The Biodegradation of Tallow by *Trichoderma harzianum* Rifai RP1.

A thesis submitted to Dublin City University
in fulfilment of the requirements for the award of the degree of
Doctor of Philosophy

by

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December 2002

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D., is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: John Fleming

I.D. No.: 95971459

Date: 12/2/2003

We may rest assured that as green plants and animals disappear one by one from the face of the globe, some of the fungi will always be present to dispose of the last remains.

B.O. Dodge, 1940.

Acknowledgements

I would like to thank my supervisor, Dr. Bríd Quilty for her help, guidance and endless patience throughout the duration of this project.

My deepest gratitude to my parents and family for supporting me in every way throughout my education. Without it I would not be where I am today.

Большое спасибо Леночке за то что была здесь со мной в течение всего этого времени. Это значит для меня всё.

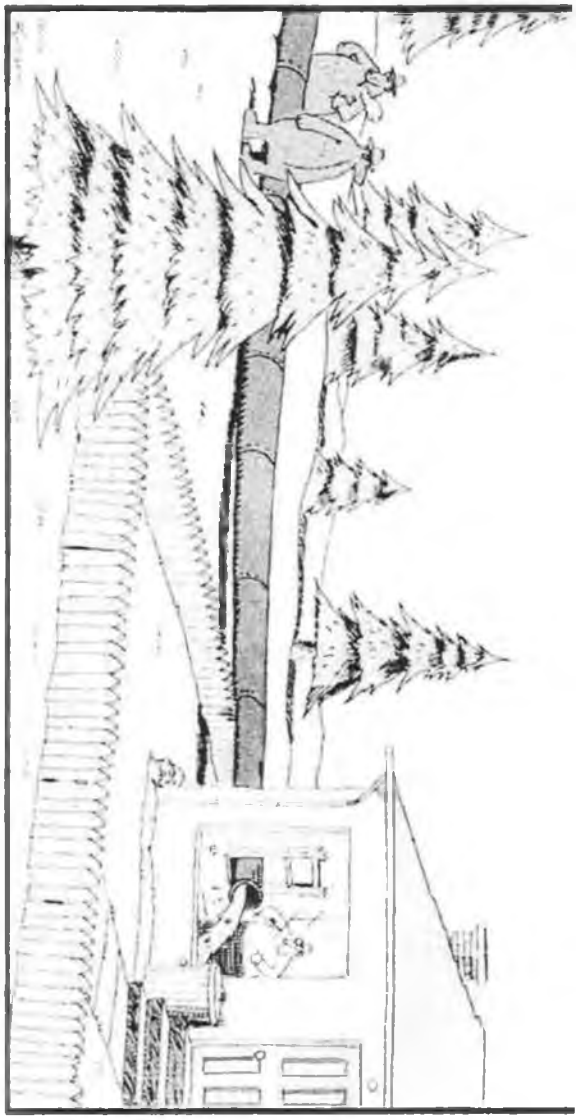
To my past and present colleagues in A112 and X174 – Alan, Henry, Sharon, Mary, Fakhruddin, Niamh, Rob, Kevin, Marianne, Sonia…thanks for putting up with me for so long (I think I should refrain from saying how long!).

To the other postgrads who made life interesting! I won't list all of you out, because there are too many! Life was never dull around DCU… (!?)

Thanks to the staff and technicians in Biotechnology for all the help and advice over my long stay in the department. A special thanks for the loan of the odd screwdriver or vicegrips to dismantle lab equipment and worry everyone else in the lab!

Thanks to Dublin Products Ltd., Dunlavin, Co. Wicklow for the wastewater and activated sludge and an endless supply of tallow.

I wish to acknowledge Enterprise Ireland for financial support for the project.



Animal Waste Management

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Abstract

The Biodegradation of Tallow by *Trichoderma harzianum* Rifai RP1.

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Wastewater, activated sludge and tallow were used as sources of organisms in enrichment cultures to screen for species capable of degrading the hard fat, tallow. A total of 58 strains were isolated, of which seven non-filamentous and two filamentous organisms removed greater than 20% of 20g/l tallow from batch cultures.

Optimum fat removal of 83% by the strain F2, identified as the fungus *Trichoderma harzianum* Rifai RP1, was observed in cultures buffered to pH 6, incubated at 25°C, shaking at 130rpm using 1g/l tallow as the sole carbon source with no added surfactant, using an inoculum of one 5-day old 8mm mycelial agar plug.

Growth followed Monod kinetics, with a k_s of 0.758g/l and μ_{max} of 1.438 day^{-1}. Glyceride hydrolysis was efficient, but free fatty acids, mainly palmitic, stearic and oleic acids, accumulated in the culture supernatant. Accumulation of intracellular lipid was observed, increasing during incubation to account for 35% - 55% of biomass. Intracellular lipid was predominantly composed of triglycerides and free fatty acids. No fatty acid preference was evident in this accumulation.

In cultures with a mixture of palmitic, stearic and oleic acids as sole carbon source, up to 97% removal was observed with 0.12g/l of the fatty acid mixture. Oleic acid was assimilated by RP1 more readily than the two saturated fatty acids. Accumulated intracellular lipid accounted for varying proportions of biomass, from 9% to 47%. Free fatty acids were the dominant lipid class intracellularly, with lower concentrations of triglycerides. Stearic acid accumulated in the intracellular lipid to a greater extent than palmitic or oleic acids.

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1. Introduction

1.1 Fungi

Fungi are a morphologically diverse group of microorganisms. They are a kingdom of eucaryotic organisms that possess β-glucan / chitin cell walls, can grow in the form of filaments, and reproduce by means of spores. They are heterotrophs that digest external organic matter and import it into the cells by active transport (Cavalier-Smith, 2001). They can be as simple as unicellular yeasts, or as complex as the fruiting bodies of mushrooms and toadstools. The most commonly encountered fungi are the yeasts and mushrooms, as well as a diverse range of moulds.

The filaments of fungi are called hyphae, which are collectively referred to as mycelia (Onions *et al.*, 1986). These hyphae may be divided into cell-like units by septa. However, where septa are present, the movement of cytoplasm and sub-cellular organelles is facilitated by pores in the septa (Griffin, 1994). Many mycelial fungi are dimorphic and can exist in a unicellular form, as yeasts. Numerous species do not display typical characteristics of fungi but have been found to belong to the kingdom Fungi using molecular biological techniques. In a similar way, some organisms which appeared to be fungi have been found not to belong to the kingdom Fungi (Cavalier-Smith, 2001).

Fungi reproduce by simple growth of the mycelium or by means of spores. Spores can be produced sexually or asexually. Sexual reproduction occurs through mating of two specialised hyphae to form a spore, genetically different from either of the parent fungi. Spores formed in this manner include the oospores and zygospores of the Mastigomycetes and Zygomycetes and the ascospores and basidiopores of the Ascomycetes and Basidiomycetes (Onions *et al.* 1986). Asexual reproduction does not involve mating and transfer of genetic material. The spores are formed on specialised hyphae in many different ways. The asexual spores of the Ascomycetes,

Basidiomycetes and Deuteromycetes, called conidia, are produced, for example, by budding, extrusion from pores or 'blowing out' of a hyphal tip (Deacon, 1984). The asexual spores of the Mastigomycetes are motile, possessing two flagella, and are called zoospores. They are produced in a sporangium by cytoplasmic cleavage. The asexual spores of the Zygomycetes are produced in a similar manner in sporangia, but are not motile (Deacon, 1984). The diversity of reproductive structures of the fungi allows them to be used as a diagnostic feature during species identification.

The taxonomy of the fungi is complex, with much debate evident in the literature on correct classifications. However, the classification by Ainsworth (1973) has been widely used (Deacon, 1984, Onions *et al.*, 1986, Berry, 1988, Griffin, 1994). In this system of classification, the Fungi are divided into the Myxomycota or slime moulds and the Eumycota or true moulds. The Eumycota include all the filamentous fungi and yeasts. The Eumycota are further divided into classes, each with distinctive characteristics. These classes are Zygomycetes, Mastigomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes. The Mastigomycetes were later referred to as Chytridiomycetes (Griffin, 1994). The Zygomycetes and Mastigomycetes are aseptate (i.e. do not have septa in their hyphae), while the Ascomycetes, Basidiomycetes and Deuteromycetes exist as septate mycelia or yeasts (i.e. have septa in their cellular structures). Also, the Basidiomycetes have clamp connections along their mycelia, readily visible as slight bulges under a low power microscope (Onions *et al.*, 1986).

The classes can be further distinguished by their reproductive structures. Mastigomycetes produce motile spores, requiring free water at some stage in their life cycles. Zygomycetes do not produce motile spores but are similar to Mastigomycetes in that they form asexual spores in a sporangium. Their sexual form of reproduction is by zygospores, formed by the fusion of the sexual organs, the dark-coloured zygospore forming between the organs. The Zygomycetes include the common fungi *Rhizopus* and *Mucor* (Griffin, 1994).

Among the septate fungi, Ascomycetes and Basidiomycetes reproduce sexually and asexually. Sexual reproduction by the Ascomycetes is by means of ascospores, which in mycelial fungi are borne internally in special structures called ascomata, which are composed of specialised mycelia. The yeasts in the class Ascomycetes bear their asci in simpler structures, usually in multiples of two. This class contains the largest number of species, estimated at 28,600, including *Saccharomyces* and *Hansenula* species (Griffin, 1994). The Basidiomycetes reproduce sexually by means of basidiospores. In this case, the spores are borne externally on basidia. Basidia can be complex structures, like mushrooms and toadstools, or simple like in rusts or smuts. This class includes the visible mushroom and toadstool species and plant parasites (Griffin, 1994). Both the Ascomycetes and Basidiomycetes can reproduce asexually by producing conidia on specialised hyphae called phialides.

The remaining class of septate fungi, the Deuteromycetes, reproduce only asexually. They reproduce by means of conidia, which are borne externally on hyphal tips and not within a reproductive structure. Many of the fungi in this class are related to those in the Ascomycetes and Basidiomycetes, but with no known sexual stage of reproduction. This class includes the common genera *Aspergillus*, *Penicillium* and *Trichoderma* among others (Griffin, 1994).

The fungi are important in nutrient cycles in the biosphere, acting as primary agents of decay of dead plants, animals and microbes (Griffin, 1994). Other fungal species are parasitic, and cause diseases in plants and animals. Their detrimental effects, as well as direct attack, also include the release of toxins (Onions *et al.*, 1986). However, fungi can also produce beneficial compounds of food and pharmaceutical value. Alcoholic drink production is carried out with the help of yeasts. Genetically engineered yeasts have been utilised to improve the quality of beers and wines (Hartmeier and Reiss, 2001). Other foods have also benefited from fungal biotechnology. The use of yeast in bread has been known since ancient times, and in modern processes environmental conditions and the strain of yeast have been

manipulated to improve flavour and texture of the bread. Yeasts have also been long used in the production of soft cheeses, while moulds have been extensively used in cheese production, for the ripening of blue and white mould cheeses (Jakobsen *et al.*, 2001). A more obvious example of food production by fungi is that of mushrooms, which are the reproductive structures of Basidomycete fungi.

Other compounds produced by fungi include organic acids, vitamins, plant hormones and antibiotics, and useful enzymes, such as cellulases, proteases, invertase, amylases and amyloglucosidase (Onions *et al.*,1986). The antibiotics are a prime example of fungal products which have revolutionised medicine since their discovery. The β-lactam and non β-lactam antibiotics have been the most important chemotherapeutic method of fighting bacterial infections since the discovery of penicillin in the early 20^{th} century. Biotechnological advances have helped produce novel antibiotics, which are important due to the emergence of antibiotic resistant bacteria (Schmidt, 2001, Anke and Erkel, 2001).

Environmental biotechnological uses of fungi have also been extensively studied, including biodegradation by the white- and brown-rot fungi, and metal biosorption by fungal biomass. The white-rot fungi have been utilised in the decolourisation of highly coloured, recalcitrant and toxic wastewaters from industries such as paper mills, textile mills, coal mines and coal-burning electricity stations. They can also biodegrade xenobiotic compounds such as chlorinated aromatics, which otherwise present a serious disposal problem (Ralph and Catcheside, 2001). The brown-rot fungi also have been shown to biodegrade xenobiotic chemicals, including polyaromatic hydrocarbons and halogenated antimicrobial compounds (Bagley and Richter, 2001). Biosorption of heavy metals from wastewater has been demonstrated in a wide range of fungi, including yeast and mycelial species (Zimmermann and Wolf, 2001).

1.1.1 *Trichoderma* species

Trichoderma fungi are filamentous fungi of the class Deuteromycetes. The species identification of *Trichoderma* fungi is difficult, due to the morphological similarity between the species. Several studies have been carried out to define the characteristics of individual species (Rifai, 1969, Bissett, 1984, 1991a, 1991b & 1991c). The classification described by Rifai has been widely used, in which nine 'species aggregates' were described. In recent years, species of *Trichoderma* have been described by molecular fingerprinting, to distinguish the species, although all species aggregates have not been studied in this manner (Gams and Meyer, 1998). Morphological characteristics remain the main method for identification and verification of species in the genus *Trichoderma* (Gams and Bissett, 1998). The *Trichoderma* have been shown to be closely related to the Ascomycete genera *Hypocrea*, *Podostroma* and *Sarawakus* by molecular and morphological investigations. In these genera, the asexual mode of reproduction is analogous to *Trichoderma* species (Gams and Bissett, 1998).

Trichoderma fungi are very widespread in soils and on decaying plant material, and are found in diverse habitats from the Antarctic to the tropics (Nevalainen and Neethling, 1998). This can be attributed to their diverse metabolic capabilities. They are capable of thriving under minimal nutritional conditions, generally grow very rapidly, and, under the correct conditions, they sporulate profusely. A wide variety of natural and xenobiotic compounds are metabolised by *Trichoderma* fungi, including hydrocarbons, sugars, polysaccharides, complex plant materials and even pesticides. The most readily utilised carbon sources include glucose, fructose, mannose, galactose, xylose and cellobiose (Kubicek-Pranz, 1998). A range of hazardous xenobiotic compounds can be biodegraded by *Trichoderma* species, including pesticides such as Arachlor, Malathion, Dalapon, DDT, dieldrin and pentachlorophenol (Katayama and Matsumura, 1993).

They are known to produce a vast array of secondary metabolites, including numerous antibiotics (Sivasithamparam and Ghisalberti, 1998). Other interesting molecules produced by *Trichoderma* include antitumour compounds (Itoh *et al.*, 1982, Gao *et al.*, 1995), plant growth regulators (Dickinson *et al.*, 1989, Merlier *et al.*, 1984) and HIV inhibitors (Qian-Cutrone *et al.*, 1996).

Trichoderma fungi are particularly well known for secreting large amounts of polysaccharases (Elad *et al.*, 1982, Klein and Eveleigh, 1998, Kubicek-Pranz, 1998, Vikineswary *et al.*, 1997). *Trichoderma* β-glucanases have found applications in wine, beer, olive oil and animal feed industries. In brewing, they are used in beer wort production and were found to be superior to those of *Penicillium emersonii*, *Aspergillus niger* and *Bacillus subtilis* (Galante *et al.*, 1998). They improved the efficiency of processing in the brewhouse. In wine production, they have improved the efficiency of clarification by hydrolysing glucans which can cause clarification and filtration problems (Galante *et al.*, 1998). During olive oil production, the addition of *Trichoderma* glucanases and hemicellulases have improved fruit maceration, increasing the efficiency of the oil production process (Galante *et al.*, 1993). In the animal feed industry, *Trichoderma* xylanases and β-glucanases have been added to improve feed conversion rates in poultry. Grain crops present in the feed contain significant levels of polysaccharides, such as β-glucans and pentosans. These are not easily digested by the poultry and slow the absorption of nutrients. The addition of the enzymes to digest the polysaccharides reduces intestinal viscosity and improves the release of nutrients from the grain (Galante *et al.*, 1998).

Although *Trichoderma* are general saprophytes, they can attack other fungi. This phenomenon is known as mycoparasitism (Hjeljord and Tronsmo, 1998). This characteristic is exploited in their use as biocontrol agents in agriculture and horticulture. When *Trichoderma* fungi are applied to crops, they can specifically attack a particular species of plant parasite. This occurs in several stages. Firstly,

Trichoderma is attracted by a chemical stimulus from the target fungus. The target is recognised by specific markers on the cell wall. The *Trichoderma* then attaches to the host and coils its hyphae around it, followed by the secretion of lytic enzymes to degrade the host's cell wall (Chet, 1990). Other mechanisms of fungal destruction by *Trichoderma* include the production of antibiotic secondary metabolites, as mentioned above, and competition for nutrients (Hjeljord and Tronsmo, 1998). Although this antifungal activity has been shown to be relatively specific, several species of pathogenic fungi are susceptible to attack by *Trichoderma* species. These include *Pythium ultimum*, which causes root rot in peas and potatoes, *Rhizoctonia solani*, a cause of rotting diseases in numerous plant species, *Sclerotium rolfsii*, an infectious disease of legumes, *Botrytis cinerae*, which appears as moulds on bean and pea pods, and *Fusarium* parasites of cereal crops (Hjeljord and Tronsmo, 1998).

Only one example of a *Trichoderma* fungus used in waste treatment has been found in the literature (Vikineswary *et al.*, 1997). In this case, the substrate was a low-oil sludge from a palm-oil processing mill. The organism grew effectively on the sludge and, in combination with *Myceliophthora thermophila*, treated the waste satisfactorily. Lipid accumulation has been studied in *Trichoderma reesei* (Brown *et al.*, 1988, Brown *et al.*, 1990, Brown and Thornton, 1993) and in *T. harzianum* and *T. viride* (Serrano-Carreon *et al.*, 1992). These studies were carried out on simple carbohydrates like glucose and sucrose. However, the biodegradation of lipids by the genus *Trichoderma* has not previously been studied, and no examination of the growth of the fungi on a lipid carbon source has been found.

