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ORIGINAL PAPER

Degradation and modification of fats, oils and grease by commercial microbial supplements

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Abstract Fats, oils and greases (FOGs) in wastewater create problems including the production of foul odours, the blockage of sewer lines and may interfere with the proper operation of sewage treatment works. Removal of FOG from wastewater is thus critically important to ensure that wastewater is disposed of efficiently and economically. In this study, the ability of commercial microbial supplements to degrade fat/oil under laboratory conditions was investigated. One of the multi-species supplements examined was capable of significantly enhancing the degradation of several fats and oils by 37–62%, in contrast to all of the single-species supplements which had no significant effect. The multi-species supplement showed no preferential cleavage or degradation of fatty acids in a range of FOGs, whilst wastewater-associated bacteria preferentially degraded octadecatrienoic acid (18:3ω3) and octadecadienoic acid (18:2ω6). A semi-solid, sticky material, likely to cause blockages in pipework and sewer lines formed when the oil was incubated in the presence of wastewater bacteria. The sticky material was enriched in saturated and mono-unsaturated fatty acids and depleted in polyunsaturated fatty acids relative to the original oil, most likely reflecting preferential fatty acid usage by the bacteria. The production of this semi-solid material by the wastewater bacteria was significantly reduced by the presence of the multi-species product, indicating that commercial supplements have the potential to minimize FOG accumulation and blockages in grease traps and sewer lines as well as enhancing FOG degradation.

Keywords Bioaugmentation · degradation · fatty acids · grease · wastewater

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Introduction

A wide variety of industries produce effluents rich in fats, oils and greases (FOGs). Effluents produced by the restaurant trade (Stoll & Gupta 1997; Wakelin & Forster 1997), the dairy industry (Vidal et al. 2000) and food processing (Cammarota et al. 2001) are a small sample of those that present potential problems in terms of wastewater management due to their high FOG content. Fats often solidify causing pipes and sewer lines to become blocked (Baig & Grenning 1976) Grease traps may also fail to retain dissolved and emulsified fats efficiently allowing them to enter the water treatment system. These lipids may then interfere with aerobic biological wastewater treatment processes by reducing oxygen transfer rates (Chao & Yang 1981) and can also reduce the efficacy of anaerobic treatment processes by reducing the transport of soluble substrates to the bacterial biomass (Rinzema et al. 1994). FOGs not properly treated by sewage works may enter rivers and oceans with potentially detrimental environmental impacts (Stams & Oude 1997). Reduction in the levels of FOGs in effluents is thus highly desirable and a good potential candidate for bioaugmentation.

Numerous microorganisms capable of degrading FOGs have been identified and may be potential

candidates for bioaugmentation products. Thermophilic vegetable-oil-degrading bacteria have been isolated, raising the possibility that commercial products could be designed to operate over a wide temperature range (Markossian et al. 2000). Mixed microbial cultures have also been shown to degrade a variety of oils indicating the potential to treat wastewater containing mixed FOGs such as that produced by the catering industry (Wakelin & Forster 1997; Tano-Debra *at al.* 1999). More recent studies have shown that mixed microbial cultures are capable of removing large amounts of FOGs from contaminated industrial effluents when used either in sand biofilms or as free living cultures demonstrating the potential versatility of bioaugmentation (El-Masry et al. 2004; El-Bestawy et al. 2005).

There are however a number of potential problems in using many of the bacteria previously shown to be FOG degraders as part of a commercial microbial supplement. Since the supplements may be used in environments where food is processed or prepared they must not represent a human health hazard. In addition, they need to have a reasonable shelf life preferably not requiring refrigeration. These criteria may explain, at least in part, why many commercial supplements contain mostly *Bacillus* sp. and closely related bacteria rather than the Gram-negative bacteria successfully used in many laboratory-based trials. The relatively small number of studies assessing the performance of commercially available FOG-degrading supplements, published in the scientific literature, has provided mixed results. Mendoza- Espinosa & Stephenson (1996) and Wakelin & Forster (1997) both found little difference in the ability of natural activated sludge and commercial additives to enhance grease degradation whilst Salome & Bonvallot (1994) showed that none of 5 bio-additives tested degraded olive oil to any significant extent. In contrast, Baig & Greening (1976) reported that bio-augmentation products improved water quality in terms of odour and grease build-up when used to treat a variety of wastewaters, although it should be noted that the data used in their study was gathered via telephone surveys and was thus not quantitative.

