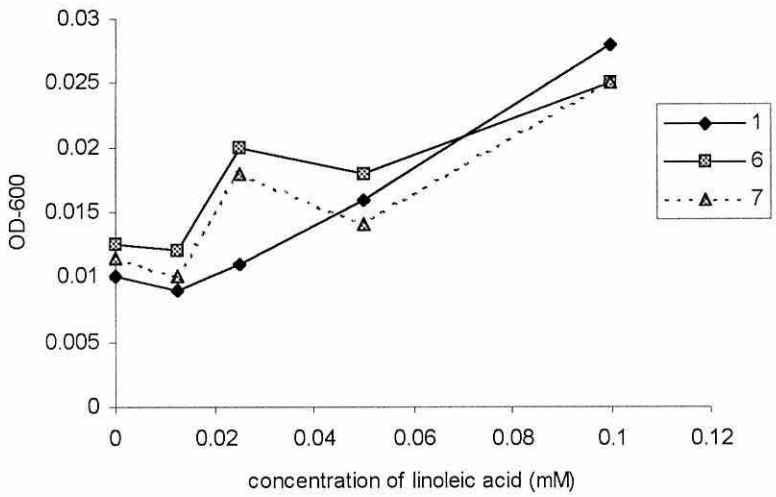


Figure 4.18. Relationship between optical density at 600nm and concentration of sodium linoleate in synthetic sewage media 2 inoculated with formulations 1, 6 and 7 incubated at 30°C with shaking at 150rpm, for 22 days.

4.2.3.2. Effect of various lipids on microbial growth

Growth of pF33 on various lipids

The following experiment was designed to determine whether the nature of the fat/oil influenced the growth of pF33. The effect of various fat/oils (non-emulsified) on the growth of pF33 in nutrient media 4 is shown in Figure 4.19.

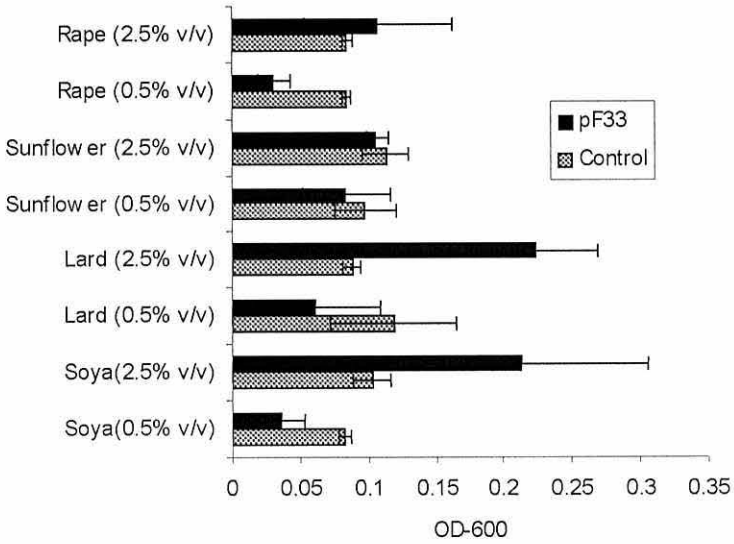


Figure 4.19. Optical densities at 600nm (mean ± SD) (a measure of microbial growth)) of nutrient media 4 supplemented with a variety of fat/oils inoculated with pF33, incubated at 30°C, with shaking at 150rpm for 11 days.

The optical densities of inoculated samples containing rapeseed (2.5% v/v), soya (2.5% v/v) and lard (2.5% v/v) were between 1.27 and 2.55 orders magnitude greater than the corresponding controls, indicating that the pF33 isolates were capable of growing on these fat/oils as the sole carbon source. The optical density of the inoculated samples containing lard was similar to that of the samples containing soya oil and approximately twice that of the samples containing rapeseed oil, which implied that lard and soya oil were more easily metabolized by pF33 than rapeseed oil. Inoculation of samples that contained the remaining fats/oils (rapeseed (0.5% v/v), lard (0.5% v/v), soya (0.5% v/v), sunflower (2.5% v/v) and sunflower (0.5% v/v)) reduced the optical densities of the samples by 5.7- 55.5% indicating that the pF33 isolates were not capable of using sunflower oil or low concentration of the other fat/oils as the sole carbon source, or that these fat/oils were inhibitory to microbial growth.

Table 4.16. Bonferoni's pairwise comparisons of the means difference of OD-600 (square rooted) in nutrient media 4 supplemented with a variety of fat/oils inoculated with pF33, incubated at 30°C, with shaking at 150rpm for 11 days. 95% confidence interval of any difference = 0.134. * significant differences

Pairwise comparison		Difference in means
Soya (0.5% v/v);	pF33 & control	-0.098
Soya (2.5% v/v);	pF33 & control	0.135*
Sunflower (0.5% v/v);	pF33 & control	-0.024
Sunflower (2.5% v/v);	pF33 & control	-0.009
Rapeseed (0.5% v/v);	pF33 & control	-0.116
Rapeseed (2.5% v/v);	pF33 & control	-0.030
Lard (0.5% v/v);	pF33 & control	-0.112
Lard (2.5% v/v);	pF33 & control	0.176*
Lard (2.5% v/v)pF33 & soya(2.5% v/v)pF33		0.017

The residuals of the transformed data (square root) were approximately normally distributed, there was no heterogeneity of variance (Levene's statistic=1.084 P=0.407) and a two-factor ANOVA indicated that there was interaction between factors (F=6.28, P<0.001) and the nature of the fat/oil significantly affected the optical densities of the samples (F=7.01, P<0.001). Although the overall effect of inoculation was not significant (F=0.02, P=0.884), Bonferoni's pairwise comparisons (Table 4.16) revealed that the optical densities of the inoculated samples of soya (2.5%) and lard (2.5%) were significantly greater than the controls. There were no significant differences between any of the other inoculated fat/oil samples and their corresponding controls or between the inoculated samples of lard (2.5%)

and soya (2.5%), which implied that the pF33 isolates were only capable of growing on lard and soya oil as the sole carbon source, provided the fat/oil was present at large concentrations.

Given that the elevated optical densities of the control samples may have been indicative of microbial contamination, the experiment was repeated. Additional samples of mixed oil were also prepared to determine whether the pF33 was able to grow more readily on mixed oil than individual fats and oils (Figure 4.20).

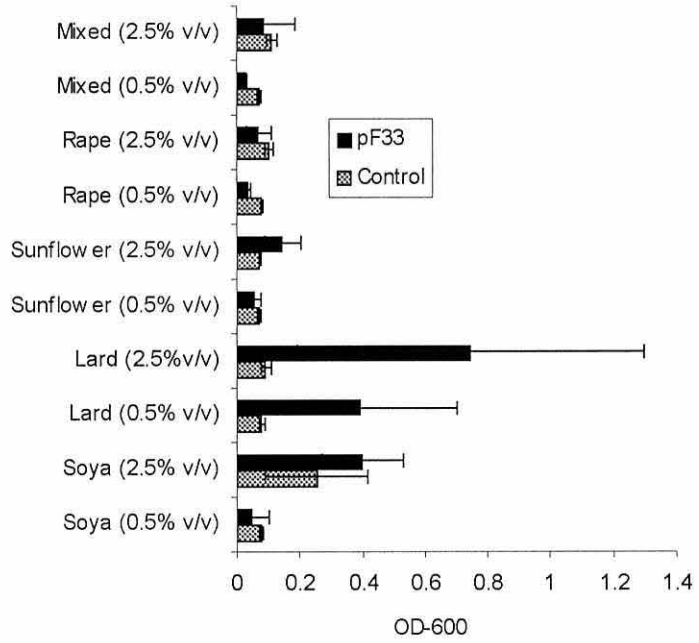


Figure 4.20. Optical densities at 600nm (mean ± SD) (a measure of microbial growth) of nutrient media 4 supplemented with a variety of fat/oils inoculated with pF33, incubated at 30°C with shaking at 150rpm, for 14 days

As was demonstrated in the previous experiment, inoculation of samples containing 2.5% of lard and soya oil raised the optical densities of the sample (by a factor of 1.6-8.2) indicating that the pF33 isolates were capable of growing on lard and soya oil (2.5%) as the sole carbon source. Inoculation also increased the optical densities of samples containing lower concentrations of lard and 2.5% of sunflower (by a factor of 2.0-5.0) and reduced the optical density of samples containing 2.5% of rapeseed oil (by 30.5%), however, indicating that the pF33 isolates were also capable of growth on lower concentrations of lard (0.5%) and 2.5% of sunflower oil but not on 2.5% of rapeseed oil. Growth of the pF33 isolates on lard (2.5%) was almost twice that on lard (0.5%) and soya oil (2.5%) and approximately 5 orders of magnitude

greater than growth on sunflower oil (2.5%). In contrast to the preliminary findings (Figure 4.19), the pF33 isolates were not capable of growing on mixed oil, as the sole carbon source.

The residuals of the transformed data ($\log_{10}$) were approximately normal, with no significant heterogeneity of variance (Levene's statistic=1.154, P=0.341). A two-factor ANOVA established that there was significant interaction between factors (F=5.90, P<0.001) and that the nature of the fat/oil significantly affected the optical densities of the samples (F=10.48, P<0.001). Although the average effect of inoculation was not significant (F=0.63, P=0.433) Bonferoni's pairwise comparisons (Table 4.17) determined that the optical densities of the inoculated samples of lard (0.5 and 2.5%) were significantly greater than the corresponding controls, suggesting that the pF33 isolates were only capable of growth on lard as sole carbon source.

Table 4.17. Bonferoni's pairwise comparisons of the mean differences of OD-600 ($\log_{10}$) in nutrient media 4 supplemented with a variety of fat/oils inoculated with pF33, incubated at 30°C with shaking at 150rpm, for 14 days. 95% confidence interval of any difference = 0.521. * significant differences

Pairwise comparisons		Difference in means
Soya (0.5% v/v);	pF33 & control	-0.275
Soya (2.5% v/v);	pF33 & control	0.237
Lard (0.5% v/v);	pF33 & control	0.590*
Lard (2.5% v/v);	pF33 & control	0.818*
Sunflower (0.5% v/v);	pF33 & control	-0.081
Sunflower (2.5% v/v);	pF33 & control	0.278
Rapeseed (0.5% v/v);	pF33 & control	-0.360
Rapeseed (2.5% v/v);	pF33 & control	-0.198
Mixed (0.5% v/v);	pF33 & control	-0.352
Mixed(2.5% v/v);	pF33 & control	-0.227
Lard(0.5% v/v)pF33 & Lard(2.5% v/v)pF33		-0.285

The impact of various fatty acids on the growth of the pF33 isolates in nutrient media 4 was also investigated (Figure 4.21). Inoculation of samples containing palmitic acid (0.1, 0.5 & 1%) and oleic acid (0.1%) raised the optical densities by a factor of 2.9-13.0 indicating that the pF33 isolates were capable of growing on palmitic acid and low concentrations of oleic acid as the sole carbon source. The density of the pF33 isolates in samples containing 0.1% of oleic acid was greater than in those containing 0.1% of palmitic acid (by a factor of ca.4) and samples containing 1% of palmitic acid resulted in greater optical densities than samples

containing 0.5% of palmitic acid (raised OD-600 by a factor of 1.8). Samples containing linoleic acid and high concentrations of oleic acid also appeared to reduce or inhibit the growth of the pF33 isolates, as indicated by the reduction in optical density (45.6-51.8%).

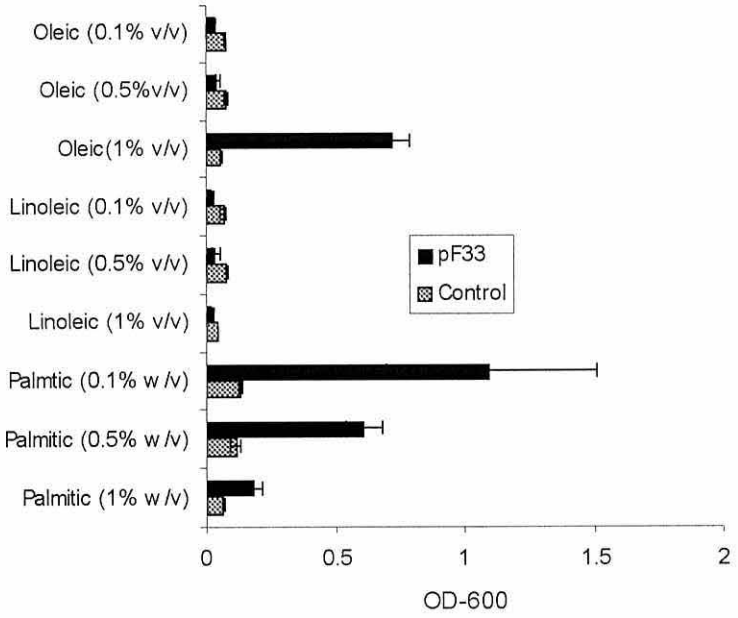


Figure 4.21. Optical densities at 600nm (mean ± SD) (a measure of microbial growth) of nutrient media 4 supplemented with a variety of fatty acids inoculated with pF33, incubated for 7 days (0.1% v/v samples) and 21 days (0.5 and 1% v/v samples) at 30°C with shaking at 150rpm.

The residuals of the transformed data ($\log_{10}$) for samples extracted after 7 and 21 days were approximately normally distributed and there was no significant heterogeneity of variance (Bartlett's χ^2=5.209-13.164, P=0.283-0.391). A two-factor ANOVA confirmed that there was significant interaction between factors (F=81.04-289.27, P<0.001) and that the optical densities of the samples were affected significantly by both inoculation (F=2.64-319.82, P<0.001) and by varying the nature of the fatty acids (F=150.91-373.93, P<0.001).

A series of Bonferoni's pairwise comparisons (Table 4.18) indicated that the growth of the pF33 isolates on all concentrations of palmitic acid and low concentrations of oleic acid (0.1%) was significant. Samples containing oleic acid (0.1%) resulted in significantly greater growth than samples containing palmitic acid (0.1%) and samples containing 1% of palmitic acid resulted in significantly greater growth than 0.5% of palmitic acid. The optical densities of the inoculated samples of the remaining fatty acid (linoleic; 0.1, 0.5 & 1%, oleic acid; 0.5 & 1%) were significantly less than the controls indicating that growth of pF33 may have been inhibited by these fatty acids.

Table 4.18. Bonferoni's pairwise comparisons of the mean difference ± 95% confidence interval of OD-600 (log_{10}) in nutrient media 4 supplemented with a variety of fatty acids inoculated with pF33, incubated for 7 days (0.1% v/v samples) and 21 days (0.5 and 1% v/v samples) at 30°C with shaking at 150rpm. *significant differences

Pairwise comparison		Difference in mean ± 95CI
Linoleic (0.1% v/v);	pF33 & control	-0.289 ± 0.126*
Oleic (0.1% v/v);	pF33 & control	1.111 ± 0.126*
Palmitic (0.1% v/v);	pF33 & control	0.454 ± 0.126*
Oleic(0.1% v/v) pF33 & palmitic (0.1% v/v) pF33		0.600 ± 0.126*
Linoleic(0.5% v/v);	pF33 & control	-0.333 ± 0.202*
Linoleic(1% v/v);	pF33 & control	-0.440 ± 0.202*
Oleic (0.5% v/v);	pF33 & control	-0.268 ± 0.202*
Oleic (0.5% v/v);	pF33 & control	-0.333 ± 0.202*
Palmitic (0.5% v/v);	pF33 & control	0.730 ± 0.202*
Palmitic (1% v/v);	pF33 & control	0.910 ± 0.202*
Palmitic(0.5% v/v)pF33 & palmitic(1% v/v)pF33		-0.240 ± 0.202*

Growth of formulations 1, 6, 7 on various lipids

The following experiments were designed to determine whether the nature of the lipid source influenced the growth of microbial formulations 1, 6 and 7. The effect of various fats and oils on the growth of formulations 1, 6 and 7 in synthetic sewage media 2 is shown in Figure 4.22.

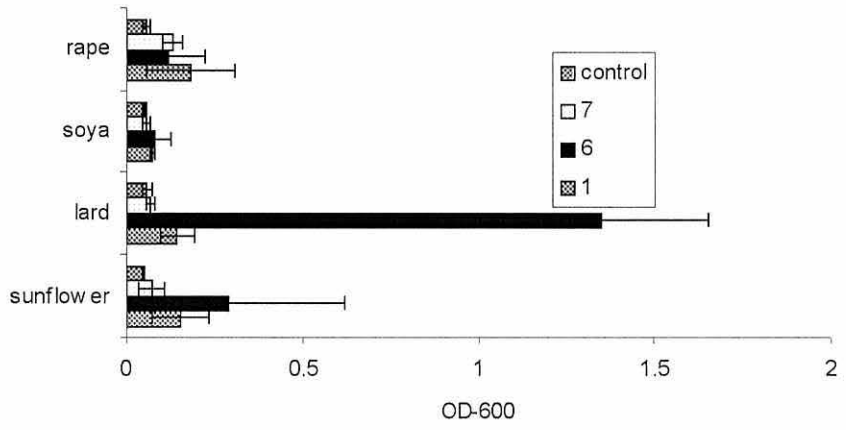


Figure 4.22. Optical densities at 600nm (mean ± SD) (a measure of microbial growth) of synthetic sewage media 2 supplemented with samples various fat/oils (2.5% v/v) inoculated with formulations 1, 6 and 7 incubated at 30°C with shaking at 130rpm for 20 days.

The optical densities of the inoculated samples were greater than those of the controls, indicating that all three microbial formulations were capable of growing on all fat/oils as the

sole carbon source. Samples of soya oil resulted in the lowest optical densities (inoculation increased OD-600 by a factor of 1.1-1.5) and the optical densities of samples of lard and sunflower oil inoculated with formulation 7 were similarly low. Samples of lard and sunflower oil inoculated with formulation 6 generated the greatest optical densities (inoculation increased OD-600 by a factor of 23.2 & 6.1, respectively) and the remaining samples of fat/oil gave rise to intermediate optical densities (inoculation increased OD-600 by a factor of 1.5-2.5).

It was also noted that there was microbial growth in samples containing both lard and formulations 1 or 6 before an inorganic nitrogen source was added to the samples. Since microbial growth was unlikely to have occurred in the absence of nitrogen, the lard must have been contaminated with a nitrogen source.

Table 4.19. Bonferoni's pairwise comparisons of the mean difference of OD-600 ($\log_{10}$) in synthetic sewage media 2 supplemented with samples various fat/oils (2.5% v/v) inoculated with formulations 1, 6 and 7 incubated at 30°C with shaking at 130rpm for 20 days. 95% confidence interval of any difference = 0.626 *significant differences.

Pairwise comparison		difference in means
Sunflower	Formulation 1 & control	0.454
	Formulation 6 & control	0.517
	Formulation 7 & control	0.153
Lard	Formulation 1 & control	0.387
	Formulation 6 & control	1.366*
	Formulation 7 & control	0.058
Soya	Formulation 1 & control	0.160
	Formulation 6 & control	0.123
	Formulation 7 & control	0.021
Rapeseed	Formulation 1 & control	0.381
	Formulation 6 & control	0.216
	Formulation 7 & control	0.333

The residuals of the transformed data ($\log_{10}$) were approximately normally distributed, there was no significant heterogeneity of variance (Levene's statistic=1.048, P=0.437) and a one-way ANOVA confirmed that there were significant differences between treatments (F=6.77, P<0.001). Bonferoni's pairwise comparisons (Table 4.19) revealed that the optical densities of only samples containing both lard and formulation 6 were significantly greater than those of

the corresponding controls, which suggested that only formulation 6 was capable of growing on lard as the sole source of carbon.

The effect of various fatty acids on the growth of formulations 1, 6 and 7 in synthetic sewage media 2 was also investigated (Figure 4.23)

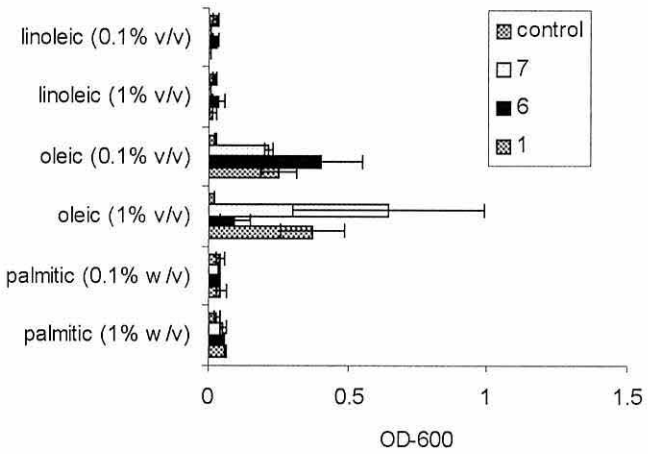


Figure 4.23. Optical densities at 600nm (mean ± SD) (a measure of microbial growth) of synthetic sewage media 2 supplemented with various fatty acids, inoculated with formulations 1, 6 and 7, incubated at 30°C with shaking at 150rpm for 12 days.

The optical density measurements indicated that only formulation 6 was capable of growth on linoleic acid (1%) and that all three microbial formulations were capable of growing on at least one samples containing oleic and palmitic acid sample as the sole carbon source (inoculation increased OD-600 by a factor of 4.1-27.6 and 1.1-1.9, respectively). Inoculation of samples containing linoleic acid generally resulted in a reduction in optical density relative to the controls (inoculation reduced OD-600 by 2.0-65.7 %), implying that all three microbial communities were incapable of growing on linoleic as the sole carbon source or that linoleic acid was inhibitory towards growth. There did not appear to be a relationship between the optical densities of inoculated samples and the concentration of palmitic acid used. However, increasing the quantity of oleic acid from 0.1 to 1% enhanced the bacterial densities of formulations 1 and 7 by a factor of 1.5 and 3.0 respectively. In contrast to previous work with pF33 isolates, the density of bacteria in samples containing 0.1 and 1% of oleic acid samples was greater than that in samples containing palmitic acid (by a factor of 1.8-12.1).

The residuals of the transformed data (square rooted) were approximately normally distributed with no significant heterogeneity of variance (Levene's statistic=1.463, P=0.132)

and a one-way ANOVA indicated that there were significant differences between some of the treatments (F=26.34, P<0.001). Bonferoni's pairwise comparisons (Table 4.20) confirmed that inoculated samples of oleic acid resulted in significantly greater optical densities than the corresponding controls, except in the case of those inoculated with formulation 6 (1% of oleic acid). Samples of oleic acid (1%) inoculated with formulation 7 also generated optical densities that were significantly greater than those inoculated with formulation 1. Furthermore, 1% v/v of oleic acid resulted in a significantly greater bacterial density than 0.1% v/v of oleic acid in those samples inoculated with formulation 7. No other fatty acids significantly enhanced the optical densities of formulation 1,6 and 7 suggesting that formulations 1, 6 and 7 were only capable of growth on oleic acid as the sole carbon source.

Table 4.20. Bonferoni's pairwise comparisons of the mean difference of OD-600 (square rooted) in synthetic sewage media 2 supplemented with various fatty acids, inoculated with formulations 1, 6 and 7, incubated at 30°C with shaking at 150rpm for 12 days. 95% confidence interval of 0.170. * significant differences

Pairwise comparison			Difference in means
Pamitic acid;	Formulation 6;	(1% v/v) & control	0.055
		(0.1% v/v) & control	0.010
	Formulation 7;	(1% v/v) & control	0.055
		(0.1% v/v) & control	-0.001
	Formulation 1;	(1% v/v) & control	0.070
		(0.1% v/v) & control	0.009
Oleic acid;	Formulation 6;	(1% v/v) &control	0.149
		(0.1% v/v)& control	0.470*
	Formulation 7;	(1% v/v) &control	0.629*
		(0.1% v/v)& control	0.307*
	Formulation 1;	(1% v/v) &control	0.452*
		(0.1% v/v)& control	0.344*
Linoleic acid;	Formulation 6;	(1% v/v)& control	0.034
		(0.1% v/v)& control	-0.004
	Formulation 7;	(1% v/v)& control	-0.062
		(0.1% v/v)& control	-0.076
	Formulation 1;	(1% v/v)& control	-0.036
		(0.1% v/v)& control	-0.083
Oleic acid (0.1% v/v);		Formulation 1 & 6	0.126
		Formulation 1 & 7	-0.036
		Formulation 6 & 7	0.162
Oleic acid (1% v/v);		Formulation 1 & 7	-0.177*
Oleic acid;		Formulation 7(0.1% v/v) & Formulation 7(1% v/v)	-0.317*
Oleic acid:		Formulation 1(0.1% v/v) & Formulation 1(1% v/v)	-0.104

4.2.4. Effect of nutrients on microbial oil degradation

In previous experiments, formulations 1, 6 and 7 were shown to have a range of lipase activities (section 4.2.2) and no significant ability to degrade vegetable oils in the absence of alternative carbon sources (section 4.2.3.2). Additional experiments were therefore carried out to determine whether the formulations were capable of degrading oil in the presence of alternative carbon sources (and other nutrients/salts derived from pF33 formulation) and whether lipase activity determinations could be used as an indication of actual oil degrading abilities (Table 4.21).

Table 4.21a. Effect of formulations 1, 6 and 7 on the degradation (mean ± SD) of soya oil (1ml) suspended in 100ml of synthetic sewage media 1(sterilized) supplemented with 1g of pF33 nutrients, incubated for [a]23 or 21[b] days at 30°C with shaking at 130 rpm.

Treatment	Oil degraded (%)
In the presence of pF33 nutrients[a]	
Formulation 1	21.74 ± 3.90
Formulation 6	64.75 ± 1.24
Formulation 7	63.52 ± 1.90
Control	17.00 ± 3.75
In the absence of pF33 nutrients[b]	
Formulation 6	36.28 ± 3.42
Control	16.44 ± 5.17

Table 4.21b. Bonferoni's pairwise comparisons of the mean difference (column-row) of soya oil degraded (%) in samples containing soya oil (1ml) and synthetic sewage media 1 (100ml), inoculated with formulations, 1 ,6, and 7. 95% confidence intervals of any difference = 9.58. *significant difference

	Control(pF33)	1 (pF33)	6 (pF33)	6
1 (pF33)	-4.74	-	-	-
6 (pF33)	-47.75*	-43.01*	-	-28.47*
7 (pF33)	-46.52*	-41.78*	1.23	-
control	-0.56	-	-	19.84*

In the presence of nutrients derived from pF33, formulations 6 and 7 resulted in similarly high levels of oil degradation (increased degradation by factor of 3.7-3.8) whilst oil degradation in samples inoculated with formulation 1 was barely greater than that of the controls (difference of 4.74%). Under conditions of limited nutrient availability, formulation 6 also appeared to be

capable of degrading oil (inoculation increased degradation by a factor of 2.2). However, the extent of oil degradation was approximately half that of the equivalent nutrient enriched samples.

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=3.611, P=0.607), and a one-way ANOVA confirmed that there were significant differences between treatments (F=124.94, P<0.001). Bonferoni's pairwise comparisons (Table 4.21) confirmed that only formulation 6 (in the presence and absence of pF33 nutrients) and formulation 7 significantly enhanced the degradation of oil relative to the appropriate control. The addition of the pF33 nutrients also significantly enhanced the ability of the formulation 6 isolates to degrade oil but did not significantly alter oil degradation in the controls.

4.2.5. Effect of nutrients on the production of particulate material

Particulate material was observed to form in nutrient media 2, which was comprised primarily of a salt solution, yeast and glucose. The effect of these three major constituents on the production of particulate material is displayed in Table 4.22.

Particulate material was observed to form in all samples, except those deficient in salts (i.e. water, yeast or glucose only). In the presence of both the contaminants and grease trap isolates, yeast extract and glucose also appeared to enhance the quantity of particulate material produced.

The residuals were normally distributed and there was no significant heterogeneity of variance (Bartlett's χ^2=3.12-6.35, P=0.096-0.374). Two one-way ANOVA's established that the effect of the salts on particulate production was significant in the case of the contaminants (F=4.05, P=0.05) but not in the case of the grease trap isolates (F=2.02, P=0.189). Tukey's pairwise comparisons (Table 4.22) revealed that those samples containing both salts and glucose produced a significantly greater quantity of particulate material than salts alone.

Table 4.22a. Quantity of suspended particulate material (mg, mean ± SD) produced in media (100-250ml) containing soya oil (1%v/v) and various combinations of salts, yeast and glucose, incubated at 30°C for 10-21 days (contaminants) and 16 days (grease trap isolates).

Treatment	Contaminants	Grease trap isolates
Salts+yeast+glucose	313.5 ± 18.4	-
Yeast extract only	absent	-
Glucose only	absent	-
Water only	absent	-
Salts + yeast +glucose	340.4 ± 84.9	267.3 ± 31.2
Salts + yeast	346.1 ± 35.9	244.3 ± 36.5
Salts +glucose	467.5 ± 9.0	285.0 ± 49.0
Salts only	295.5 ± 86.6	219.4 ± 10.1

Table 4.22b. Tukey's pairwise comparisons of the mean differences (mg) of particulate material produced in media (100-250ml) containing soya oil (1%v/v) and various combinations of salts, yeast and glucose incubated at 30°C for 21 days. 95% confidence interval of any difference = 165.8.* significant difference

Pairwise comparison	Difference in means
Salts only & salts +glucose	-172.0*
Salts only & salts +yeast	-50.6
Salts + yeast & salts + glucose	-121.4
Salts + yeast + glucose & salts only	44.9
Salts + yeast+ glucose & salts+yeast	-5.7
Salts + yeast+ glucose & salts+glucose	-127.1

Having established that the salt solution was fundamental to particulate production, the impact of individual salts was investigated (Table 4.23). The effect of the major salt constituents (KNO_3 and $MgSO_4$) was first assessed. No particulate material was produced in the absence of KNO_3 and was significantly reduced in the absence of $MgSO_4$ (T=-3.29, P=0.02) indicating that the major salt constituents played an important role in particulate production (residuals were normally distributed with no significant heterogeneity of variance; F=0.604, P=0.753)

Further experiments revealed that neither KNO_3 and $MgSO_4$ alone or in combination could induce particulate material indicating that some of the other minor salt constituents were involved in the production of particulate material. To identify whether the cationic or anionic

components of the $KNO_3$and $MgSO_4$ salts were required for particulate production K^+ and Mg^{2+} were replaced with Na^+ and Ca^{2+}, respectively. Replacing the cations reduced particulate production by approximately 50% relative to the controls (i.e. all salts).

Table 4.23a. Quantity of suspended particulate material (mg, mean ± SD) produced in media (100-250ml) containing soya oil (1%v/v) and various combinations of salts, inoculated with grease trap isolates, incubated at 30°C for 18-19 days.

	Treatment	particulate production
(I)	All salts	221.4 ± 77.7
	All Salts - KNO_3	absent
	All Salts - $MgSO_4$	34.8 ± 60.3
(II)	All salts	308.5 ± 59.9
	KNO_3 only	absent
	$MgSO_4$only	absent
	KNO_3 +$MgSO_4$	absent
	$NaNO_3$+(all salts - KNO_3)	153.6 ± 51.1
	$CaSO_4$ + (all salts - $MgSO_4$)	138.1 ± 67.4
(III)	All salts	289.5 ± 41.1
	All salts – disodium hydrogen ortho phosphate	absent
	All salts - $CaCl_2$	406.3 ± 64.8
	All salts - $MnSO_4$	238.8 ± 38.1
	All salts – ferric ammonium citrate	429.0 ± 417.0

Table 4.23b. Tukey's pairwise comparisons of the mean differences ± 95% confidence interval of particulate material produced in media (100-250ml) containing soya oil (1%v/v) and various combinations of salts, inoculated with grease trap isolates, incubated at 30°C for 18-19 days.

Pairwise comparison	Difference in means ± 95% CI
$NaNO_3$+(salts - KNO_3) & All salts	-154.9 ± 149.8*
$CaSO_4$ + (salts – $MgSO_4$) & All salts	-170.4 ± 149.8*
$NaNO_3$+(salts - KNO_3) & $CaSO_4$ + (salts – $MgSO_4$)	15.5 ± 149.8
All salts & Salts -$CaCl_2$	-116.8 ± 124.0
All salts & Salts - $MnSO_4$	50.7 ± 124.0
Salts -$CaCl_2$ & Salts - $MnSO_4$	167.5 ± 124.0*

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartlett's χ^2=0.124, P=0.94) and a one-way ANOVA confirmed that there were significant differences between some of the treatments (F=7.45, P=0.020). Tukey's pairwise comparisons (Table 4.23) established that replacing the cations in both salts significantly reduced the quantity of particulate material produced indicating that both the cationic and anionic components of KNO_3 and $MgSO_4$ were involved in particulate production.

Removing the third major constituent of the salt solution (di-sodium hydrogen ortho phosphate) also prevented the formation of particulate material, whilst the removal of relatively minor salt constituents generally altered the quantity and appearance of the particulate material produced (Table 4.23). Samples that were prepared with all the salts or salts deficient in $CaCl_2$ generated particulate material that was creamy coloured with yellow speckles. However, media that was deficient in $MnSO_4$ or ferric ammonium citrate (in the case of 2 of the 3 replicates) produced flaky creamy coloured material (salts-$MnSO_4$) or a creamy coloured material that resembled melted cheese (Salts-Ferric ammonium citrate). Particulate production was appreciably enhanced by removing both $CaCl_2$ and ferric ammonium citrate, (by 40.2% and 48.2%, respectively) and marginally reduced by removing $MnSO_4$ (by 17.5%).

The residuals were approximately normally distributed, there was no significant heterogeneity of variance (Levene's statistic=2.873, P=0.103) and a one-way ANOVA established that there were no significant differences in particulate production between treatments (F=0.55, P=0.66) Since one of the replicates samples deficient in ferric ammonium citrate failed to generated particulate material the ferric ammonium citrate data was removed from the analysis. This modification resulted in residuals that were normally distributed with no heterogeneity of variance (Bartlett's χ^2=0.577,P=0.749). A one-way ANOVA confirmed that there were significant differences between treatments (F=9.05, P=0.02) and Tukey's pairwise comparisons (Table 4.23) revealed that samples deficient in calcium chloride produced significantly more particulate material than samples deficient in manganese sulphate indicating that calcium chloride exerted a greater inhibitory effect on particulate production than magnesium sulphate.

In summary, the major salt components of the media, (i.e. potassium nitrate, magnesium sulphate, di-sodium hydrogen ortho phosphate) were essential for the production of particulate material whilst yeast extract, glucose and the minor components of the salt media (i.e. calcium chloride, manganese chloride and ferric ammonium citrate) and the potassium and magnesium constituents of the major salts were not.