Trichoderma are generally safe fungi, and are not considered pathogenic to humans or animals. There have been exceptional circumstances where immunosuppressed patients have contracted *Trichoderma* infections, but these cases are very rare. *Trichoderma* species, when used as biocontrol agents, have not caused major adverse affects to animals or humans exposed to them. Liquid fermentations do not release significant amounts of spores into the environment around them, making them

suitable for inclusion in a waste treatment bioreactor (Nevalainen and Neethling, 1998).

1.2 Lipids

Lipids are biomolecules containing as part of their structure, fatty acids or closely related compounds. The types of molecules included under this definition include glycerides, sterol esters, wax esters, phospholipids, glycosylglycerides and sphingolipids. They are composed of fatty acids bound by ester bonds to alcohols. When the alcohol involved is glycerol, the most common alcohol in lipids, glycerides, phospholipids and glycosylglycerides are formed. Other alcohols found less commonly in lipids include sterols in sterol esters and long chain alcohols in wax esters (Gunstone, 1996).

The lipids form an essential component of living cells. Lipids, such as phospholipids, sterol esters, glycosylglycerides and sphingolipids occur as structural lipids, forming cellular and organelle membranes They essentially hold biological cells together, allowing life to exist (Mathews and Van Holde, 1990). Lipids also act as energy storage molecules. The major reserve lipids in living organisms are the glycerides, mainly triglycerides (Figures 1 & 2), with mono- and diglycerides present as minor components (Gunstone, 1996).

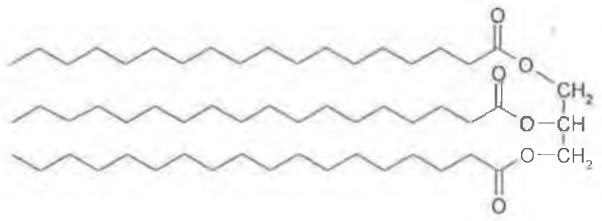

Figure 1: Tristearin, a saturated triglyceride (Mathews and Van Holde, 1990).

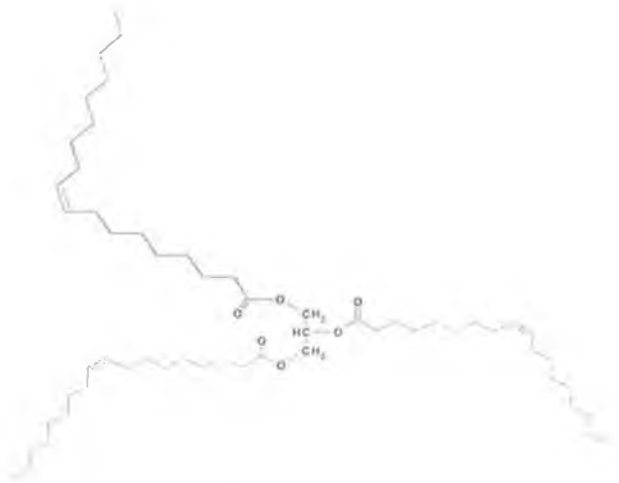

Figure 2: Triolein, a triglyceride containing unsaturated fatty acids, a major component of olive oil (Gunstone, 1996).

Glyceride lipids can exist as oils or fats, depending on the source from which they are extracted (Table 1). They are the most important lipids from a waste treatment perspective. Lipids which are solid at normal temperatures are referred to as fats, while those which are liquid are oils (Metcalf and Eddy, 1991). The constituent fatty acids determine the chemical and physical characteristics of the lipid. Animal fats consist mainly of saturated fatty acids, or with limited amounts of mono- and polyunsaturated fatty acids. Saturated glycerides pack very well together, resulting in stronger intermolecular forces, and hence the high melting point of fats. Oils differ from fats in that they contain mainly unsaturated fatty acids. The double bonds in unsaturated fatty acids are usually in the *cis* rather than the *trans* form, leading to larger molecules, which pack poorly together (Figure 2). This leads to weaker forces between molecules, and lower melting points (Morrison and Boyd, 1992).

Table 1: Major oils and fats (Gunstone, 1996)

Category	Examples
Plant sources	
vegetable oils	Soybean oil, cottonseed oil, rice bran oil, corn oil, rapeseed oil, sunflower oil
tree crop oils	olive oil, palm oil, coconut oil
industrial oils	Linseed oil, castor oil, tall oil
Animal sources	
land animals	Tallow, lard, milk fat
marine animals	Fish oil

Fats are usually of animal origin, terrestrial animals producing the hard fats such as the tallows and lard. The hardest fats, as measured by iodine value, are beef and sheep tallows (Grummer, 1992). Natural fats consist mainly of triglycerides, with lower levels of diglycerides, monoglycerides, free fatty acids, sterols, sterol esters, tocopherols and trace amounts of other components (Gunstone, 1996). Non-lipid material, in the form of moisture, impurities and unsaponifiables, can also be present at up to 4% in tallow, depending on its purity grade (Grummer, 1992). The proportions of individual fatty acids in fat glycerides vary depending on the type of fat (Table 2). In lard and beef tallow, saturated fatty acids account for approximately 40% of total fatty acids, while in mutton tallow, this can be as high as 65%. The most significant saturated fatty acids present are palmitic and stearic acids. Oleic acid is the major unsaturated fatty acid in all the fats, while linoleic also makes a significant contribution in lard, and palmitoleic appears in higher levels in beef tallow.

Table 2: Typical component fatty acids of animal fats by weight percent. Notation: 14:0 = myristic acid; 16:0 = palmitic acid; 18:0 = stearic acid; 16:1 = palmitoleic acid; 18:1 = oleic acid; 18:2 = linoleic acid. (Gunstone *et al.*, 1994)

Source	14:0	16:0	18:0	16:1	18:1	18:2	other
Lard	2	27	11	4	44	11	1
beef tallow	3	27	7	11	48	2	2
Mutton tallow	6	27	32	2	31	2	0

1.3 Lipid utilisation by microorganisms

As mentioned previously, triglyceride lipids come in the form of oils or fats, depending on their fatty acid constituents. Due to their abundance, their biodegradation by microorganisms has formed an essential subject for study. As seen in many of the studies, it is not sufficient that the microorganisms have lipolytic ability, but must also be efficient metabolisers of fatty acids. This ability is variable from species to species, some even displaying the effect of a toxic reaction to fatty acids.

1.3.1 Microbial utilisation of oils

Triglyceride oils are relatively easily utilised by a wide range of organisms and have been the subject of numerous studies. Oils are generally more easily assimilated than fats, due to their liquid nature at mesophilic temperatures, presenting an extensive surface for microbial enzyme attack. The oils studied are easily obtainable vegetable oils, offering a cheap substrate for experimentation.

Sunflower oil has lended itself to being easily metabolise by microorganisms. The fungus *Mucor circinelloides* f. *circinelloides* assimilated sunflower oil, both as sole

carbon source, and in the presence of another carbon source, sodium acetate (Jeffery *et al.*, 1999). The uptake was greatly enhanced with the additional carbon substrate, with the oil almost completely assimilated by the fungus. Other fungi from the genus *Mucorales* grew in the presence sunflower oil during the production of gamma linoleic acid (GLA), as studied by Čertík *et al.* (1997). All 48 strains examined assimilated the oil, accumulating intracellular lipid in the process.

Bacterial isolates of the genus *Acinetobacter* were found to be capable of hydrolysing sunflower oil, olive oil and synthetic triglycerides (Chappe *et al.*, 1994). The organisms, all strains of *Acinetobacter calcoaceticus*, had varying abilities to oxidise the fatty acids resulting from the hydrolysis of the triglycerides.

Olive oil, used by Chappe *et al.* alongside sunflower oil, is another abundant source of triglyceride oil. Under thermophilic conditions, the bacterium *Bacillus thermoleovorans* digested more than 90% of olive oil fed in continuous culture fed at up to 1g $l^{-1}h^{-1}$ (Becker *et al.*, 1999). When applied to wool scouring wastewater, this organism also successfully removed lipid material from continuous culture.

Soyabean oil has formed the centre of many studies. Its utilisation was studied by Koritala *et al* (1987). The range of organisms studied by these researchers included bacteria, actinomycetes, filamentous fungi and yeasts. The fungi showed the most extensive hydrolysis and removal of the oil, with some species completely hydrolysing the triglycerides. Bacteria and actinomycetes were less successful, with little removal in a lot of species, and no hydrolysis evident in the extract. Shabtai (1991) isolated a *Pseudomonas aeruginosa* strain capable of surviving under conditions with 99% soyabean oil. Other cultures grown under these conditions failed to maintain 100% viability, but some other *Pseudomonas* species and a strain of *Acinetobacter calcoaceticus* were capable of limited growth.

Various other oils have been utilised in numerous studies. A yeast capable of effectively assimilating palm oil was isolated from soil by Koh *et al.* (1983). The yeast, *Torulopsis candida*, was the only strain, of over 200 they isolated from enrichment culture, that was capable of growing on palm oil as carbon source. Useful by-products were found in the biomass of the fungus *Mortierella alpina* when grown on fish oil (Shinmen *et al.*, 1992). Polyunsaturated 20 carbon fatty acids accumulated in the biomass of the fungus, which may make it useful for animal feeds, in particular fish food. Aggelis *et al.* (1997) successfully grew *Rhodotorula* sp., *Candida tropicalis, Candida lipolytica, Candida cremoris* and *Langermania gigantea* on evening primrose oil, a polyunsaturated oil. Haba *et al.* (2000) found that several *Pseudomonas* species grew successfully on olive oil and waste frying oils. Although 36 species of microorganisms were found to grow on the oils, the *Pseudomonas* species were the most productive in terms of biomass. As a by-product of this growth, these bacteria produced considerable quantities of biosurfactant, the focus of the study.

Four organisms were found to grow on a range of oils, as well as waste grease from a restaurant, by Wakelin and Forster (1997). *Rhodococcus rubra, Nocardia amarae, Microthrix parvicella* and *Acinetobacter* sp. grew at varying degrees on oil substrates in a study to develop an inoculum for a bioreactor to treat lipid containing waste. *Acinetobacter* sp. proved to be the most effective of the isolates studies, removing up to 67% of oil added at 8g/l to the culture.

The accumulation of lipid by four fungi grown on various oils was studied by Kendrick and Ratledge (1996) They found that *Condiobolus nanodes, Entomophthora exitalis, Mortierella isabellina* and *Mucor circinelloides* grew well on most of the oils tested, accumulating various quantities of intracellular lipid during growth. During a study of lipase activity by *Fusarium solani*, Maia *et al.* (2001) successfully grew the organism on a range of oils as sole carbon source.

1.3.2 Microbial utilisation of fats

Triglyceride fats are generally derived from terrestrial animals, the most abundant being lard, from pigs, and beef and mutton tallows. Due to their solid nature at mesophilic temperatures, they have proved to be interesting subjects for study of microbial attack on lipids. Organisms have been described capable of growth on fats as carbon source, although the area has been less extensively studied than the utilisation of oils.

Tallow has been the subject of several studies, although it has not always been classified as beef or mutton tallow. The growth of three fungi directly on animal fats and in batch cultures with fats as sole carbon source was studied by Bednarski *et al.* (1993). In these studies, other fats were examined with tallow. In batch cultures containing 30g/l beef tallow or poultry fat, the highest degree of utilisation was observed on the poultry fat by the fungus *Geotrichum candidum,* which removed 49% of the added fat. The other fungi studied, *Aspergillus niger* and *Mucor miehei*, had slightly lower utilisation rates, but still grew successfully in the fat-containing media. Using surface culture on the fats, the greatest utilisation was observed on poultry tallow, with *Geotrichum candidum* again being the highest utiliser. It degraded 70% of the fat. Again the other fungi studied had lower abilities to metabolise the fats. It was also noted in this study that poultry fat was more easily digested than beef tallow.

The yeast *Saccharomycopsis lipolytica* was studied on both olive oil and animal fat by Tan and Gill (1984 & 1985). It hydrolysed and grew effectively on olive oil and showed high rates of tallow and lard utilisation. The concentrations of lipid used in these studies were, however, relatively low. The same researchers also carried out studies with the bacterium *Pseudomonas fluorescens* (Tan and Gill, 1987). Although growth occurred, degradation of olive oil, lard and tallow was less effective than in their previous studies with the yeast.

Other studies have used fats as carbon sources, but were not the primary centre of the studies. Cromwick *et al.* (1996) described the production of a potentially valuable by-product from *Pseudomonas* strains growing on tallow and tallow fatty acids. The organisms produced poly(hydroxyalkanoates), a class of naturally occurring polyesters, in batch cultures containing 2-3g/l tallow or tallow fatty acids. In a two-phase aqueous-organic system, *Pseudomonas putida* was found to achieve up to 80% hydrolysis of beef tallow, and complete hydrolysis of olive oil as substrate (Kim and Rhee, 1993). Kajs and Vanderzant (1981) successfully produced single cell protein from tallow using the yeasts *Saccharomycopsis lipolytica* and *Candida utilis*, although the degree of utilisation or hydrolysis were not reported. El-Sharkawy *et al.* (1993) found that the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces octosporus* hydrolysed lard during their studies on the lipases of these species.

The co-metabolism of fats with simpler carbohydrates has been noted in other studies. Marek and Bednarski (1996) grew the yeast *Yarrowia lipolytica*, and the filamentous fungi *Aspergillus niger, Galactomyces geotrichum* and *Rhizomucor miehei* on several fats and oils in the presence of glucose. Co-metabolism of fats was also demonstrated by Kostov *et al.* (1986). Using a mixed culture of *Candida scotii* and a *Candida* sp. previously shown to assimilate fats, the organisms effectively assimilated animal fat and sugars from continuous culture.

1.4 Biodegradation of lipids by fungi

The biodegradation of glyceride lipids consists of two steps – lipase catalysed hydrolysis of glycerides and the metabolism of the resulting fatty acids. Different enzymes systems are responsible for each step, so the ability of an organism to carry out one stage of the metabolism efficiently does not confer on it the ability to carry out the other stage as successfully. The first of these stages, the lipase catalysed hydrolysis of glycerides has been studied in detail by numerous researchers.

1.4.1 Lipase catalysed hydrolysis

The biodegradation of lipids begins by their hydrolysis by lipases, releasing free fatty acids and glycerol (Figure 3). This occurs extracellularly, as glycerides are high molecular weight molecules which cannot easily penetrate the cell wall or membrane. This breakdown to the more easily assimilated molecules of fatty acids is necessary before the organism can utilise the substrate for energy and growth (Hammer and Hammer, 2001).

Lipases are hydrolytic enzymes which are secreted by a wide array of organisms, including plants, animals and microbes. Microbial lipases can have interesting properties, depending on the organism from which they are secreted. Osmotolerant, thermophilic and psychrophilic lipases have been found (Günter and Schroder, 1992).

Due to the hydrophobic nature of their substrates, lipases act at the lipid-water interphase. This can limit the rate of hydrolysis in solid lipids such as the fats. Lipases remain inactive until it binds to an interphase, which causes a conformational change in the active site of the enzyme. This make the active site accessible to the substrate, allowing hydrolysis to be carried out (Brzozowski *et al.*, 1991, Gill and Parish, 1997). As a result of this mechanism, lipase will only work effectively on substrates which are insoluble in water.

Lipase production has been shown to be induced by the presence of extracellular lipids. Lipase of *Mucor hiemalis* was induced when oil was added to glucose medium (Akhtar *et al.*, 1983). The addition of olive oil to microbial medium was shown to increase lipase activity by the yeast *Candida rugosa* (Benjamin and Pandey, 1996). Marek and Bednarski (1996) demonstrated that oils and fats can induce activity in yeasts and fungi. A range of oils were shown to induce extracellular activity in *Fusarium solani* by Maia *et al.* (2001). The extent of lipase production varied depending on the oil used. This phenomenon was also described in early studies, which showed that lipase activity and production by *Rhizopus* fungi depended on the nature of the lipid present (Akhtar *et al.*, 1977). Sugiura *et al.* (1975) demonstrated that fatty acids can themselves induce lipase production in *Candida paralipolytica*, although the repression of lipase from *Pseudomonas aeruginosa* by oleic acid was demonstrated by Gilbert *et al.* (1991). Activity of lipase has also been influenced by other substances in the medium. *Candida rugosa* lipase has been shown to increase in the presence of sodium and calcium ions by Hernáiz *et al.* (1994).

Inhibition of lipase has also been extensively studied. High levels of simple carbohydrates have been shown to reduce activity (Marek and Bednarski, 1996). It was observed that low levels of glucose added to cultures of *Yarrowia lipolytica* and *Rhizomucor miehei* with lipid also present, increased the lipase activity, while further addition resulted in its repression. It was suggested that this pointed to lipase regulation by catabolic repression. The lipase of *Candida rugosa* was shown to be inhibited by 70% by the fatty acids produced by hydrolysis of beef tallow (Virto *et al.*, 1995). Fatty acids, alcohols and bile salts were shown to inhibit the activity of *Humicola* lipase when present in solution alone, but on addition of calcium the activity was restored. It was hypothesised that this inhibition may have been due to displacement of triglyceride substrate from the lipid-water interphase, limiting the activity (Liu *et al.*, 1973). Lipase was produced by *Candida lipolytica* in the presence of sodium dodecyl sulphate, but showed no activity in culture (Nascimento and Campos-Takaki, 1994).