In addition to the lack of consensus concerning the efficacy of commercially available bio-augmentation products in terms of enhanced FOG degradation, there is also a scarcity of information about how other, more subtle, changes to the physico-chemical nature of FOGs induced by these products may influence their subsequent behaviour in wastewater systems. The aims of the current study were thus to investigate a range of commercially available supplements both in terms of their ability to fully degrade FOGs and to induce changes in the fatty acid composition of partially degraded oil.

Materials and Methods

Gravimetric analysis of fat/oil degradation

Experiments were conducted in 250-ml conical flasks stoppered with cotton wool to encourage oxic conditions. All flasks contained 1 ml of fat/oil and 100 ml of enriched nutrient medium (yeast extract (0.2 g/l), glucose (0.1 g/l), potassium nitrate (1 g/l), magnesium sulphate-7 hydrate (0.2 g/l), di-sodium hydrogen orthophophate (0.1 g/l), calcium chloride-2-hydrate (0.01 g/l), manganese sulphate-1-hydrate (0.01 g/l), ferric ammonium citrate (0.005 g/l) or standard nutrient medium (yeast extract (0.1 g/l), glucose (0.025 g/l), potassium nitrate (1 g/l), magnesium sulphate-7 hydrate (0.2 g/l), di-sodium hydrogen orthophophate (0.1 g/l), calcium chloride-2-hydrate (0.01 g/l), manganese sulphate-1-hydrate (0.01 g/l), ferric ammonium citrate (0.005 g/l)). A number of commercial oil-degrading supplements were tested and all were used according to the manufacturers' instructions. Some flasks were autoclaved before inoculation with supplements whereas others were left to be colonized by microoganisms occurring naturally on the surface of the glassware, oil and wastewater, referred to as wastewater bacteria. Controls were prepared in the same manner but without the addition of the supplement. The flasks were incubated for 21 or 28 days at 30°C with shaking at 130 rev/min before the remaining lipids were extracted. All treatments and controls were prepared in triplicate.

Lipids were extracted by transferring the contents of the flasks into separating funnels and adding 40 ml of dichloromethane. The funnels were shaken vigorously, allowed to settle and the organic phase transferred to a florentine flask. If emulsification had occurred, separation of the two phases was achieved by centrifugation at 4500 *g* for 2–10 min assisted by the addition of a few drops of saturated sodium chloride solution. The remaining aqueous phase was re-extracted a further 3 times and the solvent phases pooled together and dried using a rotary evaporator. The dried oil was redissolved in a few ml of dichloromethane, transferred to a vial containing anhydrous sodium sulphate and then filtered through Whatman number 6 filter paper into a pre-weighed vial. The solvent was evaporated under oxygen free nitrogen at 40°C and the weight of oil determined.

Adsorption chromatography

The glyceride and free fatty acid fractions of the extracted oil were separated using adsorption chromatography. Hydrated Florisil (4%) was prepared using 10 g of 60–100 activated mesh Florisil and 0.4 ml of distilled water. The hydrated Florisil was slurried in hexane and poured into a 0.5 x 30 cm chromatography tube to achieve a column length of 10 cm. The lipid sample (2–10 mg) was applied in 2-5 ml of hexane and the column was eluted with 50 ml hexane/ethyl ether (1:1) (glyceride fraction), followed by 200 ml ethyl ether/methanol (95:5) (glyceride fraction) and 20 ml of ethyl ether/acetic acid (93:7) (fatty acid fraction) at a flow rate of 1.5-2 ml/min.

Derivatization of fatty acids

Derivatization was based upon the method of Morisson & Smith (1964). Lipid samples were dissolved in chloroform to a final concentration of 1-2 mg/ml and 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 to 100°C for 1 h, 3 ml of water and 6 ml of pentane were added to the derivatized sample and gently shaken to extract and clean the fatty acid methyl esters. The pentane layer was transferred to a new vial using a pasteur pipette and the extraction process was repeated using a further 6 ml of pentane. The pentane was then evaporated under an oxygen free nitrogen flow and the fatty acids dissolved in hexane.