4.2.6. The Growth and Adherence of Bacteria to Surfaces

Effect of nutrients and microbial supplementation on the density of adhering bacteria

Lard coated glass slides were suspended in either nutrient media 1 or water inoculated with P80 or GTL and the density of bacteria adhering to the lard-coated slides was determined at regular intervals using the DAPI staining technique. Figure 4.24. illustrates the effect of nutrients and supplementation with GTL and P80 on the density of bacteria adhering to lard.

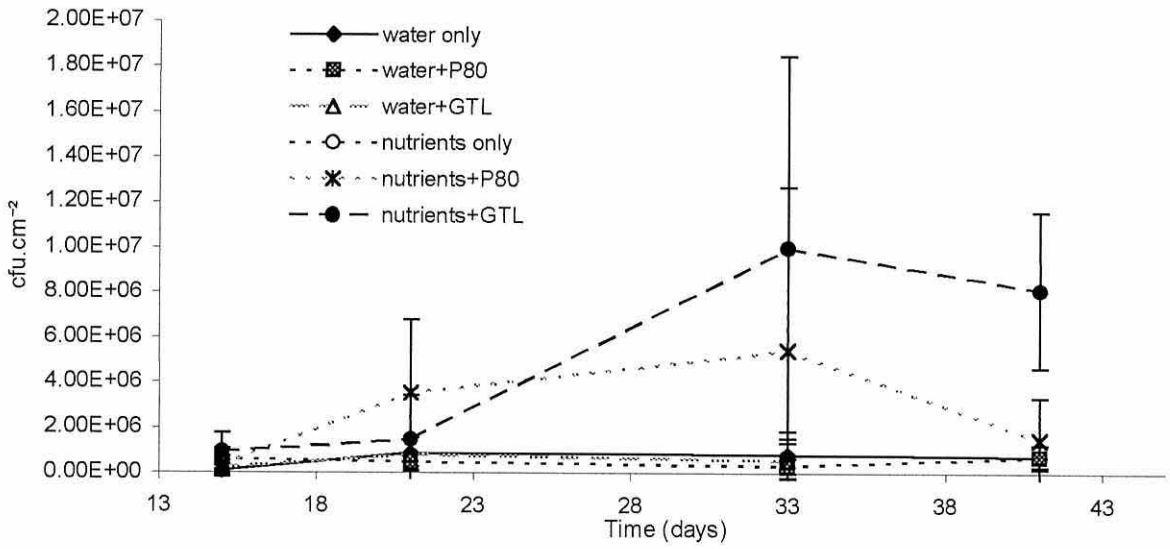


Figure 4.24. Effect of nutrients (nutrient media 1) and microbial supplementation with GTL and P80 (0.1%v/v) on the density of bacteria (mean cfu.cm^{-2} ± SD) adhering to lard coated coverslips suspended in 500ml of water/nutrient media (sterilized), incubated at room temperature.

Bacteria were observed on all lard surfaces and the large coefficients of variation (42.6-316.2%) indicated that the coverage of bacteria was uneven. Bacterial densities were low in the absence of nutrients over the entire length of the incubation period (8.68E+04 - 8.51E+05 cfu.cm^{-2}) and inoculation of water with GTL or P80 appeared to increase bacterial densities

on day 15 of the experiment (by a factor of 2.4-7.0) and have the reverse effect thereafter (bacterial densities reduced by 6.1%-60.0%, relative to water).

The addition of nutrients enhanced the bacterial densities of the inoculated samples on all sampling occasions, except day 15 (by a factor 1.8-17.3) and the effect of nutrients became more apparent as the length of the incubation period was extended (for a further 33 days), particularly in the case of GTL. On day 39 of the experiment, the effect of nutrients in both inoculated samples was less marked than on day 33, but was similar to or greater than that observed on day 15. As was the case with the lard suspended in water, the impact of supplementing the nutrient media with GTL and P80 was unclear. The data for the nutrient control samples (nutrient only) was available on day 15 only due to focussing difficulties and GTL marginally enhanced bacteria densities (by a factor of 1.5, relative to water only) whilst P80 did not (bacterial density reduced by 44.4% relative to water only).

The data was analysed in two ways; firstly, by examining how each individual treatment varied over the course of the experiment and secondly, by comparing the individual treatments at each of the sampling times. The data were transformed ($\log_{10}$) where appropriate and with the exception of the nutrients+GTL data, the residuals for all other data sets were normally distributed with no significant heterogeneity of variance (Bartlett's χ^2=1.225-6.56, P=0.161-0.521) (nutrients+GTL data, normally distributed with significant heterogeneity of variance (Bartlett's χ^2=9.747, P=0.021)).

A series of one-way ANOVA's and a Moods median test (in the case of nutrients+GTL) revealed that the density of bacteria on lard varied significantly with time in only the non-inoculated water samples and in nutrients supplemented with P80 and GTL (Table 4.24). Further one-way ANOVA's determined that there were significant differences between treatments on day 15, 33 and 41 of the experiment only.

Bonferoni's pairwise comparisons (Table 4.24) confirmed that the addition of nutrients and P80 to water significantly enhanced the density of bacteria on day 15 of the experiment only. The addition of nutrients to GTL and P80 also significantly enhanced the density of bacteria on day 15 and 33 (only in case of GTL) of the experiment and GTL resulted in a significantly

greater density of bacteria than P80 in the nutrient supplemented media on day 41 of the experiment.

Table 4.24a. F/χ^2 and P values for the one-way ANOVA's/Mood median test applied to compare the densities of bacteria adhering to lard coated coverslips suspended in 500ml of water, supplemented with nutrients, inoculated with GTL or P80 after 15-41 days incubation.

Treatment	F/χ^2	P
Water only	5.55	0.003
Water+P80	1.53	0.224
Water+GTL	2.53	0.098
Nutrients only	-	-
Nutrients+P80	5.80	0.002
Nutrients+GTL	19.55	<0.001
Day 15	4.86	0.001
Day 21	2.51	0.055
Day 33	9.75	<0.001
Day 41	22.58	<0.001

Table 4.24b. Bonferoni's pairwise comparisons of the mean difference ± 95% confidence intervals of the density of bacteria ($\log_{10}$ cfu.cm^{-2}) adhering to lard coated coverslips suspended in 500ml of water/nutrient media 1 supplemented with nutrients and inoculated with P80 and GTL incubated for 15, 33 and 41 days.* significant difference

Pairwise comparison	Day15	Day33	Day 41
Water only & Water+P80	-0.511±0.413*	0.144±0.650	0.020±0.359
Water only & Water+GTL	-0.162±0.413	0.066±0.650	-
Water+P80 & Water+GTL	0.349±0.413	-0.078±0.650	-
Nutrient only & Nutrient+P80	0.155±0.413	-	-
Nutrient only & Nutrient+GTL	-0.132±0.413	-	-
Nutrient+P80 &Nutrient+GTL	-0.287±0.413	-0.310±0.650	-0.858±0.359*
Water+P80 & Nutrient+P80	0.218±0.413	-0.817±0.650*	-0.149±0.359
Water+GTL & Nutrient+GTL	-0.418±0.413*	-1.049±0.650*	-
Water only & Nutrient only	-0.448±0.413*	-	-

Having established that nutrient supplementation significantly enhanced the density of bacteria adhering to lard on more than one occasion, a similar experiment was conducted using liquid F33 and the nutrient media provided by Organica (Figure 4.25).

As in the earlier experiment, bacterial densities fluctuated with time and coverage was uneven (coefficient of variation; 82.7-316.2%). The microbial supplement (F33) also appeared to enhance the density of bacteria relative to the control after 7 days incubation (by a factor of

1.7-16.2) and the relationship between bacterial density and time was non-linear. (Range of bacterial densities in the control and inoculated sample were 1.56E+05-5.03E+05 and 1.04E+05-2.08E+06 cfu.cm^{-2}, respectively).

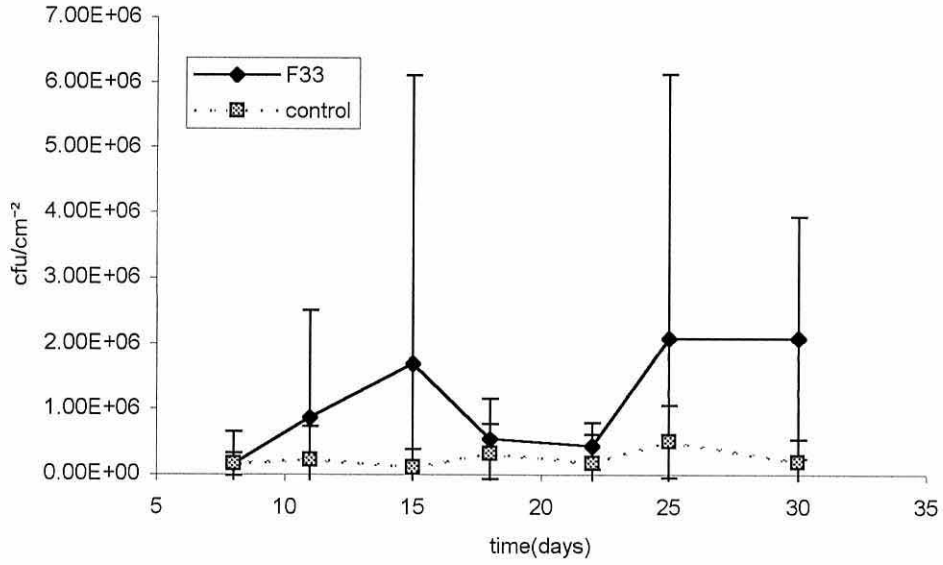


Figure 4.25. Effect of liquid F33 (0.2%v/v) on the density of bacteria (mean cfu.cm^{-2} ± SD) adhering to lard coated coverslips suspended in 500ml of sterilized Organica nutrient media (0.2%w/v) incubated at room temperature.

The residuals of the transformed data ($\log_{10}$) were approximately normally distributed and displayed no significant heterogeneity of variance (Levene's statistic=1.589, P=0.097). A two-factor ANOVA determined that there was no interaction between factors (F=1.02, P=0.413), that the average change in bacterial density with time was significant (F=2.34, P=0.035) and that the average bacterial density of the inoculated sample was significantly greater than that of the control (F=19.15, P<0.001).

Effect of surface composition and microbial supplementation on the density of adhering bacteria

Since a variety of surfaces were expected to arise in a wastewater environment, the effect of both lard and plastic surfaces on the density of adhering bacteria was investigated. Figure 4.26. illustrates the effect of surface composition and supplementation with formulation 7 and the grease trap isolates on the density of adhering bacteria.

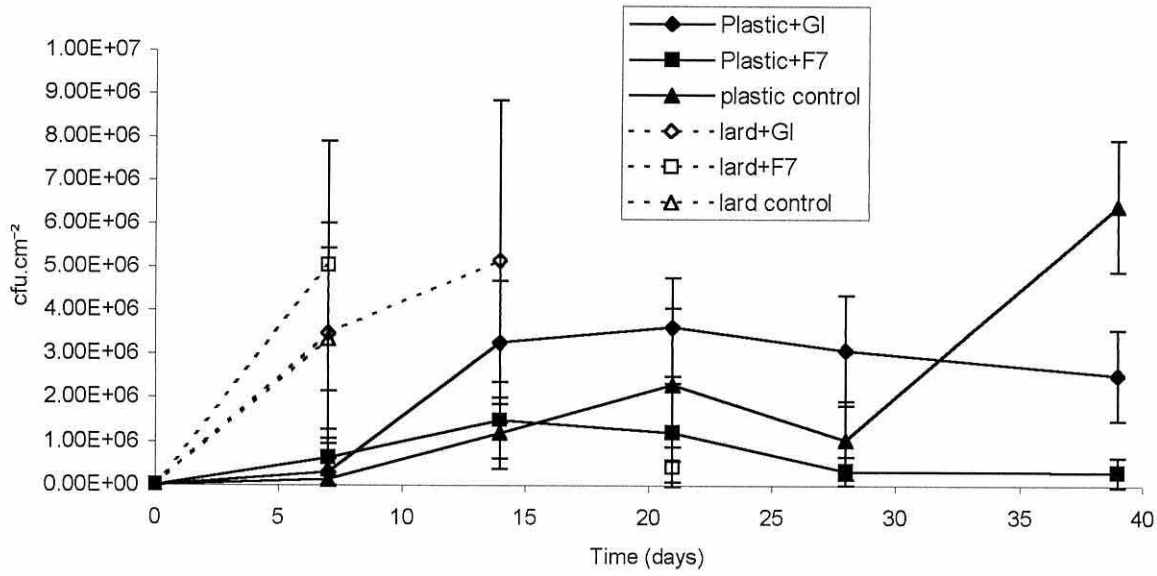


Figure 4.26. The effect of the grease trap isolates (GI) (0.05%v/v) and Formulation 7 (F7) (0.08%) on the density of bacteria adhering (cfu.cm^{-2} mean ± SD) to plastic or lard coated coverslips suspended in 500ml of synthetic sewage media 1 (sterilized) incubated at room temperature.

The density of bacteria varied considerably with the inoculum source, surface composition and incubation time and bacterial coverage on all surfaces was uneven (coefficient of variation; 31.3-132.4%). On day 7 of the experiment, the density of bacteria on lard was appreciably greater than that on the corresponding plastic treatment (by a factor of 8.0-28.3) and formulation 7 resulted in greater bacterial densities than either the grease trap isolates or the control on both lard and plastic (by a factor of 2.1-5.3 and 1.4-1.5 on plastic and lard, respectively). After day 7-14, however, observational and quantitative evidence indicated that bacterial densities of the grease trap isolates on both lard and plastic surfaces were greater than those of formulation 7 and the control (except on day 39) and that the bacterial densities of the plastic control samples were greater than those of formulation 7 (by a factor of 1.9-21.5).

The data were analysed using the approach outlined in the earlier biofilm experiment. The data was transformed where appropriate ($\log_{10}$ or square rooted) and the residuals for all treatments, except those of plastic+F33, plastic only, and day 21, were normally or approximately normally distributed with no significant heterogeneity of variance (Bartlett's χ^2=0.214-33.398, P=0.065-0.899; Levene's statistic=1.34-2.308, P=0.081-0.251). (Residuals from plastic+F33, Plastic only, and day 21 were normally or continuously distributed with significant heterogeneity of variance (Bartlett's χ^2=33.398-90.471, P<0.001; Levene's

statistic=13.6-14.528, P<0.001)). A series of one-way ANOVA's, Mood median tests and two sample t-tests (Table 4.25) revealed that the bacterial densities of all surfaces, except lard inoculated with the grease trap isolates, varied significantly with time and that there were significant differences between some of the treatments at all incubation times.

Table 4.25a. F/T/χ^2 and P values for the one-way ANOVA's, Mood median test, two sample t-tests applied to compare the densities of bacteria adhering to plastic or lard coated coverslips suspended in 500ml of synthetic sewage media 1, inoculated with the grease trap isolates (GI) or Formulation 7 (F7), incubated at room temperature for 7-29 days.

Treatment	Test statistic	P
Plastic+GI	F=78.49	<0.001
Plastic+F7	χ^2=35.76	<0.001
Plastic only	χ^2=37.72	<0.001
Lard+F7	T=8.99	<0.001
Lard+GI	T=-1.85	0.071
Lard only	-	-
Day 7	F=38.79	<0.001
Day14	F=11.59	<0.001
Day21	χ^2=51.96	<0.001
Day28	F=68.38	<0.001
Day29	F=160.75	<0.001

Table 4.25b. Bonferoni's and Tukey's pairwise comparisons of the mean difference ± 95% confidence intervals of the density of bacteria ($\log_{10}$ or square rooted cfu.cm^{-2}) adhering to plastic or lard coated surfaces suspended in 500ml of synthetic sewage media 1, inoculated with the grease trap isolates (GI) or Formulation 7 (F7), incubated at room temperature for 7-29 days.

Pairwise comparison	Day 7	Day 14	Day 28	Day 39
Plastic+F7 & Lard+F7	-0.779±0.310*	-	-	-
Plastic+GI & Lard+GI	-0.791±0.310*	-0.027±0.267	-	-
Plastic only & Lard only	-0.945±0.310*	-	-	-
Plastic+F7 & Plastic+GI	0.235±0.310	-0.379±0.267*	-2.980±0.619*	-2.705±0.747*
Plastic+F7 & plastic only	0.411±0.310*	0.072±0.267	-1.015±0.619*	-5.006±0.747*
Plastic+GI & plastic only	0.176±0.310	0.451±0.267*	1.965±0.619*	-2.301±0.747*
Lard+F7 & lard only	0.245±0.310	-	-	-
Lard+GI & lard only	0.022±0.310	-	-	-
Lard+F7 & Lard+GI	0.222±0.310	-	-	-

The various pairwise comparisons (Table 4.25) confirmed that lard significantly enhanced the density of adhering bacteria in both the control and inoculated samples on day 7 of the experiment. They also indicated that the grease trap isolates significantly enhanced the

density of bacteria adhering plastic relative to formulation 7 on day 14, 28 and 39 and relative to the control on day 14 and 28. Bacterial densities in the plastic control sample, however, were significantly greater than those inoculated with formulation 7 on day 28 and 39 and the grease trap isolates on day 39.

4.3. Discussion

The impact of a microbial community on the physico-chemical characteristics of a fat/oil will depend on (1) the capacity of the microorganisms to survive and reproduce under the environmental conditions imposed (2) the ability of the microorganisms to produce lipase (3) the capacity of the microorganism to transport the products of hydrolysis into the cell and utilise for them for growth amongst other cell functions, and in a dynamic environment (4) the capacity of the microorganisms to adhere to surfaces.

The growth of microbial formulations 1, 6 and 7 was optimum over a temperature range of 20-40°C, a pH range of 5.6-9.0 and in the presence of oxygen. Furthermore, there was evidence of microbial growth at extremes of temperature, at pH 11.2 and under conditions of oxygen limitation. Given that aerobic and anaerobic conditions arise in a wastewater environment and that a study of grease trap effluent has shown that the temperature and pH of wastewater can range from 14-52°C and 4.8-12.2 with averages of 28°C and 6.8, respectively (Holt, 1992), microbial formulations 1, 6 and 7 have the potential to survive and grow in a wastewater environment, provided adequate levels of nutrients are present.

The ability of the microbial formulations to grow over a wide variety of environmental conditions was not unexpected. All the formulations were comprised of several different microbial strains and species which were likely to have different growth ranges and optimums (Appendix VII). Formulations 1 and 7 were comprised of the same strains of bacteria but in different ratios (Appendix VII & section 5.2.3.2) which may explain why their responses to the different environmental conditions were similar. The microbial diversity of formulations 1 and 7 was also greater than that of formulation 6 (Appendix VII & section 5.2.3.2) which may account for the stronger growth of formulations 1 and 7 at extremes of temperature. Formulation 6, in contrast, was better adapted than formulations 1 and 7 for growth under

oxygenated conditions, at mesophilic temperatures and at alkaline pH's suggesting that it was designed to operate under less extreme environmental conditions.

Whilst nutrient levels in wastewater are reported to vary (Metcalf & Eddy, 1991), they are considered adequate to support microbial growth (Brock & Madigan, 1991). The synthetic sewage media employed in this study, however, induced only low levels of microbial growth in the supplements and a small but significant extent of oil degradation. Growth on synthetic sewage media was dramatically improved by adding easily metabolizable carbon sources or organic nitrogen (i.e. glucose or peptone) whilst oil degradation was enhanced by adding the pF33 nutrient supplement, which was comprised of a carbon/organic nitrogen source, salts and vitamins. These results are in agreement with those of Tano-Debrah *et al.* (1999) who also demonstrated that the extent of microbial oil degradation was influenced considerably by the nutrient composition of the media.

Nutrient additions not only improved oil degradation rates and microbial growth but also significantly enhanced the density of bacteria adhering to surfaces. These findings support the work of Heukelekian and Crosby (1955) who observed increased slime growth in sewage on addition of glucose and peptone. The adherence of organic nutrients to solid surfaces has also been known to promote the growth and attachment of bacteria (Zobell, 1943) which may account for the significantly higher bacterial densities on lard coated surfaces relative to plastic.

Given that the extent of oil degradation and density of adhering bacteria was enhanced by the presence of nutrients, it was postulated that the performance of a bioaugmentation product in wastewater may be improved by incorporating nutrients into the supplement. The inclusion of nutrients into microbial supplements is technically difficult, however, since an inhibitor must be added to prevent growth of the microorganisms during storage and the current study demonstrated that the growth of certain microoganisms was limited if the inhibitor was not adequately diluted. Furthermore, nutrient availability was shown to influence the production of particulate material by the grease trap isolates indicating that nutrient supplementation may have a detrimental effect on grease control.

Particulate material, unlike the polymers observed by Mudge *et al.* (1994), formed only when certain nutrients were present in the media. These results further supports the assumption of the earlier chapter (section 3.3), that particulate material was not simply polymerised oil. The major salt components of the media, (i.e. potassium nitrate, magnesium sulphate or di-sodium ortho phosphate) appeared to be essential for the production of particulate material whilst yeast extract and the minor components of the salt media (i.e. calcium chloride, manganese chloride and ferric ammonium citrate) and the potassium and magnesium constituents of the major salts were not. Although glucose was not essential for particulate production, the production of particulate material was significantly enhanced in its presence further indicating that particulate material was of a biological origin. It was assumed that the major salts were simply stimulating the growth of particulate producing microorganisms since they provided the basic ingredients (i.e. carbon, nitrogen and phosphorus) for microbial growth.

In addition to nutrients, wastewater is also known to embody potential growth inhibitors such as bleach and surfactants (Metcalf & Eddy, 1991; Holt, 1992). Bleach is strongly inhibitory to microbial growth and manufacturers of commercial microbial supplements usually recommend that the discharge of bleach into the drains and sewers is tightly controlled. Formulations 1, 6 and 7 were capable of growing in the presence of bleach after a dilution factor of approximately 1:10000, indicating that the application of bleach in a wastewater system which is regularly flushed with wastewater or has the capacity to retain large quantities of wastewater (for example a grease trap) may not impede the performance of commercial inocula.

Unlike bleach, commercial and domestic surfactants did not completely inhibit the growth of the microbial supplements over the concentration range investigated, but reduced growth by ca.15-60%. Increasing the concentration of the surfactants above 2 log ppm appeared to have very little effect on microbial growth, which implied that microbial formulations 1, 6 and 7 may remain viable in surfactant-rich wastewater. Whilst there are no reports of commercial or domestic surfactants inhibiting microbial growth, earlier workers (Frobisher, 1926; Hotchkiss, 1946) associated the antibacterial activities of various compounds, including those of fatty acids, with surface active effects. More recent work, in contrast, found that molecules must possess a polar end group to exert an inhibitory effect but that there was no direct correlation between surfactant and antibacterial activity (Blaxter & Czerkawaski, 1966).

Aside from growing over a wide range of environmental conditions, several of the microbial formulations were also capable of producing lipase after 24 or 48 hours incubation on a variety of lipid substrates. The majority of the non-cultured and cultured microbial formulations appeared to hydrolyse tributryn more readily than the natural fats and oils, which was not unexpected given that tributryn has been reported to be the most widely attacked of the triglycerides (Hugo & Beveridge, 1962; Alford & Steinle, 1967). Non-cultured formulations 3 and 4 were also able to hydrolyse tween-40 more readily than tween-20 and tween-80, indicating that saturated fatty acids of 16 carbon atoms in length were hydrolysed more readily than shorter chained saturated fatty acids (12:0) or monunsaturated fatty acids of a similar chain length (18:1). According to Coleman (1963), however, the rate of bacterial hydrolysis is not appreciably affected by the molecular weight of the fatty acids for chain lengths of 12 to 18 carbon atoms. Thus, tween-20 might have been expected to be hydrolysed at a similar rate to tween-40. Given that it is the accumulation of long chained fatty acids and triglycerides which lead to the formation of grease caps in grease traps (Grubbs *et al.*, 1991), the ability of some formulations to hydrolyse long chained fatty acids more readily than shorter chained ones may be advantageous in a wastewater environment.

Formulations 2 and 6 were capable of hydrolysing complex, natural fats and oils to a similar extent as tributryn. It was assumed therefore that some of the microorganisms in these formulations were producing lipase that preferentially attacks fatty acids with a double bond at the 9-10 position and/or fatty acids of 12-18 carbon atoms in length, in addition to producing lipase that attacks shorter chained fatty acids (i.e. tributryn). Microbial formulations 3, 4 and 5 only hydrolysed some of the natural fats/oils after the formulations were grown and diluted to achieve similar bacterial densities indicating that a change in bacterial density or microbial community composition during growth may have affected lipase production or activity. According to Alford and Pierce (1963), the quantity or rate of lipase production is not a function of cell growth or concentration. Thus, the change in bacterial densities was unlikely to have induced the above mentioned changes.

Formulations 2 and/or 6 were largely comprised of high lipase producing strains (Appendix VII) therefore it was not unexpected that these formulations exhibited the strongest lipolytic activities on most lipid substrates even after growth and adjustment of the starting bacterial densities. Given that formulations 2 and 6 exhibited the strongest lipase activities they were

predicted to be better equipped than formulations 1, 3, 4, 5 and 7 to degrade fat/oil in a wastewater environment, particularly if hydrolysis was the rate limiting step in the degradation process. However, one of the poor lipase producers (formulation 7) was capable of degrading a similar quantity of oil to a high lipase producer (formulation 6) implying that hydrolysis was not a rate limiting step in the oil degradation process and that lipase activity measurements alone could not predict the oil degrading potential of a commercial microbial supplement.

Cultured and non-cultured formulations 2 and/or 6 also exhibited the greatest protease and amylase activity, which indicated that formulations 2 and 6 would also be able to degrade the protein and starch present in wastewater and act on grease deposits of a heterogeneous nature.

Unfortunately, the results of the lipase activity tests were ambiguous on some occasions. Agar around the wells on tween plates often became crystalline in appearance obscuring the zone of colour intensification. Furthermore, the zone of colour intensification around adjusted formulation 2 was barely discernible from the background of the natural fat/oil agar. Although the origin of the crystalline material on the tween plates was unknown, the weak colouration of the natural fat/oil agar may been due to the production of short chained fatty acids by formulation 2 since long chained fatty acids of limited water solubility are known to give an intense blue colour, whilst short-chained fatty acids diffuse more easily through the agar to result in a weak blue colour (Alford & Steinle, 1967). The short chained fatty acid may have been the result of β-oxidation or non-lipolytic events such as carbohydrate fermentation. However, carbohydrate fermentation rather than β-oxidation has been implicated as a source of false positive results in other studies (Alford & Steinle, 1967).

The lipolytic activities of the S45 microorganisms grown at different dilutions were also investigated. Serial dilution of S45 did not give the expected pattern of microbial growth on nutrient agar and those microorganisms cultured from higher dilutions exhibited greater lipolytic activities than those grown from lower dilutions. These findings indicated that the inhibitor and/or antibiotic-producing bacteria present in S45 may have prevented the growth of the strongest lipase-producing microorganisms and may explain why S45 failed to degrade to oil in some of the earlier degradation experiments (section 3.2.2). Since microbial supplements are likely to be subjected to greater dilutions under field conditions than in the

laboratory, the presence of inhibitors may also explain why some commercial microbial supplements have failed to augment processes in the laboratory but not in the field (Stephenson & Stephenson, 1992).

In addition to hydrolysing several lipids, some of the commercial inocula investigated were capable of growth on lipids as the sole carbon source. The preliminary findings indicated that microbial growth was influenced by the concentration of lipid substrate used. Microbial growth occurred when the lipid was present at a concentration that was high enough to meet the carbon requirements of the cell but below that which was toxic.

The water soluble substrates (tween and sodium linoleate), which were not considered to be true lipids, had a varied effect on microbial growth. Tween stimulated extensive microbial growth whilst growth on sodium linoleate was poor. In both cases, the relationship between substrate concentration and microbial growth was strongly positive. The ability of tween to stimulate strong microbial growth was not unexpected given that microorganisms have been known to grow on agar at higher concentrations of tween (Alford & Steinle, 1967). Similarly, the poor growth of the formulations on sodium linoleate was fairly predictable since comparable concentrations of sodium linoleate have inhibited the growth of gram-positive microorganisms in other studies (Galbraith *et al.*, 1971).

For most of the poorly water soluble lipids investigated (non-emulsified vegetable oil, palmitic acid, oleic acid and linoleic acid) high lipid concentrations tended to reduce the extent of bacterial growth. Under these circumstances inhibition may have been due to the antibacterial effects of the fatty acids/oil or a reduction in the permeability of the surface to oxygen. Another possibility was that the larger quantities of oil/fatty acid were poorly dispersed into the media. Some of the poorly water soluble lipid substrates investigated (ie non-emulsified vegetable oil and palmitic acid) also stimulated poor microbial growth at low lipid concentrations, indicating that the carbon requirements of the microorganisms had not been met.

Given that the degradation and removal of emulsified or partially water soluble lipid substrate from the media may have masked any changes in microbial growth, optical density was

considered to be inappropriate measure of microbial growth on these lipid substrates (emulsified vegetable oil, sodium palmitate and sodium oleate).

Most of the microbial formulations investigated did not appear to utilise fat/oil as the sole carbon source. Only lard and soya oil were utilised as the sole carbon source and these results were questionable, given that growth on soya oil was not reproducible and the lard was comprised of 1% protein, in addition to triacylglerols (as indicated by the nutritional information on lard packaging). Since all the formulations exhibited lipolytic activity on the nutrient enriched natural fat/oil agar and were capable of utilising at least one fatty acid as the sole carbon source it was postulated that the microbial formulations may have been unable to hydrolyse the fat/oil in the absence of alternative carbon or organic nitrogen source. The failure of most of the microbial communities to grow on oil as the sole carbon is supported by some of the COD determinations discussed in section 3.3. and is agreement with the work of Tano-debrah *et al.* (1998). Whilst the ability of a microbial supplement to utilise fat/oil as a sole carbon source is an advantage, it is not essential, since wastewater contains alternative nitrogen/carbon sources.

Of the fatty acids investigated (16:0, 18:1ω9 and 18:2ω6), only 16:0 and/or 18:1ω9 were utilised by the microbial supplements as the sole carbon source. These results do not reflect the greater biodegradability of the unsaturated fatty acids relative to their saturated counterparts, as has been observed in other degradation studies. (Parker & Leo, 1965; Loehr & Roth, 1968; Malaney & Gerhold, 1969; Farrington & Quinn, 1971; Sun & Wakeman, 1994). Unsaturated fatty acids above a threshold concentration, are considered to be more toxic than their saturated analogs (Galbraith *et al.*, 1971), therefore the concentration of 18:2ω6 used in the current study (0.1-1%v/v) may have been inhibitory to the growth of the microbial formulations. There are reports of 18:2ω6 inhibiting bacterial growth over a concentration range of 6-30mg.l^{-1} (Galbraith *et al.*, 1971; Lalman & Bagley, 2000). However, the linoleic acid referred to in some of these studies was dissolved in water in the form of a sodium salt and was not present as the sole carbon source, therefore the results cannot be compared directly. As with linoleic acid, oleic has also been known to inhibit the growth of bacteria (Galbraith *et al.*, 1971) which may account for the growth of pF33 on 0.1% of oleic acid but not on 0.5 or 1%. According to Galbraith *et al.* (1971) and Ratledge (1994), fatty acids must be in solution to exert antibacterial activity. However, the results of this study

indicate that a high degree of water solubility may not be a requirement for antibacterial activity.

The sole carbon experiments and degradation assays further indicated that microbial community composition influenced the extent of oil degradation and microbial growth. Formulation 6, for example, was capable of significant growth on lard whilst formulation 1 and 7 were not. Similarly, formulations 6 and 7 significantly enhanced the degradation of rapeseed oil but formulation 1 did not. Since formulations 1 and 7 were comprised of the same strains of bacteria but in different ratios these findings also indicated a commercial microbial supplement must be comprised of bacteria in a certain ratio for oil degradation to occur.

As mentioned previously, some of the microbial formulations investigated (i.e. F33, formulation 7 and P80) were capable of adhering to lard or plastic surfaces on one or more occasions, which implied that some commercial inocula may remain in contact with the grease deposits in wastewater systems for long enough to exert their effects. It was also noted that the grease trap isolates significantly enhanced the density of adhering bacteria relative to formulation 7 on several occasions, indicating that commercial inocula may have to compete with high numbers of naturally occurring bacteria on the surfaces of drains, sewers and grease traps, in addition to the indigenous bacteria suspended in the wastewater.

The bacterial densities of several microbial communities on surfaces varied significantly with incubation time. Bacterial densities were observed to either fluctuate with time or increase over the first few days or weeks of the incubation period and then decline thereafter. An increase in bacteria density possibly reflected microbial growth at the surface or growth of microorganisms in the suspended state, which increased the frequency at which bacteria came into contact with the surfaces. Microbial growth on surfaces often occurs in micro-colonies (Caldwell & Lawrance, 1986), therefore the uneven coverage of bacteria on the surfaces was not unexpected. The decline in the density of adhering bacteria may have been due the biofilm sloughing off or the dispersal of attached microbial cells into the media. Bacteria often detach from surfaces following a decrease in metabolic activity brought on by starvation (Bright & Fletcher, 1983) indicating that some of the microbial communities may have depleted the nutrients in the media. The fluctuations in bacterial density may have been due to the fact that some microorganisms disseminate daughter cells into the suspended state as they complete

cell separation. After cell division, these daughter cells can then return to the surface where the process is repeated (Power & Marshall, 1988).

5. COMMUNITY DYNAMICS AND FAT/OIL DEGRADING ABILITIES OF PF33

5.1. Introduction

Several studies have shown that mixed or single strain microbial inocula are capable of enhancing the degradation of fat/oil under laboratory conditions, (Koritala *et al.*, 1987; Tano-Debrah *et al.*, 1999; Keenan & Sabelnikov, 2000; Markossian *et al.*, 2000; Mihara *et al.*, 2000). However, it has often been argued that biodegradation in the field differs from that predicted in the laboratory and a number of authors have shown that inocula fail to do in the natural environment what they can do in axenic culture. MacRae and Alexander (1965) demonstrated that a 4-(2,4-dichlorophenol) butyrate-utilising *Flavobacterium* sp. was unable enhance the degradation of the herbicide when added to soil. Similarly, Anderson *et al.* (1970) using a strain of *Mucor alternans* able to metabolize DDT (1,1,1-trichloro-2,2-bis-(4-chlorophenyl) ethane) in culture, discovered that the fungus was unable to degrade the insecticide in soil. Goldstein *et al.* (1985) also found that a *Pseudomonas* strain capable of mineralising 2,4-dichlorophenol and p-nitrophenol *in vitro* could not cause appreciable mineralisation of the compounds in sewage or lake water.