Lipases can preferentially hydrolyse certain fatty acids, or display positional specificity. El-Sharkawy *et al.* (1993) studied the specificities of lipases from *Saccharomyces cerevisiae* and *Schizosaccharomyces octosporus*, and found both expressing fatty acid specificity. This phenomenon has been exploited to hydrolyse specific fatty acids from fats containing mixtures of fatty acids. The lipase of *Geotrichum candidum* was used by Baillargeon and Sonnet (1991) to preferentially hydrolyse the *cis*-9 unsaturated fatty acids, oleic acid, from tallow, which also contains significant amounts of the saturated fatty acids, stearic and palmitic acid. Specificity for shorter chain fatty acids was shown in *Candida antartica* lipase, which preferentially hydrolyses butyric acid in place of longer chain fatty acids such as palmitic acid (Villeneuve *et al.*, 1995). Stereoselectivity of a range of microbial lipases was demonstrated by Rogalska *et al.*(1993).

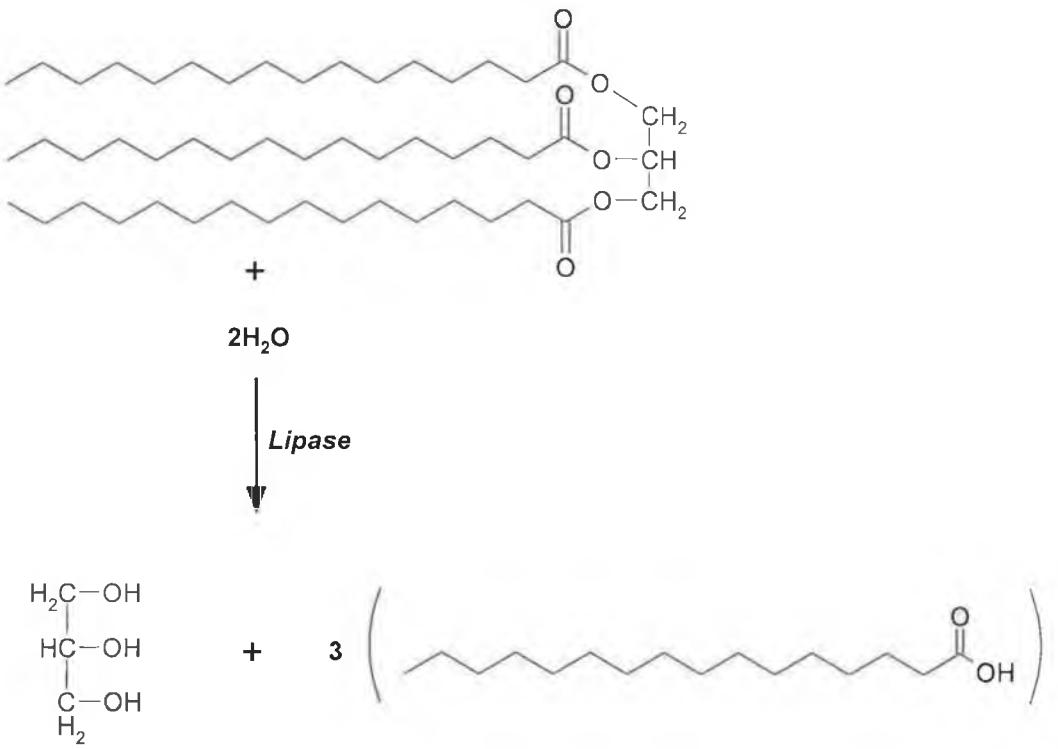

Figure 3: Hydrolysis of tripalmitin by lipase (Kallel *et al.*, 1994)

1.4.2 Metabolism of fatty acids released by hydrolysis

Once released by lipase-catalysed hydrolysis, fatty acids are assimilated by the microbial cells. The uptake of fatty acids by yeast and fungal cells is assumed to be non-specific (Kajs and Vanderzant, 1981, Ratledge, 1992). Once the fatty acids enter the cell, several fates can await them. They can be incorporated into lipid structures of the cell or stored lipid, either unchanged or following desaturation or elongation, or they can be oxidised via β–oxidation for energy and growth (Čertík *et al.*, 1997, Wakelin and Forster, 1997). Of these, β-oxidation is the pathway to complete degradation of the lipid material.

β-oxidation

β-oxidation involves the release of a series of 2-carbon acetyl-CoA units from the fatty acid molecules (Mathews and van Holde, 1990, Ratledge, 1992). Firstly the free fatty acid is bound to coenzyme A to form a fatty acyl-CoA. This molecule then undergoes a series of reactions resulting in the release of Acetyl CoA, and shortening the fatty acyl-CoA by 2 carbons (Figure 4). This shortened molecule then goes through another cycle of reactions, which is repeated until 2 molecules of acetyl CoA are released by the final cycle. This cycle of breakdown is applicable to saturated fatty acids. Unsaturated fatty acids, however, require additional steps for successful oxidation. When the double bond is encountered, firstly isomerisation occurs, if necessary, to produce a trans-double bond, which is then reduced to eliminate the double bond. The resulting saturated molecule can then be broken down by β-oxidation in the normal way. When this saturation occurs has been questioned, but according to Lalman and Bagley (2000 & 2001), it need not occur before β-oxidation commences. Intermediates of breakdown of unsaturated long chain fatty acids are sometimes themselves unsaturated, not being saturated before β–oxidation commenced. Some intermediates of breakdown, in particular saturated fatty acids

such as palmitic and myristic acids, may accumulate, due to their inhibition of their own breakdown.

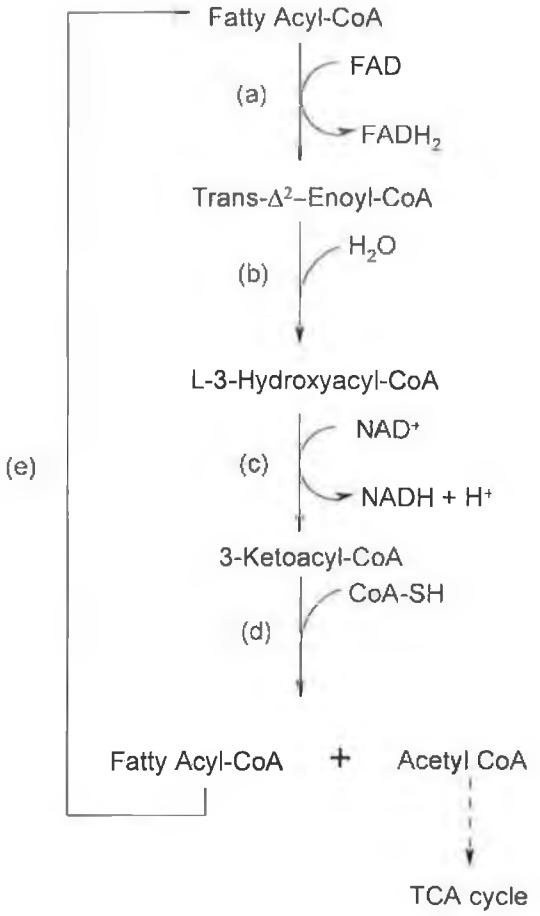

Figure 4: β-oxidation of a fatty acyl-CoA, resulting in the release of Acetyl CoA and a shortening of the fatty acyl-CoA by 2 carbons. The sequence of reactions are (a) dehydrogenation by fatty acyl CoA oxidase; (b) hydration by trans-2,3-enoyl-CoA hydratase; (c) dehydrogenation by L-3-hydroxyacyl-CoA dehydrogenase and (d) thiolytic cleavage by 3-oxoacyl-CoA thiolase. The shortened fatty acyl-CoA then starts the reaction again (e) until in the final cycle, 2 molecules of acetyl CoA are released. (Mathews and Van Holde, 1990)

Bioconversion

As an alternative pathway to complete oxidation, bioconversion of long chain fatty acids to other more useful fatty acids has been studied by several researchers. Aggelis *et al.* (1991) described the conversion of linoleic acid from sunflower oil to γ-linolenic acid (GLA) by *Mucor circinelloides*. GLA is a fatty acid which has uses in pharmaceutical and cosmetic industries. Linoleic acid, hydrolysed from the sunflower oil extracellularly, was taken into the cell, where the fatty acid not oxidised for biomass production was taken into reserve lipids. Desaturase enzymes could then act on the linoleic acid, producing GLA. The apparent conversion of linoleic acid to GLA was also observed in a range of Mucorales fungi (Čertík *et al.*, 1997). Kamisaka *et al.* (1990) studied the phenomenon in detail, using radiolabelled linoleic acid. They found that the radiolabelled carbon was incorporated in numerous lipid classes (triglycerides, phosphatidylcholine, phosphatidylserine), but when linoleic acid was desaturated to GLA, the GLA predominantly accumulated in triglycerides.

Conversion of substrate fatty acids to saturated fatty acid products has also been observed. *Yarrowia lipolytica* has demonstrated the ability to accumulate lipid intracellularly which is rich in stearic acid, despite being grown on lipid sources with a mixture of long chain fatty acids (Papanikolaou *et al.*, 2001).

Elongation of fatty acids has also been observed intracellularly (Kendrick and Ratledge, 1996, Kajs and Vanderzant, 1981). Radwan and Soliman (1988) demonstrated fatty acid chain elongation in fungi grown on C8 to C18 fatty acids. Generally, these studies have been with the lipid source as a secondary carbon source, with glucose or simple carbohydrates as the primary source. Kendrick and Ratledge (1996) have suggested that desaturase and elongase enzymes have been repressed when the sole carbon source is an oil or fat, resulting in more limited bioconversion in the biomass. Therefore the resulting intracellular lipid resembled the original substrate in fatty acid composition.

Accumulation

The accumulation of intracellular lipids by microorganisms has been observed in numerous species of microorganisms. Microorganisms displaying the ability to store reserve lipids as a significant percentage of their biomass are termed oleaginous microbes. Oleaginous microorganisms are generally algae, yeasts or filamentous fungi, although some bacteria can accumulate lipid-like material, like waxes (Řezanka, 1991). These stored lipids can have interesting properties, like high concentrations of saturated fatty acids, or fatty acids with commercial uses, like GLA. These characteristics vary from species to species, and depend on the substrate on which the organism grows. They can also be a result of failure or reduced ability of the organism to metabolise a certain fatty acid by β-oxidation, having taken it into its cells.

Many of the studies on lipid accumulation concentrate on lipid production by organisms when grown on non-lipid substrates (e.g. Aggelis, 1996, Aggelis and Komaitis, 1999, Chen and Chang, 1996, Eroshin *et al.*, 2000, Jackson *et al.*, 1998, Saxena *et al.*, 1998, Papanikolaou and Aggelis, 2002). These organisms accumulate intracellular lipid, usually on the exhaustion of a nutrient source (e.g. nitrogen), eventually using the reserve lipid itself as an energy source.

The accumulation of intracellular lipid with a lipid as carbon source has been less studied. The mechanism of lipid accumulation is different with a lipid than a non-lipid substrate. The concentration of reserve lipid with a lipid substrate appears to be independent of nitrogen concentration, and accumulated before substrate or nutrient exhaustion.

Oils have been used in the majority of cases while studying cellular lipid accumulation on lipid substrates. Aggelis *et al.* (1997) examined the uptake of polyunsaturated oil by several species. They found that although the polyunsaturated

oil was readily utilised by the organisms, the accumulated intracellular lipid profile varied from organism to organism. *Candida tropicalis* and *Candida cremoris* preferentially accumulated saturated fatty acids over unsaturated fatty acids, while *Candida lipolytica* and *Rhodotorula* sp. showed no pattern with respect to degree of unsaturation or chain length. The accumulated lipid accounted for up to 40% of cellular dry weights.

Akhtar *et al.* (1983) studied lipid accumulation in *Mucor hiemalis*, showing that the biomass accumulated up to 26% lipid in its biomass. This intracellular lipid increased rapidly during the initial stages of incubation, but depleted as the concentration of the extracellular substrate, olive oil, was consumed. Triglycerides accounted for over 50% of reserve lipids throughout incubation.

Growth of organisms has been shown to affect the composition of intracellular lipid (Kendrick and Ratledge, 1996). In this study the fungi studied accumulated 25-50% of their dry weight as lipid when grown on a range of oils. This intracellular lipid resembled the substrate oil in composition, with the organism displaying a reduced ability to synthesise polyunsaturated fatty acids, such as γ-linolenic acid.

Other researchers have demonstrated the accumulation of intracellular lipid on other oil substrates. When grown on fish oils, almost 40% of the biomass was triglyceride in the fungus *Mortierella alpina* (Shinmen *et al.*, 1992). Jeffery *et al.* (1999) found 45% of the dry weight of *Mucor circinelloides* was lipid when grown on sunflower oil as sole carbon source, but this increased up to 62% in the presence of sunflower oil with sodium acetate. Fungi from the genus Mucorales were found to accumulate 42-66% lipid in their biomass, when grown on sunflower oil (Čertík *et al.*, 1997).

Mathematical models for prediction of lipid accumulation have been proposed by Aggelis *et al.* (1995a & 1995b). Using *Mucor circinelloides* as a model oleaginous organism, lipid accumulation was linked to biomass growth and the concentration of

extracellular lipid. The organism accumulated lipid until the extracellular substrate, sunflower oil, was exhausted, after which degradation of the reserve lipid commenced. At its peak, 27% of the dry weight was made up of intracellular lipid. In the second part of this work, individual fatty acids were studied, and the model applied. The authors proposed that the fate of individual fatty acids (i.e. biotransformation or accumulation) could be determined mathematically. A later study proposed another model based on the data from these experiments (Aggelis and Sourdis, 1997). However, these models are not satisfactory for the description of intracellular lipid accumulation when animal fats are used as substrate (Aggelis, *personal communication*, 2002).

Very little work has been published on intracellular lipid accumulation using fat substrates. Growth of fungi on animal fats by Bednarski *et al.* (1993) revealed that the accumulation of intracellular lipids also occurs in these cases. In this study, it was found that the accumulation varied with the species of fungus, method of cultivation and the source of carbon used. The fatty acid composition of intracellular lipid was also greatly affected by the composition of the substrate.

Using an industrial fat as sole substrate for *Yarrowia lipolytica*, Papanikolaou *et al.* (2001) found that the yeast could accumulate up to 44% of its dry weight as intracellular lipid. This accumulated fat was mostly composed of saturated fatty acids, while unsaturated ones appeared to be consumed for growth and structural incorporation into the cell. It was suggested that the resulting intracellular lipid had a fatty acid composition similar to cocoa butter, and may be suitable as a substitute for it.

The incorporation of fatty acids from the medium into biomass was studied by Aoki *et al.* (1999). It was found that polyunsaturated fatty acids from 18 to 22 carbons in length, presented separately in cultures, were incorporated into intracellular triglycerides of *Mucor hiemalis*. When a mixture of saturated and unsaturated fatty

acids was presented to the organism, all fatty acids were utilised for growth, but saturated and monounsaturated fatty acids accumulated preferentially in intracellular lipids.

The phenomenon of lipid accumulation is important from a waste treatment point of view, as it lowers the density of the biomass, decreasing its ability to settle out in clarifiers. Also, biomass that is separated, would have a high lipid content that still requires disposal. Ideally, in a waste treatment environment, the accumulation of intracellular lipid should be as low as possible to avoid these complications.

1.5 Lipid in liquid waste streams

Fats, oils and greases present a major disposal problem in our society. They come from a wide range of sources, all contributing to the pollution load in wastewater treatment plants, both municipal and industrial. Typically fats, oils and greases are present in domestic wastewaters in the range of 50 to 150mg/l, accounting for up to 30% of COD (Hammer and Hammer, 2001). Faeces contains up to 23% lipid, making up a large part of this domestic waste lipid load (Mahlie, 1940). In industrial wastewaters, they can be present at levels of 500mg/l and higher. The types of industry that may produce such wastewater are numerous: dairy, rendering operations, oil and fat refining, slaughterhouses, meat packing and vegetable oil production industries. Restaurant and food preparation industries also generate large amounts of waste fat and oil from general kitchen activities (Bednarski *et al.*, 1993, Bridoux *et al.*, 1994, Duchène, 1980, Hammer and Hammer, 2001, Lefebvre *et al.*, 1998, Metcalf and Eddy, 1991, Petruy and Lettinga, 1997, Wakelin and Forster, 1997). Other agro-industrial operations can produce larger amounts of lipid waste, such as the wool scouring industry. Wool scouring wastewater has been known to contain up to 10,000mg/l grease (Ang and Himawan, 1994).

Fats and greases, when they enter sewers in waste streams, coat sewer networks and treatment plant piping, decreasing the effective diameter of the pipes and causing pumping problems. This accumulation is more apparent in large sewers and wastewater treatment plants than in small ones, due to the higher volume of wastewater. Also, if lipid material is not removed from the final effluent, it can interfere with biological activity in receiving waters, and is unsightly and aesthetically unpleasant (Metcalf and Eddy, 1991, Bridoux *et al.*, 1994).

Legally, all industries are responsible for ensuring the proper treatment of wastes resulting from their operations. Emissions from industries in Ireland are regulated by the Environmental Protection Agency (EPA). The EPA sets maximum levels for fat, oils and grease emissions as a single parameter. This level can vary from industry to industry, depending on the nature of the wastewater, and the ultimate disposal location of resulting effluent. The limits set for discharge into rivers are between 10 and 15mg/l. Fat, oil and grease emissions to marine environments are limited to similar levels, of around 30mg/l. Adhering to these limits is a challenge for these industries, which produce considerable amounts of lipid-containing wastewater.

1.6 Methods of lipid removal from waste streams

Fats are among the most non-degradable oleochemicals encountered in wastewater treatment. In the course of processing, use and disposal of fats and fat-containing products, they find their way eventually into waste streams, and enter wastewater treatment plants, where they have to be removed by physico-chemical or biological means. This presents a problem due to their insoluble nature and their poor biodegradability, inhibiting biological removal, or in the case of physico-chemical removal, by the large quantities recovered (Ratledge, 1992, Bridoux *et al.*, 1994).