GC-MS analysis

Fatty acids were identified using a Fisons MD 800 GC-MS with a BPX-70 column. The oven temperature programme used was as follows; Isothermal at 80°C for 2 min, 40°C/min to 160°C, 0.5°C/min to 170°C, 10°C/min to 250°C and isothermal at 250°C for 10 min. A calibration was also performed using a series of fatty acid standards which contained hexadecanioc acid (16:0), octadecanoic acid (18:0), octadec-9-enoic acid (18:1ω9), octadeca-9 c, 12 c-dienoic acid (18:2ω6) and octadeca- 9 c, 12 c,15 c-trienoic acid (18:3ω3) at concentrations of 0.2 to 16.0 μg/ml, prepared in hexane and injected in triplicate.

Microbial supplements for degrading FOGs

The product F69 was obtained from Organica (UK) Ltd, Birkenhead, Wirral, UK.

Assessment of oil solidification

Quantification of the semi-solid material generated from soya oil by the wastewater bacteria was achieved by filtering the entire contents of flasks through pre-weighed nylon mesh (porosity: 600 μm, area: 96 cm^2). The nylon mesh filter was then dried at 40°C for 18 h and re-weighed. Adherence of this material to the sides of the glass vessel was quantified by emptying the contents of the pre-weighed vials and re-weighing the vials (with the adhering material remaining) after drying at 40 °C for 18 h. The fatty acid composition of this material was determined by GC-MS, as previously described.

Results and Discussion

Effect of commercial supplements on fat/oil degradation rates

The efficacy of several commercially available FOG-degrading microbial supplements containing single or multiple bacterial species were tested. None of the single species inocula investigated (all whole cell preparations of *Bacillus subtilis*) were capable of significantly enhancing the degradation of any of the oils used (data not shown). However, one of the multi-species inocula investigated, known as F69 (Organica [UK] Ltd)(Table 1) did significantly enhance ($F = 45.03–224.27$, $P < 0.001$) the degradation of a variety of fats and oils relative to controls (Figure 1). These data are in good agreement with previous studies which have shown that microbial inocula composed of

Table 1 Composition of the microbial supplement used (F69) (data supplied by Organica [UK] Ltd)

Species	Number of strains
Bacillus subtilis	13
Bacillus megaterium	4
Bacillus thuringiensis	6
Bacillus stearothermophilus	3
Bacillus licheniformis	5
Bacillus polymyxa	4
Bacillus pumilus	2
Lactobacillus sporogenes	2
Bacillus sp.	5
Sacharomyces cerevisiae	2
Pseudomonas fluorescens	5
Pseudomonas stutzeri	3
Cellulomonas uda	1
Micrococcus sp.	1
Thiobacillus novellus	1

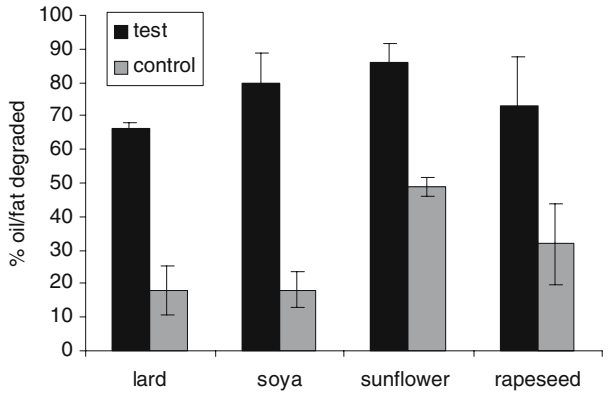

Fig. 1 Effect of microbial supplement F69 (test) on the degradation (%, mean ± SD) of various fats/oils (1 ml) suspended in standard nutrient medium (100 ml), incubated at 30°C with shaking at 130 rev/min for 21 (lard & soya oil) or 28 (sunflower and rapeseed oil) days

multiple strains of Gram-negative bacteria or yeasts were also able to degrade substantial amounts of oil. Furthermore, the enhanced performance of the multi-species inocula in comparison with single species agrees with the results of other workers (Mihara et al. 2000; El-Masry et al. 2004) indicating the way forward for the design of commercial FOG-degrading supplements.