Several workers have suggested that microorganisms able to degrade organic compounds in culture often fail when inoculated into the natural environment as a result of competition with other microorganisms (Alexander, 1984; Goldstein *et al.*, 1985; Wilderer *et al.*, 1991), the concentration of the target pollutant being too low to support growth (Goldstein *et al.*, 1985), the presence of inhibitors or unfavourable growth conditions (Alexander, 1984; Goldstein *et al.*, 1985; McClure *et al.*, 1991) the utilisation of other organic substrates rather than the target pollutant (Goldstein *et al.*, 1985; Wilderer *et al.*, 1991), or the number of microorganisms being too few to effect change (Lynch *et al.*, 1987; McClure *et al.*, 1991).

Although several authors have suggested that the growth of microbial supplements may be limited under field conditions, very little effort has been made to investigate the growth and survivability of bioaugmentation products after addition to a wastewater treatment system. Lange *et al.* (1988) reported that only 0.5% of the supplemented bacteria added to a batch scale reactor remained viable after 2 hours. Similarly, McClure *et al.* (1991) noted that the

numbers of a genetically engineered microbe introduced to a bench scale activated sludge unit survived for over eight weeks but only at a relatively low level. Both Lange *et al.* (1988) and McClure *et al.* (1991) employed culture based techniques to assess the growth and survivability of the microbial supplements. However, the capacity of these methods to quantify the structure and diversity of microbial populations was severely limited by virtue of the fact that large fractions of the microorganisms in nature are refractory to cultivation. Furthermore, the failure to mimic the original environmental parameters during cultivation may alter community structure due to the imposition of new conditions (Muyzer *et al.*, 1993; Liu *et al.*, 1997; Padmanabhan *et al.*, 1998).

An alternate method of assessing the growth and survivability of a bioaugmentation product is the nucleic acid based approach. In contrast to culture dependent methods, nucleic acid based techniques provide a powerful tool for analysing the structure and species composition of a microbial community (Muyzer, 1993; Liu *et al.*, 1997) and offer new opportunities for determining the availability and ecological function of catabolic genes that encode important pathways of xenobiotic metabolism (Padmanabhan *et al.*, 1998). Recently, a nucleic acid based technique has been developed which involves examining variations in 16S rRNA or 16S rRNA-encoding DNA (rDNA) within naturally occurring prokaryotic communities. This method has been used to assess the structure and diversity of microbial communities isolated from a range of environments including; hydrothermal vents (Moyer *et al.*, 1994), terrestrial soils (Dunbar *et al.*, 1997), marine sediments (Muyzer *et al.*, 1993), activated sludge, bioreactor sludge, aquifer sand, termite guts (Liu *et al.*, 1997) and drinking water treatment systems (Regan *et al.*, 2002). Whilst variations in 16S rRNA have never been used to monitor the growth and survivability of microbial supplements in other studies, Yu and Mohn (2001) assessed the survivability of two resin acid-degrading bacteria added to a sequencing batch reactor using another nucleic acid-based approach (ribosomal intergenic spacer analyser). These workers found that the artificially introduced bacteria were persistent in the sludge community after inoculation but did not substantially change the indigenous microbial communities composition.

The main objective of the work outlined in this chapter was to determine how environmental parameters affected the structure, diversity and oil degrading abilities of a new microbial formulation (pF33) thus allowing laboratory data to be better extrapolated to the field.

Another objective was to establish whether fat/oil degradation pathways were influenced by variations in environmental parameters.

5.2. Results

5.2.1. Factors affecting the fat/oil degrading abilities of pF33

The effect of incubation temperature, starting pH, oxygen availability and presence of grease trap isolates on the degradation of rape oil by pF33 were investigated. Due to time restraints, the phsyico-chemical controls for only the oxic treatment were prepared. Under these conditions, the extent of oil degradation was 22.93 ± 5.99% (mean ± SD, 3 observations). All samples were prepared with 1 ml of rapeseed oil, 100 ml of synthetic sewage media 1, 1g of pF33 (peanut shell carrier) and were incubated for 21 days, at 30°C with shaking at 130 rpm, at a starting pH of ca.7.3, unless stated otherwise.

Effect of oxygen availability on the ability of pF33 to degrade oil

The pF33 microorganisms were capable of degrading oil under both oxic conditions and conditions of limited oxygen availability. However, the extent of oil degradation under oxic conditions was approximately twice that experienced under conditions of limited oxygen availability (Table 5.1).

Table 5.1. Effect of oxygen availability on ability of pF33 to degrade oil (mean ± SD).

Treatment	Oil degraded (%)
Oxic conditions	82.07 ± 6.43
Limited oxygen availability	41.51 ± 11.39

The residuals of the data were normally distributed, there was no significant heterogeneity of variance (F=0.318, P=0.483) and a two sample-test confirmed that restricting the supply of oxygen to the samples significantly reduced the degradation of oil (T=5.37, P=0.006).

Plate counts on day 13 of the experiment also indicated that a large number of microorganisms were present under both oxic conditions and conditions of limited oxygen availability. However, a greater number of microorganisms were present under oxic conditions than under conditions of limited oxygen availability (Oxic conditions: 3.42 x 10^9 cf.u.ml^{-1}, conditions of limited oxygen availability; 2.7x 10^7 cf.u.ml^{-1})

Effect of starting pH on the ability of pF33 to degrade oil

The pF33 microorganisms were capable of degrading oil over a wide range of starting pH's. Oil degradation appeared to take place most readily under alkaline conditions and the extent of oil degradation was progressively reduced as the conditions became more acidic (Table 5.2).

Table 5.2. Effect of starting pH on the ability of pF33 to degrade oil (mean ± SD).

Treatment	Oil degraded (%)
pH 4	65.38 ± 14.66
pH 7	77.34 ± 14.16
pH 10	84.54 ± 2.30

The residuals were normally distributed, there was no significant heterogeneity of variance (Barltett's χ^2=4.073, P=0.131) and a one-way ANOVA confirmed that the staring pH did not significantly effect the degradation of oil (F=2.00, P=0.215).

Plate counts on day 15 of the experiment also indicated that a large number of microorganisms were present at all pH's. However, a greater number of microorganisms were present at pH 10 than at pH 4 and 7 (pH 4; 4.64 x 10^8 cf.u.ml^{-1}, pH 7; 3.64 x 10^8 cf.u.ml^{-1}, pH 10; 5.80 x 10^9 cf.u.ml^{-1})

Effect of incubation temperature on the ability of pF33 to degrade oil

The extent of oil degradation at all the temperatures investigated was considerably lower than had been observed previously (Table 5.3). Oil degradation was at a maximum at 30°C and increasing the temperature above the optimum for oil degradation appeared to reduce the extent of oil degradation more markedly (by a factor of 2.3) than lowering the temperature below the optimum (by a factor of 1.4).

Table 5.3a. Effect of temperature on the ability of pF33 to degrade oil (mean ± SD) (intermittent agitation).

Treatment	Oil degraded (%)
10°C	21.73 ± 1.98
30°C	30.23 ± 6.05
50°C	13.08 ± 2.01

Table 5.3b. Tukey's pairwise comparisons of the mean difference of oil degraded ($\log_{10}$ %). 95% confidence intervals of any difference = 0.1744. *significant differences

Pairwise comparisons	Difference in means
10°C & 30°C	-0.1383
10°C & 50°C	0.2228*
30°C & 50°C	0.3611*

The residuals of the transformed data ($\log_{10}$) were normally distributed, there was no significant heterogeneity of variance (Batlett's χ^2=1.086, P=0.581) and a one-way ANOVA confirmed that oil degradation was significantly affected by incubation temperature (F=20.55, P=0.002). Tukey's pairwise comparisons (Table 5.3) confirmed that oil degradation at 50°C was significantly lower than that at 10°C and 30°C but that oil degradation at 10°C was not significantly lower than that at 30°C.

Plate counts on day 13 of the experiment also indicated that a large number of microorganisms were present at all incubation temperatures. However, a greater number of microorganisms were present at 30°C than at 10 or 50°C (10°C; 1.30 x 10^6 cf.u.ml^{-1}, 30°C; 1.10 x 10^8 cf.u.ml^{-1} 50°C; 1.38 x 10^5 cf.u.ml^{-1}).

Effect of grease trap isolates on the ability of pF33 to degrade oil

The ability of the pF33 microorganisms to degrade oil was considerably greater than of the grease trap isolates alone or grease trap isolates combined with pF33 (by a factor of 1.5-1.6) (Table 5.4).

The residuals were normally distributed, there was no significant heterogeneity of variance (Bartletts χ^2=2.754, P=0.252) and a one-way ANOVA confirmed that oil degradation was significantly affected by the microbial communities present (F=7.88, P=0.021). Tukey's

pairwise comparisons (Table 5.4) revealed that pF33 significantly enhanced the degradation of oil relative to the grease trap microorganism alone and combined with pF33.

Table 5.4a. Effect of grease trap isolates (GI) on the ability of pF33 to degrade oil (mean ± SD).

Treatment	Oil degraded (%)
F33 only	82.07 ± 6.43
GI only	54.31 ± 4.67
F33+GI	51.64 ± 16.60

Table 5.4b. Tukey's pairwise comparisons of the mean difference of oil degraded (%). 95% confidence intervals of any difference = 26.06. * significant difference

Pairwise comparison	Difference in means
F33 & GI	27.77*
F33 & F33+GI	30.44*
GI & F33+GI	2.67

Plate counts on day 13 of the experiment indicated that microorganisms were present at similarly high numbers in all samples (GI only; 1.16 x 10^9 cf.u.ml^{-1}, F33 only; 3.42 x 10^9 cf.u.ml^{-1}, F33+GI; 2.26 x 10^9 cf.u.ml^{-1}).

Effect of lipase on the ability of pF33 to degrade oil

Oil that had been pre-treated with exogenous lipase from *Candida rugosa* (1 ml of soya oil incubated with 15ml of a 20mg.ml^{-1} lipase solution for 36 hours at 37°C with shaking at 100 rpm) was incubated with the supernatant of pF33 after soaking in nutrient media 3 (Table 5.5).

Table 5.5. Effect of lipase on the degradation (mean ± SD) of soya oil (1ml) suspended in nutrient media 3 (100ml), incubated with pF33 (10 g.l^{-1}) at 30°C, with shaking at 130rpm for 21 days.

Treatment	oil degraded (%)
F33	58.20 ± 8.43
F33+lipase	71.62 ± 4.36

The presence of lipase in the sample enhanced oil degradation by a factor of approximately 1.2. The residuals were normally distributed, there was no significant heterogeneity of

variance (F=1.404, P=0.832) and a two sample t-test confirmed that the effect of lipase was significant (T=-3.43, P=0.026).

Effect of fat/oil composition on the ability of pF33 to degrade fat/oil

Previous experiments had shown that the pF33 isolates were capable of utilising only soya and lard as the sole carbon sources (section 4.2.3.2). To determine whether the pF33 isolates were capable of degrading a variety of fat/oils in the presence of alternative carbon sources (and other nutrients/salts derived from the formulation) pF33 was incubated with various fat/oils suspended in nutrient media 3 (Figure 5.1).

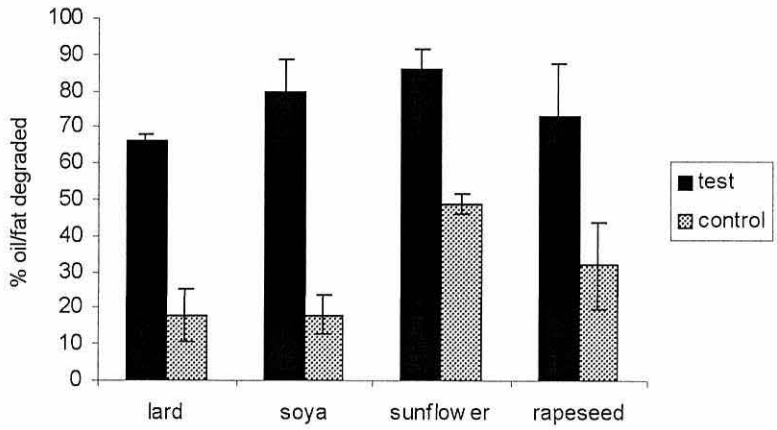


Figure 5.1. Effect of pF33 (1g) on the degradation (mean ± SD) of various fats/oils (1ml) suspended in nutrient media 3 (100ml), incubated at 30°C with shaking at 130rpm for 21([a]) or 28([b]) days.

Inoculation with pF33 enhanced the degradation of all fats and oils by differing degrees (by a factor of 1.7- 4.4, relative to controls). pF33 degraded a larger proportion of the soya oil than lard and both these fat/oils were degraded to a greater extent than rape and sunflower oil, which experienced similarly low degradation rates despite being incubated for longer.

The residuals for both lard/soya and rape/sunflower data were normally distributed and there was no significant heterogeneity of variance (Bartlett's χ^2=3.777-4.001, P=0.261-0.287). A two-factor ANOVA's confirmed that that there was no significant interaction between factors (F=0.14-3.15, P=0.114-0.722), that the effect of inoculation was significant (F=45.03-224.27, P<0.001) and that effect of the oil used was significant in the case of rape and sunflower oil (F=6.72, P=0.032) but not in the case of soya and lard oil (F=3.41, P=0.102). Bonferoni's pairwise comparisons (Table 5.6) confirmed that pF33 significantly enhanced the degradation of all fat/oils investigated and that the extent of soya oil degradation was significantly greater than that of lard (inoculated samples only).

Table 5.6. Bonferoni's pairwise comparisons of the mean difference of oil degraded (%) in samples of lard, rapeseed, soya and sunflower oil suspended in nutrient media 3 media 3 (100ml), incubated at 30°C with shaking at 130rpm for 21-28 days. 95% confidence interval of any difference = 7.40. *significant difference

Pairwise comparison		Difference in means
Lard;	F33 & control	48.57*
Soya;	F33 & control	61.62*
F33;	lard & soya	-13.32*
Control;	lard & soya	-0.270
Sunflower;	F33 & control	36.69*
Rape;	F33 & control	40.95*
F33;	Sunflower & rape	-12.87*
Control;	Sunflower & rape	17.13*

Ability of pF33 to degrade oil present at a concentration of 10%(v/v)

Oil degradation in some of the previous experiments (section 3.2.2) may have been limited by the high concentrations of oil used (10% v/v). An experiment was therefore carried out to establish whether pF33, which had previously been shown to degrade oil at a concentration of 1% (Table 5.6), was capable of degrading oil at a concentration of 10%. One of the nutrient media used in this experiment was identical to that used in the earlier degradation experiments (nutrient media 1) whilst the other (nutrient media 3) was used when pF33 significantly enhanced the degradation of oil at a concentration of 1%.

Inoculation of the samples with clay and peanut shell pF33 enhanced the degradation of oil in both nutrient media (by a factor of 1.2-1.8 relative to controls). Clay pF33 degraded considerably more oil than peanut shell pF33 in nutrient media 1 although the two microbial supplements resulted in a similar extent of oil degradation in nutrient media 3.

The residuals of the transformed data ($\log_{10}$) were normally distributed and displayed no significant heterogeneity of variance (Bartlett's χ^2=5.6663, P=0.580). A two-factor ANOVA confirmed that there was no significant interaction between factors (F=2.25, P=0.122), that the effect of inoculation was significant (F=24.95, P<0.001) and that effect of the nutrient media was not significant (F=0.91, P=0.355). Bonferoni's pairwise comparisons (Table 5.7) indicated that oil degradation was significantly enhanced by peanut shell pF33 in nutrient media 3 and by clay pF33 in nutrient media 1.

Table 5.7a. Effect of pF33 (5g) (with a peanut shell (PS) and clay carrier) on the degradation (mean ± SD) of soya oil (1ml) suspended in nutrient media 1 or 3 (10ml) incubated at 30°C with shaking at 130rpm for 19-21 days.

	Oil degraded (%)	
	Nutrient media 1	**Nutrient media 3**
PS test	17.77 ± 0.81	19.65 ± 2.14
PS control	14.57 ± 1.68	12.69 ± 0.71
Clay test	30.45 ± 5.88	23.81 ± 4.83
Clay control	16.75 ± 2.24	18.02 ± 2.32

Table 5.7b. Bonferoni's pairwise comparisons of mean difference of oil degraded (log_{10} %). 95% confidence interval of any difference = 0.1360. *significant difference

	PS Test & control	**Clay Test & control**
Nutrient media 1	0.0878	0.2568*
Nutrient media 3	0.1885*	-0.1168

5.2.2. Factors affecting the composition of rapeseed oil incubated with pF33

Lipid samples from some of the previously discussed degradation experiments (section 5.2.1) were derivatized and analysed using GC-MS to establish how the various environmental parameters affected the relative proportions of the major fatty acids in rapeseed oil incubated with pF33.

With respect to the major fatty acids, rapeseed oil that was incubated with pF33 under conditions of differing pH, oxygen availability, incubation temperature and in the presence of the grease trap isolates (Figures 5.3, 5.5, 5.6 & 5.9), experienced a decrease in the proportion of 18:2ω6 and 18:3ω3 coupled with an increase in the proportion of 16:0, 18:0 and 18:1ω7 and a variable change in the proportion of 18:1ω9.

Effect of oxygen availability on the fatty acid composition of rapeseed oil

The composition of the rapeseed oil did not vary markedly with the availability of oxygen in the samples (Figure 5.3). Minor differences included an increase in the proportion of 16:0, 18:1ω9 and 'other' fatty acids and a decrease in the proportion of 18:1ω7, 18:2ω6 and 18:3ω3

as the availability of oxygen in the sample was enhanced. 'New' fatty acids that constituted >1% of the total were detected under oxic conditions only and included 10-oxo (1.67% ± 0.73) and 10-OH (4.90% ± 1.34).

Principle component analysis of the standardized data was used to determine whether there was any relationship between the availability of oxygen in the samples and the fatty acid composition of rapeseed oil (Figure 5.2).

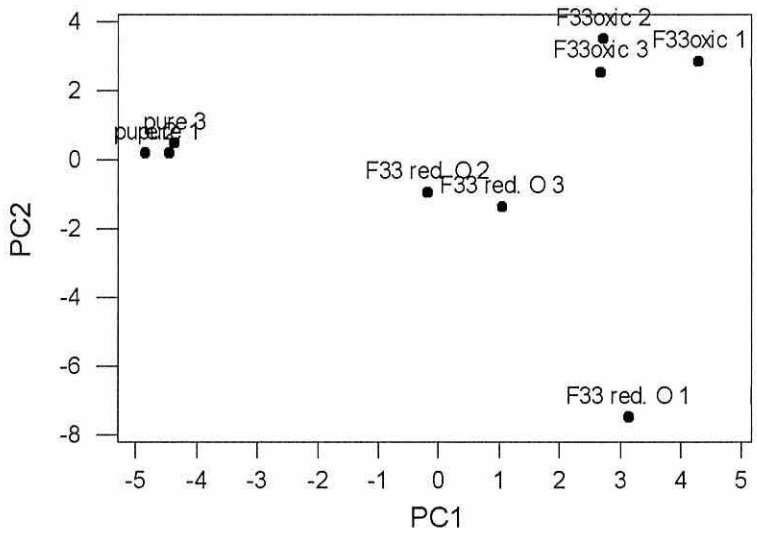


Figure 5.2. Score plot of principle components 1 and 2 for the relative proportion of all fatty acids in pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33, at 30°C with shaking at 130rpm under both oxic conditions and condition of reduced oxygen availability (F33 red. O) for 21 days.

Principle components 1 and 2 accounted for 43.0% and 34.5% of the variability, respectively. Several fatty acids influenced principle component 1 whilst nonanoic acid-9 oxo (keto), 18:1ω9 and a unidentified saturated fatty acid (sat 438) contributed to principle component 2 (Appendix III). Principle component 1 represented a high proportion of 16:0, 18:1ω7 together with a low proportions of 18:3ω3, 18:2ω6, and 18:1ω9. Principle component 2, on the other hand, represented low proportions of nonanoic acid-9 oxo (keto), 18:1ω9 and unidentified saturated fatty acid (sat 438). Principle component 1 separated pure oil samples from samples incubated with F33 indicating that principle component 1 was a measure of oil degradation. Principle component 2 also separated pF33 that was incubated under oxic conditions from pF33 that was incubated under conditions of limited oxygen availability indicating that principle component 2 may have measured the extent of an alternative degradation pathway.

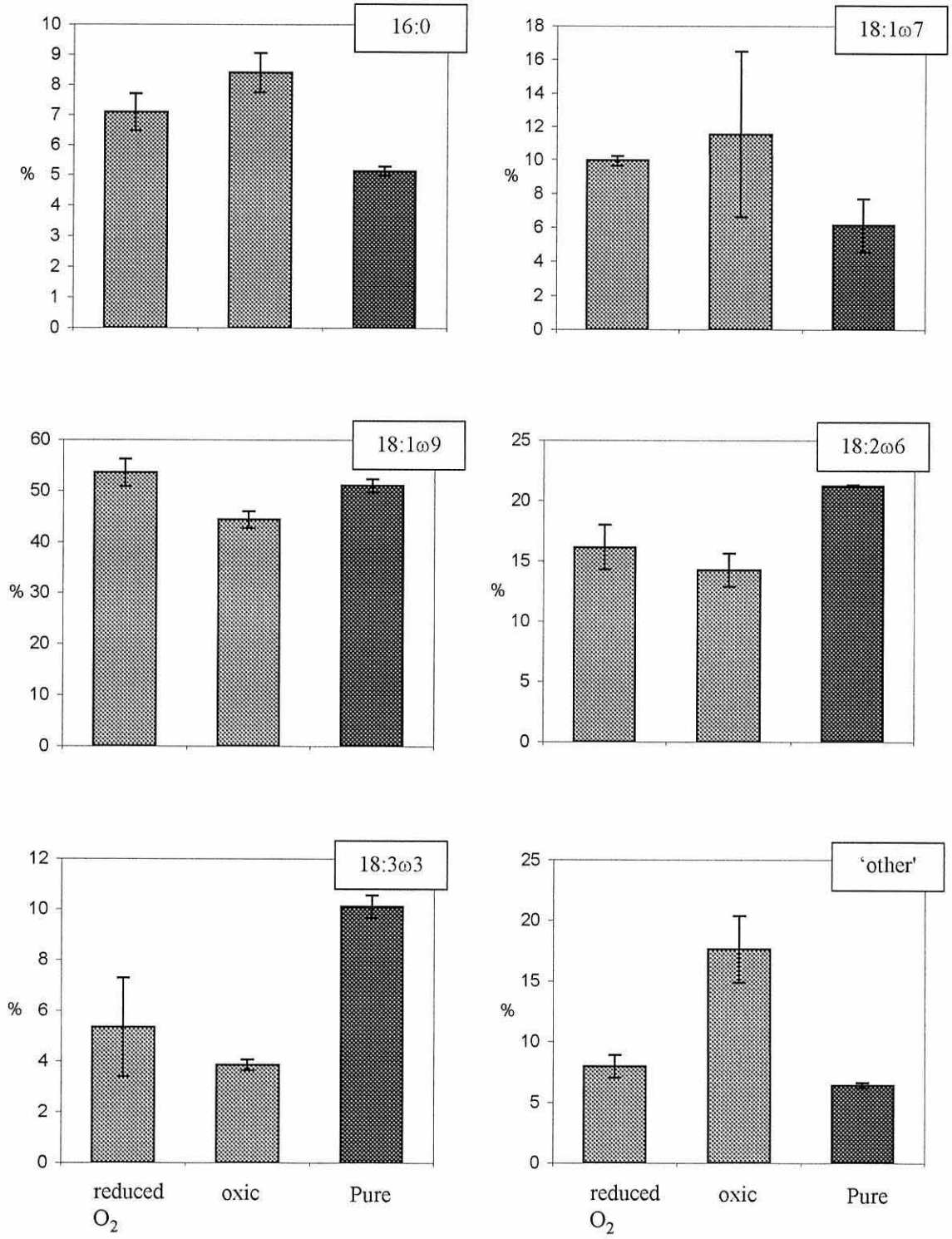


Figure 5.3. Fatty acid composition (%, mean ± SD) of pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33 (1g) incubated under oxic conditions and conditions of limited oxygen availability (reduced O_2) at 30°C with shaking at 130rpm for 21 days.

Table 5.8a. F/χ^2 and P values for the series of one-way ANOVA's and Mood median test applied to compare the relative proportions of 16:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3 in pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33 incubated under oxic conditions and conditions of limited oxygen availability at 30°C with shaking at 130rpm for 21 days.

	Test statistic	P
16:0	F = 29.61	0.001
18:1ω9	F = 17.10	0.003
18:1ω7	χ^2 = 3.60	0.165
18:2ω6	χ^2 = 6.30	0.043
18:3ω3	F = 23.76	0.001

Table 5.8b. Tukey's pairwise comparisons of the mean difference± 95% confidence interval of the proportion of 16:0, 18:0, 18:1ω7, 18ω:9, 18:2ω6 and 18:3ω3 in samples of pure rapeseed oil and rapeseed oil incubated under oxic conditions and conditions of reduced oxygen availability (red. O_2). * significant difference

	16:0	18:1ω9	18:3ω3
oxic & pure	3.273 ± 1.314*	-6.611 ± 4.953*	-6.265 ± 2.915*
red.O_2 & pure	1.970 ± 1.314*	2.530 ± 4.953	-4.781 ± 2.915*
oxic & red.O_2	1.303 ± 1.314	-9.140 ± 4.953*	1.484 ± 2.915

The residuals of all fatty acids were normally or approximately normally distributed and with the exception of 18:2ω6 and 18:1ω7 (Bartlett'sχ^2=7.480-8.063, P=0.018-0.024), there was no significant heterogeneity of variance (Bartlett's χ^2= 0.875-3.507, P=0.173-0.646) (Levene's statistic=1.901, P=0.229). A series of one-way ANOVA's and a Mood median test (Table 5.8) revealed that there were significant differences between the various sets of samples for all fatty acid tested, except 18:1ω7.

Tukey's pairwise comparisons (Table 5.8) indicated that rapeseed oil incubated under conditions of varying oxygen availability experienced a significant increase in the proportion of 16:0 coupled with a significant decrease in the proportion of 18:3ω3. The proportion of 18:1ω9 in rapeseed oil also significantly decreased under oxic conditions but did not change significantly under oxygen limited conditions.

Effect of pH on the fatty acid composition of rapeseed oil

The starting pH of the media did not affect the composition of the oil greatly (Figure 5.5).

Acidic and neutral conditions resulted in a greater proportion of 18:1ω9 and slightly lower proportion of 18:2ω6, 18:3ω3 than alkaline conditions. Acidic conditions also resulted in marginally lower proportions of 16:0, 18:1ω7 and minor fatty acids (other) than either alkaline or neutral conditions. A 'new' fatty acid (10-OH) that represented >1% of the total was also detected at pH 7 and 10 (1.66-1.73%).

Principle component analysis of the standardized data was used to determine whether there was any relationship between the pH of the media and the fatty acid composition of rapeseed oil (Figure 5.4).

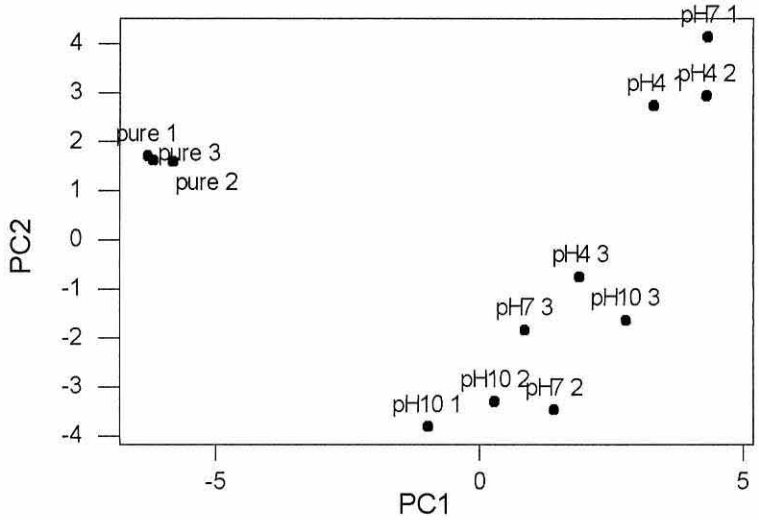


Figure 5.4. Score plot of principle components 1 and 2 for the relative proportion of all fatty acids in pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33, incubated at different starting pHs at 30°C with shaking at 130rpm for 21 days.

Principle components 1 and 2 were influenced by several fatty acids (Appendix III), which accounted for 51.6% and 25.0% of the variability, respectively. In terms of the major fatty acids, principle component 1 represented a high proportion of 16:0, 18:1ω7, 18:1ω9 together with a low proportions of 18:2ω6 and 18:3ω3, whilst principle component 2, represented a low proportion of 16:0, 18:1ω9 and 18:2ω6 and a high proportion of 18:1ω7 and 18:3ω3.

All the samples that were incubated with pF33 (pH 4-10) were separated from the pure oil by principle component 1 indicating that principle component 1 was a measure of oil degradation. In contrast, principle component 2 separated 2 of the 3 replicate samples incubated at pH 4 and one replicate sample incubated at pH 7 from the other treatments. These samples that scored positively on principle component 2 and experienced the lowest

extent of oil degradation indicating that principle component 2 was also a measure of oil degradation, perhaps by an alternative pathway.

Table 5.9a. F/χ^2 and P values for the series of one-way ANOVA's and Mood median test applied to compare the relative proportions of 16:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3 in pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33 incubated at different starting pH's, at 30°C with shaking at 130rpm for 21 days.

	Test statistic	P
16:0	F = 50.20	<0.001
18:1ω9	F = 4.58	0.038
18:1ω7	F = 12.22	0.002
18:2ω6	χ^2 = 6.67	0.083
18:3ω3	F = 237.18	<0.001

Table 5.9b. Tukey's pairwise comparisons of the mean difference ± 95% confidence interval of the proportion of 16:0, 18:0, 18:1ω7, 18:1ω9, 18:2ω6 and 18:3ω3 in samples of pure rapeseed oil and rapeseed oil incubated at different pH's. * significant difference

	16:0	18:1ω9	18:1ω7	18:3ω3
pH 4 & pure	3.332±1.190*	9.098±8.569*	3.809±3.523*	-9.126±1.334*
pH 7 & pure	4.023±1.190*	4.727±8.569	6.289±3.523*	-9.354±1.334*
pH 10 & pure	3.683±1.190*	1.396±8.569	5.041±3.523*	-8.682±1.334*
pH 4 & pH 7	-0.691±1.190	4.371±8.569	-2.480±3.523	0.229±1.334
pH 7 & pH 10	0.340±1.190	3.332±8.569	1.249±3.523	-0.673±1.334
pH 4 & pH 10	-0.351±1.190	7.702±8.569	-1.232±3.523	-0.444±1.334

The residuals for each fatty acid were normally distributed and there was no significant heterogeneity of variance (Bartlett's χ^2=0.630-6.744, P=0.081-0.890), except in the case of 18:2ω6 (Bartlett's χ^2=10.845, P=0.013). A series of one-way ANOVA's and a mood median test (Table 5.9) determined that there were significant differences between the various sets of samples for all fatty acids tested except 18:2ω6. Tukey's pairwise comparisons (Table 5.9) indicated that the starting pH of the media did not significantly effect the composition of the rapeseed oil. However, at all pHs there was a significant increase in the proportions of 16:0, 18:1ω7 and 18:1ω9 (pH 4 only) and a significant decline in the proportion of 18:3ω3 relative to the pure oil.

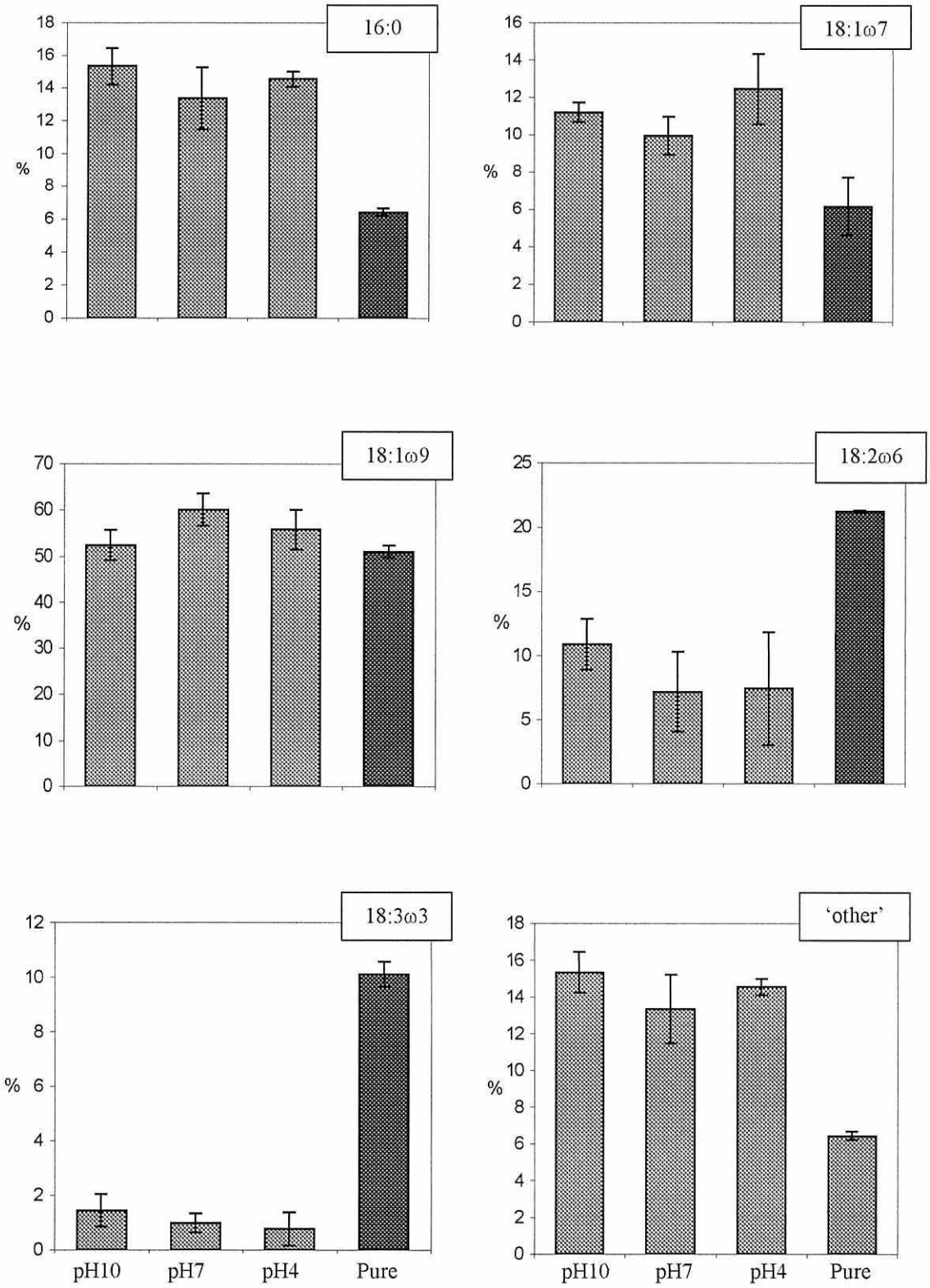


Figure 5.5. Fatty acid composition (%, mean ± SD) of pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33 (1g) incubated at different starting pH's at 30°C with shaking at 130rpm for 21 days.