1.6.1 Physical and chemical methods of lipid removal

Many wastewater treatment plants use physico-chemical primary treatment for fat removal when necessary, before passing the waste stream to the biological stage. This can be as simple as a fat trap or as complex as a Dissolved Air Flotation (DAF) system.

Fat traps are a low-technology method of removing fat from wastewater flows. Wastewater flows into the trap and passes through a series of baffles to reduce turbulence and increase residence time, causing fat to separate. The fat floats to the top of the unit due to its lower density, while the effluent is taken from the lower clarified liquid. Fat is physically removed periodically from the top of the trap. These units require constant maintenance and the recovered fat presents an additional disposal problem. Poorly maintained units are useless at clarifying wastewater, and contribute to odour and hygiene nuisances. Fat traps are considered by many users to be unsatisfactory for fat removal (Wakelin and Forster, 1997, Ansenne *et al.*, 1992, Duchène, 1980). Attempts have been made to improve conventional designs, but only slight improvements were observed (Chu and Ng, 2000). Addition of flocculants and acids can improve removal, but other chemicals were found to be detrimental to lipid removal (Ang and Himawan, 1994).

DAF systems use both physical and chemical means to remove fats, oils and greases. They operate using a combination of flocculating chemicals, and saturation of all or part of the waste stream with air under several atmospheres pressure (Figure 5). The pressurised stream enters the tank, where fine air bubbles come out of solution at atmospheric pressure. The flocculating agents, such as ferric chloride and polyelectrolytes, are added to form larger particulates which can trap air bubbles more easily. The fine bubbles float to the top of the tank, carrying the particulate aggregates, where they can be removed and collected in a receiving vessel. Variations of DAF utilise air pumped into the tank, or vacuum flotation, when a partial vacuum

is applied to air-saturated wastewater (Metcalf and Eddy, 1991). The addition of unconventional chemicals to waste flow at peak times has also been examined for enhanced fat removal, but requires continuous monitoring of fat concentrations entering the flotation unit (Steiner and Gec, 1992).

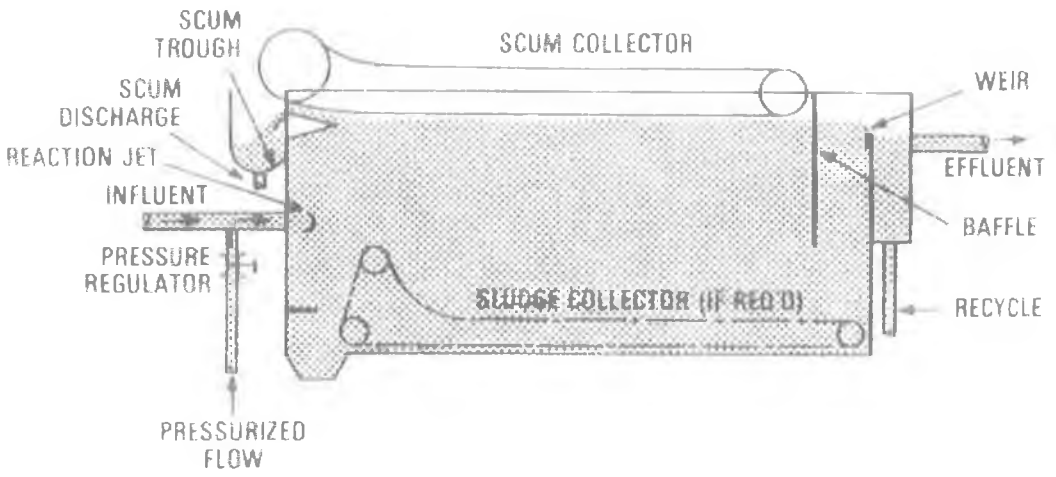

Figure 5: Dissolved Air Flotation (DAF) unit for separating fats, oils and greases from wastewater prior to biological treatment. (M^{c}Dermott, 1982)

Although effective with constant streams, these systems are prone to shock overloading, and can be ineffective with high concentrations of lipids (Chu and Hsu, 1999, Metcalf and Eddy, 1991, Forster, 1992) Also, they produce large quantities of lipid containing sludge, which presents a further disposal problem, and partial glycerides which may be present have emulsification properties, which can interfere with flocculation (M^{c}Dermott, 1982). The recovery of this lipid for re-use is often not practical because of contamination with tissue, soil and faecal matter, so disposal may be the only option (Broughton *et al.*, 1998).

In-line filtration has been used on wastewaters as an alternative to fat traps or DAF units (Bayer, 1996). Diatomaceous earth is used as the filtering agent, and it reduces fats, oils and greases, and hence insoluble BOD associated with them, to low levels. It

has not been used widely in fat wastewater treatment, and produces a high fat sludge which has to be disposed of. Alternative filtration media, such as sand or anthracite, have also been tried, but were even less effective than diatomaceous earth. These systems cannot be effectively backwashed due to the hydrophobicity of fat waste and its tendency to stick to surfaces.

Skimming devices have been used for the removal of lipids in liquid form (M^{c}Dermott, 1982). These devices work by passing a belt, made of a hydrophobic material such as a plastic, through the lipid layer in a settling tank. Hydrophobic materials stick to the belt, and are skimmed off at a collector point above the liquid level. These devices work well with liquid lipid, but in cases with fat, which is solid or semi-solid, the congealed lipids cause problems. The devices have to be operated at high temperatures to be effective.

The physico-chemical methods of fat removal produce a by-product which has to be disposed of. The dumping of lipid wastes to landfill has been banned in many countries, for example, France (Lefebvre *et al.*, 1998), so alternatives for disposal have to be found. In some industries, waste tallow is now incinerated, but the use of incinerators is controversial due to the production of by-products such as dioxins.

1.6.2 Biological removal of lipids

As an alternative to physico-chemical methods, several modes of biological treatment have been utilised. Since dumping of lipid residues is restricted and incineration is controversial, biological degradation is an attractive alternative. Both aerobic and anaerobic systems have been studied for the treatment of lipid-containing wastes. Both types of systems face a challenging substrate when fed with high concentrations of fats. The limited bioavailability of fats can inhibit breakdown. Biodegradation depends on the uptake of primary metabolites (i.e. long chain fatty acids) into the microbial cell, so hydrolysis must happen extracellularly. With fats forming

hydrophobic masses, enzymatic breakdown will be restricted, retarding the release of fatty acids. Long chain fatty acids in turn are relatively hydrophobic, providing further resistance to biodegradation (Hammer and Hammer, 2001, Lefebvre *et al.*, 1998, Loehr and Roth, 1968). However, despite this, both types of systems have been utilised in removing fats, oils and greases from waste streams.

1.6.2.1 Anaerobic biological removal of lipids

Anaerobic digestors have been extensively used in food processing industries. These reactors can be either suspended or attached growth systems. Suspended growth systems can be in the form of a simple digestion reactor, fluidised bed systems, expanded bed systems, upflow anaerobic sludge blanket (UASB) systems, baffled reactors and other variations. Attached growth systems use various types of solid media on which the microorganisms grow, or a variation of suspended growth expanded bed systems, where the biomass is attached to sand or another appropriate medium (Metcalf and Eddy, 1991). In Australia, treatment of wool-scouring wastewater, which contains up to 10000mg/l grease, is carried out by the use of open anaerobic or facultative lagoons. However, this requires large areas of land. They also emit odours and are unsightly, resulting in problems of siting in a suitable location (Ang and Himawan, 1994).

These systems have long retention times of 30-60 days for treatment of waste when used in their simplest form, unheated and unmixed. This long retention time itself creates operational problems, in particular with high fat wastewaters, in which a substantial fat layer can accumulate on the surface of the liquid (M^{c}Dermott, 1982). More advanced systems incorporating mixing and heating to mesophilic or thermophilic temperatures have lower retention times, typically 15 days or less, hence they are called 'high-rate' reactors. This can overcome the problem of a floating fat layer, and the fat remains in a liquid, and more bioavailable state in heated systems (Hansen and Mortensen, 1992, Gallert and Winter, 1999). Broughton *et al.* (1998),

however, demonstrated that although degradation of fats was technically possible in mesophilic anaerobic digestors, thermophilic anaerobic digestors were not very effective for high-fat containing waste. The fat content of the effluent from these systems was unacceptably high for a treated wastewater stream.

The problems of high lipid effluent from the reactors can be caused by a number of phenomena. Hydrolysis of triglycerides may be inhibited in anaerobic systems with high lipid content influent, slowing the biodegradation of the fats further (Vidal *et al.*, 2000). Terashima and Lin (2000) showed a large lag of solid fat removal (50 days) in a batch anaerobic reactor, with 25% of the lipid remaining after 300 days. Laboratory scale experiments with milk fat by Petruy and Lettinga (1997) show that lipid is poorly degraded in expanded granular sludge bed anaerobic reactors, with most of the lipid being absorbed onto the biomass and not being degraded. Only 22% was completely metabolised after 27 days. Coating of the granules in UASB and similar systems inhibits biodegradation and causes some washout of biomass by entrapment (Batstone *et al.*, 1997). This problem was also encountered by Hawkes *et al.* (1995) during operation of pilot scale anaerobic waste treatment systems. Although COD removal was high (from 50% to 80%), other operational problems were encountered, such as the inability of the UASB system to form biomass granules. Other studies attempted to address this problem by modifying the design of reactors, with limited success (Rinzema *et al.*, 1993). Although biomass washout was reduced, floating lipid presented an additional problem.

Even when triglyceride hydrolysis occurs, the products of breakdown, long chain fatty acids, have accumulated in anaerobic systems (Broughton *et al.*, 1998, Hanaki *et al.*, 1981, Loehr and Roth, 1968). Saturated fatty acids appear to be more recalcitrant than unsaturated fatty acids, as seen in the experiments of Lalman and Bagley (2000 & 2001). Palmitic, myristic and stearic acids accumulated for extended periods in reactors, and may have been inhibiting their own oxidation. In experiments with stearic acid as sole carbon source, it was very slowly degraded, with over 50%

remaining after 50 days. Oleic, linoleic and palmitoleic acids were readily converted to saturated fatty acids when presented to the biomass. In all these cases, it appeared that β-oxidation of the fatty acids was inhibited.

Accumulated long chain fatty acids present an additional problem to anaerobic waste treatment systems. They are toxic to the biomass, inhibiting not only their own breakdown, but that of other nutrients in the waste stream. This occurs by the toxic effect they have on methanogenic and acetogenic bacteria (Becker *et al.*, 1999, Hanaki *et al.*, 1981). Repression of milk-fat degradation by inhibition of fatty acid breakdown was demonstrated by Hanaki *et al.* (1981). When milk fat was added to the reactor at 4000mg/l, it was hydrolysed easily but the resulting fatty acids produced apparently retarded their own β-oxidation. Chappe *et al.* (1994) found that long chain fatty acids are toxic to certain microorganisms present in bioadditives, in particular *Bacillus* species. Oleic acid specifically has been shown in numerous studies to be responsible for the failure of treatment systems. In anaerobic fixed-bed systems, it has been shown to be toxic above levels as low as 80mg/l (Alves *et al.*, 2001). Acclimated sludges were more tolerant to long chain fatty acids. Acclimation times were, however, about 100 days before tolerance to oleic acid increased. In suspended biomass systems, the toxic concentration was found to be as low as 30mg/l oleic acid, although stearic acid was found to be safe up to 100mg/l (Lalman and Bagley, 2001). Other studies demonstrated that the type of reactor, and hence the form of the biomass, had an effect on the toxicity of long chain fatty acids (Pereira *et al.*, 2002). It was found that the resistance to oleic acid toxicity was ten times greater in an expanded granular sludge bed reactor than in an anaerobic system with suspended biomass. The toxic effect of oleic acid has also been shown in anaerobic fixed bed systems by Alves *et al.* (2001).

1.6.2.2 Aerobic biological removal of lipids

Aerobic biological removal of lipids has been shown to be more promising. Mendoza-Espinosa and Stephenson (1996) studied activated sludge and a commercial bioaugmentation product in laboratory scale batch experiments. They reported that 70% removal of up to 2.3g/l lard was achieved after 16 days by both activated sludge and the bioadditive cultures, when acclimated to its presence for extended periods of time. No difference in the removal abilities by the activated sludge or the bioadditive was noted.

Wakelin and Forster (1997 & 1998) compared activated sludge and an undefined mixed culture, called MC1, isolated from restaurant grease traps. Pure cultures of *Acinetobacter* sp., *Rhodococcus rubra*, *Nocardia amarae* and *Microthrix parvicella* were also compared with MC1 and activated sludge. The isolates removed from 17% to 67% of 8g/l lipid, depending on the type of oil and the species of organism. In comparison, MC1 removed from 29% to 72%, while activated sludge achieved from 52% to 98% under the same conditions. Further studies on MC1 and activated sludge in a specially designed reactor found that the mixed culture could achieve up to 83% removal of grease from a fast food restaurant, but acclimated activated sludge produced the highest results, with over 90% removal. The system involved was operated in a fill and draw fashion, with influent grease at 20g/l, operated with a 4 day hydraulic retention time. The use of a preliminary biological system with these microbial communities produced a 'single phase effluent' suitable for discharge in sewers, and further treatment in a conventional waste treatment system.

The use of defined mixed cultures in removal of lipid waste was studied by Chigusa *et al.* (1996). They isolated nine yeast species, mostly *Candida* species, from wastewater treatment systems of food manufacturing plants. Using a mixture of the 9 yeast strains, in a reactor fed with wastewater containing an average of 10,000mg/l oil, the effluent concentration of lipid was reduced to about 100mg/l. On treatment of

the resulting effluent in an activated sludge system, this level was reduced to below 2mg/l. Tano-Debrah *et al.* (1999) also used a defined mixed culture, in this case of 15 bacterial isolates. The isolates were not identified, but were determined to be non-pathogenic. The percentage removal of 100g/l lipid varied with pure fat samples from 24% to 73% after 14 days. With a mixture of several fats and oils, manipulation of the environmental conditions by supplementing with additional carbon sources and variation of the pH led to 95% removal.

Grulois *et al.* (1992 & 1993) developed a biological system capable of handling fat-containing wastewater, called the 'Biomaster G' system. The system consisted of an aeration basin into which the wastewater was pumped, and an inoculum of a defined consortium of microorganisms was added. Greater than 80% removal was achieved using the system on a municipal wastewater flow, containing up to 2g/l lipid material.

Hrudey (1981) demonstrated that low concentrations of emulsified lipid could be effectively removed by activated sludge, but above 0.25g lipid day^{-1} g^{-1} mixed liquor suspended solids, the effluent quality deteriorated. The removal of saponified lipid material was demonstrated by Kallel *et al.* (1994) and Lefebvre *et al.* (1998). The lipid was first hydrolysed chemically using sodium hydroxide, followed by introduction of the resulting effluent into activated sludge reactors. This replaced the biological hydrolysis of lipid with a chemical process. Kallel *et al.* studied the saponification of the lipid material in a wastewater treatment plant. With an influent lipid concentration corresponding to 2 to 3g/l.d COD, effluent COD after saponification and activated sludge treatment was less than 1g/l. Lefebvre *et al.* found that at laboratory scale, saponification of lipid from domestic wastewater increased its biodegradation by activated sludge to greater than 98%, of an initial concentration of 0.4g/l. Without saponification, removal was 87%. These studies reported advantages of pre-saponification of lipid residues, such as larger contact surface for the biological stage of treatment and easier handling of lipid material. However, foaming problems were also noted.

The ability of several commercial bioadditives to biodegrade lipid was studied by Chappe *et al.* (1994). Using 50g/l emulsified olive oil as the substrate, none of the bioadditives were capable of completely removing the added lipid, although the percentage removal rates were not reported. Also, they made the observation that Gram positive bacteria were inhibited or destroyed by the presence of free fatty acids.

The effective removal of lipid from olive-oil containing wastewater by activated sludge was described by Velioğlu *et al.* (1992). Using laboratory scale activated sludge systems, 70% removal of up to 1.2g/l lipid was noted when operated at a steady state. The wastewater was fed at 0.25l/day, into a reactor of 2l volume. However, the wastewater feed contained other nutrient sources as well as the lipid.

An early study by Young (1979) claimed that conventional biological waste treatment plants can successfully deal with grease and oil containing wastewaters. This study, however, did not report on problems that may have occurred in these plants as a result of grease and oil input.

As with anaerobic systems, aerobic biological treatment is affected by the presence of lipid waste in significant quantities. It has been reported that fats, oils and greases contribute to the formation of stable foams in the aeration tanks. Filamentous organisms associated with foaming, such as *Microthrix parvicella*, can utilise and store lipid, giving a hydrophobic nature to the cell surfaces. *Rhodococcus rubra*, also associated with aeration tank foaming, has been shown to produce foam when grown in the presence of an oily substrate. It has been hypothesised that metabolites of lipid breakdown, which have high surfactant properties, carry the biomass they are associated with into the foam zone. The gas bubbles in the foam are more rigid and static than free-moving bubbles, and therefore subject the microorganisms in it to nutrient and oxygen starvation. Further degradation of the lipid metabolites would then be restricted. The additional problem of foam in the final settling tanks may then occur, releasing it with the final effluent (Forster, 1992). Other earlier studies

suggested however that the formation of a stable foam did not appear to be dependent on the concentrations of lipid in the aeration tank (Goddard and Forster, 1986).

The loss of sludge from secondary clarifiers may also be caused by high lipid concentrations (Hydroscience, 1971). Biomass with high levels of lipid will have lower specific gravity, and will not settle as effectively in the clarifiers. Other organisms in activated sludge systems are also affected by the excess presence of lipid. Protozoan levels have been shown to be decreased in a waste treatment system subjected to influent with high lipid concentrations (Hrudey, 1982). Little work has been carried out on the toxicity of long chain fatty acids on aerobic systems.