The extent of oil degradation by F69, ranged from 37% to 62% relative to the controls. Previous work has also shown that the nature of fat/oil can influence microbial degradation rates, as was found in the current study (Groenwold et al. 1982; Tano-Debrah et al. 1999). Since Sun & Wakeham (1994) demonstrated that unsaturated fatty acids are degraded more readily than their saturated counterparts, the lower degradation of lard could be explained by the reduced unsaturated fatty acid content relative to the other oils studied. However, work by Tano-Debrah et al. (1999) and Groenwold et al. (1982) indicated that there was no relationship between the biodegradability of the fat/oil and the degree of saturation, suggesting that other factors may be important. Another explanation for the reduced degradation rate of lard, may therefore be that solid fats are less likely to be as well dispersed in the

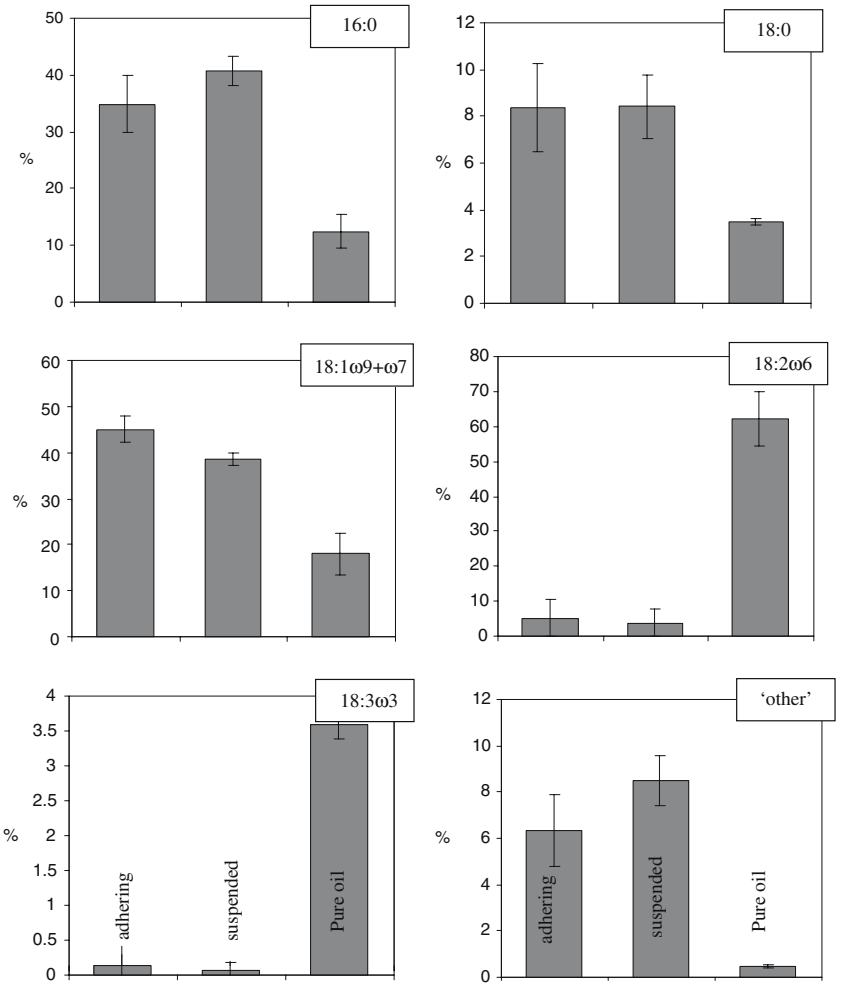

Fig. 2 Composition (%) of pure soya oil and the suspended and adhering semi-solid material produced by the wastewater bacteria in nutrient medium (135 ml) containing mechanically emulsified soya oil (0.6 %v/v) incubated for 14 days at 30°C with shaking at 150 rev/min