Effect of incubation temperature on the fatty acid composition of rapeseed oil

Incubation temperature affected the relative proportions of fatty acids in rapeseed oil considerably (Figure 5.6). Some of the fatty acid compositional changes reported earlier (i.e. increase in the proportion of 16:0, and minor fatty acids and decrease in the proportion of 18:2ω6 and 18:3ω3) became more marked as the temperature was raised whilst the increase in the proportion of 18:1ω9 and 18:1ω7 was greater at 30°C than at extremes of temperature (10°C or 50°C).

'New' fatty acids that constituted greater than 1% of the total were detected at an incubation temperature of 50°C only. These fatty acids included azaleic acid (2.82 ± 0.30%), an unidentified saturated fatty acid (sat 410; 2.24 ± 0.63%) and a possible monounsaturated fatty acid (monunsat 2078; 3.56 ± 3.13%).

Principle component analysis of the standardized data was used to establish whether there was any relationship between the incubation temperature and the fatty acid composition of rapeseed oil (Figure 5.7).

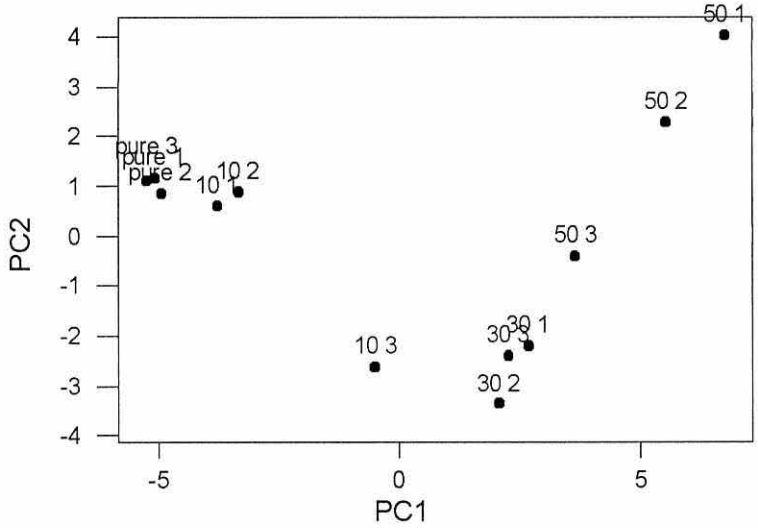


Figure 5.7. Score plot of principle components 1 and 2 for the relative proportion of all fatty acids in pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33 incubated at 10°C, 30°C or 50°C with intermittent shaking.

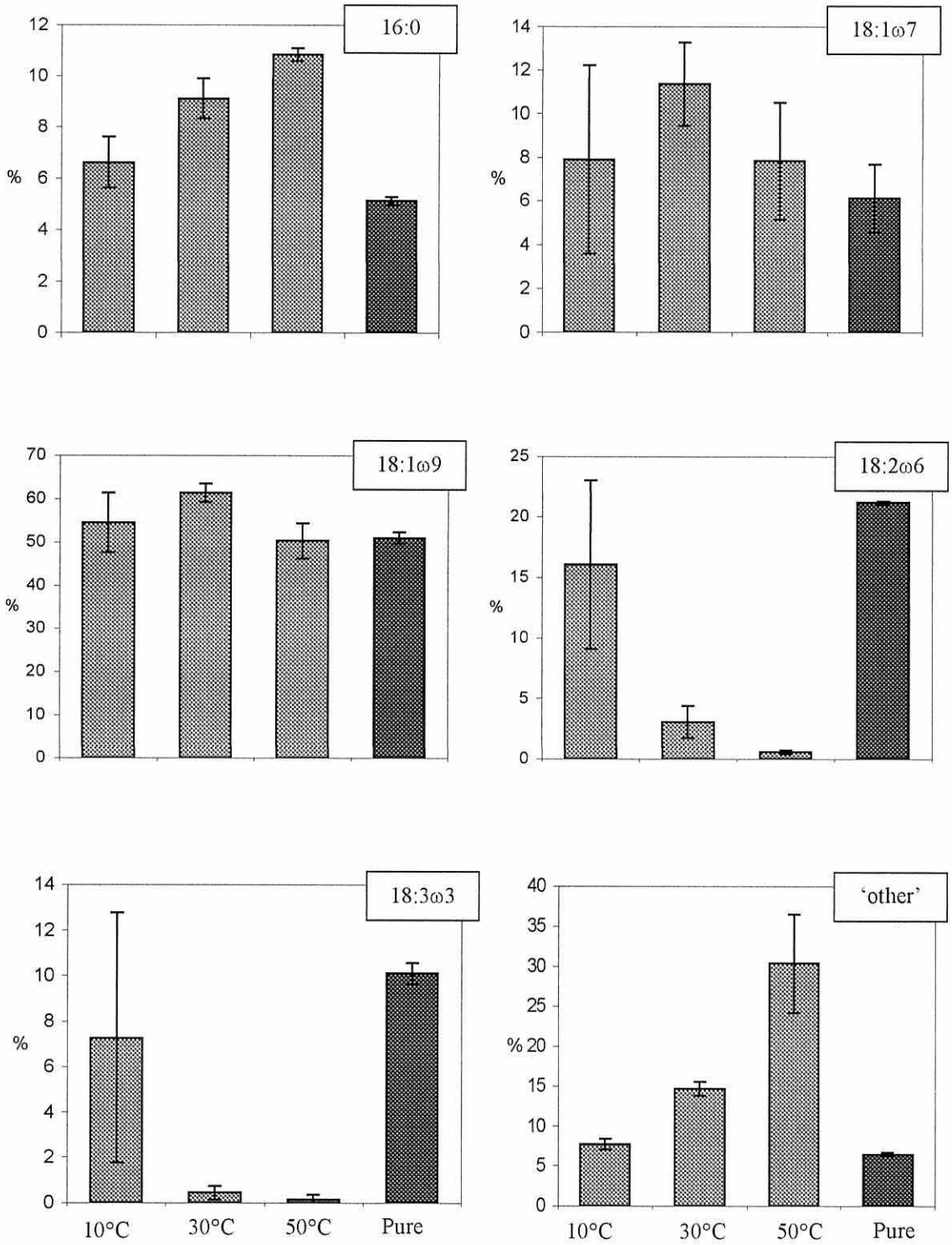


Figure 5.5. Fatty acid composition (%, mean ± SD) of pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33 (1g) incubated at different temperatures with occasional shaking for 21 days.

Principle components 1 and 2 accounted for 61.5% and 16.1% of the variability, respectively. Several fatty acids had a similar influence on principle component 1 (Appendix III) whilst a monounsaturated fatty acid (monunsat 1628), 18:1ω9 and 18:1ω7 contributed to principle component 2. With respect to the major fatty acids, principle component 1 represented a high proportion of 16:0, 18:1ω7, 18:1ω9.together with a low proportions of 18:2ω6 and 18:3ω3. Principle component 2, on the other hand represented a high proportion of monounsaturated fatty acid (monunsat 1628) and a low proportion of 18:1ω9 and 18:1ω7. Principle component 1 was a measure of temperature whilst principle component 2 did not resolve the samples at all. There did not appear to be any relationship between the extent of oil degradation and score on either principle component 1 and 2.

Table 5.10a. F and P values for the series of one-way ANOVA's applied to compare the relative proportions of 16:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3 in pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33 incubated at different temperatures with intermittent shaking for 21 days.

	F	P
16:0	45.88	<0.001
18:1ω9	4.36	0.043
18:1ω7	1.80	0.225
18:2ω6	23.83	<0.001
18:3ω3	9.79	0.005

Table 5.10b. Tukey's pairwise comparisons of the mean difference± 95% confidence interval of the proportion of 16:0, 18:0, 18:1ω7, 18ω:9, 18:2ω6 and 18:3ω3 in samples of pure rapeseed oil and rapeseed oil incubated at different temperatures. * significant difference

	16: 0	18:1ω9	18:2ω6	18:3ω3
10°C & pure	1.481±1.702	3.489±11.005	-5.145±9.262	-2.852±7.216
30°C & pure	3.074±1.702*	10.367±11.005	-18.142±9.262*	9.675±7.216*
50°C & pure	5.712±1.702*	-0.745±11.005	-20.620±9.262*	-9.958±7.216*
10°C & 30°C	-2.493±1.702*	-6.878±11.005	12.997±9.262*	-6.823±7.216
30°C & 50°C	-3.477±1.702*	11.112±11.005*	2.478±9.262	0.283±7.216
10°C & 50°C	-4.232±1.702*	4.236±11.005	15.474±9.262*	7.106±7.216

The residuals for the major fatty acids were normally or approximately normally distributed, there was no heterogeneity of variance (Barltett's χ^2=0.657-5.866, P=0.118-0.883, Levene's statistic=1.005-1.021, P=0.433-0.439) and a series of one-way ANOVA's (Table 5.10)

determined that there were significant differences between the various sets of samples for all fatty acids tested except 18:1ω7.

Effect of grease trap isolates on the fatty acid composition of rapeseed oil

The effect of the different microbial communities on the fatty acid composition of oil was similar (Figure 5.9). Oil that was incubated with the grease trap isolates (either alone or in combination with pF33) contained lower proportions of 16:0, 18:1ω7 and the minor fatty acids (other) and greater proportions of 18:1ω9 and 18:3ω3 relative to oil incubated with pF33 alone. 'New' fatty acids that constituted >1% of the total were detected in the presence of pF33 only and included 10-oxo (1.67 ± 0.73%) and 10-OH (4.90 ± 1.34%).

Principle component analysis of the standardized data was used to determine whether there was any relationship between the microbial community composition and the fatty acid composition of rapeseed oil (Figure 5.8).

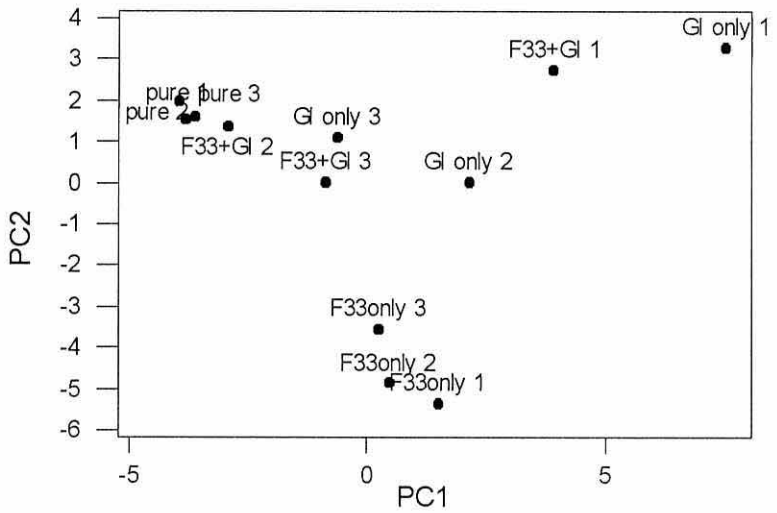


Figure 5.8. Score plot of principle components 1 and 2 for the relative proportion of all fatty acids in pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33, grease trap isolates (GI) and pF33+grease trap isolates incubated at 30°C with shaking at 130rpm for 21 days.

Principle components 1 and 2 accounted for 38.9% and 27.9% of the variability, respectively. Several fatty acids had a similar influence on principle component 1 whilst 22:0, 10-oxo and 10-OH contributed to principle component 2 (Appendix III).

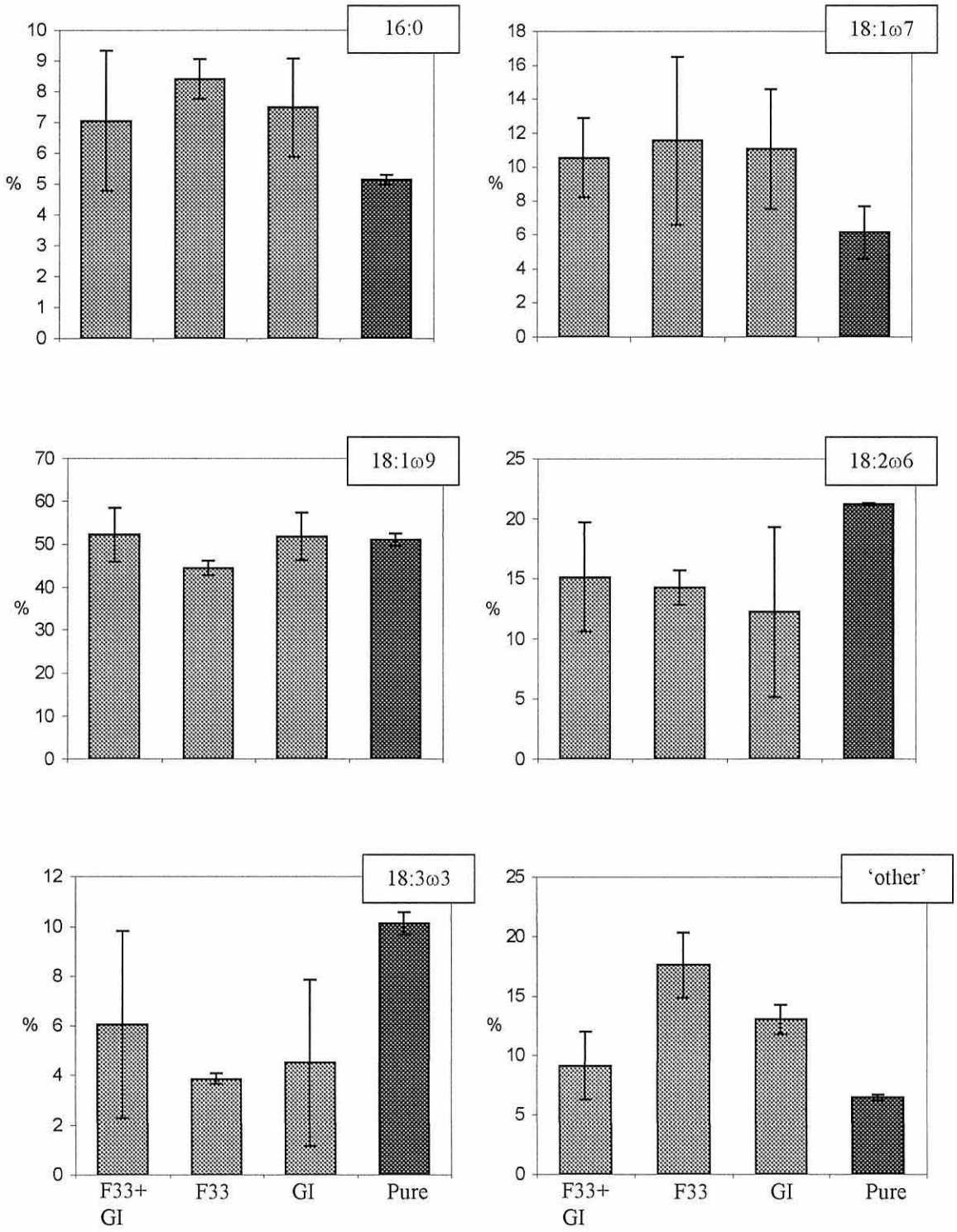


Figure 5.9. Fatty acid composition (%, mean ± SD) of pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33, grease trap isolates (GI) and pF33+grease trap isolates incubated at 30°C with shaking at 130rpm for 21 days.

With respect to the major fatty acids, principle component 1 represented a high proportion of 16:0, 18:1ω7 and 18:1ω9 together with a low proportions of 18:2ω6 and 18:3ω3. Principle component 2, on the other hand represented low proportions of 22:0, 10-oxo and 10-OH. Principle component 2 separated samples incubated with F33 alone from those of the pure oil and oil incubated with the grease trap isolates alone and grease trap isolates combined with pF33. Those samples that scored negatively on principle component 2 (i.e. F33 only) experienced the greatest extent of oil degradation.

The residuals for each fatty acid were normally or approximately normally distributed with no significant heterogeneity of variance (Bartlett's χ^2=2.72-5.056, P=0.165-0.518) (Levene's statistic=1.310, P=0.337) except in the case of 16:0 and 18:2ω6 (Bartlett's χ^2=8.030-14.407, P=0.002-0.045). A series of one-way ANOVA's and Mood median tests (Table 5.11) revealed that inoculation did not significantly affect the composition of the major fatty acids in rapeseed oil and the effect of the different microbial communities on the fatty acid composition of oil was similar in terms of the major fatty acids.

Table 5.11. F/χ^2 and P values for the series of one-way ANOVA's and Mood median test applied to compare the relative proportions of 16:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3 in pure rapeseed oil and rapeseed oil (1ml) suspended in synthetic sewage media 1 (100ml) inoculated with pF33, grease trap isolates or grease trap isolates+pF33 incubated at 30°C with shaking at 130rpm for 21 days.

	Test statistic	P
16:0	χ^2 = 4.00	0.261
18:1ω9	F = 2.13	0.175
18:1ω7	F = 1.65	0.253
18:2ω6	χ^2 =6.67	0.083
18:3ω3	F = 3.70	0.062

Impact of pF33 on the fatty acid composition of various fat/oils

To ascertain if the degradation pathways of the various fats/oils broken down by pF33 differed, the fatty acid compositions of the inoculated fat/oil samples, controls and pure oil were investigated using GC-MS.

The relative proportions of the major fatty acids present in pure rapeseed, soya, sunflower and lard oil and these fat/oils incubated with or without pF33 are illustrated in Figure 5.10. The fatty acid composition of both the control (without pF33) and inoculated samples differed

from those of the pure fat/oils. Compositional changes were generally more marked in the inoculated samples of the fat/oils (except 18:2ω6, 18:1ω7, 18:1ω9 in sunflower, 18:1ω7 in rapeseed and 18:0 in lard) and were also most extensive in sunflower and rapeseed oil. In the majority of fat/oils investigated, the control and inoculated samples contained proportionally less 18:3ω3 and 18:2ω6 than the pure oil but were enriched in the minor fatty acids (other), 16:0, 18:0 and 18:1ω7 (except 16:0 in soya oil control and lard samples, 18:0 in inoculated lard sample and 18:1ω7 in inoculated lard samples). The proportion of 18:1ω9 in the fat/oil samples did not vary in a consistent manner and in all of the fat/oils except sunflower oil the changes were marginal. 'New' fatty acids that represented >1% of the total were detected in both the inoculated and control samples of rapeseed, sunflower and soya oil but not in lard. These 'new' fatty acids included azaleic acid, found in the rapeseed control, (1.75 ± 0.21%) and 10-OH, found in the inoculated rapeseed (6.62 ± 5.69%), soya (1.58 ± 2.44%) and sunflower oil samples (5.35 ± 5.74%).

After the appropriate transformation ($\log_{10}$ in case of 16:0 and 18:0 in rapeseed oil) the residuals were approximately normally distributed and with the exception of 18:1ω9, 18:2ω6 and 18:3ω3 in rapeseed oil and 18:2ω6 in soya oil (Bartlett's χ^2=7.59, P=0.001-0.006) displayed no significant heterogeneity of variance (Levene's statistic=1.486-3.345, P=0.106-0.299, Bartlett's χ^2=1.173-5.653, P=0.06-0.556). A series of one-way ANOVA's and Mood median tests (Table 5.12) determined that only the rapeseed and sunflower oil experienced significant fatty acid compositional changes during the experiment (for all fatty acids except 18:1ω9).

Tukey's pairwise comparisons (Table 5.12) indicated that with the exception of 18:2ω6 in the rapeseed oil control sample, both the inoculated and control samples of sunflower and rapeseed oil contained a significantly greater proportion of 16:0, 18:0 and 18:1ω7 and a significant smaller proportion of 18:2ω6 and 18:3ω3 than the raw oil. Despite the fact that pF33 reduced the quantity of extractable oil considerably (section 5.2.1), Tukey's pairwise comparisons determined that pF33 did not have a significant impact on the fatty acid composition of the oil. These results imply that physico-chemical processes were responsible for the compositional changes observed and that the microbial supplement in some cases may have enhanced these changes (but not significantly).

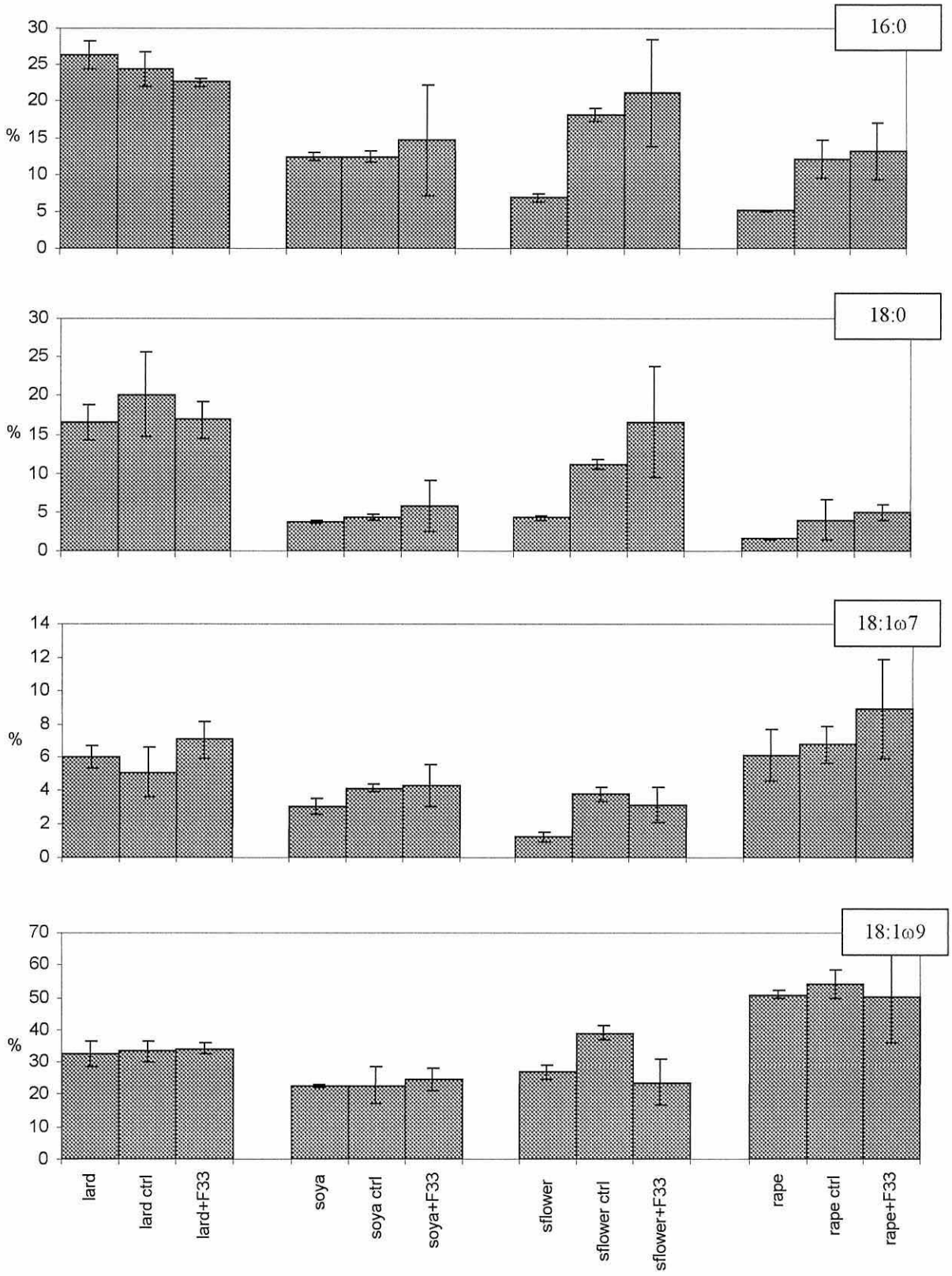


Figure 5.10. Effect of pF33 and physico-chemical process (control) on the fatty acid composition (%, mean ± mean) of various fats and oils (1ml) suspended in nutrient media 3 (100ml), incubated at 30°C with shaking at 130rpm for 21-28 days.

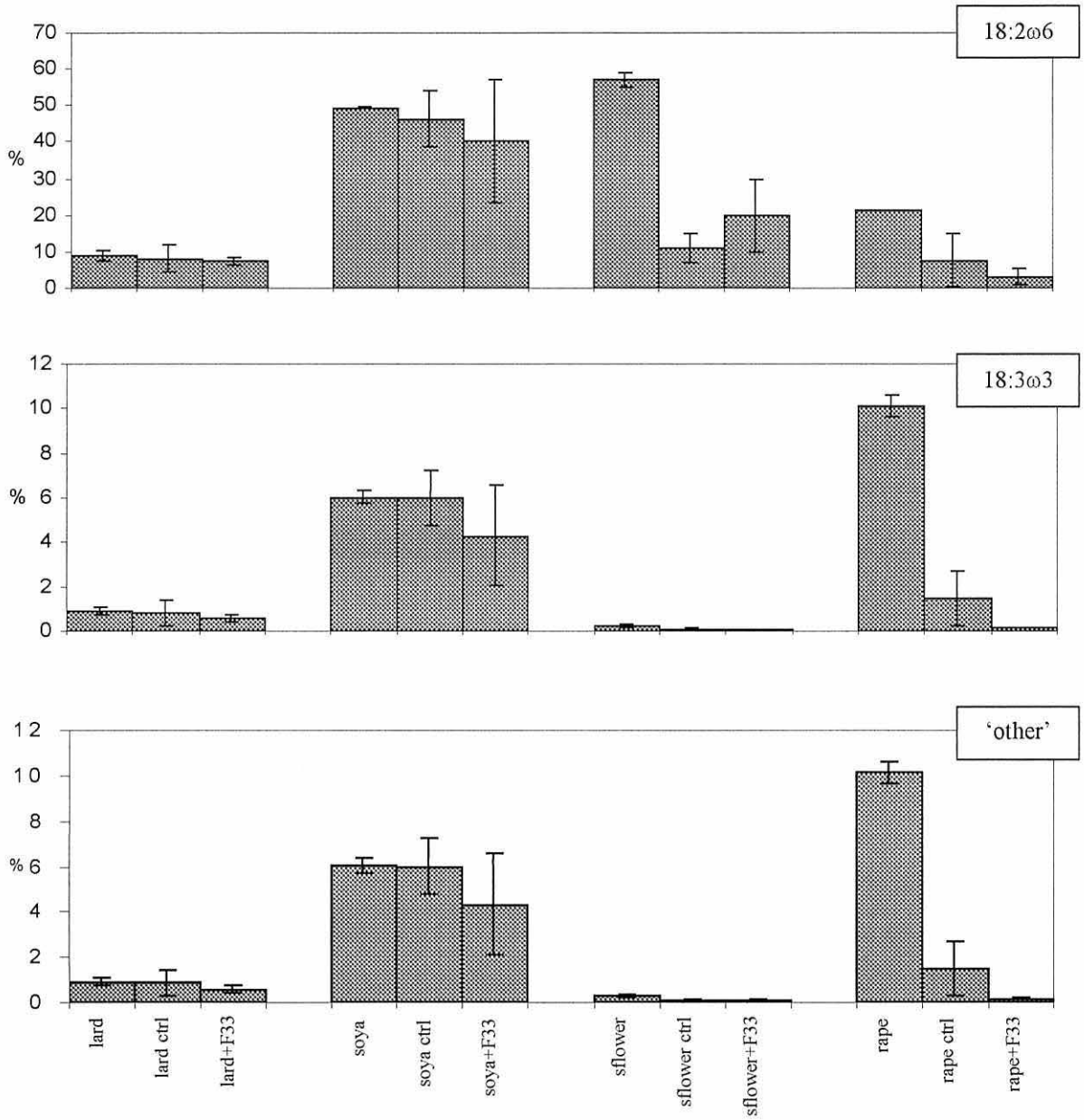


Figure 5.10. Continuation

Table 5.12a. F/χ^2 and P values for the series of one-way ANOVA's and Mood median tests applied to compare the relative proportions of 16:0, 18:1ω9, 18:1ω7, 18:2ω6 and 18:3ω3 in pure fat/oil and fat/oil (1ml) suspended in nutrient media 3 (100ml) inoculated with pF33 incubated at 30°C with shaking at 130rpm for 21-28 days.

	lard		soya		rapeseed		sunflower	
	F	P	F/χ^2	P	F/χ^2	P	F	P
16:0	3.51	0.098	0.28	0.763	16.89	0.003	9.23	0.015
18:0	0.85	0.471	0.88	0.463	8.04	0.020	6.67	0.030
18:1ω9	0.20	0.826	0.19	0.828	3.60	0.165	3.06	0.121
18:1ω7	2.21	0.190	2.30	0.181	1.50	0.295	12.50	0.007
18:2ω6	0.28	0.765	0.90	0.638	6.30	0.043	45.05	<0.001
18:3ω3	0.69	0.536	1.31	0.338	6.30	0.043	9.01	0.016

Table 5.12b. Tukey's pairwise comparisons of the mean difference± 95% confidence interval of the proportion of 16:0, 18:0, 18:1ω7, 18ω:9, 18:2ω6 and 18:3ω3 ($\log_{10}$ % for 16:0 and 18:0 in rapeseed oil) in samples of pure sunflower (Sf) and rapeseed (Rp) oil, the control (C) fat/oil samples and fat/oil incubated with pF33. *significant difference

	16:0	18:0	18:1ω7	18:2ω6	18:3ω3
Sf C & pF33	-2.98 ±10.79	-5.40±10.37	0.68±1.67	-8.85±15.87	-0.01±0.14
Sf C & pure	11.33±10.79*	6.91±10.37*	2.63±1.67*	-46.07±15.87*	-0.17±0.14*
Sf pF33 & pure	14.32±10.79*	12.32±10.37*	1.94±1.67*	-37.22±15.87*	-0.16±0.14*
Rp C & pF33	-0.03±0.23	-0.06±0.39	-	-	-
Rp C & pure	0.37±0.23*	0.41±0.39*	-	-	-
Rp pF33 & pure	0.40±0.23*	0.46±0.39*	-	-	-

Effect of clay and peanutshell pF33 on the fatty acid composition of soya oil

In previous work, peanutshell pF33 significantly enhanced the degradation of oil in nutrient media 4 whist clay pF33 had not (section 5.2.1). To determine whether the clay pF33 was capable of altered the relative proportions of fatty acids in the oil without significantly reducing the quantity of oil present, the samples from this experiment were analysed using GC-MS (Figure 5.11).

Despite the fact that peanutshell pF33 mineralised some of the oil, the composition of oil that was incubated with F33 was almost identical to that in the control. In contrast, oil that had been incubated with clay PF33 experienced major compositional changes and was characterised by high proportions of the minor fatty acids (other), 16:0, 18:0 and 18:1ω9 and low proportions of 18:2ω6 and 18:3ω3, relative to the control. A 'new' fatty acid with a

relative proportion greater than 1% (1.30±0.13%) was also detected in the oil samples inoculated with clay pF33. This 'new' fatty acid was identified as an 18:2ω? isomer and was observed to have a retention time greater than that of 18:2ω6.

The residuals were normally distributed, there was no heterogeneity of variance (Bartlett's χ^2=1.776-6.122, P=0.106-0.620) and a series of two-factor ANOVA's (Table 5.13) determined that there were significant differences between some of the treatments for all fatty acids tested.

Bonferoni's selective pairwise comparisons (Table 5.13) revealed that were significant difference between the clay test and control samples but not between the peanut shell test and control samples for all fatty acids tested indicating that clay pF33 significantly altered the composition of soya oil but peanut shell pF33 did not. As with some of the other microbial communities (section 3.2.5), clay pF33 also degraded 18:2ω6 and 18:3ω3 more readily than 16:0, 18:0 and 18:1ω9.