The resolution of these operational problems is a priority for wastewater treatment plant operators. A range of biological products are available in Ireland and abroad which have been specially formulated for the treatment of fat, oil and grease containing wastewaters. These are microbial preparations sometimes with enzymes added, used to supplement existing populations in wastewater treatment plants, and to introduce populations where the natural populations are very low (e.g. in fat traps). These formulations are periodically added to the wastewater treatment systems, fat traps, sewers and other areas with lipid containing wastewaters.

In Ireland, the preparations BFL 5000FG, BFL 5050VF and BFL 5150PS are marketed by Biofuture Ltd. These have been formulated for the degradation of animal fats and greases, vegetable fats and greases, and grease and fat in pumps and sumps respectively. They are preparations based on natural, harmless bacteria, specific for the application stated (Biofuture Ltd., 2002).

Several preparations are available in Britain from different suppliers. Bio-Sock is a loose weave sock containing dehydrated bacteria which produce grease degrading enzymes, produced by Copa Ltd. The sock is floated in the location desired, operating under aerobic conditions (Copa Ltd., 2002). Hegtrap is a powder and liquid

formulation containing biodegradable surfactants and a population of fat digesting bacteria, from Oil Cleaning Bio-products Ltd. The powder, containing the dormant bacteria, is added to the specially formulated liquid at the time of use (Oil Cleaning Bio-products Ltd., 2002). HEB is a blend of aerobic and facultative bacteria, selected to biodegrade animal fats and greases, as well as petroleum oils. This formulation, sold by Bioproductions Ltd., can be used in soil and land reclamation, as well as wastewater treatment (Bioproductions Ltd., 2002).

Although products such as these are available on the market, tallow and other animal fats still present a significant problem in wastewater treatment systems. In one rendering plant's system, bioadditives were used, but failed to eliminate problems associated with the high concentrations of tallow in the waste stream. Physical and chemical systems only change the disposal problems, as the recovered lipid residue has to be dealt with. Dumping of fat residues in landfill is no longer an option and other methods such as incineration are controversial. The addition of a preliminary bioreactor for fat removal would solve operational and compliance difficulties where such waste exists.

A bioreactor for the treatment of lipid-containing waste should contain microbial populations which can completely biodegrade lipids without producing problematic residues. Hard fats such as tallow have the additional difficulty of being in solid form at normal waste treatment temperatures. Therefore, the isolation of microorganisms capable of digesting tallow and other animal fats is the first stage in the development of such a reactor. The breakdown of fat by these organisms would then be of interest to identify problematic steps in the biodegradation, if any exist.

In this project, the aims were:

- to isolate an organism or organisms capable of growing on tallow as a sole carbon source;

- to optimise the environmental conditions for growth of a selected organism on tallow;

- to investigate the biodegradation of tallow by a selected lipolytic isolate, and determine if any of the steps involved were limiting lipid breakdown.

2. Materials and Methods

2.1 Materials

2.1.1 Organisms

The organisms used were isolated from the wastewater treatment plant of a commercial rendering operation, Dublin Products Ltd., Dunlavin, Co. Wicklow, and from unsterilised tallow from the same operation. Wastewater and activated sludge were used as sources from the wastewater treatment plant.

2.1.2 Source of chemicals

Chemicals were obtained from a number of sources including Reidel-de-Haen, Sigma, Aldrich, Fluka and Supelco. Mixed tallow was obtained from Dublin Products Ltd., Dunlavin, Co. Wicklow.

2.1.3 Buffers

0.1M citrate phosphate buffer

This buffer was prepared by adding x ml of 0.2M Na_2HPO_4 to (100-x) ml of 0.1M citric acid to give the desired pH, as per the following table:

pH	3.0	4.0	5.0	6.0	7.0
x	20.6	38.6	51.5	63.2	82.4

Phosphate buffer (for pH studies)

Phosphate buffer was prepared by adding x ml 0.2M Na_2HPO_4 to (50-x) ml 0.2M NaH_2PO_4, and diluting to 100ml.

pH	6.0	7.0
x	6.15	30.5

KH Phthalate NaOH buffer

To prepare this buffer at pH 5.0, 50ml 0.1M KH Phthalate was added to 22.6ml 0.1M NaOH, and made up to 100ml with distilled water.

KH Phthalate HCl buffer

This was prepared by adding 50ml 0.1M KH Phthalate to x ml 0.1M HCl, and diluting to 100ml. The pH was determined by x, the volume of HCl as follows:

pH	3.0	4.0
x	22.3	0.1

Sodium phosphate buffer (for inoculum preparation)

0.01M Sodium phosphate buffer was prepared by dissolving Na_2HPO_4 (0.01M) and NaH_2PO_4 (0.01M) in distilled water. This was then adjusted to pH 7.0.

2.1.4 Media

Minimal medium

The culture medium used was a modification of that described by Shikoku-Chem (1994). The minimal medium was made up in distilled water with the following components added:

	g/l
Yeast extract	0.1
$(NH_4)_2SO_4$	2.0
K_2HPO_4	0.9
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.05
$FeSO_4.7H_2O$	0.02

Enrichment medium

For enrichment cultures, minimal medium was adjusted to pH 7.0 and autoclaved at 121°C for 15 minutes. The carbon source was tallow, added at 20g/l after autoclaving.

Unbuffered liquid medium

The unbuffered medium used for other studies consisted of the minimal medium supplemented with tallow. Tallow was added to the minimal medium before autoclaving at 121°C for 15 minutes. The tallow concentration used was 20g/l unless otherwise stated.

Buffered liquid medium

The buffered media were prepared by adding the medium components of the minimal medium to the appropriate buffers. Tallow was then added before autoclaving at 121°C for 15 minutes. The tallow concentration was 20g/l unless otherwise stated.

Olive oil agar

Olive oil agar was prepared by supplementing unbuffered minimal medium with 20g/l olive oil and 2%(w/v) agar. This was autoclaved at 121°C for 15 minutes.

Olive oil antibiotic agar

This was prepared by supplementing olive oil agar with 0.5% chloramphenicol after autoclaving before the medium began to solidify.

Other media

Other media were obtained from Oxoid and prepared as per the manufacturer's instructions. They were autoclaved at 121°C for 15 minutes, except for malt extract agar, which was autoclaved at 115°C for 10 minutes.

2.2 Methods

2.2.1 Measurement of growth

Measurement of growth of non-filamentous organisms

Growth of non-filamentous organisms was monitored by measuring the Optical Density (OD) at 660nm using a Unicam 8625 UV/VIS Spectrophotometer. The spectrophotometer was blanked against distilled water.

Measurement of growth of filamentous organisms

Growth of filamentous organisms was monitored by dry weight. The medium was vacuum-filtered through pre-weighed Whatman no. 1 filter paper. These were dried to constant weight in a drying oven at 60°C, and re-weighed. From this the dry weight was calculated.

2.2.2 Measurement of pH

The pH was measured using an Orion 920A pH meter

2.2.3 Culture conditions

The culture media were added at 100ml to 250ml conical flasks. The carbon source, tallow, was added at 20g/l. Incubation was at 25°C, maintained in a warm room, and shaking at 130rpm (Jeio Tech SK-71 shaker table). These conditions were used for all studies unless otherwise stated. Uninoculated controls were also run in parallel.

Isolation of organisms

The organisms were isolated from enrichment cultures. The enrichment medium was inoculated with activated sludge or wastewater at 2%(v/v), or with unsterilised tallow as the sole source of microorganisms. Various incubation conditions were used for each inoculum, 30°C and ambient temperature, both shaking at 130rpm and non-shaking.

On days 3 and 15, samples from the enrichment cultures were streaked onto olive oil agar. These were incubated at the temperature of their parent flask. Non-filamentous organisms were purified by sub-culturing onto olive oil agar until pure cultures were obtained. Filamentous fungi were isolated by sub-culturing onto olive oil antibiotic agar repeatedly until pure colonies were obtained. Following isolation, non-filamentous organisms were maintained on nutrient agar, and filamentous fungi on malt extract agar.

On day 21, samples from the enrichment cultures were streaked onto nutrient agar. Isolates were purified on nutrient agar and malt extract agar, and maintained on these media.

Screening of non-filamentous isolates

The inocula for screening of non-filamentous isolates were overnight cultures in nutrient broth resuspended in sodium phosphate buffer to an OD of 0.9-1.2. These were used at 2%(v/v) in 100ml medium with 20g/l tallow. Incubation was at their isolation temperature.

Screening of filamentous isolates

The inocula used during screening of filamentous isolates were 8mm mycelial agar plugs of the fungi, from the edge of 5-day cultures on malt extract agar. One mycelial plug was added to each flask.

Growth condition and biodegradation studies

The inocula for growth conditions and biodegradation studies were 8mm agar plugs from the edge of malt extract agar lawns of *T. harzianum* Rifai RP1. One mycelial plug from a 5-day old lawn was used, except where the age and size of the inoculum was being studied. When the age of the inoculum was being studied, one plug from 2, 5 or 8-day old cultures were used. When inoculum size was being studied, 1, 5 or 10 plugs from a 5-day old culture were used.

Where temperature variation was being studied, 4°C, 25°C, 30°C and 37°C were maintained in temperature controlled rooms at the appropriate temperature. A temperature of 55°C was achieved in a water bath (Julabo SW-20C).

When the addition of Tween 80 was being studied, it was added at 2%(v/v) prior to autoclaving.

pH studies were carried out by buffering the medium to pH values of 3, 4, 5, 6 and 7. An unbuffered culture with an initial pH of 7 was run in parallel. The buffered media were prepared by adding the minimal medium components to the appropriate buffer instead of to water.

The rate of agitation was studied by running cultures in parallel on different shakers at rates of 0, 100, 130 and 200rpm.

During biodegradation studies, tallow was studied at 1, 2, 10 and 20g/l. Since analysis involved the destruction of the cultures, separate flasks were analysed at the different time points.

The studies on the metabolism of the fatty acid mixture were carried out by adding the mixture in the place of tallow at the appropriate concentrations. The fatty acids were added to the media prior to autoclaving.

2.2.4 Morphological examination of isolates

The macroscopic morphology of the isolates was examined using a 10x magnification stereomicroscope (Hertel & Reuss Optik Kassel).

2.2.5 Identification of the fungus

The filamentous fungus, *Trichoderma harzianum* Rifai, selected for further study, was identified based on physiological and morphological characteristics. The organism was examined after growth on malt extract agar, wood shavings and paper. The colony macroscopic characteristics were examined using a stereomicroscope (Hertel & Reuss Optik Kassel). For microscopic examination of the agar cultures, temporary wet mounts were prepared (Fassatiová, 1986). A small area of mycelia was taken with a needle and transferred with a small amount of agar to a glass slide. A drop of water was added and a cover slip was pressed onto it. For examination of wood shaving and paper grown cultures, a small amount of mycelium was taken with a needle and treated in the same way. Examination was carried out with a Nikon Optiphot microscope. Images were achieved with a digital camera with a Planachromat 40X 0.95mm numerical aperature.

The identity was determined from information and keys in Onions *et al.* (1986), (reproduced in Appendix A), and Barnett and Hunter (1972) (reproduced in Appendices B and D). A malt extract agar slant of the fungus was also submitted to Deutsche Sammlung von Mikroorganismen und Zellculturen GmbH (DSMZ), Braunschweig, Germany for identity confirmation.

2.2.6 Growth of the fungus on paper and wood shavings

The paper was prepared by mincing Whatman no. 1 filter paper in a blender until it formed a fine powder. This paper was autoclaved at 121°C for 10 minutes, and approximately 1g was added to each petri dish. To this, sterile water was added at the rate of approximately 2ml. per gram of paper.

The woodshavings were from softwood, obtained from a local cabinetmaker's workshop. They were autoclaved at 121°C for 10 minutes and approximately 2g was added to each petri dish. Sterile water was added at the rate of approximately 5ml per 2g of woodshavings.

The inocula for the woodshavings and paper were prepared by growing the fungus in 20ml aliquots of malt extract broth for 5 days. These were centrifuged at 4000rpm for 5 minutes, the liquid decanted off, and resuspended in 10ml phosphate buffer. Centrifugation was then repeated, the liquid decanted, and the pellet resuspended in a further 10ml phosphate buffer. This was used to inoculate the paper and woodshavings at the rate of 2ml in each petri dish.

2.2.7 Dubois assay

The Dubois assay was used to determine the amount of carbohydrate in a plug of malt extract agar. The method was as described by Dubois *et al.* (1956). 0.5ml of an aqueous sample containing the malt extract agar was mixed with 0.5ml of a 0.53M

phenol solution (5g of phenol in 100ml water) in a test tube. To this, 2.5ml of sulphuric acid reagent was added rapidly and the resulting solution vortexed. The sulphuric acid reagent consisted of 2.5g hydrazine sulphate in 500ml sulphuric acid. The solution was incubated in the dark for 1 hour, and the absorbance measured at 490nm. A standard curve of glucose was used as a reference (Figure 6).

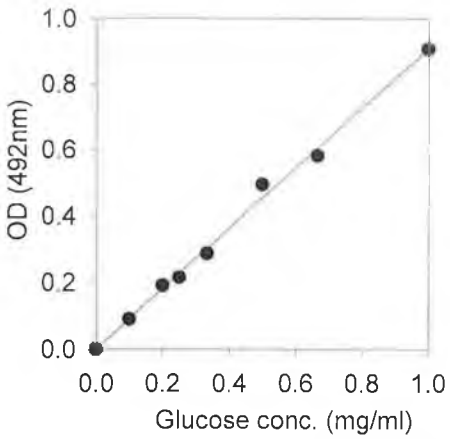

Figure 6: Standard curve for carbohydrate determination by the Dubois assay.

2.2.8 Measurement of extracellular lipid by n-hexane extraction-gravimetric method

The extraction and measurement of extracellular lipid was based on methods used by Shikoku-Chem (1994) and Koritala *et al.* (1987). The sample was first acidified to pH 2.0 or lower. This was then heated to approximately 50°C to melt the tallow, allowing easier extraction. The mixture was then transferred to a separating funnel, its container rinsed with hexane, and the washings added to the separating funnel. Approximately 20ml hexane was then added to the funnel, and shaken vigorously. The layers were allowed to separate and the lower aqueous layer drained off. If an emulsion formed, the emulsion was drained into a glass universal, and centrifuged at 4000rpm for 3 minutes.

The separated layers were then treated as for the main extraction solvents. The hexane layer was drained into a conical flask containing a small amount of anhydrous sodium sulphate. This was then filtered through Whatman no. 1 filter paper to remove the sodium sulphate into a clean pre-weighed 250ml round-bottom flask. The extraction was then repeated with the aqueous fraction. When filamentous biomass was present, the biomass was washed with hexane, and the washings combined with hexane extracts from the previous steps. The solvent was removed using a rotary evaporator. The flasks were then placed in an oven at 50°C to ensure all the solvent was removed until a constant weight was observed. From this the amount of lipid remaining could be determined by subtracting the weight of the flask from the total weight of the flask and extracted lipid.

2.2.9 Measurement of intracellular lipid by chloroform methanol extraction gravimetric method.

Intracellular lipid was extracted by the method described by Bligh and Dyer (1959). Dried biomass was added to 38ml of a chloroform/methanol/water (1:2:0.8) mixture. This mixture was monophasic. The samples were shaken for 30 minutes, after which 10ml of chloroform and 10ml of water were added to the mixture. This gave a chloroform/methanol/water ratio of 2:2:1.8, which was a biphasic mixture. The bottom chloroform layer was drained off, dried over sodium sulphate, and filtered into clean pre-weighed 50ml round-bottom flasks. The solvent was removed using a rotary evaporator. The flasks were then placed in an oven at 50°C to ensure all the solvent was removed, and then re-weighed. From this the amount of lipid remaining could be determined by subtracting the weight of the flask from the total weight of the flask and extracted lipid.

2.2.10 Glyceride analysis by High Performance Liquid Chromatography

The glyceride content of extracted lipids was analysed by High Performance Liquid Chromatography (HPLC). The HPLC procedure was based on the method used by Ritchie and Jee (1985). Analysis was carried out on a Varian HPLC system comprising a Varian inert 9012 HPLC pump, Varian Star 9040 refractive index detector, a Varian Al200 autosampler with column oven and Varian Star Chromatography software on a 400MHz Pentium II PC with 64Mb RAM. Solvents were filtered and degassed prior to use. The column used was a Waters Spherisorb 5μm silica 4.6X250mm analytical column, with heptane/tetrahydrofuran/formic acid (80/20/0.5) mobile phase. Samples were dissolved in the mobile phase containing 50ppm butylated hydroxytoluene (BHT) as an antioxidant. The sample injection volume was 100μl, the mobile phase flow rate was 2.0ml/min, and the column was held at 35°C in a column oven. Standards of mono-, di- and triglycerides and fatty acids were injected to confirm the retention times.

Glyceride species and free fatty acids were quantified using external standards. Standard curves of mono-, di- and triglycerides and fatty acids were constructed (Figures 7-10), and the weight concentration in each sample calculated.

Fractions were collected from the detector at times 0.33 minutes after the peak retention times. These fractions were used for fatty acid analysis of the glyceride and free fatty acids by gas chromatography.