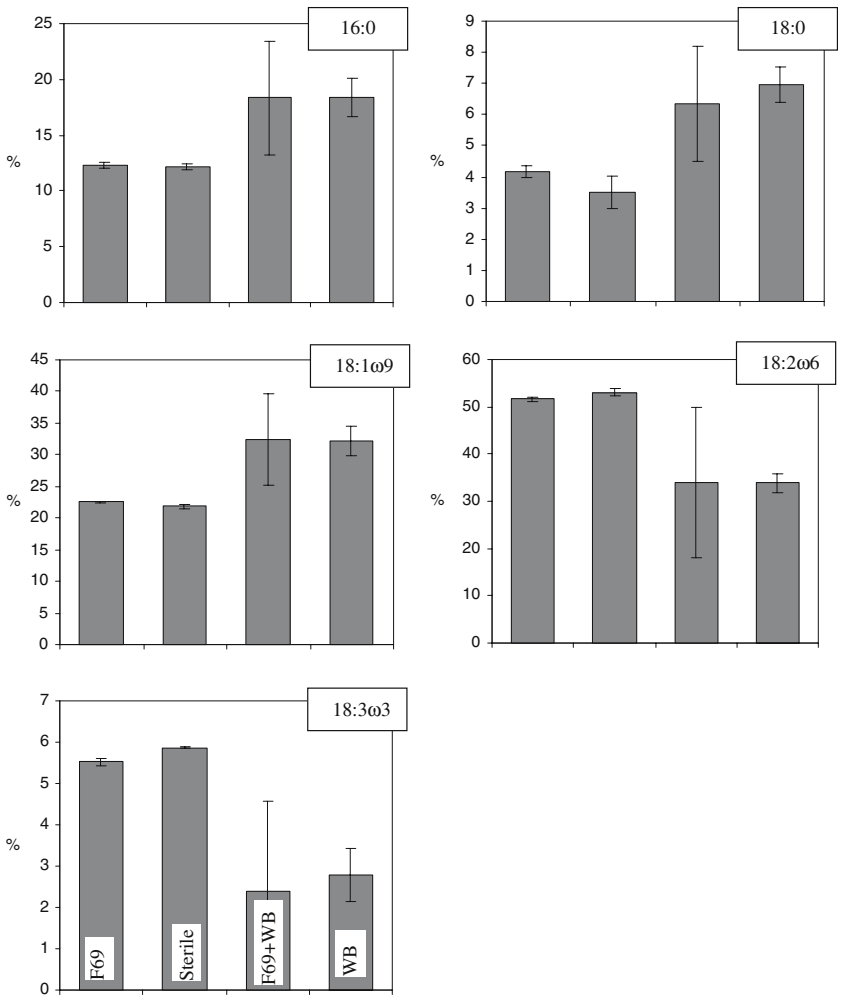

Fig. 3 Effect of F69 and the wastewater bacteria (WB) on the relative proportions (%, mean ± SD) of the major fatty acids in the glyceride fraction of soya oil (1 ml) suspended in enriched nutrient medium (200 ml) incubated for 21 days at 30°C with agitation on a regular basis

media as liquid oils and fats, and oils must be dispersed in the media for successful microbial growth to occur (Ratledge 1994).

The levels of oil degradation reported here are lower than some other studies (El-Bestawy et al. 2005; Tano-Debrah et al. 1999). The use of dichloromethane as an extraction solvent may explain these differences, at least in part, since it is more effective at extracting slightly polar material such as fatty acids than hexane which has been widely used in previous work (El-Bestawy et al. 2005; Tano-Debrah

et al. 1999). The high levels of apparent degradation in the sterile controls (18-50%) illustrate the importance of properly assessing factors such as extraction efficiency when measuring FOG degradation, something often omitted in similar studies. Auto-oxidation processes such as polymerization (Pereira et al. 2003) make the oil less amenable to extraction and account for the apparent degradation in the sterile controls.

Effect of commercial supplement F69 on the production of semi-solid, sticky material

When oil was incubated with the wastewater bacteria an opaque, semi-solid sticky material was formed. Some of this material stuck to the sides of the culture vessels (10 ± 2 mg (mean ± Standard deviation) in the controls; 5 ± 2 mg in flasks supplemented with F69) and some remained suspended in the culture medium (333 ± 49 mg control; 46 ± 49 mg F69). The fatty acid composition of the suspended and adhering semi-solid material was similar, but however quite different from the original oil (Figure 2). A series of one-way ANOVA's ($F = 31.2–489.0$ $P < 0.001$) and subsequent Tukey's pairwise comparisons showed that they contained significantly greater proportions of 16:0, 18:0, 18:1ω7 and 18:1ω9 and significantly lower proportions of 18:2ω6 and 18:3ω3, than the original oil.