Table 5.13a. F and P values for the two factor ANOVA's applied to compare the relative proportions of 16:0, 18:0, 18:1ω9, 18:2ω6 and 18:3ω3 for soya oil (1ml) suspended in nutrient media 3 (10ml) inoculated with clay or peanutshell pF33 (5g) incubated at 30°C with shaking at 130rpm for 19-21 days. (Average effect of inoculation and formulation used shown)

	Inoculation		**Formulation used**		**Interaction**	
	F	**P**	**F**	**P**	**F**	**P**
16:0	149.59	<0.001	172.88	<0.001	166.13	<0.001
18:0	302.98	<0.001	290.79	<0.001	334.19	<0.001
18:1ω9	32.90	<0.001	49.37	<0.001	15.08	0.005
18:2ω6	158.27	<0.001	183.95	<0.001	141.5	<0.001
18:3ω3	207.94	<0.001	184.75	<0.001	155.33	<0.001

Table 5.13b. Bonferoni's selective pairwise comparisons of the mean difference (± 95% confidence interval) of the proportion of 16:0, 18:0, 18:1ω7, 18ω:9, 18:2ω6 and 18:3ω3 in soya oil incubated with clay and peanut shell (PS) pF33. * significant difference

	16:0	**18:0**	**18:1ω9**	**18:2ω6**	**18:3ω3**
Clay control & test	-13.351*	-4.960*	-12.811*	33.627*	5.803*
	±2.047	±0.559	±3.906	±5.003	±0.807
PS control & test	0.483	-0.051	1.296	-1.263	0.172
	±2.047	±0.559	±3.906	±5.003	±0.807

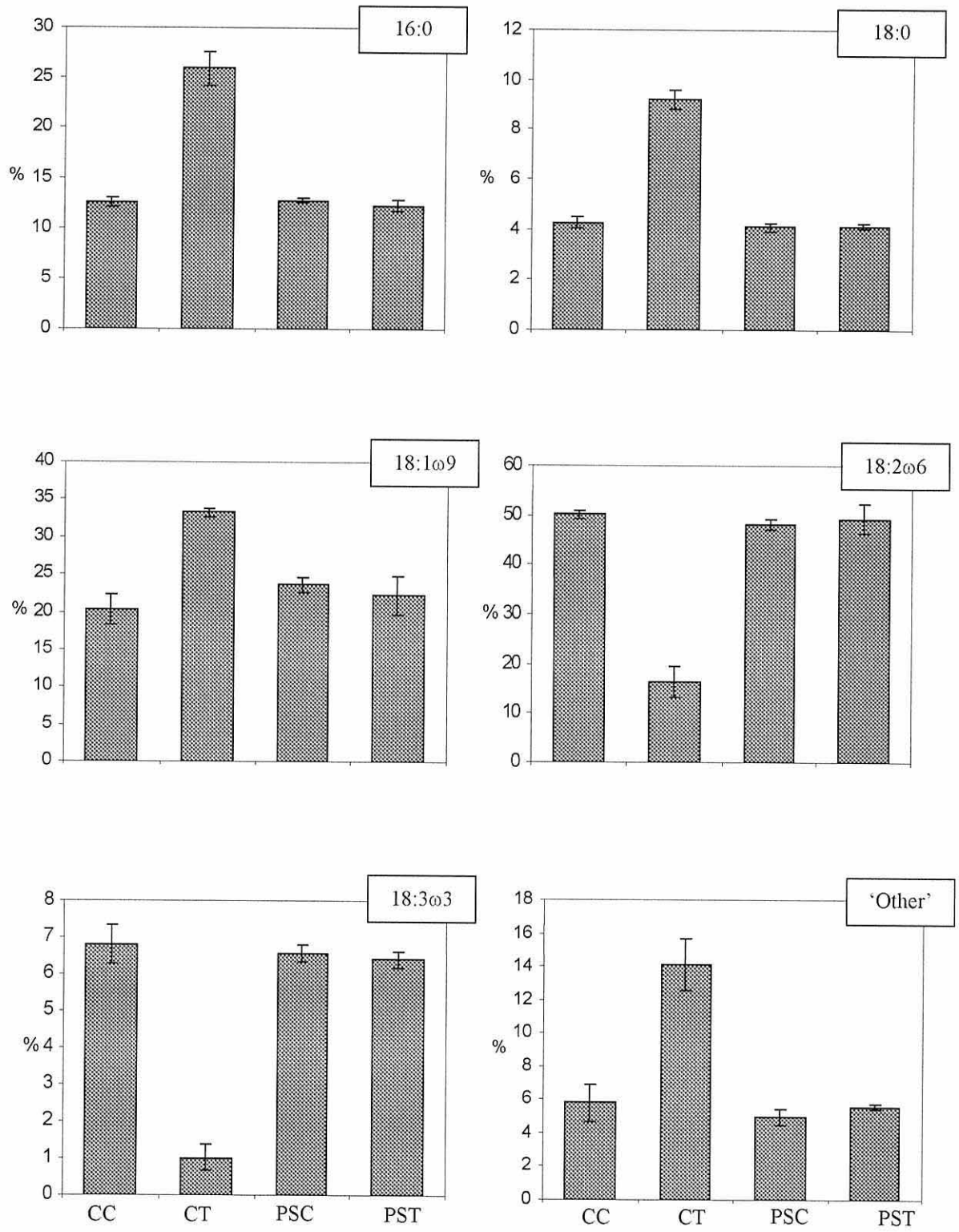


Figure 5.11. Effect of peanut shell powdered pF33 (PST) and clay pF33 (CT) on the fatty acid composition (%, mean ± SD) of soya oil (1ml) suspended in nutrient media 3 (10ml) incubated at 30°C with shaking at 130rpm for 19-21 days. Controls were prepared in the same manner but were autoclaved after inoculation with clay and peanut shell pF33 (peanutshell pF33 control (PSC) and clay pF33 control (CC)).

5.2.3. T-RFLP analysis of pF33 community dynamics during oil degradation

T-RFLP analysis of PCR amplified portions of the 16S rRNA gene was used to determine how the microbial community structure of pF33 changed during incubation at different temperatures, pH's, oxygen levels and in the presence of the grease trap isolates.

The average number of unique 3' and 5' terminal restriction fragments (ribotypes) found in a sample for each enzyme digest is shown in Table 5.14. Digests of the PCR products with *Rsa*I resolved fewer ribotypes than either *Msp*I or *Hha*I therefore only the *Msp*I and *Hha*I digests were used to interpret microbial population dynamics.

Table 5.14. Average number (± SD) of unique 3'and 5' ribotypes generated when digesting PCR amplified portions of 16S rRNA.

	3'	5'
***Rsa*I**	1.16 ± 0.96	2.03 ± 1.62
***Hha*I**	3.24 ± 1.82	3.43 ± 2.74
***Msp*I**	4.68 ± 3.01	4.57 ± 3.48

5.2.3.1. Factors affecting pF33 community dynamics during oil degradation

Effect of oxygen availability on the microbial community structure of pF33

The change in the community composition, as indicated by the dominant terminal restriction fragments for each enzyme digest, and diversity, as indicated by total number of unique terminal restriction fragments present for each enzyme digest, of pF33 with time under conditions of differing oxygen availability is summarised in Table 5.15 (see Appendix IV for further details).

The dominant terminal restriction fragments for all enzyme digests varied with time (generally between week 1 and 2) under oxic conditions but not under conditions of limited oxygen availability, indicating that the community composition of pF33 was less stable under oxic conditions than under conditions of limited oxygen availability.

Table 5.15. Dominant terminal restriction fragment (base pair) and total number of unique terminal restriction fragments (shown in brackets) for PCR amplified 16S rRNA from pF33 incubated with soya oil (1%v/v) and synthetic sewage media 1, under conditions of differing oxygen availability at 30°C with shaking at 130rpm for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

	***Hha*I(3')**	***Hha*I(5')**	***Msp*I(3')**	***Msp*I(5')**
Oxic T=1	345 (2)	215 (1)	298 (6)	69 (3)
Oxic T=2	163 (3)	240 (8)	365 (10)	147 (12)
Oxic T=3	163 (3)	240 (6)	365 (12)	142 (12)
Limited O_2 T=1	345 (3)	215 (1)	298 (5)	69 (4)
Limited O_2 T=2	345 (10)	215 (2)	298 (8)	69 (6)
Limited O_2 T=3	345 (6)	215 (4)	298 (7)	69 (5)

The number of unique terminal restriction fragments for each enzyme was generally greater under oxic conditions than under conditions of limited oxygen availability (except in the case of the Hha(3') T=1, 2, 3; Msp(3') T=1) indicating that the microbial population of pF33 was generally more diverse under oxic conditions compared to conditions of limited oxygen availability.

Cluster analysis of the data was also performed and indicated that under oxic conditions the community structure of pF33 changed markedly between week 1 and 2 of the experiment (13 % similarity) but less dramatically between week 2 and 3 (70% similarity). In contrast, the community composition of pF33 incubated under conditions of limited oxygen availability, changed only marginally over the entire length of experiment (week 1 cf.. 2; 82% similarity, weeks 2 cf.. 3; 89% similarity).

Cluster analysis also showed that after 7 days incubation, there appeared to be little difference in microbial community composition between those samples that were incubated under oxic conditions and conditions of limited oxygen availability (89% similarity). Following both the second and third week of the experiment, however, the community structures of pF33 incubated under oxygen limited and non-oxygen limited conditions were dissimilar (13% similarity).

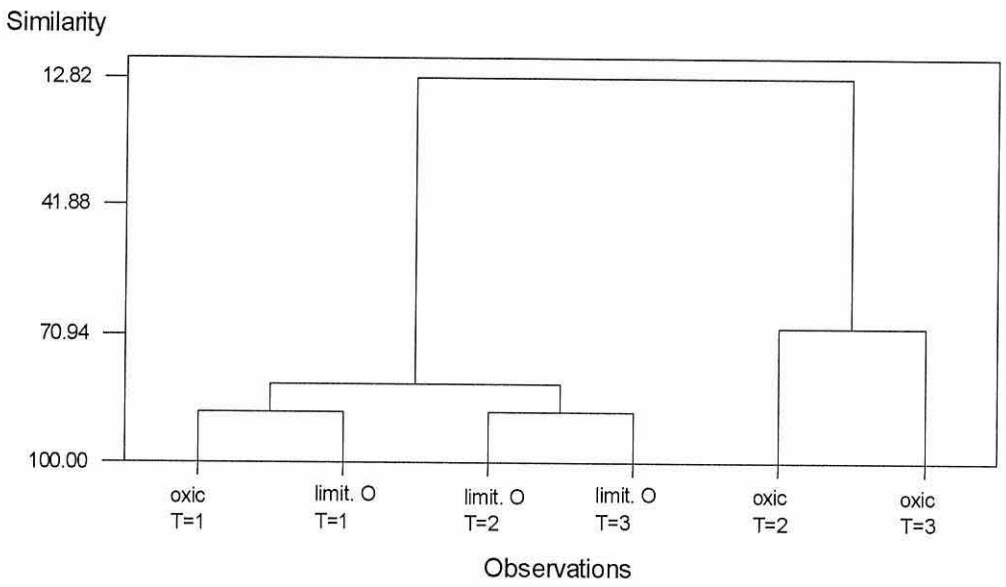


Figure 5.12. A dendrogram produced by cluster analysis of the 5' and 3' T-RF lengths from both *Msp*I and *Hha*I enzyme digests of PCR amplified 16S rRNA from pF33 incubated with soya oil (1%v/v) and synthetic sewage media 1, under oxic conditions and conditions of limited oxygen availability (limit. O) at 30°C with shaking at 130rpm for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

Principle component analysis of the standardized T-RFLP data was also carried out (Figure 5.13). Principle components 1 and 2 accounted for 41.8% and 27.3% of the variability, respectively and numerous terminal restriction fragments contributed to both principle components 1 and 2 (Appendix III). All of the dominant terminal restriction fragments were amongst the major contributors to principle component 1 whilst terminal restriction fragments which constituted less than 25% of the total digested amplified DNA such as Hha(3')227, Hha(5')141, Msp(3')124, Msp(5')142 had the greatest weighting in principle component 2. Principle component 1 (in terms of the dominant ribotypes) represented an increase in the proportion of Hha(3') 163, Hha(5')240, Msp(3')365 and Msp(5')147 and a decrease in the proportion of Hha(3')345, Hha(5')215, Msp(3')298, Msp(5')69 whilst principle component 2 represented an increase in the proportion of (or rather the presence of) all the aformentioned contributing fragments.

Principle component analysis also indicated that the community composition of pF33 was less stable under oxic conditions than under conditions of limited oxygen availability. pF33 incubated under conditions of limited oxygen availability scored negatively on both principle components over the course of the experiment. In contrast, pF33 that was incubated under oxic conditions, scored negatively on both principle components 1 and 2, during the first week of the experiment, positively on principle component 1 and negatively on principle

component 2 during the second week and positively on both components 1 and 2 during the third week.

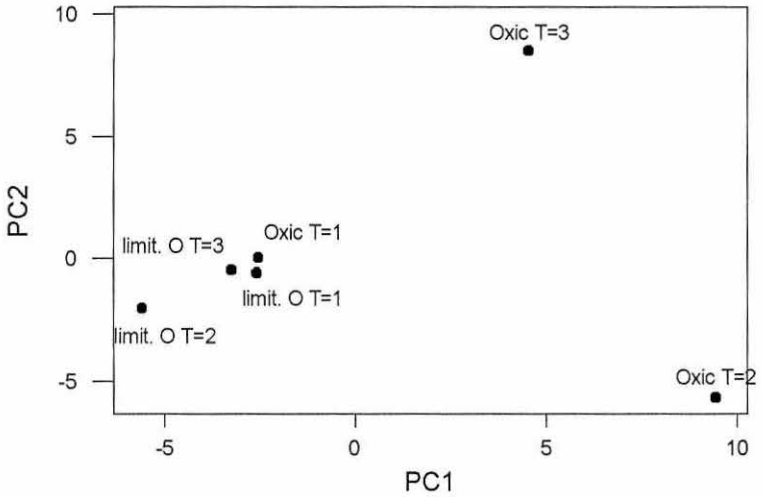


Figure 5.13. Score plot of principle components 1 and 2 for the 5' and 3' T-RF lengths from both *Msp*I and *Hha*I enzyme digests of PCR amplified 16S rRNA from pF33 incubated with soya oil (1%v/v) and synthetic sewage media 1,under conditions of differing oxygen availability at 30°C with shaking at 130rpm for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

Effect of grease trap isolates on the microbial community structure of pF33

The community composition and diversity of the pF33 microorganisms, grease trap isolates and pF33 and grease trap isolates combined is shown in Table 5.16 (see Appendix IV for further details).

Table 5.16. Dominant terminal restriction fragment (base pair) and total number of unique terminal restriction fragments (shown in brackets) for each enzyme digest of PCR amplified 16S rRNA from pF33 only, pF33+ grease trap isolates (GI) and grease trap isolates only (GI) incubated with soya oil (1%v/v) and synthetic sewage media 1 at 30°C with shaking at 130rpm for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

	***Hha*I(3')**	***Hha*I(5')**	***Msp*I(3')**	***Msp*I(5')**
F33 only T=1	345 (2)	215 (1)	298 (6)	69 (3)
F33 only T=2	163 (3)	240 (8)	365 (10)	147 (12)
F33 only T=3	163 (3)	240 (6)	365 (12)	142 (12)
F33+GI T=1	163 (2)	215 (1)	367(2)	144(2)
F33+GI T=2	163 (5)	215 (5)	365 (4)	69/135(4)
F33+GI T=3	163 (4)	242 (10)	364 (5)	137(9)
GI only T=1	65 (3)	57 (4)	294(6)	395(3)
Gi only T=2	65 (3)	57 (4)	294 (5)	394(3)
GI only T=3	65 (2)	57 (4)	294 (3)	394(3)

The dominant terminal restriction fragments varied with time for all enzyme digests of the pF33 microbial community (generally between week 1 and 2) and for three enzyme digests of the combined microbial community (*Hha*I(5'), *Msp*I(3'), *Msp*I(5')). In contrast, the community structure of the grease trap isolates was stable over the incubation period with only the dominant *Msp*I(5') digest changing with time.

There was no clear trend between the microbial inocula used and the total number of unique terminal restriction fragments for the *Hha*I(5') and *Hha*I(3') digests. However, the number of terminal restriction fragments for *Msp*I(3') and *Msp*I(5') digests were generally greater in pF33 than in the grease trap isolates or grease trap isolates combined with pF33 indicating greater diversity.

Cluster analysis of the data (Figure 5.14) indicated that the composition of all three microbial communities changed over the incubation period. The community structure of both the grease trap isolates and pF33 changed considerably from week 1 to 2 (47% and 48% similarity, respectively) and then remained stable from week 2 to 3 (74% and 73% similarity, respectively) whilst the community composition of the grease trap isolates and pF33 combined changed markedly over the entire length of the incubation period (week 1 cf.. 2, weeks 2 cf. 3; 48% similarity).

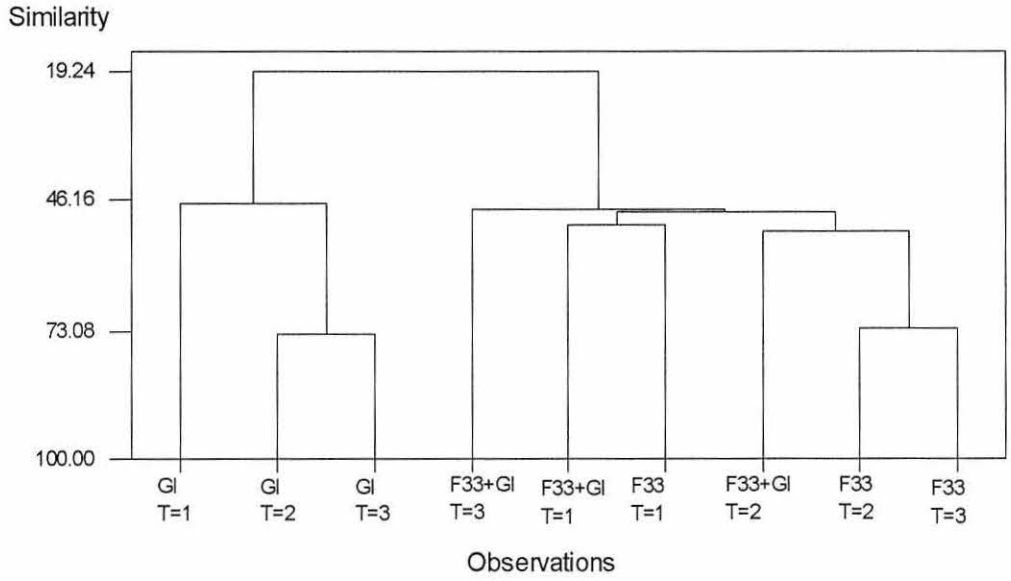


Figure 5.14. A dendrogram produced by cluster analysis of the 5' and 3' T-RF lengths from both *Msp*I and *Hha*I enzyme digests of PCR amplified 16S rRNA from pF33, grease trap isolates and pF33+grease trap isolates incubated with soya oil (1%v/v) and synthetic sewage media 1 at 30°C with shaking at 130rpm for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

Cluster analysis also indicated that the community structure of all three isolates were distinct from each other on each sampling occasion. However, pF33 combined with the grease trap isolates was more similar to that of pF33 alone (51%, 53%, 48% similarity after weeks 1, 2 and 3 respectively) than the grease trap isolates alone (19% similarity after 1, 2 and 3 weeks incubation).

Principle component analysis of the standardized T-RFLP data was also performed (Figure 5.15). Principle components 1 and 2 explained 24.8% and 20.0% of the variability respectively. Both principle components were influenced by a number of terminal restriction fragments (Appendix III). Only 1 of the dominant terminal restriction fragments (Hha(5')240) was a major contributor to principle component 1. The other major contributors, constituted either less than 6% of the total digested amplified DNA (Msp(3')317 and Msp(5')365) or as much as 5-39% of the total digested amplified DNA (Msp(5')134 and Msp(5')147). Principle component 2, was influenced largely by Hha(5')169, Msp(5')118 Msp(5')150. None of these terminal restriction fragments were dominant in any samples and constituted less than 27% of the total digested amplified DNA. Both principle components 1 and 2 represented an increase in the proportion of all of the major contributing terminal restriction fragments.

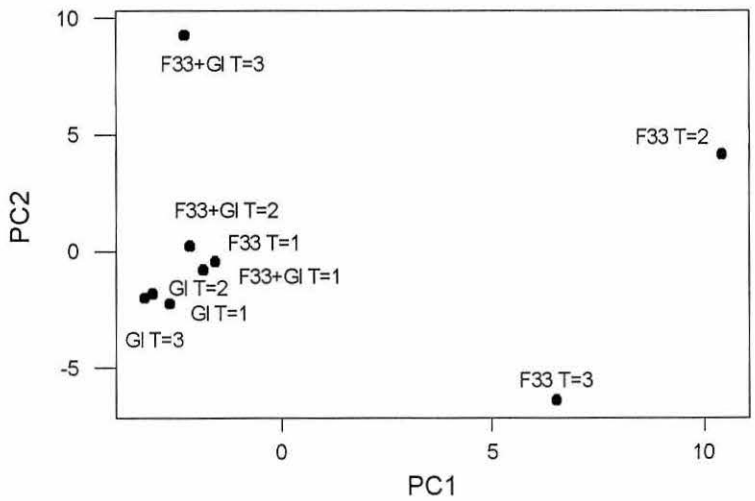


Figure 5.15. Score plot of principle components 1 and 2 for the 5' and 3' T-RF lengths from both *Msp*I and *Hha*I enzyme digests of PCR amplified 16S rRNA from pF33, grease trap isolates and pF33+grease trap isolates incubated with soya oil (1%v/v) and synthetic sewage media 1 at 30°C with shaking at 130rpm for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

In general, samples inoculated with the grease trap isolates were clustered more tightly than those inoculated with pF33, indicating that a fraction of the microbial population in pF33 was

more dynamic than in the grease trap isolates. The score plots for the grease trap isolates were tightly clustered in the negative regions of both principle components for weeks 1-3. However, pF33 alone scored positively on principle components 1 and negatively on principle component 2 after 7 days incubation, positively on principle components 1 and 2 after 14 days incubation and positively on principle component 1 and negatively on principle component 2, after 21 days incubation. pF33 combined with the grease trap isolates, in contrast, scored negatively on principle component 1 over the entire length of the experiment but scored more positively on principle component 2 after 2 to 3 weeks incubation.

Effect of starting pH on the microbial community structure of pF33

The change in community composition and diversity of pF33 with time under different starting pH's is given in Table 5.17.

Table 5.17. Dominant terminal restriction fragment (base pair) and total number of unique terminal restriction fragments (shown in brackets) for each enzyme digest of PCR amplified 16S rRNA from pF33 incubated with soya oil (1%v/v) and synthetic sewage media 1 at 30°C with shaking at 130rpm, at different starting pH's for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

	***Hha*I(3')**	***Hha*I(5')**	***Msp*I(3')**	***Msp*I(5')**
pH 4 T=1	345 (4)	215 (2)	298 (2)	69 (2)
pH 4 T=2	163 (4)	226 (8)	365 (5)	150 (11)
pH 4 T=3	163 (2)	222 (8)	298 (4)	116 (9)
pH 7 T=1	345 (5)	215 (7)	298 (5)	69 (8)
pH 7 T=2	345 (5)	215 (2)	298 (10)	69 (7)
pH 7 T=3	163 (5)	240 (5)	298 (12)	134 (5)
pH 10 T=1	65 (4)	340 (5)	294 (6)	398 (7)
pH 10 T=2	163 (2)	240 (5)	294 (3)	398 (6)
pH 10 T=3	65 (2)	340 (2)	294 (1)	398 (3)

The dominant terminal restriction fragments for all four enzyme digests varied at least once over the incubation period under acidic conditions (generally between week 1 and 2). In contrast, the dominant terminal restriction fragment for only two or three enzyme digests varied over the incubation period under alkaline and neutral conditions, respectively.

The total number of unique terminal restriction fragments for each enzyme was generally lower at pH 10 than at pH 4 or 7 after 2-3 weeks incubation indicating that the microbial population was less diverse at pH 10.

Cluster analysis of the data (Figure 5.16) indicated that the microbial community composition of pF33 varied with time at all pH's. At pH 7, the change in community structure was less marked between week 1 and 2 (65% similarity) than between week 2 and 3 (44% similarity) whilst under under acidic conditions the reverse trend was observed (week 1 cf. 2; 44% similarity, week 2 cf. 3; 61% similarity). In contrast, the extent of charge in the microbial community composition in pF33 incubated under alkaline conditions was the same between week 1 and 2 as between week 2 and 3 (51% similarity) and the samples incubated at pH 10 formed a distinct cluster. Cluster analysis of the data also indicated that after 7 days incubation, the community structure at pH 7, was more closely related to that at pH 4 (65% similarity) than at pH 10 (44% similarity). After 2 or 3 weeks incubation, however, none of the microbial communities were particularly similar (43-44% similarity).

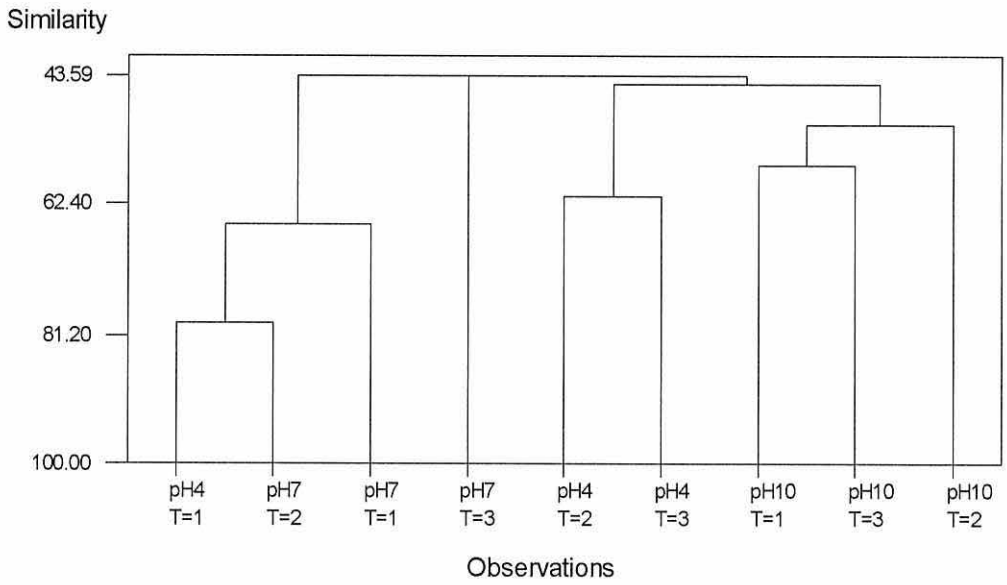


Figure 5.16. A dendrogram produced by cluster analysis of the 5' and 3' T-RF lengths from both *Msp*I and *Hha*I enzyme digests of PCR amplified 16S rRNA from pF33 incubated with soya oil (1%v/v) and synthetic sewage media 1 at 30°C with shaking at 130rpm, at different starting pH's for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

The standardized T-RFLP data was also examined using principle component analysis (Figure 5.17). Principle components 1 and 2 accounted for 22.7 and 20.8% of the variability respectively. Several terminal fragments contributed to both principle components 1 and 2 (Appendix III). One of the dominant terminal restriction fragments (Msp(5')134) was amongst the greatest contributors to principle component 1. The other terminal restriction fragments that accounted for most of the variability in principle component 1 constituted less than 7% of the total digested amplified DNA (Hha(3')115, Hha(3')142, Hha(3')148,

Hha(5')382, Msp(3')81-138, Msp(5')166, Msp(5')169) or as much as 38-42% of the total digested amplified DNA (Hha(5')240, Msp(5')137). Principle component 2 was influenced largely by two of the dominant terminal restriction fragments (Hha(3')345 & Msp(3')298) and by two terminal restriction fragments that constituted less than 15% of the total digested amplified DNA (Msp(3')260 & Msp(5')62). Principle component 1 represented an increase in the proportion of all the aformentioned terminal restriction fragments whilst principle component 1 represented a decline in the proportion of Hha(3')345, Msp(3')298, Msp(3')260 and Msp(5')62.

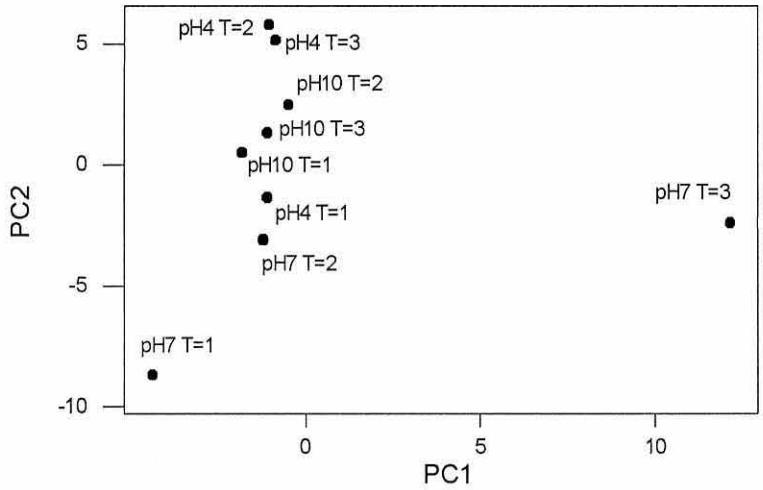


Figure 5.17. Score plot of principle components 1 and 2 for the 5' and 3' T-RF lengths from both *Msp*I and *Hha*I enzyme digests of PCR amplified 16S rRNA from pF33 incubated with soya oil (1%v/v) and synthetic sewage media 1 at 30°C with shaking at 130rpm, at different starting pH's for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

In general, those samples incubated at extremes of pH (ie pH 4 and 10) were clustered more tightly than those samples incubated at pH 7, indicating that a fraction of the microbial population was more dynamic under neutral conditions than at extremes of pH. The score plots of samples that were incubated under alkaline conditions were tightly clustered in the negative region of principle component 1 and positive region of principle component 2. Those samples that were incubated at pH 4, however, scored negatively on both principle components 1 and 2 after 1 weeks incubation but scored positively on principle component 2 after 2-3 weeks incubation. In contrast, samples incubated at pH 7 scored negatively on principle component 2 over the entire length of the incubation period but scored negatively on principle component 1 during the first 2 weeks of the experiment and positively during the third week.

Effect of incubation temperature on microbial community composition

The change in community composition and diversity of pF33 with time under different incubation temperatures is shown in Table 5.18.

Table 5.18. Dominant terminal restriction fragment (base pair) and total number of unique terminal restriction fragments (shown in brackets) for each enzyme digest of PCR amplified 16S rRNA from pF33 incubated with soya oil (1%v/v) and synthetic sewage media 1at different temperatures for 7, 14 and 21 days (T=1, T=2 & T=3, respectively). *enzymes failed to digest 16S rRNA /PCR reaction failed to amplify 16S rRNA

	***Hha*I(3')**	***Hha*I(5')**	***Msp*I(3')**	***Msp*I(5')**
10°C T=1	163 (2)	*	367 (2)	144 (1)
10°C T=2	163 (3)	57 (1)	367 (3)	144 (5)
10°C T=3	163 (2)	*	367 (2)	144 (1)
30°C T=1	345 (4)	215 (2)	298 (4)	69 (1)
30°C T=2	163 (3)	238 (3)	365 (2)	144 (3)
30°C T=3	163 (5)	238 (9)	365 (7)	144 (11)
50°C T=1	163 (1)	367 (1)	*	*
50°C T=2	65 (1)	*	294 (1)	*
50°C T=3	*	*	*	*

The dominant terminal restriction fragments varied at least once over the incubation period for all enzyme digests of pF33 incubated at 30°C (weeks 1 to 2) but not for pF33 incubated at 10°C. Most of the restriction enzymes failed to digest the 16S rRNA amplified from samples incubated at 50°C. However, the dominant terminal restriction fragment of the Hha(3') enzyme digest varied between weeks 1 and 2.

The number of unique terminal restriction fragments at 50°C was considerably lower than at 10 or 30°C indicating that pF33 was less diverse at 50°C than at 10 or 30°C.

Cluster analysis of the data (Figure 5.18) indicated that the community structure of pF33 changed with time at all incubation temperatures. At 30°C, the change in community composition was most marked over weeks 1 and 2 (12% cf. 74% similarity of weeks 2 and 3) whilst at 10°C the extent of charge was the same between week 1 and 2 as between week 2 and 3 (60% similarity). At 50°C, the change in community composition between week 1 and 2 was almost as extensive as at 30°C (22% similarity).

Cluster analysis of the data also implied that after 1 weeks incubation, the community structure at 10°C was more similar to that at 50°C (41% similarity) than at 30°C (12% similarity). After 2-3 weeks incubation, however, this situation was reversed. (week 2, 30°C cf. 50°C; 22% similarity, 10°C cf. 30°C; 41% similarity, week 3, 30°C cf. 10°C; 41% similarity)

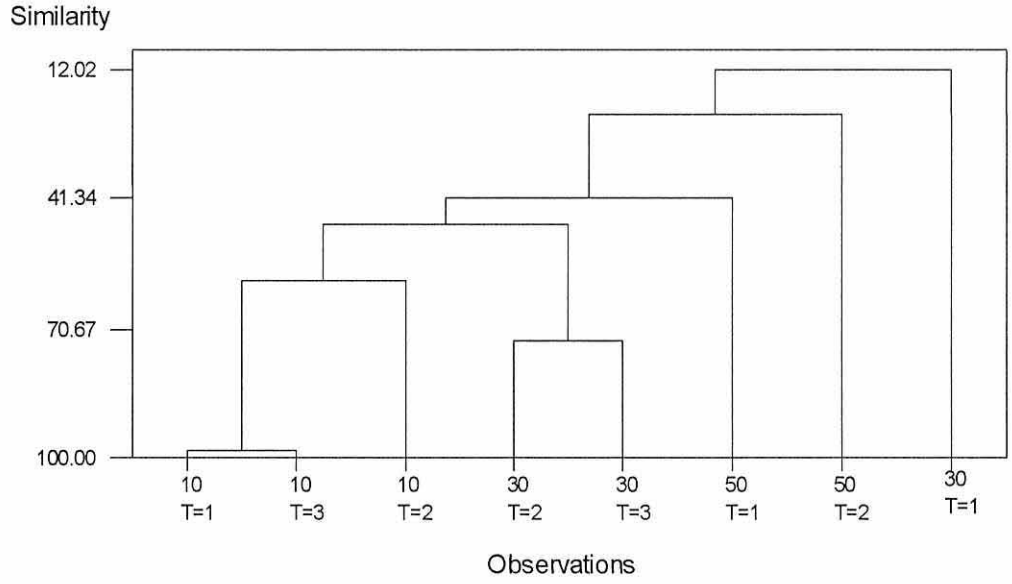


Figure 5.18. A dendrogram produced by cluster analysis of the 5' and 3' T-RF lengths from both *Msp*I and *Hha*I enzyme digests of PCR amplified 16S rRNA from pF33 incubated with soya oil (1%v/v) and synthetic sewage media 1 at different temperatures for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

The standardized T-RFLP data was also examined using Principle component analysis (Figure 5.19). Principle components 1 and 2 accounted for 53.5% and 18.0% of the total variability respectively. Several terminal restriction fragments contributed to both principle components 1 and 2 (Appendix III). Most of the major contributors to principle component 1 constituted less than 3% of the total digested amplified DNA (e.g Hha(3')57, Hha(3')142, Hha(5')173, Msp(3')143) although some comprised as much as 10-22% of the total digested amplified DNA (Hha(5')226, Msp(3')295, Msp(3')368, Msp(5')150) Principle component 2 was influenced largely by four dominant terminal restriction fragments (Hha(3')345, Hha(5')215, Msp(3')298 Msp(5')69) and some minor fragments (Hha(3')298, Hha(5')69, Msp(3')62) that constituted less than 7% of the total digested amplified DNA. Principle component 1 represented a decline in the proportion of the aformentioned terminal restriction fragments wheras principle component 2 represented an increase in the proportion of Hha(3')345, Hha(5')215, Msp(3')298 Msp(5')69, Hha(3')298, Hha(5')69 and Msp(3')62.

In general, those samples incubated at extremes of temperature (ie 10°C & 50°C) were clustered more tightly than those samples incubated at intermediate temperature (ie 30°C), indicating that a fraction of the microbial population was more dynamic under intermediate temperatures than extremes of temperatures. Those samples that were incubated at 30°C scored negatively on principle component 1 and positively on principle component 2 after 1 weeks incubation, negatively on both components 1 and 2 after 2 weeks incubation and negatively on principle component 1 and positively on principle component 2, after 3 weeks incubation.