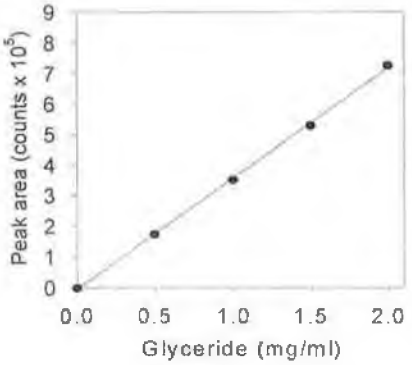

Figure 7: Standard curve for monoglyceride determination by HPLC

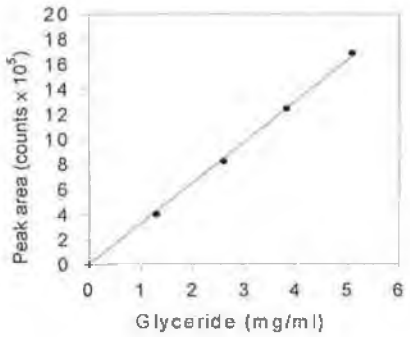

Figure 8: Standard curve for diglyceride determination by HPLC

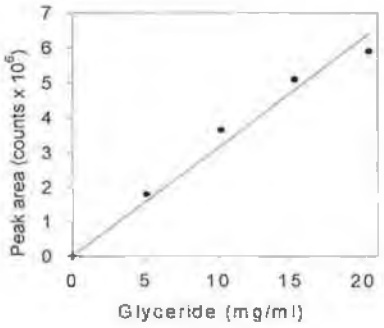

Figure 9: Standard curve for triglyceride determination by HPLC

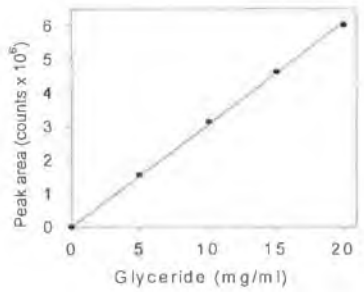

Figure 10: Standard curve for free fatty acid determination by HPLC

2.2.11 Fatty acid analysis in lipid fractions by Gas Chromatography

Fractions obtained from HPLC were transferred to 50ml round-bottomed flasks, and the solvent evaporated off over a water bath. Derivatisation of fatty acids to fatty acid methyl esters was carried out as described by Firestone and Horwitz (1979). Glyceride fractions were hydrolysed by refluxing in the presence of 5ml 0.5N methanolic sodium hydroxide for 5 minutes. After this stage, both fatty acid and glyceride fractions were treated in the same manner. 0.1ml of an approximately 0.5M solution of Tridecanoic acid in n-Hexane was added to all the samples as an internal standard for relative retention time confirmation in the Gas Chromatography. The samples were boiled under reflux for 2 minutes after the addition of 5ml 14% boron trifluoride-methanol. 5ml Hexane was then added through the condenser and reflux continued for a further minute. The flask was then removed from heat, and 10-15ml saturated sodium chloride solution added. The flask was swirled gently and further saturated NaCl added to float the hexane layer into the neck of the flask. From this, 4.5ml of the hexane layer was removed to a test tube and the solvent evaporated, and the residue resuspended in 0.05ml hexane. A small amount of anhydrous sodium sulphate was added to remove any water present.

The methyl esters were then analysed by Gas Chromatography. This was based on the method described by Yabuuchi and Moss (1982). 8µl aliquots of the samples were injected onto a Carlo Erba HRGC 5300 equipped with a flame ionisation detector. The column was an Alltech EC-WAX 30m X 0.32mm with a film thickness of 0.25µm. The injector temperature was maintained at 250°C and the detector at 300°C. The column temperature was programmed from 190°C to 250°C at 6°C /min with a final hold of 8 min at 250°C. Chromatograms were recorded on a Carlo Erba Mega Series integrator, which facilitated peak integration for quantitative analysis.

Fatty acids were identified by comparison of the relative retention times of their methyl esters with those of known standards. The relative retention times were relative to the internal standard, Tridecanoic acid methyl ester, and were calculated by the formula:

$$r = \frac{tr_x - tr_u}{tr_s - tr_u}$$

where:

r = relative retention time

tr_x = retention time of component of interest

tr_u = retention time of unretained component

tr_s = retention time of internal standard.

The percentages of each fatty acid methyl ester were calculated from the peak areas. Internal normalisation was used, as described in Standard Methods for the Analysis of Oils, Fats and Derivatives (Paquot and Hautfenne, 1987). By internal normalisation, it is assumed that all the components of interest are represented on the chromatogram. For total elution of these components, the sum of the areas under the

peaks equals 100%. Since significant amounts of components below C8 were absent, the content of a given constituent was determined by expressing the area of its peak as a percentage of the sum of the areas of all the peaks of interest :

$$\%_i = \frac{A_i X 100}{\sum A}$$

$\%_i$ = mass % of component *i* expressed as methyl ester

A_i = area of peak corresponding to component *i*

ΣA = sum of the areas of peaks of components of interest

2.2.12 Data Analysis

Standard errors

Standard errors (se) were calculated from the formula:

$$se = \frac{s}{\sqrt{n}}$$

where s was the standard deviation of the range and n was the number of sampling points in the range.

Regression analysis

Regression analysis was carried out using the scientific graph program SigmaPlot, version 1.02 (Jandel Corporation)

3. Results

3.1 Isolation and screening of organisms capable of growth on tallow from the waste-treatment system of a rendering operation.

In order to isolate organisms capable of degrading tallow, sources were selected that had a high probability of containing such organisms. The sources were tallow, wastewater and activated sludge from a commercial rendering operation, which produced substantial quantities of fat as waste. They were used to inoculate enrichment cultures, which consisted of minimal medium supplemented with 20g/l tallow. Isolations were carried out at various times on agar media. Olive oil agar was used as a selective medium for isolations on days 3 and 15 of incubation, while nutrient agar was used on day 21. The isolates were broadly divided based on macroscopic morphology, into filamentous and non-filamentous strains, where appropriate. These organisms were then screened with respect to their ability to grow on and remove tallow when grown in pure culture.

3.1.1 Isolation of organisms

Enrichment culture

During the incubation of the enrichment cultures, general observations were made of the culture behaviour. The pH of the enrichment cultures was observed as a sign of tallow hydrolysis, while the optical densities (OD) were noted as an indication of microbial growth. Although the nature of microbial growth and the tallow substrate may have caused interferences in OD measurements, it gave an indication of biomass production. In most cases, a drop in pH was accompanied by a rise in OD (Table 3).

In cultures with unsterilised tallow as the source of organisms, the drop in pH was slightly greater in the non-shaking cultures at 30°C, where a drop of 1.00 pH unit was

observed. In the shaking cultures, the change in pH was 0.39 units. However, there was not as significant a difference at ambient temperature. While the drop in non-shaking cultures was 0.55 pH units, that in shaking cultures was slightly greater at 0.78 pH units. It was also noted that the OD rise was slightly higher in non-shaking flasks. Rises of 0.086 and 0.069 were noted at ambient and 30°C respectively, while in shaking flasks, these rises were 0.012 and 0.044 respectively.

The pH changes were significantly higher with the wastewater inocula. In shaking cultures, drops of 4.93 and 4.82 pH units were seen at ambient temperature and 30°C respectively. In non-shaking cultures, the drops were 4.28 and 4.71 respectively. Shaking cultures also underwent greater increases in OD. At ambient temperature, an increase of 3.374 was observed, while at 30°C, the increase was 2.979. The increases were lower in non-shaking cultures, where rises of 0.119 and 1.007 were noted at ambient and 30°C respectively.

Activated sludge inoculated enrichment cultures displayed the greatest pH changes. The largest drop was observed in the shaking culture at ambient temperature, where a drop of 5.12 pH units was recorded. At 30°C, the drop was 4.74 units. In non-shaking cultures, the decreases were slightly less. At ambient temperature a drop of 4.21 pH units was recorded, while at 30°C, this decrease was 4.40 pH units. The shaking cultures also yielded the highest increases in OD. At ambient temperature, the shaking cultures rose by 3.772, while at 30°C, the rise was 2.475. Lower increases were seen in the non-shaking cultures, with no increase being recorded at ambient temperature. The rise of 0.746 at 30°C was less than that recorded in the corresponding wastewater inoculated culture.

The microbial populations from activated sludge had a greater ability to grow on tallow than those from wastewater or tallow under shaking conditions. This greater growth was also accompanied by larger drops in pH, indicating hydrolysis of tallow glycerides.

Table 3: Changes in optical density and pH of enrichment cultures following 21 days incubation under various culture conditions. (* = based on 1:2 dilution)

Source of microbes	Culture conditions	ΔOD (660nm)	ΔpH (initial pH = 7.0)
Tallow	Ambient, shaking	0.012	-0.78
Tallow	Ambient, non-shaking	0.086	-0.55
Tallow	30°C, shaking	0.044	-0.39
Tallow	30°C, non-shaking	0.069	-1.00
Wastewater	Ambient, shaking	3.374*	-4.93
Wastewater	Ambient, non-shaking	0.119	-4.28
Wastewater	30°C, shaking	2.979*	-4.82
Wastewater	30°C, non-shaking	1.007	-4.71
Activated sludge	Ambient, shaking	3.772*	-5.12
Activated sludge	Ambient, non-shaking	-0.008	-4.21
Activated sludge	30°C, shaking	2.475*	-4.74
Activated sludge	30°C, non-shaking	0.746	-4.40

Microbial isolation

A range of filamentous and non-filamentous organisms were isolated from the three sources (Figures 11 & 12). They were each given a code, beginning with 'NF' for non-filamentous isolates, and 'F' for filamentous fungi. Ambient temperature yielded 28 isolates altogether, while 30 were isolated at 30°C. Among these, activated sludge was the richest source, from which 28 isolates were recovered. Of these, 16 of them were found at ambient temperature, while 14 of them were present at 30°C (Table 4). Two of the fungi, F3 and F9 were present at both temperatures. Wastewater yielded 21 isolates in all, 7 of which were found at ambient temperature, with 14 at 30°C. When tallow was the only source of microorganisms, only 13 isolates were recovered between both temperatures, 5 at ambient temperature and 8 at 30°C. While non-filamentous organisms were found in all 3 sources, no filamentous fungi were

isolated from tallow alone. Also, the filamentous fungi which were recovered, were all found in shaking cultures and not in non-shaking cultures.

Table 4: Number of isolates recovered from enrichment cultures with various sources of inocula, at ambient temperature and at 30°C.

Source	Ambient temperature	30°C
Unsterilised tallow	5	8
Wastewater	7	14
Activated sludge	16	14

With tallow as the sole source of microorganisms, the isolates obtained were all non-filamentous (Figures 11 & 12). In shaking flasks, at ambient temperature, no isolates were seen on day 3, while one organism was isolated on each of days 15 (NF49) and 21 (NF50). At 30°C, one isolate was obtained on each of days 3 (NF23) and 15 (NF24), while three were found on day 21 (NF25, NF26 and NF27). In non-shaking flasks, one organism was isolated at ambient temperature (NF40) and 30°C (NF13) on day 3. Similarly on day 15, one isolate each was recovered at ambient temperature and 30°C (NF41 and NF14 respectively). Day 21 also yielded one isolate at each temperature (NF42 and NF15 respectively).

With the wastewater inoculum, both filamentous and non-filamentous organisms were recovered (Figures 11 & 12). In shaking flasks, no non-filamentous organisms were isolated at ambient temperature. However, one fungus was found on day 3 (F6), two on day 15 (F6 and F14) and one (F14) on day 21. At 30°C, three non-filamentous isolates (NF19, NF20 and NF21) were obtained on day 3, and on day 21, one (NF22) was found. No non-filamentous organisms were isolated on day 15. Also, one filamentous fungus (F11) was isolated on day 3, three on day 15 (F2, F3 and F5) and one on day 21 (F3). In non-shaking flasks at ambient temperature, two non-filamentous organisms (NF35 and NF36) were isolated on day 3, one (NF37) on day

15 and two (NF38 and NF39) on day 21. At 30°C, wastewater yielded one non-filamentous organism (NF7) on day 3, one (NF8) on day 15 and four (NF9, NF10, NF11 and NF12) on day 21. No filamentous fungi were isolated from non-shaking cultures at either temperature.

Activated sludge yielded the greatest diversity of isolates (Figures 11 & 12). In shaking cultures, the number of non-filamentous isolates obtained at ambient temperature was slightly more than at 30°C. On day 3, ambient temperature yielded four organisms (NF43, NF44, NF45 and NF46), while 30°C yielded none. On day 15, none was isolated at ambient and two at 30°C (NF16 and NF17), while on day 21, two were found at ambient (NF47 and NF48), and one at 30°C (NF18). No filamentous fungi were isolated at days 3 and 21 at ambient temperature, but three were found at day 15 (F3, F5 and F9). At 30°C, one fungus (F11) was found on day 3, three on day 15 (F2, F3 and F9) and three (F2, F6 and F9) on day 21. In non-shaking cultures, the difference between the number of non-filamentous isolates at 30°C and ambient was less. On day 3, ambient yielded three organisms (NF28, NF29 and NF30), while 30°C produced two (NF1 and NF2). On day 15, two organisms were obtained at ambient temperature (NF31 and NF32), and one at 30°C (NF3). Ambient temperature also yielded two organisms on day 21 (NF33 and NF34), while 30°C yielded three (NF4, NF5 and NF6). No filamentous fungi were isolated from non-shaking flasks.

The morphologies of the isolated organisms are noted in Tables 5 and 6. Of the 50 non-filamentous isolates recovered, 32 of them were white while 14 were opaque, with only four coloured strains. The shape of the colonies varied from small discrete circular colonies to large diffuse shapeless colonies, with flat and domed profiles. The greatest diversity in morphologies was noted in those organisms isolated from wastewater and activated sludge. Of the seven filamentous isolates, five were white, while the other two displayed powdery green aerial growth.

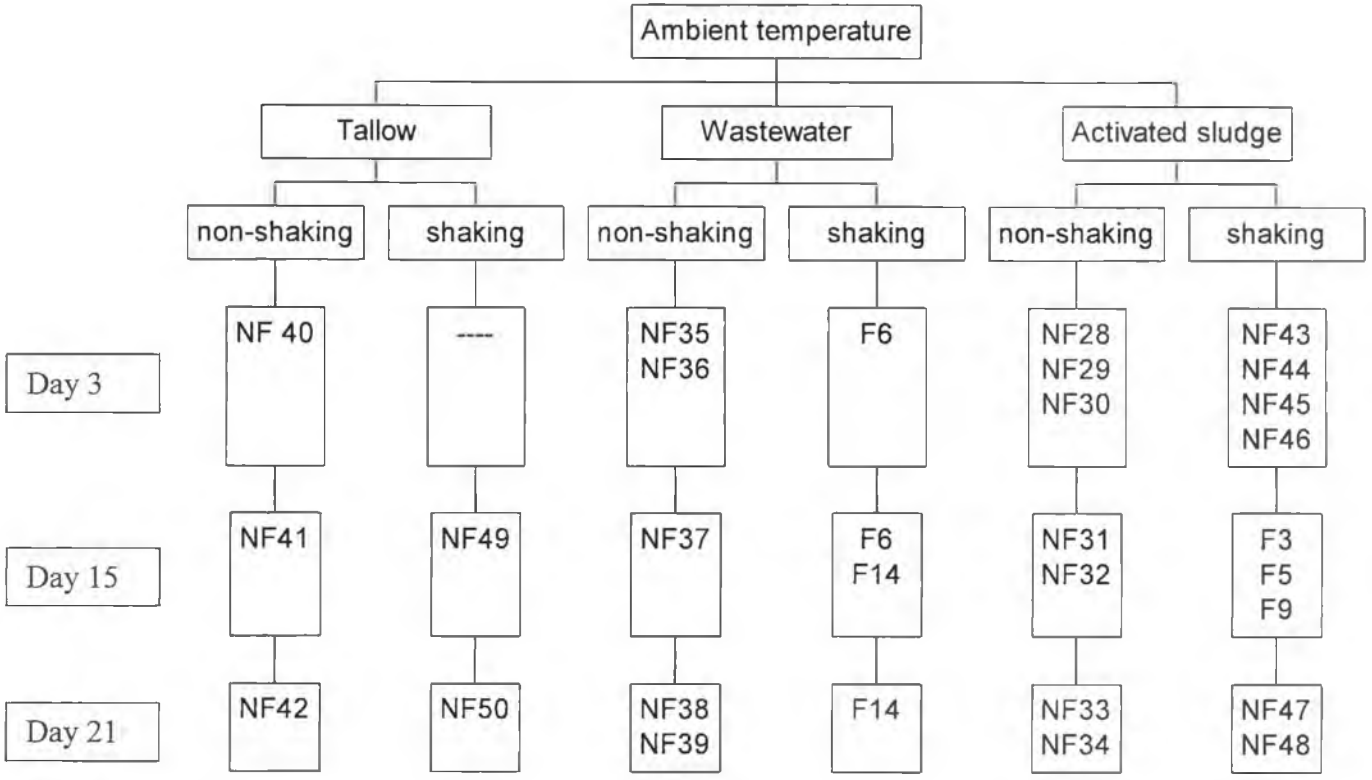

Figure 11: Isolates recovered from shaking and non-shaking enrichment cultures at ambient temperature, with various inoculum sources.