The semi-solid material was initially considered to be polymerized oil, as oil has been observed to solidify

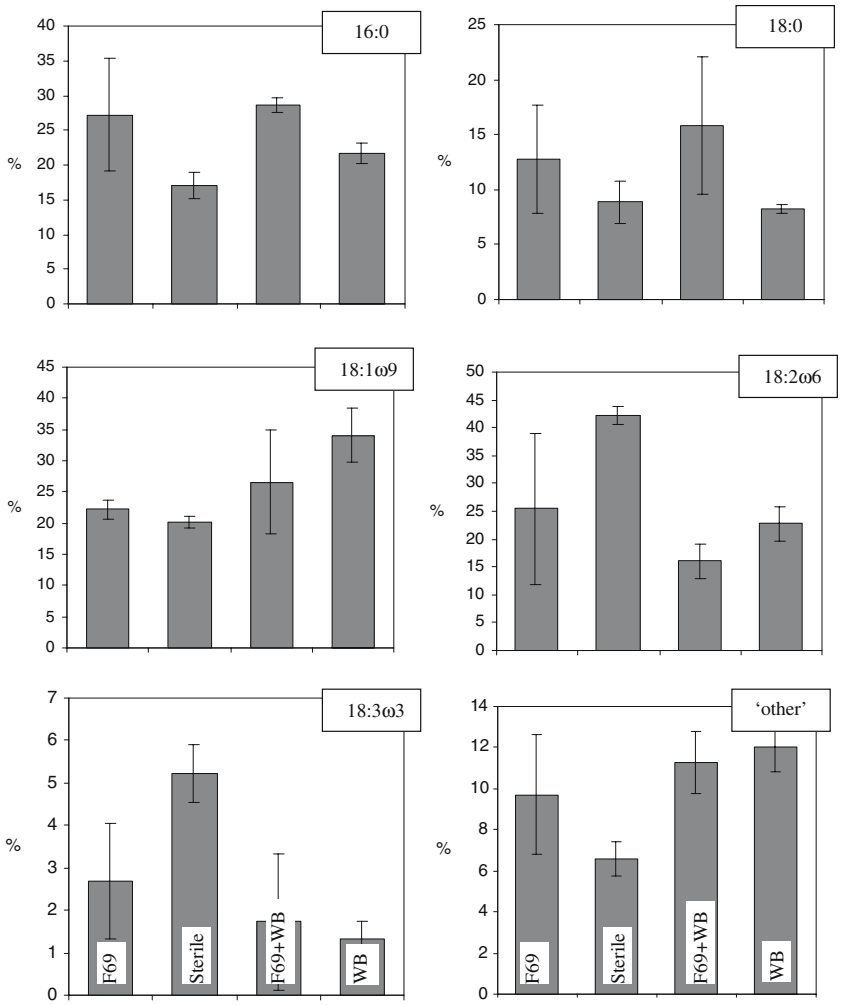

Fig. 4 Effect of F69 and the wastewater bacteria (WB) on the relative proportions (%, mean ± SD) of the major fatty acids in the free fatty acid fraction of soya oil (1 ml) suspended in enriched nutrient medium (200 ml) incubated for 21 days at 30°C with agitation on a regular basis

via polymerization in other studies (Mudge et al. 1994; Pereira et *al.* 2003). Furthermore, GC-MS analysis revealed that this semi-solid material, like the polymers examined by Mudge et al. (1994), was enriched in 16:0 and 18:1ω9 relative to the original oil. Unlike the polymers observed by Mudge et al. (1994) however, the semi-solid material developed only in the presence of the wastewater bacteria and was not of a 'used chewing gum-like' appearance. Production of the semi-solid material was therefore more likely to be a consequence of microbial activity than polymerization, which is an autoxidation process. The production of such material by bacteria associated with wastewater is likely to contribute to the well-documented problem of sewer and pipeline blockage caused by FOGs (Chao & Yang 1981).

F69 inhibited the production of the semi-solid material suspended in the medium and adhering to the culture vessels by over 80% and 50% respectively. T-tests confirmed that these reductions were significant (T = 5.69–10.08; $P < 0.001$) and demonstrates a potential role for microbial inocula in keeping sewer lines free of blockages as well as enhancing oil degradation.