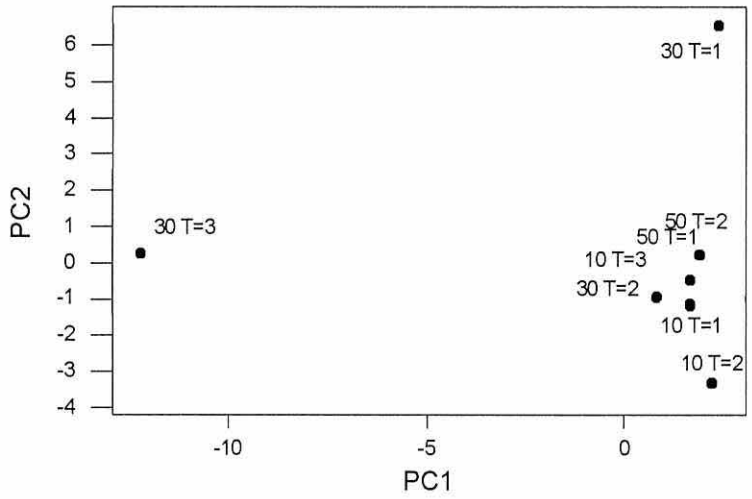


Figure 5.19. Score plot of principle components 1 and 2 for the 5' and 3' T-RF lengths from both *Msp*I and *Hha*I enzyme digests of PCR amplified 16S rRNA from pF33 incubated with soya oil (1%v/v) and synthetic sewage media 1at different temperatures for 7, 14 and 21 days (T=1, T=2 & T=3, respectively).

5.2.3.2. Identification of the pF33 microorganisms

A condensed version of the Ribosomal Database Project (Maidak *et al.*, 2001) was prepared (Appendix V) consisting of terminal restriction fragments (TRF's) for *Rsa*I, *Msp*I and *Hha*I digest of 16S rRNA from only microorganisms that were expected to be found in pF33 (Appendix VII) and in a grease trap environment. Unfortunately, several of the predominating T-RF's in some of the samples were absent from the database and those that were present often provided little information on microbial community composition due to the absence of other indicative terminal restriction fragments in the samples. Nevertheless, it was possible to identify some of the microorganisms that predominated in pF33 and thus highlight some of the microorganisms which may have had an important role in the oil degradation process.

pF33 microorganisms present under conditions of varying oxygen availability

After 7 days incubation, under both oxic conditions and conditions of limited oxygen availability the pF33 microbial community was dominated largely by *Lactobacillus* lm-17, (as indicated by the presence of the dominant fragments; Msp(3')298 and Hha(3') 345). Under conditions of limited oxygen availability, the *Lactobacillus* lm-17 strain remained dominant over the remainder of the incubation period whilst other lactobacillus strains such as *L.casei* and/or *L.paracasei* (fragments; Hha(3')298 and Msp(3')367) which were not detected after 1 weeks incubation became more important. Under oxic conditions, in contrast, the proportion of *Bacillus* sp. and *Paenibacillus* sp. in F33 increased over the remainder of the incubation period (dominant fragments Msp(3')365 and Hha(3')163) whilst the proportion of *Lactobacillus* lm-17 declined (dominant fragments; Msp(3')298 and Hha(3')345). A *Bacillus* strain, possibly *Bacillus pseudomycoides*, which was not detected after 7 days incubation also became more important under oxic conditions during the second and third week of the experiment (indicative fragment; Msp(5')147).

pF33 microorganisms present in samples inoculated with the grease trap isolates

The microbial community composition of the grease trap isolates was not resolved. *Eubacterium limosum* and/or *Lactobacillus* lm-17 may have been present over the entire length of the incubation period (fragments; Msp(3')294 and Hha(3')345). However, if these species were predominanting in the sample, both the Hha(3')345 and Msp(3')294 fragments might be expected to constitute a large proportion of the total digested amplified DNA, which they did not. The absence of *Bacillus* species markers such as Msp(3')365 and Hha(3')163 also suggested that *Bacillus* sp. did not constitute a large proportion of the microbial community.

In contrast, the pF33 community after 1 weeks incubation appeared to be dominated largely by L*actobacillus* lm-17 (dominant fragments; Msp(3') 298 and Hha(3') 345). After a further 1-2 weeks incubation, however, *Bacillus* sp. and *Paenibacillus* sp. consituted the largest proportion of pF33 (dominant fragments; Msp(3')365 and Hha(3') 163). A *Bacillus* strain possibly *Bacillus pseudomycoides* that was not detected after 1 weeks incubation also became more important during the second and third week of the experiment (fragment; Msp(5')147).

Unlike pF33, the combined microbial community (pF33 & grease trap isolates) appeared to be dominated by the *Bacillus* and *Paenibacillus* sp. over the entire length of the incubation period (dominant fragments; Msp(3')364-367, Hha(3')163). Given that the size of the dominant Msp(3') fragment in the combined microbial community differed from that in the pF33 community by 1-2 base pairs, the *Bacillus* and *Paenibacillus* sp. present in the combined microbial community may have been distinct from those found in pF33. There was also evidence to suggest that *Paenibacillus chondroitins* (Msp(5')150, Msp(3')297-299, Hha(5')226, Hha(3')163) and *Paenibacillus macerans* (Msp(5')135-137, Msp(3')297-299, Hha(5')242, Hha(3')133) became more important towards the end of the incubation period in the combined microbial community but not in pF33. Furthermore, *Bacillus pseudomycoides* (Msp(5')147) appeared to be less important in the combined microbial population than in pF33.

pF33 microorganisms present at different incubation temperatures

Lactobacillus lm 17 appeared to dominate after 7 days incubation at 30°C (dominant fragments Msp(3')298 and Hha(3')345) whilst *Bacillus* and *Penibacillus* sp may have dominated at 10°C (dominant fragments Msp(3')367 and Hha(3')163). After a further 1 to 2 weeks incubation the *Bacillus* and *Paenibacillus* sp. were thought to be predominant at both incubation temperatures. Given that the dominant Msp(3') fragment at 10°C differed from that at 30°C by 2 base pairs (after 2-3 weeks incubation), the dominant *Bacillus* and *Paenibacillus* species at 30°C may have been distinct from those at 10°C. There was also evidence to suggest that one or several of the following strains were important after 2 or 3 weeks at 30°C but not at 10°C: *Brevibacillus choshinensis*, *Brevibacilus agri* (fragments Msp(5')152, Msp(3')298, Hha(5')226, Hha(3')163, Rsa(5')445), *Paenibacillus chondroitinus* (fragments; Msp(5')152, Msp(3')295, Hha(5')226, Hha(3')163) *Bacillus circulans* (Fragments Msp(5')152, Msp(3')365, Hha(5')238, Hha(3')163and Rsa(5')453) and *Bacillus benzovorans* (Fragments Msp(5')152, Msp(3')365, Hha(5')238 and Hha(3')163). Too few terminal restriction fragments were resolved at 50°C to identify the microbial species present.

pF33 microorganisms present at different starting pH;s

Lactobacillus lm-7 dominated at pH 4 and 7 after the first week of the incubation period (dominant fragments Msp(3')298 and Hha(3')345). However, an unidentified fraction of the

microbial community dominated at pH 10. *Lactobacillus fermentum* (fragment; Msp(3')68), *Lactobacillus reuteri* and *Lactobacillus pontis* (fragments; Msp(3')68, Hha(3')345) may have also present at pH7 but were not at pH 4 and pH 10. After 2 weeks incubation, in contrast, *Bacillus*, *Penibacillus* and *Brevibacillus* species were predominant at pH 4 and pH 10 (dominant fragment Msp(3')365/294 and Hha(3')163), whilst *lactobacillus* lm-7 remained the predominant species at pH 7 (dominant fragments; Msp(3')298 and Hha(3')345). *Bacillus circulans* (Fragments Msp(5')150, Msp(3')365, Hha(5')238, Hha(3')163 and Rsa(5')452) and *Bacilus benzovorans* (Fragments Msp(5')150, Msp(3')365, Hha(5')238 and Hha(3')163) may have also been present at pH 4 but not at pH10 or 7. Over the remainder of the incubation period, *Bacillus*, *Brevibacillus* and *Penibacillus* species were dominant at pH 7 (dominant fragments; Msp(3')298 and Hha(3')163) whilst *Penibacillus* and *Brevibacillus* (dominant fragments; Msp(3')295 and Hha(3')163) were dominant at pH 4. At pH 10, the unidentified fragment of the microbial community was dominant, once again.

5.2.3.3. T-RFLP analysis of formulations 1, 6 and 7

The community compositions and diversities of formulations 1,6 and 7 before and after growth in lab M nutrient E broth are shown in Table 5.19.

Table 5.19. Dominant terminal restriction fragment and total number of unique terminal restriction fragments (shown in brackets) for enzyme digest of PCR amplified 16S rRNA for each of formulation 1, 6 and 7 (F1, F6 and F7) before and after growth in Lab M nutrient media for 24 hours at 30°C with shaking at 130rpm *enzymes failed to digest PCR amplified 16S rDNA/PCR reaction failed to amplify 16S rDNA.

	***Hha*I(3')**	***Hha*I(5')**	***Msp*I(3')**	***Msp*I(5')**
F1	290 (6)	213 (5)	242 (7)	460 (7)
F1(grown)	163 (1)	240 (2)	365 (2)	144 (2)
F6	308 (1)	369 (1)	62 (1)	*
F6(grown)	163 (1)	336 (2)	145 (1)	*
F7	65 (3)	213 (2)	242 (5)	460 (4)
F7(grown)	164 (4)	238 (3)	367 (5)	145 (5)

The data in Table 5.19 indicates that the community compositions of the three formulations were modified by growth in Lab M nutrient E broth. Three of the four enzyme digests indicated that the community structure of formulation 1 may have been similar to that of formulation 7 before growth in Lab M nutrient E broth but not afterwards.

The community composition of formulation 6 was distinct from that of formulations 1 and 7 and was also less diverse. Formulation 6 may have appeared less diverse than the other formulations since the yield of amplified DNA from formulation 6 was less than that from the other formulations (as indicated by intensity of band on agarose gel). The lower DNA yield was thought to be the result of the lipase accelerant present in formulation 6, which may have inhibited the PCR reaction.

5.3. Discussion

The concentration and nature of fat/oil is likely to vary in wastewater, therefore the ability of pF33 to degrade various fat/oils over a wide concentration range was investigated. pF33 was capable of significantly enhancing the degradation of several fats and oils, which varied in their physico-chemical properties. The extent of fat/oil degradation was variable (35-60% after taking the control samples into account) and soya oil was degraded to a significantly greater extent than lard. These findings are in agreement with those of Tano-Debrah *et al.* (1999) and Groeneworld *et al.* (1982) who utilised a different selection of fat/oils but also noted that the nature of the fat/oil influenced microbial degradation rates.

Although the work of Tano-Debrah *et al.* (1999) and Groeneworld *et al.* (1982) did not indicate that there was a relationship between the biodegradability of the fat/oil and the degree of saturation, a number of other studies have shown that unsaturated fatty acids are degraded more readily than their saturated counterparts (Parker & Leo 1965; Loehr & Roth, 1968; Malaney & Gerhold, 1969; Farrington & Quinn 1971; Sun & Wakeman 1994). The greater degradation of soya oil shown in the current study may have been due to the higher proportion of 18:2ω6 and 18:3ω3 compared to lard. Another explanation for the reduced degradation rate of lard, was that solid fats were less likely to be as well dispersed in the media as liquid oils and according to Ratledge (1994), fats and oils must be dispersed in the media for successful microbial growth to occur.

In contrast to literature suggesting a relationship between fatty acid degradation rates and degree of saturation, the pF33 microorganisms (peanut shell carrier) degraded the major fatty acids at a similar rate (at an oil concentration of 1%). Of the four types of fat/oil investigated only rapeseed and sunflower oil experienced significant changes in the relative proportions of

the major fatty acids and these changes were the result of abiotic processes, rather than biological activity. The abiotic changes observed; an increase in the proportion of 16:0 and decrease in the proportion of 18:2ω6 and 18:3ω3, were similar to those induced by the microbial communities discussed in chapter 3 and may have been the result of autoxidation. Lard and soya oil were incubated for a shorter period of time than rapeseed and sunflower oil, which may explain why these fat/oils did not experience a significant change in the relative proportion of the major fatty acids. Given that the rate of autoxidation increases with the number of double bonds present in the molecule (Gunstone, 1958; Gere, 1982) and that lard and soya oil were enriched in saturated and mono-unsaturated fatty acids relative to rapeseed and sunflower oil, lard and soya oil may have also been less affected by the autoxidation processes than the other oils.

The failure of the pF33 microorganisms to utilise 18:2ω6 and 18:3ω3 more readily than 16:0 and 18:1ω9 (at oil concentrations of 1%) was unexpected and contradicts much of the existing literature (Parker & Leo 1965; Loehr & Roth, 1968; Malaney & Gerhold, 1969; Farrington & Quinn 1971; Sun & Wakeham 1994). Unsaturated fatty acids may be degraded more readily than their saturated derivatives either because the long chain acyl-CoA synthetases are more active towards the unsaturated fatty acids (Gurr & Harwood, 1991) or because the unsaturated fatty acids are more soluble and thus bioavailable relative to the saturated fatty acids (Loehr & Roth, 1968). Another explanation is that microorganisms convert 18:3 successively to 18:2, 18:1 and even 18:0 since the antibacterial effects of the fatty acids increase with the degree of unsaturation and the preferential removal of the highly unsaturated fatty acids reduces the toxicity of the media (Kemp & Lander, 1984; Kemp *et al.*, 1984; Pereira, 1999). In this study, physico-chemical processes reduced the proportion of 18:3ω3 and 18:2ω6 in the oil considerably. Thus if the later theory held true the pF33 microorganisms may have had less need to eliminate 18:3ω3 and 18:2ω3 from the media.

pF33 was also capable of significantly enhancing the degradation of soya oil that was present at high oil concentrations. However, the extent of degradation at high oil concentrations was considerably less than at low oil concentrations. As in chapter 2, the low levels of degradation were thought to be the result of the poor dispersal of oil in the media and/or oxygen limitation. Although the clay carrier contained an oxygen generator, oxygen availability was

still considered to be a limiting factor since the oxygen generator was unlikely to function over the entire length of the incubation period and its effectiveness was unknown.

The impact of nutrient availability on oil degradation was not examined directly. However, it was observed that pF33 with the clay carrier significantly enhanced oil degradation only when the yeast/glucose concentrations were raised whilst pF33 with the peanut shell carrier and thus additional nutrients behaved in the converse manner. These findings imply that there may be an optimum nutrient concentration for microbial oil degradation, in agreement with the work of Tano-Debrah *et al.* (1999) and COD work illustrated in chapter 3.

Although unable to significantly enhance the degradation of oil that was present at a concentration of 10% in reduced nutrient media, pF33 with a clay carrier was capable of significantly altering the fatty acid composition of oil whilst pF33 with a peanut shell carrier was not. The compositional changes induced were similar to those brought about by the microbial communities discussed in chapter 3 and the autoxidation process described above. Given that the only difference between the microbial supplements was the carrier used, the carrier was likely to be responsible for the fatty acid compositional changes observed. The clay carrier contained only an oxygen generator whilst the peanut shell contained only nutrients, which implies that the lower nutrients levels and/or high oxygen levels enhanced process such as autoxidation, hydrogenation and/or the partial β-oxidation of 18:2ω6 and 18:3ω3

Aside from degrading a variety of fats and oils over a wide concentration range a microbial supplement must also be capable of operating under the fluctuating oxygen levels, pH's and temperatures encountered in a wastewater environment. pF33 was capable of significantly enhancing the degradation of oil under both oxic and oxygen limited conditions. However, the extent of oil degradation under oxygen-limited conditions was significantly less than under oxic conditions. Anaerobic degradation is less efficient at yielding energy than aerobic decomposition thus the reduced rate of oil degradation at low oxygen concentrations was not unexpected (Westermann, 1996). Work with marine sediments has also indicated that fatty acid degradation in oxic environments is more rapid than in anoxic environments (Harvey *et al.*, 1995). However, several workers have reported that the anoxic decomposition of organic matter in sediments can occur at rates comparable to or faster than oxic decomposition

(Westrich & Berner, 1984; Henrichs & Reeburgh, 1987; Lee, 1992) in contrast to the findings of the current work..

The reduction in oil degradation under oxygen limited conditions may have been due to a change in the predominating fatty acid degradation pathway. A significantly greater proportion of 18:1ω9 was detected in rapeseed oil incubated under oxygen limited conditions and a new fatty acid (10-OH) was produced under oxic conditions but not under conditions of oxygen limitation. The decline in the proportion of 18:1ω9 with increasing oxygen concentrations may have been due to the growth of bacteria that were able to degrade 18:1ω9 more readily and/or an increase in the rate of hydrogenation, hydroxylation or oxidation of 18:1ω9. Another possibility was that the increase in oxygen availability enhanced the rate at which autoxidation took place, as was observed by Gere (1982).

10-OH was also detected in the inoculated soya, rapeseed and sunflower samples discussed earlier but was not evident in the physico-chemical controls. These findings indicate that 10-OH was produced by the pF33 microorganisms rather than abiotic process. Given that 10-OH was detected under oxic conditions only, the microorganisms responsible for the production of this fatty acid were most active (or present only) under oxic conditions. The 10-OH in question may have been 10-hydroxy-octadecanoic acid which has been observed as a product of oleic acid (18:1ω9) degradation in other studies (Koritala & Bagby, 1992). The production of this fatty acid from 18:1ω9 may thus account for the reduction in the proportion of 18:1ω9 under oxic conditions.

Oil degradation rates in the oxygen limited environment may have been lower due to the reduction in bacterial numbers and/or diversity of the microbial population, as indicated by T-RFLP analysis. The microorganisms that predominated under oxygen limited conditions may have also been less active or less efficient at degrading the oil. Another explanation for the reduced oil degradation rate was that the community composition of pF33 fluctuated considerably with time under oxic conditions but not under oxygen limited conditions. The fluctuations in community structure indicated that several microbial species were involved in the degradation of oil and that a dynamic population may be required for the efficient degradation of oil The dynamics of an oil-degrading microbial community has never been reported on before, however, numerous microbial species are known to be involved in the

degradation of other organic substrates such as phenol and rice straw and the structures of these microbial communities can remain fairly constant (Weber *et al.*, 2001) or change with time (Guieysse *et al.*, 2001).

Although the identities of the oil-degrading microorganisms were not fully resolved using T-RFLP analysis there was evidence to suggest that under oxic conditions a *Lactobacillus* strain dominated over the first week of the experiment, whilst *Bacillus* and *Paenibacillus* species dominated over the remainder of the incubation period. In contrast, under conditions of limited oxygen availability a *Lactobacillus* strain remained dominant over the entire length of the incubation period.

Whilst *Bacillus* strains have proved to be efficient oil-degraders in other studies (Becker *et al.*, 1999; Becker & Markl, 2000; Markossian *et al.*, 2000) Koritala *et al.*, (1987) reported that the oil-degrading capacities of *Bacillus* microorganisms compare poorly with those of yeasts and fungi. There appear to have been no studies that have compared the oil-degrading capacities of *Bacillus*, *Paenibacillus* and *Lactobacillus* directly and unlike *Bacillus* sp., the oil-degrading abilities of *Lactobacillus* and *Paenibacillus* sp. have never been investigated in any detail. Interestingly, *Lactobacillus* strains have been known to produce 10-hydroxyoctadenanoic acid from oleic acid (Wanikawa *et al.*, 2002) whilst *Bacillus* strains have not. Given that 10-OH was detected only under oxic conditions and that *Lactobacillus* predominated under conditions of oxygen limitation, there was no evidence of a relationship between the proportion of *Lactobacillus* in the microbial community and the occurrence of 10-hydroxyoctadecanoic acid in the samples.

The impact of microbial community composition on oil degradation rates was also highlighted by T-RFLP analysis of formulations 1 and 7. According to the manufacturers formulations 1 and 7 were identical in composition. However, formulation 7 enhanced the degradation of oil whilst formulation 1 did not. T-RFLP analysis indicated that formulations 1 and 7 were similar in composition before growth in Lab M nutrient E broth but not afterwards suggesting that growth in Lab M nutrients prior to inoculation of the oil samples enabled one formulation to enhance the degradation of oil but not the other. These findings confirm the reports of other workers that culture based methods can modify the structure of a microbial community (Muyzer *et al.*, 1983; Liu *et al.*, 1997; Padmanabhan *et al.*, 1998) and also suggest

that subtle differences in microbial community composition can have a marked impact on oil degradation rates.

The starting pH of the media did not significantly influence the ability of pF33 to degrade oil. However, there was a relationship between the extent of oil degradation and starting pH of the media with a 20% increase in oil degradation from pH 4 to 10. Similarly, Tano-Debrah *et al.* (1999) demonstrated that the extent of oil degradation increased by 55% as the initial pH of the media was raised from pH 4.5 to 9.5 but failed to test the significance of this relationship. These authors proposed that oil degradation was more rapid at higher pH's since emulsion formation in alkaline media is greater than in acidic media. Ratledge (1994) also reported that oil degradation could only take place if the pH was maintained at 5.5 or above but suggested that at high pH's the concentration of undissociated fatty acid in the media is reduced and thus the antibacterial effects of the fatty acids are minimized (Ratledge, 1994). pH may have failed to significantly impact oil degradation rates in the current study because the nutrients present in pF33 were buffering the pH of the media. Another possibility was that the pF33 microorganisms were capable of adjusting the pH of the media, as observed in earlier work.

The relationship between pH and oil degradation may have arisen because of variations in fatty acid degradation pathways. Although the starting pH of the media did not appear to have a great impact on the relative proportions of the major fatty acids in rapeseed oil, a 10-OH fatty acid was detected at pH 7 and 10 only, implying that the microorganisms responsible for the production of this fatty acid were most active (or present only) at neutral to alkaline pH's. Oil degradation rates may have also been influenced by bacterial numbers since a greater number of bacteria were present at pH 10 than an at pH 7 or 4. pH appeared to have little impact on the level of microbial diversity but there were differences in microbial community composition at the various pH's and perhaps those microorganisms predominating at higher pH's were more active and/or efficient at degrading oil.

The capacity of pF33 to degrade oil over all incubation temperatures in non-continuously shaken cultures was poor. However, the extent of oil degradation at 50°C was significantly lower than at 10°C and 30°C indicating that the optimum temperature for oil degradation lay between 10°C and 50°C. Tano-Debrah *et al.* (1999) using a multi-strained inocula also reported that the optimum temperature for microbial oil degradation lay between 10 and 50°C

(20°C). However, appreciable oil degradation occurred at extremes of temperature (i.e. 15°C and 42°C) and oil samples were shaken at 120 rpm over the incubation period, in contrast to the samples in the current work.

The decline in oil degradation at 50°C may have been due to disparities in the fatty acid degradation pathways. An increase in incubation temperature resulted in a significant reduction in the proportion of 18:2ω6 coupled with a significant increase in the proportion of 16:0. The rise in incubation temperature may have promoted the growth of bacteria that were able to degrade 18:2ω6 more readily than 16:0 or induced reactions such as hydrogenation and/or β-oxidation of 18:2ω6. According to Neidleman, (1987) microbes often increase the proportion of saturated fatty acids when the environmental temperature is increased therefore the hydrogenation of 18:2ω6 by pF33 was not unexpected. The rate of autoxidation may have also increased with the rise in temperature since an autoxidation product was detected (azaleic acid) at 50°C but not at 10°C or 30°C.

Oil degradation rates were unlikely to have been influenced by absolute bacterial numbers since the number of bacteria present at 50 and 10°C were similar. T-RFLP analysis indicated, however, that there was a reduction in the level of bacterial diversity at 50°C, which may have influenced degradation rates. There was also evidence to suggest that different groups of microorganism predominated at the various incubation temperatures and those microorganisms present at 50°C may have been less active or less efficient at degrading the oil.

In addition to operating under conditions of varying oxygen availability and over a wide pH an temperature range, a commercial inocula must also be capable of competing successfully with the indigenous microbial population. pF33 degraded a significantly greater proportion of the oil than the grease trap isolates but was unable to enhance oil degradation when the two communities were combined. All three microbial communities were comprised of a similarly high number of microorganisms therefore the reduced oil degrading capacity of the combined microbial community was likely to be the result of differences in community structure and diversity.

It has been suggested by several authors that bioaugmentation products may fail in the field because they are unable to compete with the indigenous microbial community (Goldstein *et*

al, 1985; Horsfall, 1979; Stephenson & Stephenson, 1992.). T-RFLP analysis revealed, however, that the structure of the combined microbial community was more similar to that of pF33 than the grease trap isolates, indicating that some of pF33 microorganisms were competing successfully with the grease trap isolates.

The pF33 microorganisms may have failed to enhance the degradation of oil in the presence of the grease trap isolates because the diversity of the combined microbial population was less than that of pF33 alone. Another explanation for the reduced oil degradation rate of the combined microbial community was that the grease trap isolates unbalanced the dynamics of the pF33 populations. In fact, PCA analysis of the T-RFLP data indicated that part of the pF33 population was extremely dynamic when cultured alone but not in the presence of the grease trap isolates. Interestingly, 10-OH, a potential indicator of high oil degradation rates, was only detected when pF33 was cultured alone. The dynamic part of the pF33 community may thus have been responsible for both the production of 10-OH and the faster rate of oil degradation.

6. GENERAL DISCUSSION AND CONCLUSIONS

Bioaugmentation of fat/oil degradation

Bioaugmentation has been described as the process of introducing non-indigenous micro-organisms into a system with the principal purpose of enhancing process performance (Stephenson & Stephenson, 1992; Lange *et al.*, 1998). In theory, bioaugmentation of grease degradation is achieved by the introduction of single or multiple strains of grease degrading bacteria. However, the results of quantitative studies examining the efficacy of such treatments have been mixed.

In general, bioaugmentation studies conducted under field conditions were successful. Several authors reported improvements in terms of fat/oil concentrations and/or COD and BOD levels. (Baig & Greening, 1976; Grubbs *et al.*,1991; Holt, 1992; Maes, 1994; Keenan & Sabelnikov, 2000). However, results were questionable due the lack of a proper control. The best that was normally achieved was a comparison between the same system before and after a bioaugmentation regime was adopted. However, other factors may have changed to cause the improvements in wastewater quality.

Laboratory-based investigations, which allow proper comparisons between treatment and control systems, have shown that several single and multi-strained inocula are capable of enhancing oil degradation (Koritala *et al.*, 1987; Tano- Debra *et al.*, 1999; Markossian *et al.*, 2000; Mihara *et al.*, 2000; Keenan & Sabelnikov, 2000). However, the efficacy of commercially available inocula under laboratory conditions has generally been poor and these studies normally failed to replicate the non steady state conditions found within the field.

Given that the effectiveness of bioaugmentation remains largely inconclusive the principle objective of this work was to determine using laboratory-based experiments whether commercial microbial supplements were likely to enhance oil degradation in the field. In order to accomplish this it was necessary to assess the performance of commercial inocula under a wide range of environmental conditions and examine other microbial characteristics (such as amylase and protease activity, ability to attach to surfaces) that may influence the performance of a bioaugmentation product in the field.

Fat/oil degradation was assessed gravimetrically and by using COD determinations. However, both methods were beset by number of problems. The COD determinations were generally hampered by inherent difficulties in obtaining a truly representative sample, particularly when the oil polymerized and/or particulate material formed. Furthermore, the partial degradation of fat/oil by the commercial inocula, may have actually increased the apparent COD of the sample, since shorter chain fatty acids are more soluble and thus available for chemical oxidation (Grubbs, 1983). Gravimetric analysis was considered the most absolute measure of fat/oil content but was largely hampered by the formation of emulsions during solvent extraction of the fat/oil.

Despite these difficulties, it was possible to determine gravimetrically that some commercial inocula were capable of significantly enhancing oil degradation under laboratory conditions (F33, pF33, formulation 6 & 7) whilst other formulations were not (P80, GTL, G40, S45 & formulation 1). Some of the formulations investigated may have failed to enhance oil degradation because they lacked sufficient nutrients in the media (G40). Other supplements (P80 & GTL) may have performed poorly due to high oil concentrations which prevented adequate mixing of the media. A number of formulations, however, (S45 & formulation 1) were simply unable to degrade oil even when sufficient levels of nutrients were present and oil concentrations were such that the oil was well dispersed in the media. These supplements may have failed to degrade oil because the inhibitors present were not sufficiently diluted, the number of microoganisms present in the supplements were too few, the microoganisms lacked the genetic capacity to degrade fat/oil or the microbial community composition of the formulation was incorrect.

Another reason why the single strained supplements may have failed to enhance oil degradation was that a consortia of organisms rather than an individual microorganism was required to degrade oil. Indeed, several studies have shown that combined inocula achieve higher levels of oil degradation than single strains alone (Keenan and Sabelnikov, 2000; Mihara *et al.*, 2000) and it has been suggested that the end products resulting from the activity of one group of organisms may serve as substrates for another group of organisms during oil degradation (Pereira, 1999). In the current study, efficient oil degradation was observed only when there were clear changes in community structure with time thus indicating that the pF33 may be degrading the fat/oil in the sequential manner proposed by Pereira (1999) .

Unlike multi-strained/species products, the conditions over which a single-strained/species product can operate may also be limited. Multi-strained/species products are comprised of several different groups of microorganisms which are likely to exhibit a variety of growth ranges and optimums and therefore operate over a wide range of environmental conditions. Distinct groups of pF33 microorganisms were observed to dominate under different environmental conditions, which may account for the growth/oil degradation of the multi-strained/species products (pF33, formulation 1, 6 and 7) over a wide temperature and pH range and under conditions of varying oxygen availability.

Fat/oil degradation rates

Several factors were shown to significantly influence the rate of fat/oil degradation. Nutrients, particularly, organic carbon/nitrogen were essential for successful growth on fat/oil. Very few of the commercial supplements investigated were capable of utilising any fat/oil as the sole carbon source and yeast/glucose proved to be essential for the microbial degradation of soya oil present at high concentrations (0.3-0.6 %v/v) but not a low concentrations (0.1 %v/v). There was also evidence to suggest that very high nutrient levels were actually inhibitory to microbial fat/oil degradation implying that optimum nutrient concentrations for microbial oil degradation must exist. These results are in agreement with those of Tano-Debrah *et al.*, (1999) who reported that the presence of organic nitrogen (peptone) enhanced fat/oil degradability considerably (from 12 to 85%) but that increasing the peptone concentration from 1 to 2% significantly lowered the quantity of oil/fat degraded. It was also postulated that nutrient availability may have an indirect effect on fat/oil degradation rates given that peptone additions improved the growth of formulations 1, 6 and 7 in synthetic sewage media and that nutrients, including lard, significantly enhanced the number of microorganisms adhering to surfaces.

The nature of the lipid source was also demonstrated to have a significant effect on the extent of oil/fat degradation. pF33 degraded soya oil at a significantly greater rate than lard and was able to grow on palmitic acid (16:0) and oleic acid (18:1ω9) but not linoleic acid (18:2ω6) as the sole carbon source. When presented with natural fats and oils, however, pF33 generally degraded the major fatty acids at a similar rate. Formulations 1, 6 and 7, in contrast to pF33, were capable of growing on oleic acid but not on palmitic or linoleic acid as the sole carbon source. The failure of the microbial supplements to grow on linoleic acid more readily than

oleic and palmitic acid was unexpected given that other workers have found that fatty acid reactivity/biodegradability increases with the degree of unsaturation (Parker & Leo 1965; Loehr & Roth, 1968; Malaney & Gerhold, 1969; Farrington & Quinn, 1971; Sun & Wakeman, 1994). Unsaturated fatty acids are also known to exert greater antimicrobial effects than the equivalent saturated fatty acid (Galbraith *et al.*,1971). Thus, the growth of the microbial formulations on the various fatty acids may have reflected a balance between these two factors.

The ability of pF33 to degrade palmitic and oleic acid more readily than linoleic under some conditions but not others, indicates that the presence of alternative carbon/organic nitrogen sources and other triglycerides/fatty acid may influence the relative rates at which the fatty acids are degraded. The rate at which 18:2ω6 was degraded in rape oil was also enhanced by raising incubation temperatures whilst increasing the availability of oxygen enhanced the rate at which 18:1w9 was degraded.

Incubation temperature was also observed to have a significant effect on oil degradation rates. A significantly greater proportion of the oil was degraded at 30°C and 10°C relative to 50°C. Tano-Debrah *et al.*, (1999) observed a similar relationship between incubation temperature and oil degradation. However, it is important to note that aerobic thermophilic microorganisms have been isolated that display strong oil degrading capacities at temperatures as high as 65°C (Becker & Markl, 2000). Furthermore, anaerobic reactors, which often treat grease-rich wastewater, may operate effectively over a temperature range of 30-60°C (Pavlostathis & Giraldo-Gomez, 1991).

Oxygen availability was also observed to have a significant impact on oil degradation rates with oxic conditions promoting a significantly greater extent of oil degradation than conditions of limited oxygen availability. Sediment studies have also shown that the degradation of fatty acids under aerobic conditions is greater than in an anaerobic environment (Sun *et al.*, 1993; Harvey *et al.*, 1995; Sun *et al.*, 1997). However, aerobic and anaerobic biological grease wastewater treatment process have been known achieve similarly high grease removal efficiencies, perhaps because anaerobic digesters are often heated.

The presence of the grease trap isolates and environmental contaminants in the samples also affected oil degradation rates. The performance of both powdered and liquid F33 was significantly hampered by the presence of the environmental isolates despite the fact that the environmental isolates were capable of significantly enhancing the degradation of oil. These results support the theory proposed by several authors that commercial inocula may fail do in nature what they successfully do in axenic culture due to competition with the indigenous microoganisms (Horsfall, 1979; Goldstein *et al.*, 1985; Stephenson & Stephenson, 1992.). It was also observed that the presence of the commercial inocula occasionally reduced the oil-degrading capacity of the environmental isolates, suggesting that bioaugmentation can be detrimental to grease control.

In contrast to the findings of Ratledge, (1994) and Tano-Debrah *et al.*, (1999), oil degradation in this work was not significantly affected by the starting pH of the media. One reason why pH may not have had a significant impact on fat/oil degradation was that the pF33 microorganisms were capable of changing the pH of the media. Indeed, several of the formulations investigated were able to alter the pH of the media during microbial growth (formulation 1,6 and 7) or oil degradation (F33). However, the exact mechanism by which this was achieved was unknown.

The microbial community compositions of the bioaugmentation products also influenced oil degradation rates. Efficient oil degradation only occurred when the supplements were comprised of certain microoganisms in particular ratios. Another requirement for efficient oil degradation was a diverse microbial community structure, which changed markedly with time. Unfortunately, the identities of the microorganisms responsible for oil degradation were not fully resolved. However, T-RFLP analysis indicated that in the case of pF33 incubated under ideal conditions, a *Lactobacillus* strain dominated over the first week of the experiment whilst *Bacillus* and *Paenibacilus* strains dominated over the remaining two weeks. By contrast, under less ideal environmental conditions, (i.e. lower temperatures, reduced oxygen levels, presence of the grease trap isolates) one of the aformentioned microbial groups dominated over the entire length of the incubation period. It was never established whether a specific group of the pF33 microoganisms was responsible for the degradation of the oil or whether several of the pF33 microorganisms degraded the oil in a sequential manner, as suggested by Pereira (1999).