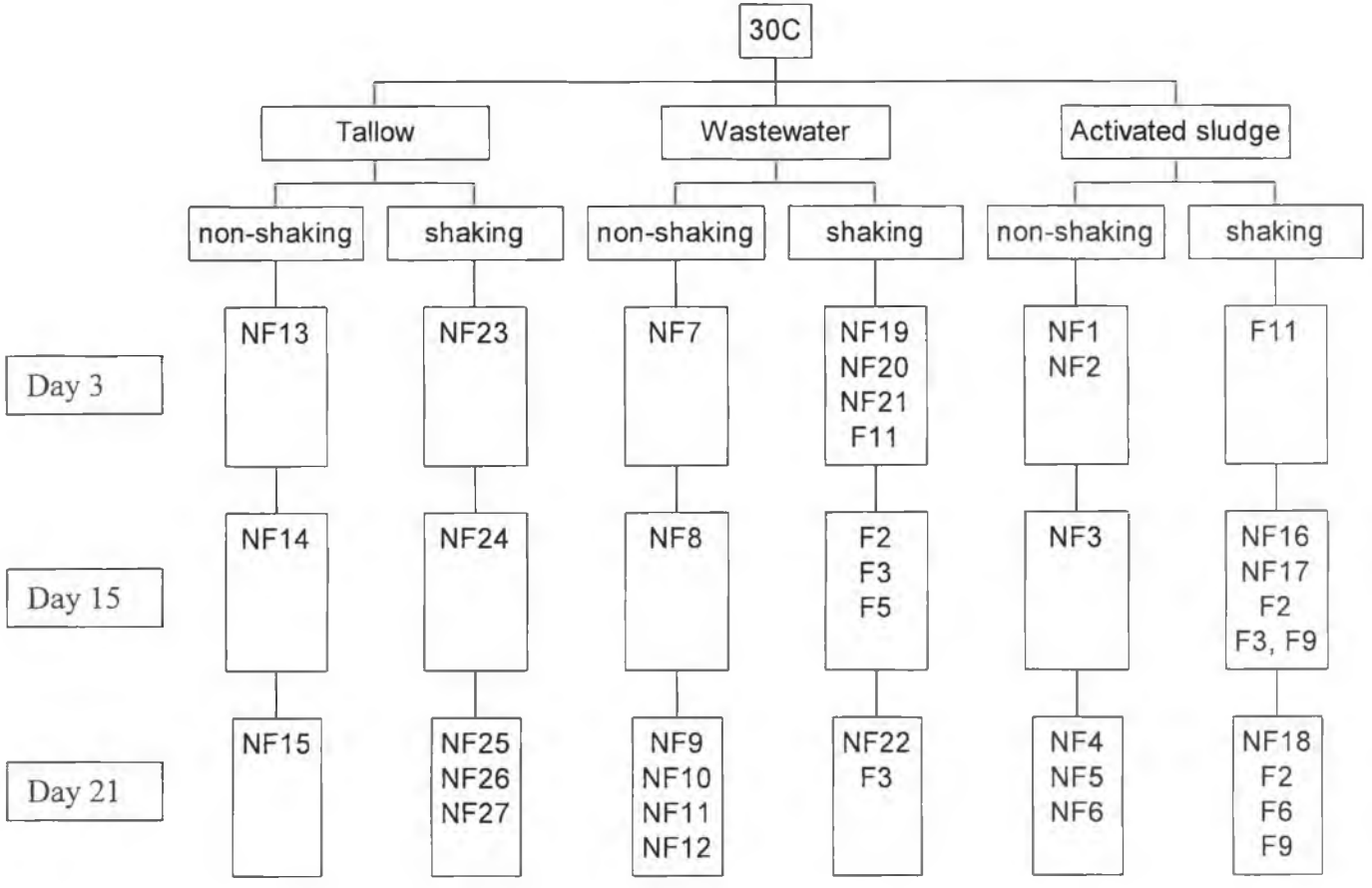

Figure 12: Isolates recovered shaking and non-shaking enrichment cultures at 30^{O}C, with various inoculum sources.

Table 5: Colony morphology of organisms isolated at ambient temperature from enrichment cultures with various inoculum sources.

Organism code	Source	Colony morphology
NF40	tallow	Flat, opaque, matt, irregular
NF41	tallow	Flat, opaque, matt, irregular
NF42	tallow	Flat, white, matt, circular
NF49	tallow	Flat, opaque, matt, irregular
NF50	tallow	Flat, white, matt, circular
NF35	wastewater	Domed, opaque, shiny, circular
NF36	wastewater	Flat, yellow, shiny, irregular
NF37	wastewater	White, diffuse
NF38	wastewater	Domed, white, shiny, circular
NF39	wastewater	Flat, opaque, shiny, irregular
NF28	activated sludge	Domed, opaque, shiny, circular
NF29	activated sludge	Flat, opaque, shiny, irregular
NF30	activated sludge	Domed, white, shiny, circular
NF31	activated sludge	White, diffuse
NF32	activated sludge	Domed, white, shiny, circular
NF33	activated sludge	Domed, white, shiny, circular
NF34	activated sludge	White, matt, undulate margins
NF43	activated sludge	Domed, white, shiny, circular
NF44	activated sludge	Flat, opaque, matt, irregular
NF45	activated sludge	Domed, opaque, shiny, circular
NF46	activated sludge	flat, opaque, matt, irregular
NF47	activated sludge	White, matt, undulate margins
NF48	activated sludge	White, matt, circular
F14	wastewater	White, filamentous aerial
F3	wastewater/activated sludge	Light green, powdery aerial growth
F5	wastewater/activated sludge	White, filamentous aerial, dense growth
F6	wastewater/activated sludge	White, filamentous aerial
F9	activated sludge	Dark green, powdery aerial growth

Table 6: Colony morphology of organisms isolated at 30°C from enrichment cultures with various inoculum sources.

Organism code	Source	Colony morphology
NF13	tallow	Flat, opaque, matt, irregular
NF14	tallow	Flat, opaque, matt, irregular
NF15	tallow	Flat, white, shiny, diffuse
NF23	tallow	White, matt, undulate margins
NF24	tallow	Flat, opaque, matt, irregular
NF25	tallow	Flat, white, shiny, circular
NF26	tallow	Flat, "off-white", matt, circular
NF27	tallow	Flat, white, matt, irregular
NF7	wastewater	Domed, white, shiny, circular
NF8	wastewater	White, diffuse
NF9	wastewater	Domed, white, semi-matt, circular
NF10	wastewater	Domed, white, matt, irregular
NF11	wastewater	Flat, white, matt, irregular
NF12	wastewater	Flat, white, matt, irregular
NF19	wastewater	Domed, yellow, shiny, circular
NF20	wastewater	Domed, white, shiny, circular
NF21	wastewater	Domed, white, shiny, circular
NF22	wastewater	White, matt, irregular
NF1	activated sludge	Domed, opaque, shiny, circular
NF2	activated sludge	Flat, white, matt, circular
NF3	activated sludge	White, diffuse
NF4	activated sludge	Flat, white, shiny, diffuse
NF5	activated sludge	Flat, white, matt, undulate margin
NF6	activated sludge	Domed, white, matt, circular
NF16	activated sludge	Brown, matt, circular
NF17	activated sludge	White, matt, circular
NF18	activated sludge	White, matt, diffuse
F2	wastewater/activated sludge	Clear mycelia, white aerial filaments
F3	wastewater/activated sludge	Light green, powdery aerial growth
F5	wastewater/activated sludge	White, filamentous aerial, dense growth
F11	wastewater/activated sludge	White, filamentous aerial growth
F6	activated sludge	White, dense, filamentous aerial growth
F9	activated sludge	Dark green, powdery aerial growth

3.1.2 Screening of the isolates for their ability to grow on tallow in pure culture

The isolates were screened by growing them in pure culture, in minimal medium with 20g/l tallow as the sole carbon source. Growth of the non-filamentous organisms was monitored by measuring OD (660nm), while dry weights were used as a measure of the growth of the filamentous organisms. Changes in pH and extracellular tallow removal were also measured (Tables 7 to 10).

The ability of the isolates to remove tallow varied greatly. While some isolates demonstrated no ability, up to 35% removal was observed with others. The majority of those with higher tallow removal capabilities were isolated at 30°C. Taking those isolates that removed greater than 20% of the tallow, eight were found at 30°C, while only two were isolated at ambient temperature. It was also noted that activated sludge yielded the greatest number of the higher tallow degraders. It produced six, while three were found in wastewater and only one in tallow. In order of tallow removal ability, these organisms were F2>NF6 >F9> NF10 > NF3 > (NF17 = NF32) > (NF12 = NF23).

Since the filamentous fungus F2 removed the highest percentage of tallow from culture, it was selected for further studies. Also, it presented a novel subject for study, as little work had been published to date on tallow degradation by filamentous fungi.

Table 7: Growth and percentage tallow removal by non-filamentous organisms isolated at ambient temperature.

Organism code	ΔpH (initial pH = 7.0)	ΔOD	% fat removal
NF32	-3.78±0.11	1.96±0.04	21±0%
NF48	-3.71±0.05	1.94±0.05	18±3%
NF42	-3.33±0.25	1.81±0.47	14±5%
NF37	-3.36±0.21	2.03±0.30	13±6%
NF50	-3.07±0.02	1.57±0.13	13±1%
NF35	-3.06±0.50	1.67±0.45	8±6%
NF46	-0.12±0.24	0.02±0.04	8±1.5%
NF41	-3.01±0.01	0.83±0.12	6±2%
NF34	-1.35±0.06	0.65±0.01	6±2%
NF36	-0.07±0.23	0.01±0.00	5±2%
NF45	-0.10±0.02	-0.02±0.00	4±1%
NF31	-3.63±0.02	1.83±0.05	3±3%
NF39	-3.60±0.08	2.17±0.17	2±2%
NF43	-0.16±0.15	0.69±0.04	1±0.5%
NF49	-2.11±0.02	0.19±0.14	1±1%
NF44	-1.00±0.10	0.73±0.12	0±1%
NF30	0.34±0.04	0.01±0.01	0±1%
NF38	-2.45±0.13	0.80±0.17	0±0%
NF29	-1.52±0.08	0.58±0.03	0±0%
NF40	-1.55±0.59	-0.03±0.01	0±0%
NF33	0.61±0.06	0.14±0.06	-
NF28	0.68±0.34	0.06±0.07	-
NF47	-0.42±0.33	0.01±0.02	-

Table 8: Growth and percentage tallow removal by non-filamentous organisms isolated at 30°C.

Organism code	ΔpH (initial pH = 7.0)	ΔOD	% fat removal
NF6	-3.82±0.07	2.19±0.08	31±4%
NF10	-3.11±0.55	2.03±0.20	26±0%
NF3	-3.68±0.09	2.12±0.05	25±5%
NF17	-3.75±0.11	2.28±0.02	21±1%
NF12	-4.00±0.11	2.45±0.08	20±6%
NF23	-2.69±0.24	2.32±0.05	20±3%
NF19	-3.00±0.09	0.89±0.11	17±1%
NF14	-3.39±0.02	0.98±0.06	16±2%
NF27	-3.83±0.04	2.14±0.10	16±7%
NF21	-3.21±0.09	1.15±0.36	15±5%
NF20	-3.03±0.15	0.55±0.07	14±1%
NF16	-3.37±0.00	1.06±0.10	14±1%
NF8	-4.00±0.04	2.17±0.12	10±1%
NF24	-3.50±0.04	1.92±0.03	10±3%
NF18	-3.95±0.12	2.17±0.02	7±2%
NF26	-3.49±0.04	1.97±0.03	7±1%
NF22	-0.03±0.01	-0.06±0.01	5±2%
NF9	-0.09±0.01	-0.04±0.03	3±1%
NF5	0.32±0.01	0.06±0.03	3±1%
NF2	-1.84±0.14	1.05±0.21	0.5±0%
NF1	-1.88±0.01	1.01±0.01	0±0%
NF7	-1.24±0.13	0.52±0.01	0±0%
NF13	-0.08±0.01	0.28±0.09	0±0%
NF15	0.46±0.05	0.19±0.15	-
NF25	0.50±0.07	0.06±0.06	-
NF4	0.68±0.09	0.02±0.03	-
NF11	0.61±0.12	-0.01±0.04	-

Table 9: Growth and percentage tallow removal by filamentous fungi isolated at ambient temperature.

Organism Code	ΔpH (initial pH = 7.0)	Δdry weight (g)	% fat removal
F9	-3.45±0.11	0.15±0.01	27±1%
F3	-3.67±0.05	0.25±0.00	18±3%
F14	-3.00±0.25	0.18±0.01	15±2%
F5	-3.53±0.08	0.17±0.02	12±1%
F6	-2.96±0.02	0.10±0.01	10±2%

Table 10: Growth and percentage tallow removal by filamentous fungi isolated at 30°C.

Organism Code	ΔpH (initial pH = 7.0)	Δdry weight (g)	% fat removal
F2	-3.53±0.21	0.15±0.02	35±5%
F9	-3.45±0.11	0.15±0.01	27±1%
F3	-3.67±0.05	0.25±0.00	18±3%
F11	-2.28±0.19	0.17±0.03	14±2%
F5	-3.53±0.08	0.17±0.02	12±1%
F6	-2.96±0.02	0.10±0.01	10±2%

3.2 The identification and optimisation of culture conditions of the isolate, F2.

3.2.1 The identification of F2

The filamentous fungus, F2, grew well on malt extract agar at 25°C. It formed a mycelial mat almost filling the plate within 5 days (Figure 13). The growth at the edge of the plate was aerial and filamentous, while the older growth at the centre of the plate lacked the aerial hyphae and was hyaline. The hyphae were found to be septate and hyaline when examined microscopically (Figure 14).

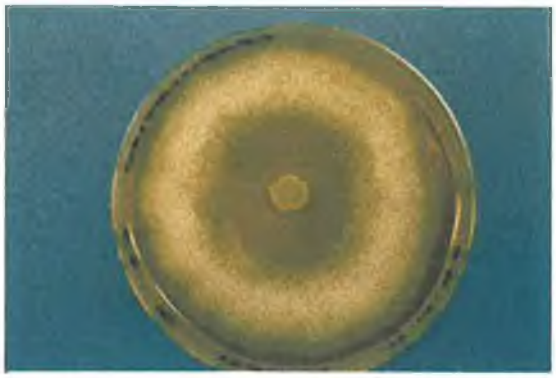

Figure 13: Filamentous fungus F2 following 5 days growth at 25°C on malt extract agar.

Figure 14: Septate hyphae of filamentous fungus, F2. (bar = 10μm)

For identification of a filamentous fungus, the reproductive structures are examined. However, sporulation in F2 was absent under the conditions described above. Therefore, a range of incubation conditions were examined to determine if sporulating areas could be produced by F2. Temperatures of 4°C, 25°C, 37°C, 55°C and ambient laboratory temperature were examined, with incubation both in darkness and in daylight. After 21 days of incubation at ambient temperature in daylight, sporulating areas were visible as light green tufted areas around the edges of the colony on malt extract agar (Figure 15). Growth at 25°C was as seen previously, with no obvious sporulating areas present. No growth was seen at 4°C, 37°C or 55°C.

Figure 15: Filamentous fungus F2 following growth for 21 days at ambient temperature in daylight on malt extract agar. Sporulating areas were visible as light green areas around the edge of the colony.

The reproductive structures were distinctive (Figure 16). These structures were conidiophores, which are asexual reproductive structures. The conidiophores were well developed and distinct from the rest of the mycelium. They were irregularly branched. The conidia were produced from the apex of the branches, where budding was evident. The conidia were single-celled, hyaline and spherical or ellipsoidal in shape. They were present singly, not in chains, and separated from the conidiophores.

There was no evidence of sexual reproductive structures, which indicated that F2 belonged to the Deuteromycetes. The keys of Onions *et al.* (1986) (Appendix A), and Barnett and Hunter (1972) (Appendix B), were followed based on microscopic observations. Using these keys the identity was determined, which was confirmed by DSMZ as *Trichoderma harzianum* Rifai (Appendix C).

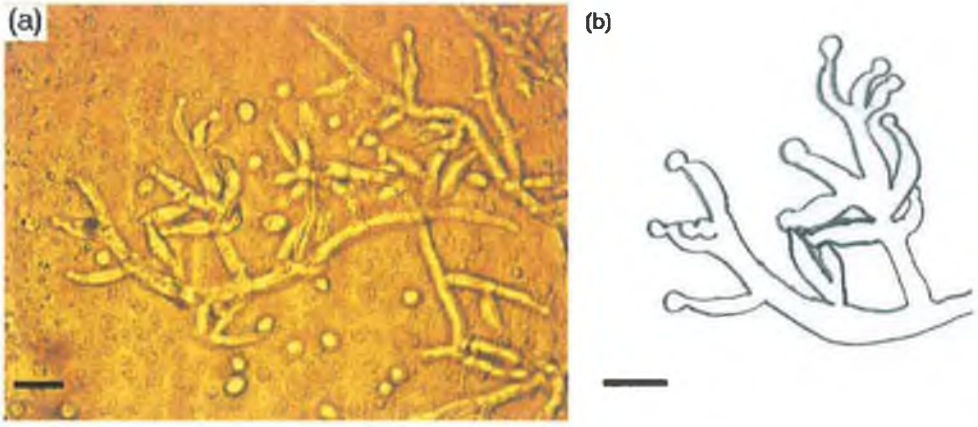

Figure 16: Reproductive structures of filamentous fungus, F2, (a) under the microscope (bar = 10μm) and (b) drawing of the reproductive structure viewed in (a) (bar = 10μm).

It was noted by DSMZ that sporulation was achieved on wood chips. It was decided to check if more extensive sporulation of F2 could be achieved in this way. The organism was inoculated onto wood chips and paper as outlined in section 2.2.6.

Sporulating areas were found, but were very limited (Figure 17). These were green and tufted, and microscopically the reproductive structures were identical to those observed previously on malt extract agar.

In this work, F2 was referred to as *T. harzianum* Rifai RP1, as it was isolated from the waste treatment system of a rendering plant.