Effect of the commercial supplements on the fatty acid compositions of fats and oils

To investigate fatty acid preferences of different groups of microbes, soya oil was incubated under sterile conditions and with various combinations of the wastewater bacteria and F69. After 3 weeks incubation, the oil was extracted and both the glyceride and free fatty acid fraction of the oil analysed using GC-MS (Figures 3 & 4).

In the case of the glyceride fraction, oil incubated with F69 alone was similar to the sterile controls suggesting that the lipase produced by the F69 microorganisms was non-specific. By contrast, the wastewater bacteria induced high proportions of 16:0, 18:0 and 18:1ω9 and low proportions of 18:2ω6 and 18:3ω3 relative to the sterile controls implying potential lipase

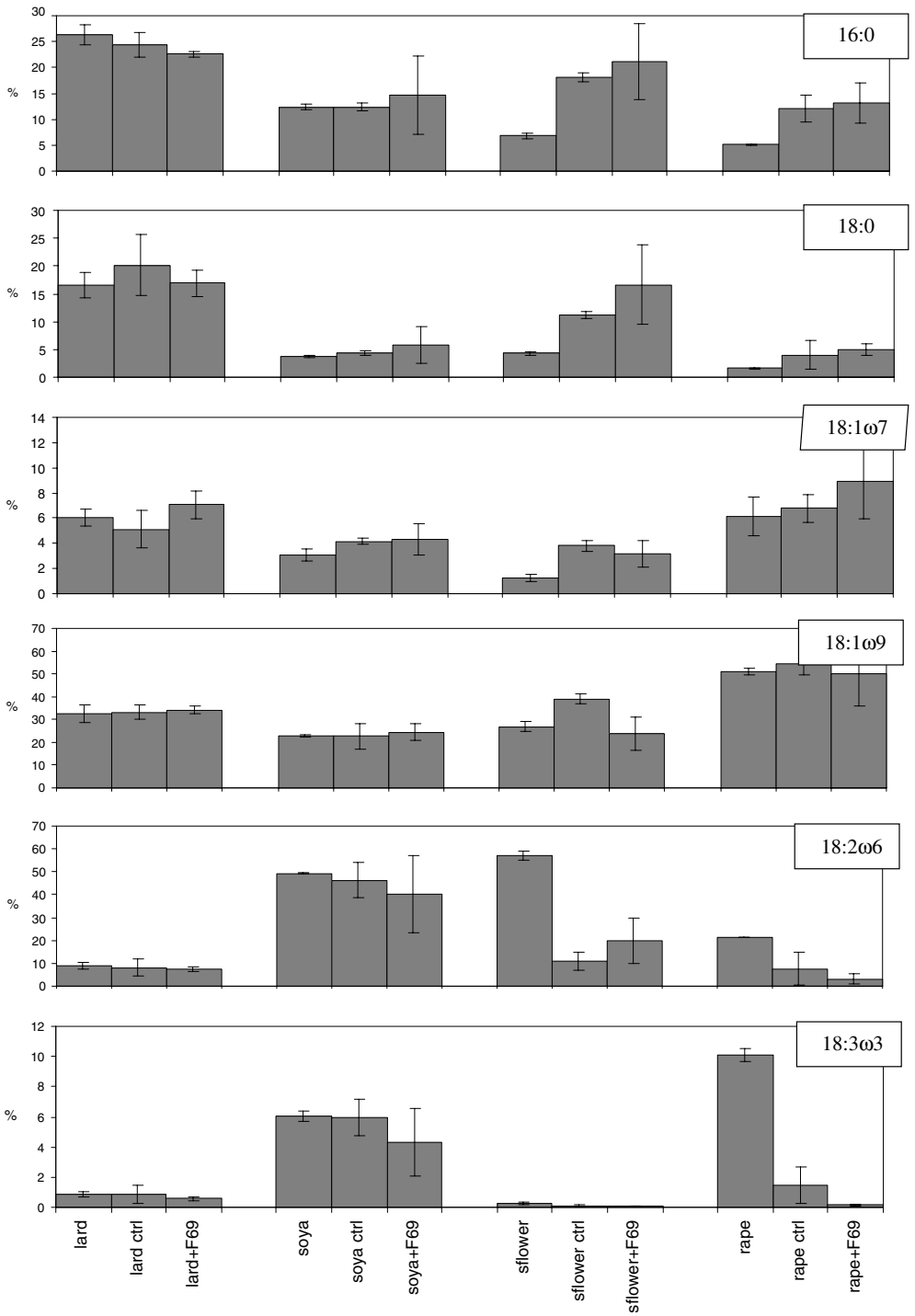

Fig. 5 Effect of F69 and physico-chemical processes (ctrl) on the fatty acid composition (%, mean ± SD) of various fats and oils (1 ml) suspended in standard nutrient medium (100 ml), incubated at 30°C with shaking at 130 rev/min for 21–28 days

specificity in terms of either cleavage position or substrate. When F69 and the wastewater bacteria were both present the fatty acid profile on average was similar to that produced by the wastewater bacteria alone but there was far greater variability between the replicates than with the wastewater bacteria alone. The increased variability in the fatty acid composition of the glyceride fraction of the oil most likely indicates that different microbial flora dominated the different replicates.

The apparent selectivity of the lipase produced by the wastewater bacteria is unusual and may indicate that they possess hitherto undescribed enzymes. Fungal lipases have been identified which catalyse the

hydrolysis of fatty acyl substituents with a double bond at the 9,10 position (Ratledge 1994). However, it is unlikely that this type of lipase was responsible for the changes observed since 18:1ω9 would have been hydrolysed at a similar rate to 18:2ω6 and 18:3ω3. Lipases specific for the position at which the majority of 18:2ω6 and 18:3ω3 fatty acids were situated in the triglyceride molecule, usually the central position (Gurr & Harwood 1991), have not been previously isolated from bacteria.