The impact of chemical surfactants/emulsifiers on oil degradation rates was also investigated but results were inconclusive due to an overall lack of oil degradation in the samples. Surfactants/emulsifiers were shown to contribute considerably the COD of samples, however, indicating that the addition of surfactants to wastewater may result in an overall deterioration of water quality.

The effect of lipase on oil degradation rates was also inconclusive in the earlier degradation experiments. However, lipase additions significantly enhanced the performance of pF33 in later work, indicating that hydrolysis may have been a rate limiting step in the degradation process. In contrast, formulation 7, which exhibited low lipase activity, degraded rape oil to the same extent as a highly lipolytic formulation (formulation 6), implying that hydrolysis was not a rate limiting step in the degradation process. These findings are in agreement with the existing literature which has shown that hydrolysis may or may not be the rate limiting steps in the oil degradation process (Hsu *et al.*, 1983; Becker & Markl, 2000).

Fat/oil degradation pathways

Fat/oil that is incubated with a commercial microbial supplements may enter numerous biotic and abiotic degradation pathways (Figure 6.1). The first stage in the biotic fat/oil degradation process was hydrolysis, catalysed by lipase. Several of the commercial microbial inocula investigated were capable of producing lipase. However, their lipolytic activities varied widely. Some of the formulations hydrolysed triglycerides comprised of shorter chained acids (ie tributryn) more readily than those comprised of longer chained fatty acids (ie natural fats and oils) whilst other formulation appeared to hydrolyse these two groups of triglycerides at a similar rate. The lipase produced by some microbial supplements (liquid-F33) also appeared to be non-fatty acid specific whilst there was evidence to suggest that the lipase secreted by the environmental contaminants was specific for 18:3ω3 and 18:2ω6.

A number of the microbial supplements were also capable of mineralizing fat/oil which indicated that β-oxidation must have been taking place. The presence of new fatty acids such as 16:1ω? and 14:2ω? further implied that β-oxidation was occurring and that the fatty acids may not have been hydrogenated prior to the β-oxidation pathway. There was no evidence that the commercial microbial inocula were capable of α or ω-oxidation. However, mid-

chained hydroxlations/oxidations appeared to have taken place to yield 3-hydroxy, 10-hydroxy and 10-oxo fatty acids.

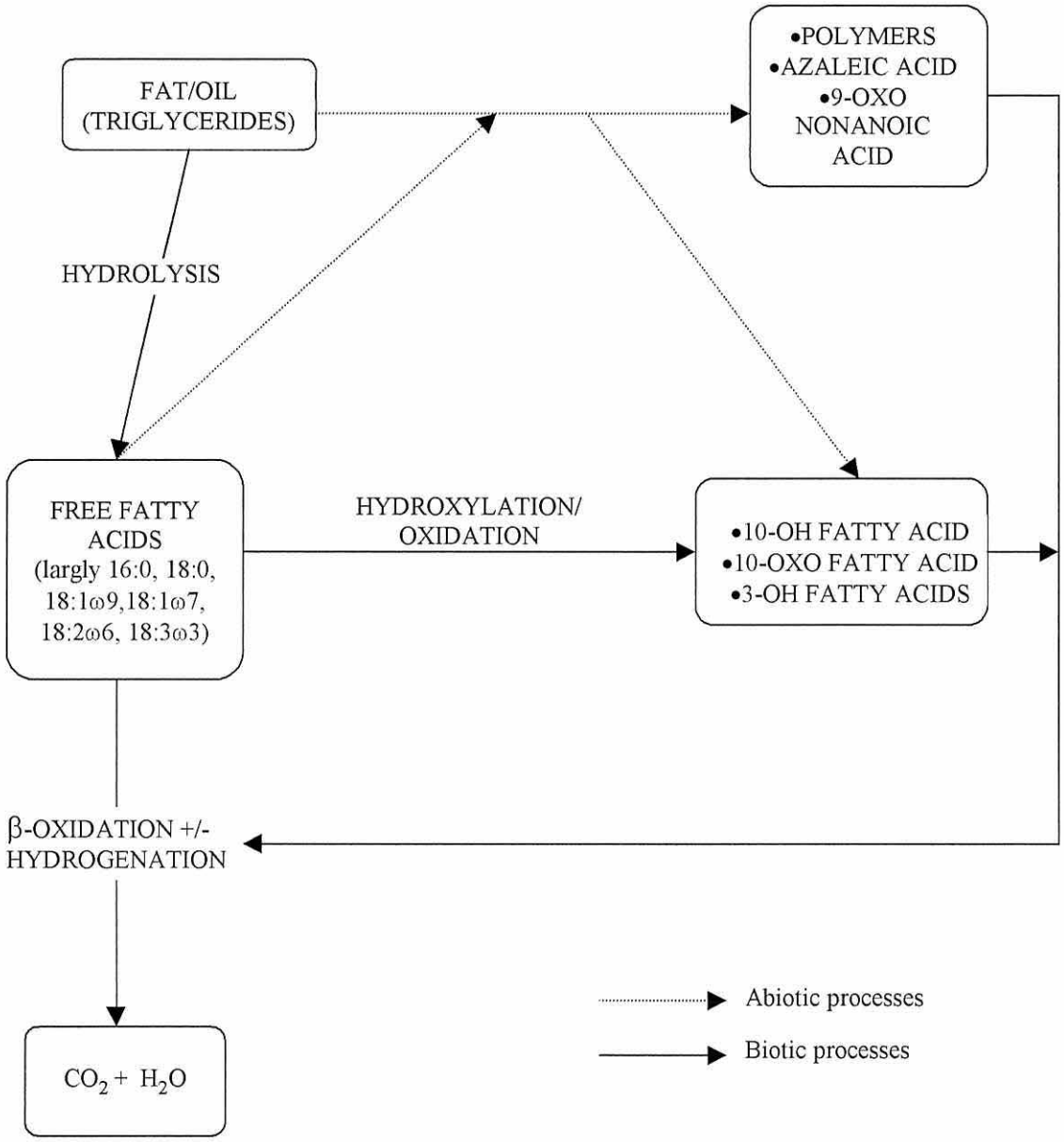


Figure 6.1. Schematic representation of possible pathways of degradation for fat/oil incubated with commercial microbial formulations

The major abiotic oil degradation pathway taking place was autoxidation. Autoxidation of the triglycerides and/or free fatty acids was thought to result in the formation of polymers, azaleic acid, 9-oxo nonanoic acid, 10-hydroxy, 10-oxo and possibly 3-hydroxy fatty acids. In later experiments, abiotic processes induced significant changes in the relative proportion of the major fatty acids whilst biological activity did not, indicating that autoxidation was an

important oil degradation pathway. Interestingly, autoxidation induced fatty acid compositional changes that were similar to those brought about by the environmental isolates in earlier work, suggesting that many of the fatty acid compositional changes observed in field studies (Parker & Leo, 1965; Loehr & Roth, 1968; Malaney & Gerhold, 1969; Farrington & Quinn, 1971; Sun & Wakeman, 1994; Pereira, 1999) may have been the result of autoxidation rather than biological activity.

Several factors such as temperature, oxygen availability, pH, microbial community composition, and nutrients appeared to influence the relative rates at which the various fat/oil degradation pathways were taking place. The importance of the autoxidation reaction appeared to increase with incubation temperature since an autoxidation product (azaleic acid), was detected at 50°C but not at 30°C or 10°C. A rise in the incubation temperature also resulted in significant decline in the proportion of 18:2ω6 coupled with an increase in 16:0 indicating that the rate of autoxidation or possibly hydrogenation and/or first few cycles of β-oxidation of 18:2ω6 may have been enhanced.

Under oxic conditions the decline in the proportion of 18:1ω9 was more rapid than under conditions of limited oxygen availbility suggesting that the rate of hydrogenation, hydroxylation or oxidation of 18:1ω9 was raised. In addition, a new 10-OH fatty acid was detected under oxic conditions, at pH 7 and 10 only indicating that the mid-chain hydroxylation reaction was only active under oxic conditions, at neutral to alkaline pH's.

In earlier degradation experiments, there was little evidence to suggest that the commercial microbial inocula were hydrogenating unsaturated fatty acids. However, new 18:2ω? and 18:3ω? isomers were detected in samples inoculated with environmental isolates, which were indicative of isomerisation, the first stage in the hydrogenation process. Furthermore, the particulate material produced by these microbial communities was highly enriched in saturated fatty acids (18:0 and 16:0) relative to the raw oil, which implied that the hydrogenation reaction and/or first few cycles of β-oxidation may have been predominating. By contrast, the presence of the environmental isolates did not appear to effect the relative proportions of the major fatty acids (and thus major degradation pathways) in later degradation experiments but did inhibit the mid-chain hydroxylation reactions.

It was also noted that certain nutrients (ie potassium nitrate, magnesium sulphate or di-sodium ortho phosphate) were essential for the production of 'particulate' material. These nutrients may have encourage the growth of bacteria responsible for the production of particulate material and thus enhanced reaction such as hydrogenation and/or the first few β-oxidation cycles of 18:2ω6 and 18:3ω3.

Summary and conclusions

In summary, the current work demonstrated that some commercial available inocula are capable of significantly enhancing fat/oil degradation under ideal laboratory conditions whilst others are not. The ability of the commercial supplements to enhance oil degradation in the field was not tested. However, the results of several laboratory-based experiments indicated that commercial microbial supplements have the potential to enhance fat/oil degradation in the field;

- Growth/oil degradation occurred over a wide range of environmental conditions
- Several different fats and oils were degraded.
- Protease and amylase, were produced in addition to lipase.
- The microorganisms were capable of adhering to plastic and/or lard surfaces
- The quantity of suspended and adhering particulate material produced by environmental contaminants was reduced.
- The combined microbial community (i.e. grease trap isolates + pF33) was more similar in composition to that of pF33 than the grease trap isolates.

By contrast, there was also evidence to suggest that some commercial inocula may fail to enhance fat/oil degradation in the field;

- Growth of the supplements on synthetic sewage media/oil degradation was poor in the absence of additional nutrients
- The ability of the supplements to degrade oil was significantly hampered by the presence of environmental isolates
- The quantity of pF33 used relative to the quantity of oil/nutrient media present was higher than would be used in the field

Clearly, further work is required to establish whether commercial microbial supplements are capable of degrading fat/oil in the field. Ideally, the performance of the microbial supplements should be examined at full scale rather than in the laboratory. However, if adequate control and test facilities are not available, this may not be feasible. Future laboratory-based investigations must also ensure that the non-steady state conditions found in the field are replicated and that commercial inocula are dosed as they would be in the field.

Whilst parameters such as COD, BOD and FOG (fats, oils and grease) often fail to illustrate the true impact of a bioaugmentation programme in the field, work developed here provides a novel adjunct to monitoring the performance of commercial supplements in field. GC-MS analysis may provide a means of tracking potential oil degradation markers such as 10-OH whilst the nucleic acid approach may be used to examine the survivability and progress of a commercial inocula in a wastewater system.

APPENDIX

I. Nutrient media compositions

	Media 1	Media 2	Media 3	Media 4
Yeast extract ($g.l^{-1}$)	2.000	0.200	0.100	0.000
Glucose ($g.l^{-1}$)	1.000	0.100	0.025	0.000
Potassium nitrate ($g.l^{-1}$)	1.000	1.000	1.000	1.000
Magnesium sulphate-7 hydrate ($g.l^{-1}$)	0.200	0.200	0.200	0.200
Di-sodium hydrogen othophophate ($g.l^{-1}$)	0.100	0.100	0.100	0.100
Calcium chloride-2-hydrate ($g.l^{-1}$)	0.010	0.010	0.010	0.010
Manganese sulphate-1-hydrate ($g.l^{-1}$)	0.010	0.010	0.010	0.010
Ferric ammonium citrate ($g.l^{-1}$)	0.005	0.005	0.005	0.005

II. Synthetic sewage media compositions

	Media 1	Media 2	Media 3
Potassium nitrate ($g.l^{-1}$)	0.000	1.000	1.000
Peptone ($g.l^{-1}$)	0.160	0.000	0.500
Urea ($g.l^{-1}$)	0.030	0.000	0.000
Sodium Chloride ($g.l^{-1}$)	0.007	0.007	0.007
Calcium Chloride ($g.l^{-1}$)	0.006	0.006	0.006
Magnesium sulphate-7 hydrate ($g.l^{-1}$)	0.056	0.056	0.056
Di-sodium hydrogen ortho phosphate ($g.l^{-1}$)	0.028	0.028	0.028

III. Loading factors derived from Principle Component Analysis

Experiment examining the effect of F33 and the contaminants on the relative proportion of fatty acids in soya oil (Section 3.2.5.)

	Glyceride fraction		Free fatty acid fraction	
Variable	PC1	PC2	PC1	PC2
14:0	0.189	0.528	0.266	0.125
16:0	0.284	-0.095	0.377	-0.141
18:0	0.278	-0.006	0.333	0.090
20:0	0.273	0.005	0.346	0.042

	Glyceride fraction		Free fatty acid fraction	
Variable	**PC1**	**PC2**	**PC1**	**PC2**
22:0	0.280	-0.040	0.283	0.114
17:0	0.190	0.285	0.251	0.119
15:0	0.228	0.310	0.352	0.200
keto	0.146	-0.556	0.106	-0.309
18:1ω9	0.285	-0.030	-0.090	-0.416
18:1ω7	0.163	-0.391	-0.149	-0.402
18:2ω6	-0.284	0.120	-0.284	0.297
18:2ω?	0.273	-0.126	-0.003	-0.175
18:3ω3	-0.285	0.047	-0.168	0.409
18:3ωx?	-0.276	-0.019	0.126	0.148
18:3ωy?	-0.244	-0.192	0.171	0.047
azaleic	0.255	-0.011	0.129	-0.332
unknown 1	-	-	-0.276	0.193

Experiment examining the effect of S45 and F33 on the relative proportions of fatty acids in soya oil inoculated with grease trap isolates (section 3.2.5.)

Fatty acid	**PC1**	**PC2**
14:0	0.124	-0.107
15:0	-0.099	-0.380
16:0	-0.212	0.214
17:0	-0.216	0.152
18:0	-0.233	0.042
20:0	-0.233	0.032
22:0	-0.240	-0.014
sat(306)	-0.235	0.106
sat(326)	-0.209	0.161
18:1ω9	-0.241	0.004
18:1ω7	-0.193	-0.220
keto	-0.204	0.149
mono(1413)	-0.179	0.111
mono(1419)	-0.175	0.060
18:2ω6	0.238	-0.075
18:2(804)	0.121	-0.155
18:2(824)	0.127	0.007
18:2(1093)	-0.238	-0.014
18:2(1046)	-0.126	-0.378
18:2(1052)	-0.099	-0.343
18:3ω3	0.241	0.011
18:3(967)	0.230	0.028
18:3(997)	0.215	-0.012
polyunsat(1328)	-0.122	-0.385
polyunsat(1342)	-0.106	-0.344
polyunsat(1354)	-0.095	-0.288
azaleic	-0.236	0.087

Experiments examining the effect of incubation temperature (temp), pH, oxygen availability (oxygen) and grease trap isolates (GI) on the relative proportion of fatty acids in rape oil incubated with pF33 (section 5.2.2.)

	Temp		pH		Oxygen		GI	
Fatty acid	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
sat(165)	0.225	0.062	0.222	-0.079	0.149	-0.167	0.158	0.016
sat(227)	0.164	0.010	0.175	-0.123	0.223	0.004	0.164	-0.077
sat (267)	0.197	0.191	0.183	-0.115	0.153	0.076	0.212	-0.072
16:0	0.225	-0.023	0.218	-0.159	0.256	0.066	0.200	-0.164
17:0?	0.216	0.077	0.177	-0.190	0.235	0.113	0.142	-0.260
18:0	0.223	0.024	0.169	-0.249	0.229	0.154	0.162	-0.270
18:1ω9	0.037	-0.432	0.191	0.208	-0.090	-0.271	0.120	0.269
18:1ω7	0.087	-0.345	0.182	-0.129	0.161	0.035	0.122	-0.045
20:0	0.226	0.058	0.101	-0.279	0.197	0.181	0.111	-0.293
22:0	0.209	-0.074	0.152	-0.280	0.198	0.176	0.086	-0.300
keto	0.153	-0.278	0.166	0.235	0.076	-0.292	0.235	0.180
azaleic	0.202	0.175	0.213	0.156	0.168	-0.220	0.251	0.145
sat (850)?	0.165	0.285	0.086	0.168	0.195	0.093	0.189	-0.102
sat (438)?	0.212	-0.038	0.162	0.249	0.097	-0.270	0.197	0.120
monunsat(476)	-0.155	0.121	-0.220	0.122	-0.172	0.103	-0.047	0.137
monunsat(832)	-0.127	0.107	-0.183	0.100	0.077	0.166	0.002	-0.205
monunsat(1368)	0.112	-0.263	0.190	-0.071	0.233	-0.083	0.255	0.025
monunsat(1628)	-0.061	0.359	-0.132	-0.139	-0.062	0.177	0.006	0.038
10-oxo	0.097	0.015	0.016	-0.234	0.180	0.188	0.043	-0.307
18:2ω6	-0.220	0.108	-0.245	-0.028	-0.259	0.005	-0.274	-0.003
18:2(1400)	0.213	0.134	0.211	0.153	0.131	-0.263	0.253	0.149
18:2(1307)	0.213	0.129	0.154	0.204	0.088	-0.261	0.238	0.161
18:2(1317)	0.198	0.162	0.185	0.218	0.088	-0.261	0.207	0.173
18:2(1326)	0.199	0.113	0.177	0.200	0.088	-0.261	0.090	0.029
18:2(1335)	0.209	0.138	0.185	0.228	0.088	-0.261	0.238	0.150
18:2(1349)	0.214	0.057	0.177	0.189	0.088	-0.261	0.178	0.167
18:3ω3	-0.210	0.155	-0.237	0.099	-0.268	0.009	-0.263	0.088
18:3(1181)	-0.204	0.162	-0.236	0.109	-0.261	-0.074	-0.255	0.086
18:3(1241)?	-0.100	0.060	-0.100	-0.008	0.229	0.019	0.007	-0.273
18:3(1245)?	-0.161	0.128	-0.230	0.128	-0.255	0.021	-0.189	0.118
10-OH?	0.034	-0.212	0.008	-0.270	0.177	0.207	0.034	-0.319

Experiments examining the effect of incubation temperature (temp), pH, oxygen availability (oxygen) and grease trap isolates (GI) on relative proportion of *Hha* I and *Msp* I digests of pF33 (section 5.2.3.)

Grease trap isolates

Variable	PC1	PC2
Hha(3') 57	-0.069	-0.061
Hha(3') 65	-0.094	-0.077
Hha(3') 142	0.161	0.079
Hha(3') 163	0.131	0.098
Hha(3') 227	0.100	-0.122
Hha(3') 262	-0.034	0.005
Hha(3') 298	-0.036	0.177
Hha(3') 345	-0.026	0.023
Hha(3') 347	-0.047	-0.035
Hha(3') 348	-0.046	0.069
Hha(5') 57	-0.092	-0.077
Hha(5') 65	-0.092	-0.076
Hha(5') 69	-0.036	0.177
Hha(5') 136	-0.036	0.177
Hha(5') 141	0.100	-0.122
Hha(5') 150	-0.036	0.177
Hha(5') 152	0.161	0.079
Hha(5') 163	0.179	0.060
Hha(5') 169	0.061	0.204
Hha(5') 173	0.161	0.079
Hha(5') 178	-0.034	0.005
Hha(5') 196	-0.036	0.177
Hha(5') 210	0.100	-0.122
Hha(5') 215	-0.054	-0.007
Hha(5') 222	0.100	-0.122
Hha(5') 224	0.161	0.079
Hha(5') 226	-0.036	0.177
Hha(5') 336	-0.074	-0.055
Hha(5') 239	-0.036	0.177
Hha(5') 240	0.201	-0.018
Hha(5') 242	-0.052	0.153
Hha(5') 250	0.161	0.079
Hha(5') 261	-0.034	0.005
Hha(5') 337	-0.041	-0.043
Hha(5') 338	-0.047	-0.035
Hha(5') 382	-0.036	0.177
Hha(5') 400	-0.034	0.005
Msp(3') 56	0.100	-0.122
Msp(3') 58	0.100	-0.122
Msp(3') 62	-0.040	0.034
Msp(3') 68	-0.029	-0.015

Oxygen availability

Variable	PC1	PC2
Hha(3') 57	-0.038	-0.012
Hha(3') 65	-0.048	-0.010
Hha(3') 116	-0.083	-0.046
Hha(3') 142	0.140	-0.127
Hha(3') 148	-0.083	-0.046
Hha(3') 154	-0.083	-0.046
Hha(3') 161	-0.083	-0.046
Hha(3') 163	0.168	0.042
Hha(3') 172	-0.083	-0.046
Hha(3') 195	-0.083	-0.046
Hha(3') 227	0.067	0.193
Hha(3') 298	-0.106	-0.046
Hha(3') 308	-0.106	-0.048
Hha(3') 341	-0.048	-0.010
Hha(3') 345	-0.147	-0.062
Hha(5') 69	-0.104	-0.044
Hha(5') 83	-0.048	-0.010
Hha(5') 141	0.067	0.193
Hha(5') 144	-0.048	-0.010
Hha(5') 152	0.140	-0.127
Hha(5') 163	0.154	-0.097
Hha(5') 169	0.140	-0.127
Hha(5') 173	0.140	-0.127
Hha(5') 210	0.067	0.193
Hha(5') 215	-0.156	-0.048
Hha(5') 222	0.067	0.193
Hha(5') 224	0.140	-0.127
Hha(5') 240	0.168	0.029
Hha(5') 250	0.140	-0.127
Msp(3') 56	0.067	0.193
Msp(3') 58	0.067	0.193
Msp(3') 62	-0.048	-0.010
Msp(3') 65	-0.083	-0.046
Msp(3') 68	-0.142	-0.058
Msp(3') 70	-0.058	-0.017
Msp(3') 73	-0.083	-0.046
Msp(3') 84	0.067	0.193
Msp(3') 123	0.067	0.193
Msp(3') 124	0.067	0.193
Msp(3') 125	-0.083	-0.046
Msp(3') 129	0.067	0.193

Grease trap isolates

Variable	PC1	PC2
Msp(3') 70	-0.034	0.005
Msp(3') 84	0.100	-0.122
Msp(3') 123	0.100	-0.122
Msp(3') 124	0.100	-0.122
Msp(3') 129	0.100	-0.122
Msp(3') 139	0.161	0.079
Msp(3') 143	0.161	0.079
Msp(3') 145	0.161	0.079
Msp(3') 257	-0.041	-0.043
Msp(3') 291	-0.050	-0.038
Msp(3') 294	-0.091	-0.077
Msp(3') 295	0.101	0.176
Msp(3') 298	-0.040	0.000
Msp(3') 302	-0.029	-0.015
Msp(3') 308	0.110	-0.091
Msp(3') 317	0.200	-0.023
Msp(3') 321	0.161	0.079
Msp(3') 364	-0.036	0.177
Msp(3') 365	0.148	-0.046
Msp(3') 367	-0.033	-0.006
Msp(3') 395	-0.041	-0.043
Msp(3') 415	-0.053	-0.040
Msp(3') 416	0.154	-0.039
Msp(3') 448	-0.050	-0.038
Msp(5') 69	-0.049	-0.002
Msp(5') 116	0.100	-0.122
Msp(5') 118	-0.027	0.182
Msp(5') 125	-0.036	0.177
Msp(5') 134	0.198	0.023
Msp(5') 135	-0.034	0.005
Msp(5') 137	-0.036	0.177
Msp(5') 142	0.100	-0.122
Msp(5') 144	-0.017	-0.035
Msp(5') 147	0.190	0.062
Msp(5') 148	-0.034	0.005
Msp(5') 150	0.007	0.198
Msp(5') 151	0.100	-0.122
Msp(5') 152	-0.036	0.177
Msp(5') 154	-0.029	-0.015
Msp(5') 166	0.184	0.052
Msp(5') 169	-0.036	0.177
Msp(5') 279	0.100	-0.122
Msp(5') 290	0.161	0.079
Msp(5') 295	-0.041	-0.043
Msp(5') 297	0.100	-0.122
Msp(5') 354	0.161	0.079
Msp(5') 361	0.161	0.079
Msp(5') 365	0.202	-0.011

Oxygen availability

Variable	PC1	PC2
Msp(3') 139	0.140	-0.127
Msp(3') 143	0.140	-0.127
Msp(3') 145	0.140	-0.127
Msp(3') 260	-0.083	-0.046
Msp(3') 294	-0.057	-0.002
Msp(3') 295	0.165	-0.062
Msp(3') 298	-0.161	-0.055
Msp(3') 302	-0.038	0.002
Msp(3') 308	-0.049	0.109
Msp(3') 317	0.167	0.037
Msp(3') 321	0.140	-0.127
Msp(3') 365	0.153	0.088
Msp(3') 367	-0.123	-0.029
Msp(3') 416	0.169	0.019
Msp(5') 62	-0.088	-0.041
Msp(5') 69	-0.165	-0.056
Msp(5') 116	0.067	0.193
Msp(5') 118	0.140	-0.127
Msp(5') 125	-0.083	-0.046
Msp(5') 134	0.169	-0.039
Msp(5') 142	0.067	0.193
Msp(5') 144	-0.027	0.162
Msp(5') 147	0.164	-0.064
Msp(5') 150	0.140	-0.127
Msp(5') 151	0.067	0.193
Msp(5') 154	-0.092	-0.015
Msp(5') 166	0.146	-0.091
Msp(5') 279	0.067	0.193
Msp(5') 290	0.140	-0.127
Msp(5') 297	0.067	0.193
Msp(5') 298	-0.097	-0.049
Msp(5') 308	-0.083	-0.046
Msp(5') 354	0.140	-0.127
Msp(5') 361	0.140	-0.127
Msp(5') 365	0.170	0.017
Msp(5') 370	0.140	-0.127
Msp(5') 373	0.140	-0.127
Msp(5') 472	0.067	0.193

Grease trap isolates

Variable	PC1	PC2
Msp(5') 370	0.161	0.079
Msp(5') 373	0.161	0.079
Msp(5') 389	-0.050	-0.038
Msp(5') 394	-0.073	-0.054
Msp(5') 395	-0.041	-0.043
Msp(5') 396	-0.047	-0.035
Msp(5') 416	-0.064	-0.059
Msp(5') 448	-0.050	-0.038
Msp(5') 472	0.100	-0.122

Starting pH

Variable	PC1	PC2
Hha(3') 57	-0.031	0.010
Hha(3') 65	-0.038	0.047
Hha(3') 115	0.207	-0.044
Hha(3') 142	0.207	-0.044
Hha(3') 148	0.207	-0.044
Hha(3') 163	0.070	0.164
Hha(3') 215	-0.075	-0.162
Hha(3') 298	-0.051	-0.096
Hha(3') 301	-0.075	-0.161
Hha(3') 308	-0.019	-0.025
Hha(3') 345	-0.020	-0.172
Hha(5') 57	-0.036	0.043
Hha(5') 65	-0.031	0.010
Hha(5') 69	-0.019	-0.025
Hha(5') 163	0.052	0.136
Hha(5') 178	-0.018	0.109
Hha(5') 191	-0.075	-0.161
Hha(5') 196	-0.025	0.154
Hha(5') 215	-0.053	-0.150
Hha(5') 222	-0.020	0.127
Hha(5') 226	-0.020	0.120
Hha(5') 231	-0.015	0.096
Hha(5') 238	-0.021	0.123
Hha(5') 240	0.152	0.008
Hha(5') 242	0.207	-0.027
Hha(5') 294	-0.015	0.096
Hha(5') 301	-0.075	-0.161
Hha(5') 329	-0.075	-0.161
Hha(5') 335	-0.031	0.010
Hha(5') 337	-0.075	-0.161
Hha(5') 340	-0.038	0.048
Hha(5') 344	-0.078	-0.168
Hha(5') 350	-0.075	-0.161
Hha(5') 382	0.207	-0.044
Msp(3') 60	-0.031	0.010

Incubation temperature

Variable	PC1	PC2
Hha(3') 57	-0.201	0.013
Hha(3') 65	0.040	0.004
Hha(3') 142	-0.201	0.013
Hha(3') 163	-0.039	-0.251
Hha(3') 298	0.039	0.320
Hha(3') 345	0.014	0.320
Hha(3') 347	-0.201	0.013
Hha(3') 489	0.013	-0.047
Hha(5') 57	0.036	-0.162
Hha(5') 69	0.039	0.320
Hha(5') 163	-0.201	0.013
Hha(5') 169	-0.201	0.013
Hha(5') 173	-0.201	0.013
Hha(5') 196	-0.201	0.013
Hha(5') 215	0.038	0.320
Hha(5') 222	-0.201	0.013
Hha(5') 226	-0.201	0.013
Hha(5') 238	-0.114	-0.033
Hha(5') 242	-0.201	0.013
Hha(5') 367	0.027	-0.023
Hha(5') 489	0.013	-0.047
Msp(3') 62	0.039	0.320
Msp(3') 143	-0.201	0.013
Msp(3') 294	0.041	0.004
Msp(3') 295	-0.201	0.013
Msp(3') 298	0.035	0.319
Msp(3') 308	-0.201	0.013
Msp(3') 317	-0.201	0.013
Msp(3') 365	-0.092	-0.037
Msp(3') 367	0.064	-0.180
Msp(3') 368	-0.201	0.013
Msp(5') 69	0.038	0.320
Msp(5') 116	-0.201	0.013
Msp(5') 118	-0.201	0.013
Msp(5') 135	-0.201	0.013

Starting pH

Variable	PC1	PC2
Msp(3') 65	-0.021	-0.057
Msp(3') 68	-0.021	-0.057
Msp(3') 69	-0.075	-0.161
Msp(3') 70	0.123	-0.077
Msp(3') 72	-0.021	-0.057
Msp(3') 73	-0.021	-0.057
Msp(3') 81	0.207	-0.044
Msp(3') 84	0.207	-0.044
Msp(3') 102	0.207	-0.044
Msp(3') 106	0.207	-0.044
Msp(3') 110	0.207	-0.044
Msp(3') 116	0.207	-0.044
Msp(3') 122	0.207	-0.044
Msp(3') 124	0.207	-0.044
Msp(3') 136	0.207	-0.044
Msp(3') 138	0.207	-0.044
Msp(3') 203	-0.015	0.096
Msp(3') 260	-0.081	-0.179
Msp(3') 294	-0.038	0.048
Msp(3') 295	-0.024	0.147
Msp(3') 298	0.077	-0.172
Msp(3') 308	-0.074	-0.091
Msp(3') 317	-0.009	0.046
Msp(3') 362	-0.018	0.109
Msp(3') 365	-0.024	0.141
Msp(3') 367	-0.050	-0.086
Msp(3') 395	-0.031	0.010
Msp(3') 398	-0.031	0.010
Msp(5') 60	-0.031	0.010
Msp(5') 62	-0.076	-0.172
Msp(5') 69	-0.048	-0.133
Msp(5') 74	-0.018	0.109
Msp(5') 83	-0.021	-0.057
Msp(5') 116	-0.022	0.135
Msp(5') 118	-0.018	0.109
Msp(5') 120	-0.019	0.025
Msp(5') 125	-0.015	0.096
Msp(5') 134	0.207	-0.044
Msp(5') 135	-0.026	0.154
Msp(5') 137	0.207	-0.044
Msp(5') 144	-0.046	0.059
Msp(5') 146	-0.012	0.069
Msp(5') 148	-0.015	0.096
Msp(5') 150	-0.023	0.136
Msp(5') 154	-0.024	0.091
Msp(5') 157	-0.015	0.096
Msp(5') 166	0.199	-0.029
Msp(5') 169	0.207	-0.044

Incubation temperature

Variable	PC1	PC2
Msp(5') 137	-0.201	0.013
Msp(5') 144	-0.024	-0.210
Msp(5') 148	-0.201	0.013
Msp(5') 150	-0.201	0.013
Msp(5') 152	-0.164	-0.019
Msp(5') 154	0.036	-0.162
Msp(5') 166	0.036	-0.162
Msp(5') 173	0.036	-0.162
Msp(5') 290	-0.201	0.013
Msp(5') 365	-0.201	0.013
Msp(5') 396	0.036	-0.162

	Starting pH	
Variable	**PC1**	**PC2**
Msp(5') 173	-0.009	0.046
Msp(5') 282	-0.075	-0.161
Msp(5') 290	-0.018	0.109
Msp(5') 292	-0.075	-0.161
Msp(5') 295	-0.022	0.137
Msp(5') 298	-0.077	-0.168
Msp(5') 304	-0.075	-0.161
Msp(5') 310	-0.075	-0.161
Msp(5') 318	-0.009	0.046
Msp(5') 394	-0.022	0.047
Msp(5') 395	-0.031	0.010
Msp(5') 398	-0.038	0.051
Msp(5') 416	-0.031	0.010
Msp(5') 420	-0.031	0.010

IV. Size and relative proportions fragments generated using *Rsa* I, *Hha* I and *Msp* I

Rsa I (3')	58	120	283	344	357	434	435	438	453	490	491
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
pH4 T=2	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
pH4 T=3	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
pH7 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=2	25.2	0.0	0.0	0.0	0.0	0.0	0.0	74.8	0.0	0.0	0.0
pH7 T=3	0.0	0.0	0.0	2.3	0.0	0.0	72.8	0.0	0.0	10.1	14.9
pH10 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
pH10 T=2	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
pH10 T=3	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
10°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	94.3	0.0	0.0	5.7
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
30°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
30°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	66.4	15.6	17.9	0.0	0.0
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	17.8	0.0	0.0	82.2	0.0	0.0	0.0
F33+GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	82.8	17.2	0.0	0.0	0.0
F33+GI T=3	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0

***Rsa* I (3')**	**58**	**120**	**283**	**344**	**357**	**434**	**435**	**438**	**453**	**490**	**491**
F33oxic T=2	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
F33oxic T=3	0.0	0.0	10.1	0.0	0.0	0.0	89.9	0.0	0.0	0.0	0.0
F33limO T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
F33limO T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0

***Rsa* I (5')**	**55**	**71**	**90**	**100**	**104**	**105**	**120**	**306**	**344**	**356**	**437**	**440**	**445**	**452**
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.2	40.7	0.0	23.3
pH4 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.8	0.0	0.0	2.9
pH7 T=1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=2	77.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=3	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.5	0.0	0.0	0.0
pH10 T=1	0.0	0.0	0.0	42.6	0.0	50.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=2	0.0	0.0	0.0	18.1	0.0	36.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=3	0.0	0.0	0.0	39.8	0.0	60.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.5	10.6	0.0
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	46.2	0.0	0.0	0.0	0.0	53.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=2	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=3	0.0	0.0	0.0	56.9	43.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