In the free fatty acid fraction of the oil, the presence of both F69 and the wastewater bacteria induced an increase in the proportion of 16:0, 18:0 and a decline in 18:2ω6 and 18:3ω3 relative to the control. The effect of F69 was much more variable however, and unlike the wastewater bacteria did not significantly influence the compositions of major fatty acids. Preferential degradation of 18:2ω6 and 18:3ω3 by the wastewater bacteria supports the findings of other studies which have shown that unsaturated fatty acids are more reactive than saturated fatty acids of a similar chain length (Sun & Wakeham 1994). It has been 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 (Novak & Carlson 1970). The rise in the proportion of 16:0, 18:0 and sometimes 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 (Pereira 1999; Lalman & Bagley 2001) and is thought to indicate that either the capacity of the microorganisms to process intermediates has been saturated or that other microorganisms are required to degrade the intermediates produced (Lalman & Bagley 2001). In contrast to the wastewater bacteria F69 did not exhibit significant preferential degradation of fatty acids suggesting that the F69 microorganisms were better able to process the intermediates produced perhaps due to their high level of diversity.

The effect of F69 on the fatty acid composition of a range of fats and oils was also investigated and is shown in Figure 5. For all fat/oils investigated the sterile controls and inoculated samples contained proportionally less 18:3ω3 and 18:2ω6 than the pure oil and were often enriched in 16:0, 18:0 and 18:1ω7. The fatty acid compositional changes were significant in the case of sunflower (18:2ω6) and rapeseed oil (18:3ω3) only. Since polyunsaturated fatty acids are known to be more reactive than their more saturated counterparts (Sun & Wakeham 1994) these changes are not surprising given the high levels of 18:2ω6 and 18:3ω3 in sunflower and rapeseed oils respectively. The fact that F69 did not have a significant impact on the fatty acid composition of the oil relative to the sterile controls, demonstrates that physico-chemical rather than biological factors were responsible for the fatty acid changes in the fat/oil. Furthermore, since no semi-solid, sticky material was observed in any of the samples these data indicate that the commercial supplement F69 has the potential to degrade a variety of oils without producing material likely to block sewer lines.

Conclusions

Bacteria associated with wastewater preferentially degrade unsaturated fatty acids producing semi-solid, sticky material likely to block sewers. Multi-species microbial inocula can degrade significant amounts of a variety of fats and oils without significantly modifying the fatty acid composition and may thus help keep sewer lines free of grease deposits. Further work should investigate the efficacy of commercial inocula under a range of environmental conditions.

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