***Rsa* I (5')**	**55**	**71**	**90**	**100**	**104**	**105**	**120**	**306**	**344**	**356**	**437**	**440**	**445**	**452**
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
F33+GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	23.1	0.0	0.0
F33oxic T=1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=2	12.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	87.6	0.0	0.0
F33oxic T=3	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=3	36.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

***Rsa* I (5')**	**453**	**461**	**469**	**474**	**480**	**488**	**490**	**492**	**493**
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0
pH4 T=2	0.0	3.5	0.0	9.3	0.0	12.1	0.0	0.0	2.9
pH4 T=3	0.0	0.0	69.6	0.0	11.9	0.0	0.0	5.8	0.0
pH7 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	22.5	0.0
pH7 T=3	0.0	0.0	0.0	0.0	0.0	0.0	98.2	0.0	0.0
pH10 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.8	0.0
pH10 T=2	0.0	0.0	0.0	0.0	0.0	0.0	45.3	0.0	0.0
pH10 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0
10°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0
30°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=2	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=3	68.4	0.0	0.0	5.5	0.0	7.1	0.0	1.9	0.0
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

***Rsa* I (5')**	**453**	**461**	**469**	**474**	**480**	**488**	**490**	**492**	**493**
50°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=3	0.0	0.0	0.0	2.9	0.0	0.0	74.0	0.0	0.0
F33oxic T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0
F33limO T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	63.4	0.0

***Hha* I (3')**	**57**	**65**	**115**	**116**	**142**	**148**	**154**	**161**	**163**	**172**	**195**	**215**	**227**	**262**	**298**	**301**	**308**	**341**
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.9	0.0	0.0	0.0	0.0	0.0	17.4	0.0	4.8	0.0
pH4 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	96.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	91.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0	0.0	25.1	0.0	0.0	6.6	1.4	0.0	0.0
pH7 T=2	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	7.3	0.0	0.0	0.3	0.0	0.0	3.5	0.0	0.0	0.0
pH7 T=3	0.0	0.0	0.7	0.0	0.7	0.8	0.0	0.0	61.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=1	0.9	51.8	0.0	0.0	0.0	0.0	0.0	0.0	3.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=2	0.0	39.1	0.0	0.0	0.0	0.0	0.0	0.0	60.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=3	0.0	82.3	0.0	0.0	0.0	0.0	0.0	0.0	17.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	7.8	0.0	0.0	0.0	0.0	0.0	0.0	92.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=2	0.0	4.7	0.0	0.0	0.0	0.0	0.0	0.0	92.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=3	0.0	8.8	0.0	0.0	0.0	0.0	0.0	0.0	91.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Hha I (3')	57	65	115	116	142	148	154	161	163	172	195	215	227	262	298	301	308	341
30°C T=1	0.0	3.2	0.0	0.0	0.0	0.0	0.0	0.0	2.3	0.0	0.0	0.0	0.0	0.0	6.9	0.0	0.0	0.0
30°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	88.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=3	1.1	0.0	0.0	0.0	1.6	0.0	0.0	0.0	84.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	2.4	94.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=2	2.1	93.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=3	0.0	94.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	58.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=2	0.0	3.1	0.0	0.0	0.0	0.0	0.0	0.0	51.5	0.0	0.0	0.0	0.0	1.4	0.0	0.0	0.0	0.0
F33+GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	74.7	0.0	0.0	0.0	0.0	0.0	3.5	0.0	0.0	0.0
F33oxic T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=2	0.0	0.0	0.0	0.0	3.3	0.0	0.0	0.0	78.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	71.7	0.0	0.0	0.0	19.8	0.0	0.0	0.0	0.0	0.0
F33limO T=1	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=2	0.0	0.0	0.0	0.7	0.0	1.0	0.5	0.6	1.8	1.1	0.7	0.0	0.0	0.0	18.2	0.0	3.9	0.0
F33limO T=3	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	9.8	0.0	0.0	0.0	0.0	0.0	15.4	0.0	2.8	0.8

Hha I (3')	343	345	347	348	489
pH4 T=1	0.0	62.8	0.0	0.0	0.0
pH4 T=2	0.0	3.7	0.0	0.0	0.0
pH4 T=3	0.0	8.9	0.0	0.0	0.0
pH7 T=1	0.0	65.5	0.0	0.0	0.0
pH7 T=2	0.0	87.7	0.0	0.0	0.0
pH7 T=3	0.0	36.3	0.0	0.0	0.0
pH10 T=1	0.0	43.9	0.0	0.0	0.0
pH10 T=2	0.0	0.0	0.0	0.0	0.0

***Hha* I (3')**	**343**	**345**	**347**	**348**	**489**
pH10 T=3	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0
10°C T=2	0.0	2.7	0.0	0.0	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0
30°C T=1	0.0	87.7	0.0	0.0	0.0
30°C T=2	0.0	10.0	0.0	0.0	1.5
30°C T=3	0.0	12.0	0.7	0.0	0.0
50°C T=1	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	0.0	0.0	0.0	0.0
GI T=1	0.0	3.5	0.0	0.0	0.0
GI T=2	0.0	4.8	0.0	0.0	0.0
GI T=3	0.0	0.0	5.4	0.0	0.0
F33+GI T=1	0.0	41.4	0.0	0.0	0.0
F33+GI T=2	0.0	38.1	0.0	5.9	0.0
F33+GI T=3	0.0	19.6	0.0	2.2	0.0
F33oxic T=1	0.0	90.2	0.0	0.0	0.0
F33oxic T=2	0.0	18.0	0.0	0.0	0.0
F33oxic T=3	0.0	8.5	0.0	0.0	0.0
F33limO T=1	0.0	96.9	0.0	0.0	0.0
F33limO T=2	0.0	71.4	0.0	0.0	0.0
F33limO T=3	0.0	70.0	0.0	0.0	0.0

***Hha* I (5')**	**57**	**65**	**69**	**83**	**90**	**136**	**141**	**144**	**150**	**152**	**163**	**169**	**173**	**178**	**191**	**196**	**210**	**215**
pH4 T=1	0.0	0.0	23.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	76.1
pH4 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	0.0	0.0	1.1	0.0	6.5	0.0	0.8
pH4 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.7	0.0	0.0	0.0	0.0	4.8	0.0	4.5
pH7 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	58.7

***Hha* I (5')**	**57**	**65**	**69**	**83**	**90**	**136**	**141**	**144**	**150**	**152**	**163**	**169**	**173**	**178**	**191**	**196**	**210**	**215**
pH7 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	96.6
pH7 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.0	0.0	0.0	14.6
pH10 T=1	16.6	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	30.7
pH10 T=2	10.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=3	32.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=2	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=1	0.0	0.0	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	92.9
30°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5
30°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	0.4	1.4	0.0	0.0	2.2	0.0	0.9
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	75.4	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.6
GI T=2	73.1	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1
GI T=3	70.8	3.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
F33+GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.3	0.0	0.0	0.0	47.5
F33+GI T=3	0.0	0.0	2.2	0.0	0.0	8.5	0.0	0.0	6.0	0.0	0.0	2.0	0.0	0.0	0.0	6.6	0.0	9.0
F33oxic T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
F33oxic T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	10.0	1.3	1.4	0.0	0.0	0.0	0.0	2.5
F33oxic T=3	0.0	0.0	0.0	0.0	0.0	0.0	23.8	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	5.3	2.6
F33limO T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
F33limO T=2	0.0	0.0	18.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	81.3
F33limO T=3	0.0	0.0	18.2	1.0	0.0	0.0	0.0	2.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	78.3

***Hha* I (5')**	**222**	**224**	**226**	**231**	**336**	**238**	**239**	**240**	**242**	**250**	**261**	**294**	**301**	**329**	**335**	**337**	**338**	**340**
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=2	19.0	0.0	33.5	0.0	0.0	32.5	0.0	0.0	3.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=3	64.6	0.0	0.0	12.4	0.0	4.6	0.0	4.0	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.6	0.0	2.3	0.0	0.0
pH7 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	43.7	36.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.6	0.0	0.0	41.7
pH10 T=2	0.0	0.0	7.9	0.0	0.0	0.0	0.0	42.7	5.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	33.7
pH10 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	67.5
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=2	0.0	0.0	0.0	0.0	0.0	92.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=3	3.1	0.0	21.2	0.0	0.0	66.4	0.0	0.0	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	19.5	0.0	0.0
GI T=2	0.0	0.0	0.0	0.0	21.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=3	0.0	0.0	0.0	0.0	18.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.0	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	31.2	0.0	5.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=3	0.0	0.0	14.5	0.0	0.0	0.0	7.4	0.0	40.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=2	0.0	7.0	0.0	0.0	0.0	0.0	0.0	70.8	0.0	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=3	8.0	0.0	0.0	0.0	0.0	0.0	0.0	58.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Hha I (5')	222	224	226	231	336	238	239	240	242	250	261	294	301	329	335	337	338	340
F33limO T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Hha I (5')	344	350	367	382	400	489
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=2	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=3	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=1	33.6	3.8	0.0	0.0	0.0	0.0
pH7 T=2	3.4	0.0	0.0	0.0	0.0	0.0
pH7 T=3	0.0	0.0	0.0	3.5	0.0	0.0
pH10 T=1	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=2	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=3	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=2	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=1	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=2	0.0	0.0	0.0	0.0	0.0	1.8
30°C T=3	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=1	0.0	0.0	100.0	0.0	0.0	0.0
50°C T=2	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	0.0	0.0	0.0	0.0	0.0	0.0
GI T=2	0.0	0.0	0.0	0.0	0.0	0.0
GI T=3	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=2	0.0	0.0	0.0	0.0	4.1	0.0
F33+GI T=3	0.0	0.0	0.0	2.9	0.0	0.0

***Hha* I (5')**	**344**	**350**	**367**	**382**	**400**	**489**
F33oxic T=1	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=2	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=3	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=1	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=2	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=3	0.0	0.0	0.0	0.0	0.0	0.0

***Msp* I (3')**	**56**	**58**	**60**	**62**	**65**	**68**	**69**	**70**	**72**	**73**	**75**	**81**	**84**	**102**	**106**	**110**	**116**	**122**
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=1	0.0	0.0	0.0	0.0	0.0	0.0	16.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=2	0.0	0.0	0.0	0.0	0.5	2.7	0.0	2.3	1.3	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0	2.1	1.6	1.4	1.1	1.6	1.2	1.3
pH10 T=1	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=1	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Msp I (3')	56	58	60	62	65	68	69	70	72	73	75	81	84	102	106	110	116	122
GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=2	0.0	0.0	0.0	3.9	0.0	0.0	0.0	3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=3	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=1	0.0	0.0	0.0	0.0	0.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=3	1.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0
F33limO T=1	0.0	0.0	0.0	0.0	0.0	1.6	0.0	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=2	0.0	0.0	0.0	0.0	1.2	3.4	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=3	0.0	0.0	0.0	0.4	0.0	0.9	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Msp I (3')	123	124	125	129	136	138	139	143	145	202	203	257	260	291	294	295	298	302
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	77.1	0.0
pH4 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	48.1	0.9	0.0
pH4 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	78.9	5.3	0.0
pH7 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.8	0.0	0.0	0.0	62.1	0.0
pH7 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.5	0.0	1.0	0.0	67.0	0.0
pH7 T=3	0.0	1.5	0.0	0.0	3.4	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	81.1	0.0
pH10 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	62.5	0.0	31.1	0.0
pH10 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	50.3	0.0	0.0	0.0
pH10 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.9	0.0	0.0	0.0
10°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.0	0.0	1.4	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.2	0.0	0.0	0.0
30°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	0.0	89.6	0.0
30°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.8	0.0
30°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	19.6	2.5	0.0

Msp I (3')	123	124	125	129	136	138	139	143	145	202	203	257	260	291	294	295	298	302
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.0	0.0	91.2	0.0	4.2	0.0
GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	89.1	0.0	3.7	0.0
GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	58.6	0.0	1.8	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	31.6	0.0
F33+GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	31.3	0.0
F33+GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	37.8	10.1	0.0
F33oxic T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4	0.0	76.4	0.9
F33oxic T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.6	1.5	0.7	0.0	0.0	0.0	0.0	0.0	0.0	29.8	7.9	0.0
F33oxic T=3	1.1	1.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0	6.1	0.0
F33limO T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	89.3	0.0
F33limO T=2	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.7	0.0	0.0	0.0	79.1	0.0
F33limO T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4	0.0	79.7	0.0

Msp I (3')	308	317	321	362	364	365	367	368	395	398	415	416	448
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0	22.9	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=2	1.6	0.0	0.0	0.7	0.0	48.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=3	0.0	0.0	0.0	0.0	0.0	14.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=1	2.0	0.0	0.0	0.0	0.0	0.0	4.7	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=2	2.5	0.0	0.0	0.0	0.0	0.0	15.1	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=1	0.0	0.0	0.0	0.0	0.0	0.0	4.5	0.0	0.7	0.7	0.0	0.0	0.0
pH10 T=2	0.0	0.9	0.0	0.0	0.0	48.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	92.1	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	92.6	0.0	0.0	0.0	0.0	0.0	0.0

Msp I (3')	308	317	321	362	364	365	367	368	395	398	415	416	448
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	89.8	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	4.1	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=2	0.0	0.0	0.0	0.0	0.0	94.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=3	3.0	1.4	0.0	0.0	0.0	52.9	0.0	19.7	0.0	0.0	0.0	0.0	0.0
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0	0.9	0.0
GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5	0.0	1.0
GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	39.5	0.0	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	68.4	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=2	0.0	0.0	0.0	0.0	0.0	61.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=3	0.0	0.0	0.0	0.0	49.1	0.0	2.4	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=1	1.6	0.0	0.0	0.0	0.0	0.0	16.2	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=2	0.9	1.2	0.9	0.0	0.0	55.3	0.0	0.0	0.0	0.0	0.0	1.1	0.0
F33oxic T=3	1.7	1.0	0.0	0.0	0.0	74.4	0.0	0.0	0.0	0.0	0.0	0.8	0.0
F33limO T=1	0.7	0.0	0.0	0.0	0.0	0.0	6.6	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=2	1.8	0.0	0.0	0.0	0.0	0.0	6.5	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=3	1.3	0.0	0.0	0.0	0.0	0.0	15.6	0.0	0.0	0.0	0.0	0.0	0.0

Msp I (5')	60	62	69	74	75	83	116	118	120	125	134	135	137	142	144	146	147	148
pH4 T=1	0.0	0.0	79.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	20.2	0.0	0.0	0.0
pH4 T=2	0.0	0.0	0.0	0.8	0.0	0.0	18.4	10.0	0.0	0.0	0.0	4.9	0.0	0.0	17.0	4.9	0.0	0.0
pH4 T=3	0.0	0.0	3.2	0.0	0.0	0.0	47.8	0.0	0.0	7.9	0.0	3.2	0.0	0.0	5.1	0.0	0.0	16.1
pH7 T=1	0.0	0.8	43.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0
pH7 T=2	0.0	0.6	85.7	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.7	0.0	0.0	0.0
pH7 T=3	0.0	0.0	10.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	40.2	0.0	38.6	0.0	0.0	0.0	0.0	0.0
pH10 T=1	0.5	0.0	18.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	0.0	0.0	0.0

Msp I (5')	60	62	69	74	75	83	116	118	120	125	134	135	137	142	144	146	147	148
pH10 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.7	0.0	0.0	0.0	23.8	0.0	0.0
pH10 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
10°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	79.4	0.0	0.0	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
30°C T=1	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=2	0.0	0.0	4.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	89.4	0.0	0.0	0.0
30°C T=3	0.0	0.0	0.7	0.0	0.0	0.0	3.9	3.1	0.0	0.0	0.0	1.6	1.3	0.0	62.9	0.0	0.0	0.9
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=1	0.0	0.0	32.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	67.8	0.0	0.0	0.0
F33+GI T=2	0.0	0.0	33.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	33.8	0.0	0.0	20.8	0.0	0.0	11.6
F33+GI T=3	0.0	0.0	6.6	0.0	0.0	0.0	0.0	7.7	0.0	1.5	0.0	0.0	43.7	0.0	3.6	0.0	5.0	0.0
F33oxic T=1	0.0	0.0	79.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	17.0	0.0	0.0	0.0
F33oxic T=2	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	36.8	0.0	0.0	0.0	0.0	0.0	38.1	0.0
F33oxic T=3	0.0	0.0	1.2	0.0	0.0	0.0	3.4	0.0	0.0	0.0	16.6	0.0	0.0	18.5	18.2	0.0	12.6	0.0
F33limO T=1	0.0	1.4	89.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.8	0.0	0.0	0.0
F33limO T=2	0.0	1.0	91.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0
F33limO T=3	0.0	0.0	77.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.4	0.0	0.0	0.0

Msp I (5')	150	151	152	154	157	166	169	173	199	279	282	290	292	295	297	298	304	308
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=2	34.3	0.0	0.0	4.9	0.0	1.7	0.0	0.0	0.0	0.0	0.0	1.7	0.0	1.5	0.0	0.0	0.0	0.0
pH4 T=3	10.3	0.0	0.0	0.0	2.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.5	0.0	0.0	0.0	0.0

Msp I (5')	150	151	152	154	157	166	169	173	199	279	282	290	292	295	297	298	304	308
pH7 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	6.0	0.0	0.0	32.6	4.7	0.0
pH7 T=2	0.0	0.0	0.0	1.4	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0
pH7 T=3	0.0	0.0	0.0	0.0	0.0	6.7	4.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=2	0.0	0.0	0.0	9.0	0.0	3.8	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=2	0.0	0.0	6.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=3	10.3	0.0	8.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.2	0.0	0.0	0.0	0.0
GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=3	26.5	0.0	1.7	0.0	0.0	0.0	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=1	0.0	0.0	0.0	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=2	7.1	0.0	0.0	0.0	0.0	6.1	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=3	0.0	4.9	0.0	0.0	0.0	1.4	0.0	0.0	0.0	18.2	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0
F33limO T=1	0.0	0.0	0.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.3	0.0	1.0
F33limO T=3	0.0	0.0	0.0	3.7	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0

Msp I (5')	310	318	354	361	365	370	373	389	394	395	396	398	416	420	448	472	489
pH4 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH4 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=1	9.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH7 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pH10 T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	34.7	0.0	39.9	2.0	2.3	0.0	0.0	0.0
pH10 T=2	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	18.2	0.0	0.0	38.8	0.0	0.0	0.0	0.0	0.0
pH10 T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	33.1	0.0	0.0	62.0	0.0	0.0	0.0	0.0	0.0
10°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.8	0.0	0.0	0.0	0.0	0.0	0.0
10°C T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30°C T=3	0.0	0.0	0.0	0.0	5.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50°C T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	91.0	0.0	0.0	3.8	0.0	0.0	0.0	0.0
GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.1	93.7	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0
GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	66.0	0.0	31.4	0.0	2.6	0.0	0.0	0.0	0.0
F33+GI T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33+GI T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=2	0.0	0.0	0.5	1.3	5.1	0.6	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33oxic T=3	0.0	0.0	0.0	0.0	3.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0

Msp I (5')	310	318	354	361	365	370	373	389	394	395	396	398	416	420	448	472	489
F33limO T=1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F33limO T=3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

V. Condensed version of Ribosomal Database

*Msp*I(5')	*Msp*I(3')	*Hha*I(5')	*Hha*I(3')	*Rsa*I(5')	*Rsa*I(3')	Species
0	416	0	70	0	0	*Alcaligenes*
0	300	0	165	0	0	*Bacillus*
0	368	0	164	0	0	*Bacillus* ida216
145	302	240	0	0	0	*Bacillus* 168
0	367	0	0	0	0	*Bacillus acidovorans*
0	368	0	164	0	0	*Bacillus* ak1
0	369	0	165	0	0	*Bacillus alcacophilus*
0	370	0	166	0	0	*Bacillus alcaliinulinus*
0	370	0	166	0	0	*Bacillus alcalophilus* 1
165	369	239	166	485	0	*Bacillus alcalophilus* 2
145	301	240	0	486	0	*Bacillus amyloliquefaciens*
0	302	0	166	0	0	*Bacillus azotoformans* 1
155	302	242	166	458	0	*Bacillus azotoformans* 2
148	0	244	0	460	0	*Bacillus badius* 1
0	370	0	166	0	0	*Bacillus badius* 2
146	370	241	166	487	0	*Bacillus badius* 3
153	0	240	0	456	0	*Bacillus benzoevorans* 1
0	370	0	166	0	0	*Bacillus benzoevorans* 2
153	370	240	166	456	0	*Bacillus benzoevorans* 3
0	302	240	166	485	0	*Bacillus* c125
136	369	242	165	488	0	*Bacillus caldolyticus*
0	373	0	166	0	0	*Bacillus caldotenax*
136	139	142	165	488	0	*Bacillus caldovelox*
147	370	0	166	488	0	*Bacillus cereus*
151	0	238	0	454	0	*Bacillus circulans* 1
151	370	238	166	454	0	*Bacillus circulans* 2
0	367	0	165	0	0	*Bacillus coagulans* 3
0	368	0	166	0	0	*Bacillus coagulans* 4
133	370	228	166	475	0	*Bacillus coagulans* 5
0	370	0	166	0	0	*Bacillus cohnii*
0	370	0	166	0	0	*Bacillus cycrophilus*
0	370	0	166	0	0	*Bacillus denitrificans*
0	302	0	166	0	0	*Bacillus firmus* 1
146	302	242	166	458	0	*Bacillus firmus* 2
0	367	0	166	0	0	*Bacillus flavothermus* 1
0	371	0	166	0	0	*Bacillus flavothermus* 2
147	369	242	165	476	0	*Bacillus fusiformis* 1
147	370	242	166	458	0	*Bacillus fusiformis* 2
153	367	229	166	452	0	*Bacillus fusiformis* 3
138	368	244	165	488	0	*Bacillus* strain gli

***Msp*I(5')**	***Msp*I(3')**	***Hha*I(5')**	***Hha*I(3')**	***Rsa*I(5')**	***Rsa*I(3')**	**Species**
0	370	0	166	0	0	*Bacillus globisporis*
139	245	246	166	492	0	*Bacillus* icps10
0	368	0	164	0	0	*Bacillus* ida629
0	302	0	166	0	0	*Bacillus infernus*
0	370	0	166	0	0	*Bacillus insolitus*
0	368	0	166	0	0	*Bacillus* k1
0	369	0	165	0	0	*Bacillus laevolacticus*
0	370	0	166	0	0	*Bacillus lentus*
0	302	0	166	0	0	*Bacillus licheniformis* 1
145	302	241	166	457	0	*Bacillus licheniformis* 2
0	300	0	166	0	0	*Bacillus longisporus*
153	370	240	166	456	0	*Bacillus macroides*
153	370	240	166	455	0	*Bacillus maroccanus*
147	370	0	166	488	0	*Bacillus medusa*
0	370	0	166	0	0	*Bacillus megaterium* 1
167	370	0	166	458	0	*Bacillus megaterium* 2
134	370	240	166	456	0	*Bacillus methanolicus*
0	370	0	166	0	0	*Bacillus mycoides* 1
147	370	0	166	488	0	*Bacillus mycoides* 2
0	369	0	165	0	0	*Bacillus myxolacticus*
0	370	0	166	0	0	*Bacillus pallidus*
148	370	242	166	458	0	*Bacillus pasteurii*
0	370	0	166	0	0	*Bacillus pseudomegaterium*
147	0	0	0	0	0	*Bacillus pseudomycoides*
149	302	236	166	452	0	*Bacillus psychrosaccharolyticus*
81	302	240	0	190	0	*Bacillus pumilis*
0	368	0	164	0	0	*Bacillus racemilacticus*
0	302	0	166	0	0	*Bacillus silvestris*
0	370	0	166	0	0	*Bacillus smithii*
153	367	230	166	453	0	*Bacillus sphaericus* 1
153	367	231	166	454	0	*Bacillus sphaericus* 2
153	367	232	166	455	0	*Bacillus sphaericus* 3
154	366	231	166	454	0	*Bacillus sphaericus* 4
154	367	234	166	457	0	*Bacillus sphaericus* 5
0	368	0	352	0	0	*Bacillus sphaericus* 6
0	370	0	166	0	0	*Bacillus sporothermodurans*
0	369	0	165	0	0	*Bacillus stearothermophilus* 1
136	370	244	166	490	0	*Bacillus stearothermophilus* 2
144	302	239	0	455	0	*Bacillus subtilis* 168 1
145	302	240	0	455	0	*Bacillus subtilis* 168 2
145	302	240	0	456	0	*Bacillus subtilis* 168 3
145	302	240	0	475	0	*Bacillus subtilis* 168 4
145	299	239	468	285	0	*Bacillus subtilis* a405
145	301	240	0	456	0	*Bacillus subtilis* ncdo1769

***Msp*I(5')**	***Msp*I(3')**	***Hha*I(5')**	***Hha*I(3')**	***Rsa*I(5')**	***Rsa*I(3')**	**Species**
145	300	240	0	486	0	*Bacillus subtilis* tb11
145	302	240	0	475	0	*Bacillus subtilis* 1
143	303	238	0	454	0	*Bacillus subtilis* 2
145	302	240	0	455	0	*Bacillus subtilis* 3
145	302	241	0	457	0	*Bacillus subtilis* 4
0	245	0	166	0	0	*Bacillus* ta2a1
0	370	0	166	0	0	*Bacillus thermoalcophilus*
136	370	242	166	476	0	*Bacillus thermoameolvorans*
0	370	0	166	0	0	*Bacillus thermocatenuatus*
0	302	0	166	0	0	*Bacillus thermocloace*
0	370	0	166	0	0	*Bacillus thermodenitrificans* 1
0	372	0	166	0	0	*Bacillus thermodenitrificans* 2
132	0	238	0	484	0	*Bacillus thermoleovorans* 1
0	372	0	166	0	0	*Bacillus thermoleovorans* 2
0	0	0	166	0	0	*Bacillus thuringiensis* 1
0	370	0	166	0	0	*Bacillus thuringiensis* 2
147	370	0	166	488	0	*Bacillus thuringiensis* 3
138	0	244	0	491	0	*Bacillus tipchiralis*
0	300	0	164	0	0	*Bacillus* tp84
0	205	0	165	0	0	*Bacillus tusciae*
0	370	0	166	0	0	*Bacillus vedderi*
0	299	0	166	0	0	*Bacillus viscosus*
150	369	244	166	491	440	*Bacillus vortex*
0	368	0	164	0	0	*Bacillus* wn13
165	370	240	166	455	0	*Bacillus fastidiosus*
81	370	240	166	455	0	*Bacillus.* sp 4830
0	416	0	0	0	72	*Borrelia*
0	299	0	166	0	0	*Brevibacillus agri* 1
153	299	228	166	444	0	*Brevibacillus agri* 2
153	299	197	166	444	0	*Brevibacillus borstelensis*
122	299	228	166	444	0	*Brevibacillus brevis* 1
153	299	0	166	463	0	*Brevibacillus brevis* 2
294	299	228	166	444	0	*Brevibacillus centrosporus*
153	299	228	166	444	0	*Brevibacillus choshinensis*
153	199	228	166	444	0	*Brevibacillus reuszeri*
0	299	0	166	0	0	*Brevibacillus thermoruber*
0	416	0	0	0	435	*Ehrlichia sennetsu*
136	201	0	0	0	294	*Eubacterium biforme*
0	294	0	342	0	0	*Eubacterium limosum* 1
220	294	376	342	467	0	*Eubacterium limosum* 2
440	367	61	0	100	0	*Eubacterium tenue*
269	0	0	0	101	0	*Helicobacter pametensis*
217	364	233	66	0	0	*Lactobacillus*
0	370	0	296	0	0	*Lactobacillus casei*

***Msp*I(5')**	***Msp*I(3')**	***Hha*I(5')**	***Hha*I(3')**	***Rsa*I(5')**	***Rsa*I(3')**	**Species**
279	74	230	0	475	0	*Lactobacillus catenaformis*
0	370	0	0	493	0	*Lactobacillus farciminis*
0	68	0	0	0	0	*Lactobacillus fermentum* 1
0	68	406	0	0	0	*Lactobacillus fermentum* 2
0	302	68	348	0	0	*Lactobacillus fructivorans*
0	296	0	342	0	0	*Lactobacillus* lm-17
0	370	0	296	0	0	*Lactobacillus paracasei*
0	68	0	348	0	0	*Lactobacillus reuteri*
0	68	0	348	0	0	*Lactobacillus pontis*
482	419	360	0	100	0	*Methylophilus methylotrophus*
149	0	336	0	417	0	*Methylosinus sporium*
0	299	0	0	0	0	*Micrococcus species*
0	367	0	165	0	0	*Paenibacillus* 1
0	368	0	166	0	0	*Paenibacillus* 2
0	369	0	166	0	0	*Paenibacillus* 3
152	369	246	166	491	0	*Paenibacillus* 4
0	297	0	164	0	0	*Paenibacillus alginolyticus* 1
160	297	235	164	0	0	*Paenibacillus alginolyticus* 2
0	369	244	166	489	0	*Paenibacillus alvei*
147	368	241	165	486	0	*Paenibacillus apiarius*
150	369	244	166	0	0	*Paenibacillus azotofixans*
150	369	244	166	0	0	*Paenibacillus chibansis*
0	297	0	164	0	0	*Paenibacillus chondroitinus* 1
153	297	228	164	0	0	*Paenibacillus chondroitinus* 2
169	369	244	166	0	0	*Paenibacillus curdlanolyticus*
150	315	244	169	489	0	*Paenibacillus glucanolyticus*
0	299	0	166	0	0	*Paenibacillus gordonae*
150	299	244	166	290	0	*Paenibacillus illinoisensis*
145	302	241	166	457	0	*Paenibacillus lautus* 1
150	369	244	166	489	0	*Paenibacillus lautus* 2
150	369	244	347	489	0	*Paenibacillus lautus* 3
145	302	240	348	486	0	*Paenibacillus Lentimorbus*
135	299	240	166	485	0	*Paenibacillus macerans* atc8244
138	299	244	166	0	0	*Paenibacillus macerans* jcm250
148	312	241	166	486	0	*Paenibacillus macquariensis* 1
152	299	246	166	491	0	*Paenibacillus macquariensis* 2
150	299	244	166	489	0	*Paenibacillus pabuli*
150	421	0	166	0	0	*Paenibacillus peoriae*
0	369	0	166	0	0	*Paenibacillus polymyxa* 1
150	369	242	166	487	0	*Paenibacillus polymyxa* 2
132	239	238	160	483	0	*Paenibacillus validus* 1
140	299	234	166	479	0	*Paenibacillus validus* 2
492	0	209	0	72	0	*Pseudomonas*
0	370	0	166	0	0	*Stearothermophilus*

*Msp*I(5')	*Msp*I(3')	*Hha*I(5')	*Hha*I(3')	*Rsa*I(5')	*Rsa*I(3')	Species
67	207	470	230	454	0	*Streptomyces vellosus*
149	312	256	166	501	0	*Thermobacillus xylanolyticus*
147	312	0	0	0	0	*Thiobacillus halophilus*

V.I. Parameters obtained from the linear regression of FAME concentrations in standards

The GC-MS was calibrated on three separate occasions. On the first 2 occasions the GC-MS was calibrated without using an internal standard whilst on the third occasion an internal standard was used

Run 1

	<20 μg.ml^{-1}			>20 μg.ml^{-1}			
	m	**F**	**P**	**m**	**c**	**F**	**P**
16:0	940116	1077.7	<0.01	9827706	475530	386.1	<0.01
18:0	820120	1193.7	<0.01	7935914	437697	300.2	<0.01
18:1	481273	889.5	<0.01	4024167	323568	801.3	<0.01
18:2	441842	686.8	<0.01	4535537	282203	527.2	<0.01
18:3	481218	1361.3	<0.01	5178720	264395	1456.0	<0.01

Run 2

	<20 μg.ml^{-1}			>20 μg.ml^{-1}			
	m	**F**	**P**	**m**	**c**	**F**	**P**
16:0	138505	259.4	<0.01	219881	-1538162	1164.7	<0.01
18:0	114598	175.9	<0.01	175783	-1114363	1248.9	<0.01
18:1	24882	225.8	<0.01	37398	-210389	1763.2	<0.01
18:2	56888	180.6	<0.01	76215	-21128	533.6	<0.01
18:3	59772	214.5	<0.01	86095	-167355	1009.4	<0.01

Run 3

	<5 μg.ml^{-1}			>5 μg.ml^{-1}			
	m	**F**	**P**	**m**	**c**	**F**	**P**
16:0	1.38	1143.8	<0.01	2.15	-3.59	192.6	<0.01
18:0	1.20	957.5	<0.01	1.58	-1.37	402.2 0	<0.01
18:1	0.502	1119.0	<0.01	0.68	-0.70	1445.5	<0.01
18:2	0.511	776.2	<0.01	0.72	-0.80	183.5	<0.01
18:3	0.524	633.7	<0.01	0.75	-0.89	237.0	<0.01

VII. Microbial product characteristics

The following information was supplied by the manufacturers of the microbial supplements:

G40 - 2 strains of *Bacillus subtilis* suspended in molasses (liquid formulation).
P80 - as above but without the molasses (liquid formulation).
GTL - (liquid formulation)

Name of strain	Number of strains
Bacillus subtilis	3
Bacillus polymyxa	2
Bacillus licheniformis	2
Pseudomonas fluorescens	1
Bacillus stearothermophilus	2
Bacillus sp.	1
Bacillus megaterium	2
Bacillus sphericus	1
Bacillus pumulis	1

pF33- (powdered formulation)

Name of strain	Number of strains
Bacillus subtilis	13
Bacillus megaterium	4
Bacillus thurengesis	6
Bacilus stearothermophilus	3
Bacillus lichenformis	5
Bacillus polymyxa	4
Bacillus pumulis	2
Lactobacillus sporogenes	2
Bacillus sp.	5
Sacchormyces cerevisiae	2
Pseudomonas fluorescens	5
Pseudomonas stutzeri	3
Lactobacillus aeruginosa	1
Cellulomonas uda	1
Micococcus sp	1
Thiobacillus novellus	1

pF33 with the clay carrier contained an oxygen generator whilst pF33 with the peanut shell carrier contained a nutrient supplement.

F33 - Composition similar to above but number and relative proportions of the various strains/species differed (liquid formulations).

S45 - as above but also contained an inhibitor

Formulations 1-7 – similar to above. Formulations 1 and 7 were more diverse than formulation 6. However, formulations 6 and 2 contained high lipase producing microorganisms.

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