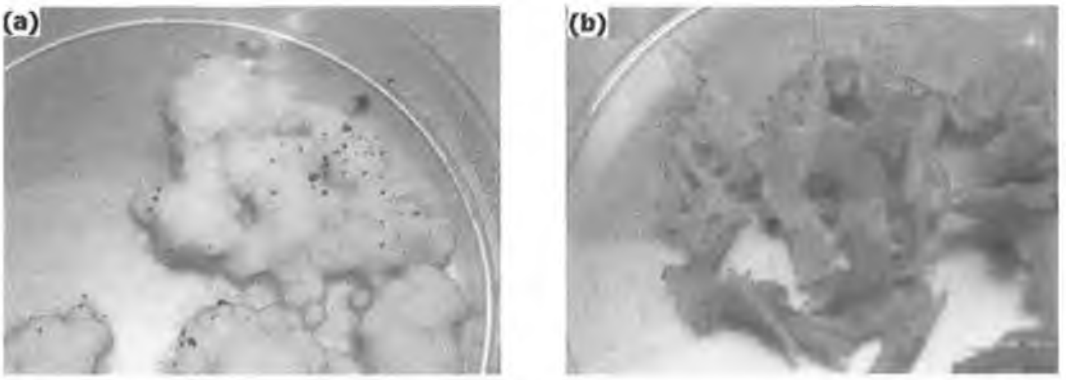

Figure 17: Sporulating areas of *Trichoderma harzianum* Rifai as observed on (a) paper and (b) wood shavings

3.2.2 The optimisation of environmental conditions for tallow removal by *Trichoderma harzianum* Rifai RP1 when grown on tallow as a sole carbon source.

T. harzianum Rifai RP1 was studied in shake flask cultures at 25°C, with minimal medium as described in section 2.1.2 supplemented with 20g/l tallow as the sole carbon source. These conditions were used throughout, unless otherwise stated.

The environmental conditions under which the fungus was grown were varied in order to determine the optimum conditions for removal of extracellular lipid from culture. The age of the inoculum, the physical nature of the substrate, the control of pH, the temperature of incubation, the size of the inoculum, the rate of agitation and the concentration of the tallow substrate were examined.

3.2.2.1 The influence of the age of the inoculum

Mycelial agar discs were used as inocula for studies of growth of *T. harzianum* Rifai RP1 with tallow as the sole carbon source. The age of the agar culture from which the plugs were taken was examined, to determine if it influenced the growth of the organism and its removal of tallow from culture. One 8mm diameter mycelial agar plug of 2, 5 or 8 days old were used as inocula for the study.

The pH was monitored during incubation. The pH change did not vary significantly with varying inoculum ages, the decrease being similar in all cases (Figure 18). With 2, 5 and 8 day inocula, the pH dropped by 3.8, 3.8 and 3.5 pH units respectively. The pH in the uninoculated controls remained constant.

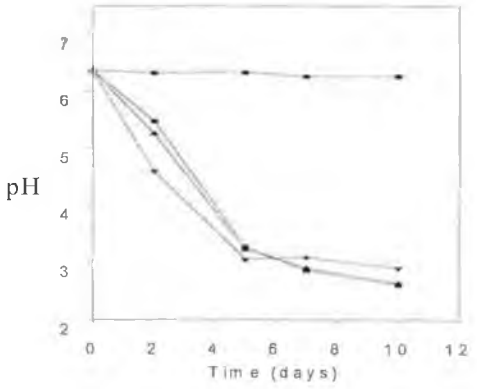

Figure 18: pH of cultures of *T. harzianum* Rifai RP1 grown on 20g/l tallow with (■) 2 day old, (▲) 5 day old and (▼) 8 day old inocula (● = control).

There was not a significant difference in dry weight increases between the inocula ages, over the 10 days of incubation. The dry weight increase for the 2-day inoculum was 1.1g/l, with slightly lower yields for the 5-day and 8 day inocula (Figure 19). These produced 1.0g/l and 0.8g/l respectively.

Removal rates of tallow also were not significantly different. With 20g/l initial tallow, the 2, 5 and 8-day inocula removed 15%, 14% and 15% respectively (Figure 20).

No significant differences existed between the performance of the inocula of different ages. The 2-day old cultures only half filled the agar plates, while with the 5- and 8-day old cultures, the plates were filled. Therefore, it was decided that 5-day old cultures would be used for inocula for future experiments, as it provided a greater area suitable for inocula, without the need for extended incubation.

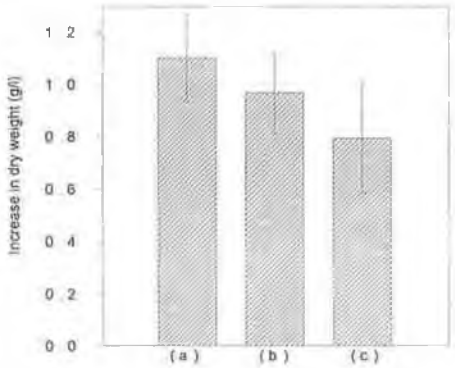

Figure 19: Dry weight increase by *T. harzianum* Rifai RP1 grown on 20g/l tallow with (a) 2 day old inoculum; (b) 5 day old inoculum and (c) 8 day old inoculum.

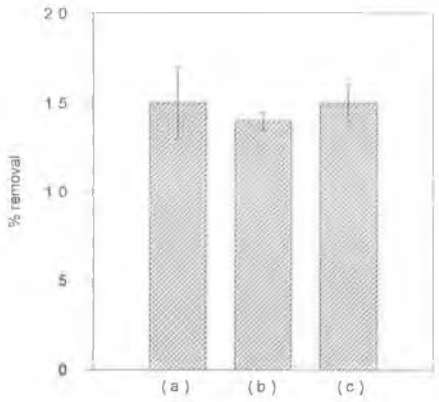

Figure 20: Percentage tallow removal by *T. harzianum* Rifai RP1 grown on 20g/l tallow with (a) 2 day old inoculum; (b) 5 day old inoculum and (c) 8 day old inoculum.

3.2.2.2 The influence of the physical nature of the tallow

The tallow substrate was solid at normal incubation temperatures. This reduced its bioavailability significantly. The changing of the physical nature of the tallow might increase its surface area, exposing a greater proportion to enzymatic attack. The tallow melted over a broad range, with liquefaction gradually taking place from approximately 40°C to 50°C. Up to 40°C, the tallow became softer as the temperature increased. The dispersion of tallow with a surfactant might also increase bioavailability, due also to increased surface area.

The effects of elevating the temperature and adding a surfactant were studied to determine if tallow removal could be enhanced, by increasing its bioavailability.

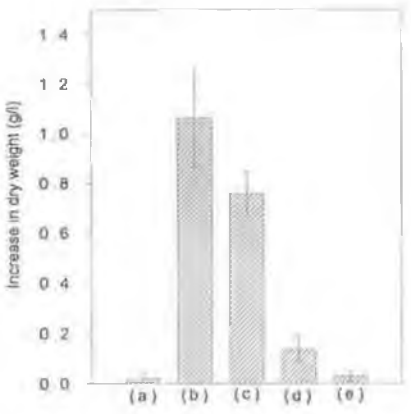

Figure 22: Dry weight increase of *T. harzianum* Rifai RP1 grown on 20g/l tallow at (a) 4°C; (b) 25°C; (c) 30°C; (d) 37°C and (e) 55°C.

The greatest decreases in pH were observed at 25°C and 30°C, the temperatures at which tallow removal was greatest. The pH dropped by 3.5 and 3.3 pH units respectively at these temperatures (Table 11). At 37°C, the pH drop was lower at 0.9 pH units. At 4°C and 55°C, the pH remained within 0.1 pH units of the controls.

Table 11: Changes in pH in cultures of *T. harzianum* Rifai RP1 following 10 days incubation at various temperatures. (initial pH = 7.0)

Temperature of incubation	**ΔpH**
4°C	0.1±0.02
25°C	-3.5±0.19
30°C	-3.3±0.17
37°C	-0.9±0.24
55°C	-0.1±0.04

The elevation of the temperature of incubation did not improve removal of tallow, despite the increased bioavailability. This was due to lack of growth of RP1 at the elevated temperatures, and at 4°C. The greatest removal was at 25°C, which displayed the greatest increase in biomass and the sharpest drop in pH. At 30°C, removal was slightly less, and this was reflected in biomass production and pH drop.

3.2.2.2.2 The influence of a surfactant

The melting of the tallow did not improve its removal, despite increased bioavailability. Adding a surfactant to the culture medium would increase surface area of the tallow, and hence bioavailability, without the inhibitory effects of temperature extremes. The effect of adding a surfactant on tallow removal was therefore examined.

The surfactants Tween 80 and Triton X-100 were selected as possible emulsifiers. Preliminary experiments found that 2%(w/v) Tween 80 or 0.5%(w/v) Triton X-100 were required to effectively disperse the tallow. The effect of the surfactants at these concentrations on the hexane extraction and measurement of extracellular tallow was examined before use in culture. Although Tween 80 had little impact on the recovery of tallow, Triton X-100 interfered with the assay of tallow. It was therefore decided to use Tween 80 as the surfactant in culture. The effect of its addition to cultures of *T. harzianum* Rifai RP1 grown on tallow was examined.

It was found that Tween 80 reduced the removal of tallow by *T. harzianum* Rifai RP1 over 10 days incubation. From 20% removal without Tween, removal dropped to 11% in its presence. However, it was found that biomass increase was greater with Tween added (Figure 23). In its absence, dry weight increase was 1.5g/l, but 4.5g/l with the surfactant. This suggested that RP1 could utilise Tween as a carbon source. This was confirmed with Tween 80 as the sole carbon source, when dry weight increased by 2.8g/l.

A similar pH drop was noted for cultures with tallow and with and without Tween (Figure 24).

The addition of the surfactant inhibited the removal of tallow from culture by RP1. It was decided to carry out further studies without the surfactant.

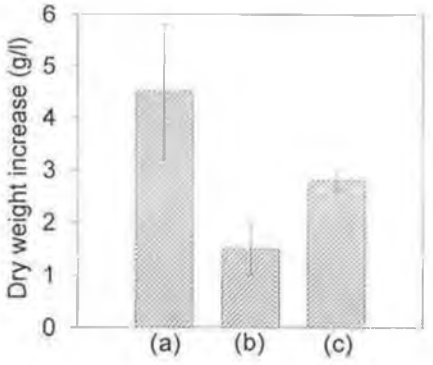

Figure 23: Dry weight increase of *T. harzianum* Rifai RP1, with (a) tallow + Tween 80; (b) tallow only and (c) Tween 80 only.

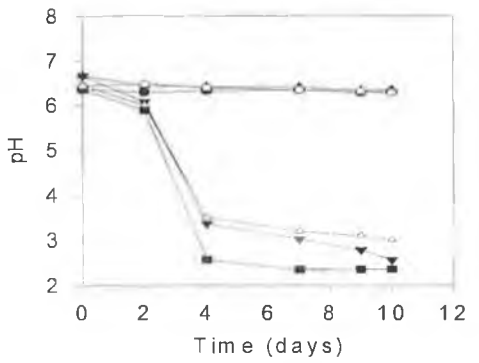

Figure 24: pH of cultures of *T. harzianum* Rifai RP1 with tallow alone, Tween 80 alone, and a combination of both. Symbols: ● tallow + Tween 80, control; ■ tallow + Tween 80, inoculated; ▲ tallow only, control; ▼ tallow only, inoculated; □ Tween 80 only, control; Δ Tween 80 only, inoculated.

3.2.2.3 The effect of pH control on tallow removal

During the growth of *T. harzianum* Rifai RP1 on tallow, large drops in pH were observed. The influence of buffering of the medium on removal of tallow, and on biomass production was examined. A range of pH values were chosen – buffering to pH 3, 4, 5, 6 and 7 were compared.

3.2.2.3.1 The effect of buffering with citrate phosphate buffer

0.1M citrate phosphate buffer was used at pH's of 3, 4, 5, 6 and 7 in the medium, over an incubation period of 10 days. This buffer was chosen as it could be used over this wide pH range. Again, tallow removal and growth of RP1 were recorded.

It was found that the optimum pH for tallow removal was pH 6, with 34% of 20g/l initial extracellular tallow removed after 10 days (Figure 25). Lower removals of

tallow were observed at other pH's. 26%, 21%, 17% and 14% removal were achieved at pH's 5, 4, 7 and 3 respectively. In the unbuffered culture, only 11% removal was observed.

The largest increases in dry weight occurred in cultures with the highest tallow removal (Figure 26). The greatest increase occurred at pH 6, at 3.6g/l, with less biomass produced at other buffered pHs. The increase was 2.5g/l at pH 5, 1.8g/l at pH 4, 1.5g/l at pH 3 and 1.4g/l at pH 7. The lowest dry weight increase occurred in unbuffered culture, at 0.9g/l.

The optimum pH for tallow removal and growth of RP1 was buffered to pH 6. However, it was noticed that some growth occurred on the citrate buffer in the absence of tallow. Therefore, it was decided to confirm the optimum pH on other buffers.

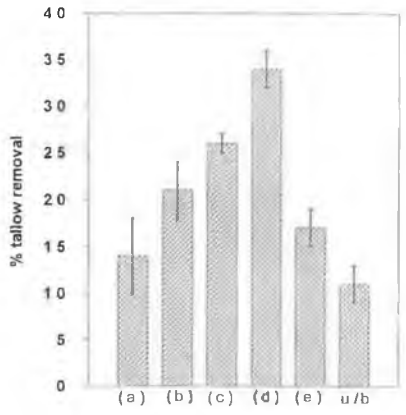

Figure 25: Tallow removal by *T. harzianum* Rifai RP1 in 0.1M citrate buffered cultures at (a) pH 3; (b) pH 4; (c) pH 5; (d) pH 6; (e) pH 7; and u/b unbuffered.

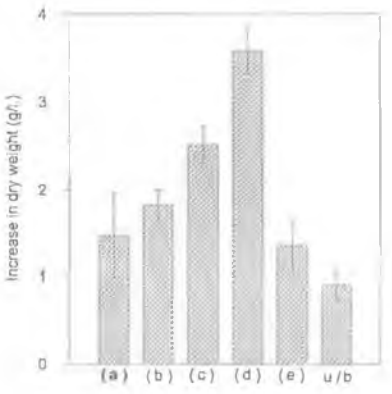

Figure 26: Dry weight increase in cultures of *T. harzianum* Rifai RP1 in 0.1M citrate buffered cultures at (a) pH 3; (b) pH 4; (c) pH 5; (d) pH 6; (e) pH 7; and u/b unbuffered.

3.2.2.3.2 The effect of buffering with Phosphate and KH phthalate buffers

To confirm findings with citrate phosphate buffer, the effect of buffering with phosphate and KH phthalate buffers was studied. Phosphate buffer was used at pH 6 and 7, KH phthalate HCl buffer was used at pH 5, and KH phthalate NaOH buffer was used at pH 3 and 4.

As was found with citrate phosphate buffer, the optimum pH for tallow removal was pH 6, at 40% (Figure 27). The order of tallow removal was slightly different for other pH values. pH 7, 5, 4 and 3 facilitated removal of 23%, 10%, 9% and 5% respectively.

Dry weight increases followed the same trend, with the highest at pH 6, of 2.6g/l (Figure 28). Increases of 2.3, 0.8, 0.5 and 0.4g/l were observed at pH's of 7, 5, 4 and 3 respectively.

Since the optimum pH was confirmed at pH 6 and RP1 did not grow on the phosphate buffer in the absence of an added carbon source, it was decided that it would be used for subsequent experiments.

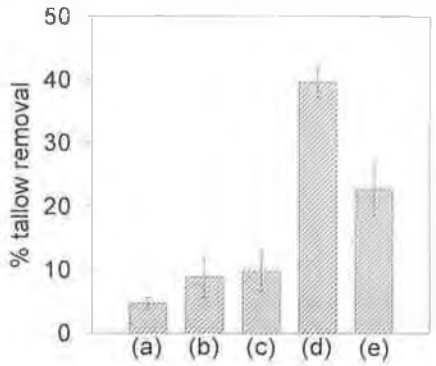

Figure 27: Tallow removal by *T. harzianum* Rifai RP1 in phosphate and KH phthalate buffered cultures at (a) pH 3; (b) pH 4; (c) pH 5; (d) pH 6 and (e) pH 7.

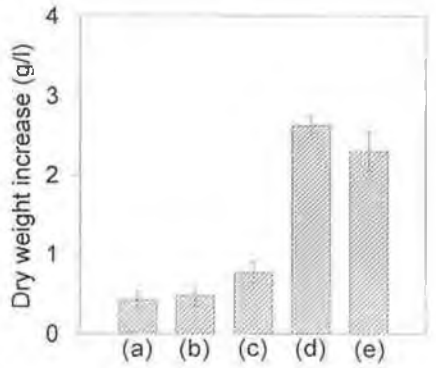

Figure 28: Dry weight increase by T. harzianum Rifai RP1 in phosphate and KH phthalate buffered cultures at (a) pH 3; (b) pH 4; (c) pH 5; (d) pH 6 and (e) pH 7.

3.2.2.4 The effect of temperature on growth and tallow removal

It was noted in earlier experiments that the temperature of incubation had a significant effect on the growth of *T. harzianum* Rifai RP1 in unbuffered cultures. At higher temperatures, the biomass production was greatly reduced. The optimum temperature of incubation for growth and enzyme activity can vary from species to species. To determine if the temperature of incubation had an effect on growth and tallow removal in buffered culture, the effect of incubating at temperatures of 4°C, 25°C, 30°C, 37°C and 55°C was examined.

The greatest tallow removal was observed at 25°C, at 43%, followed by 30°C with 29% (Figure 29). No removal was observed at 4°C or 37°C, and just 2% was seen at 50°C.

The greatest increase in dry weight was also observed at 25°C, with 2.8g/l being produced (Figure 30). At 30°C, 2g/l was produced, while no increase was observed at 37°C or 50°C. An increase of 0.1g/l was seen at 4°C. pH held to within 0.2 pH units of the original value throughout the run.

The optimum temperature for growth and tallow removal in buffered and unbuffered cultures was 25°C. This temperature was therefore used for subsequent studies.

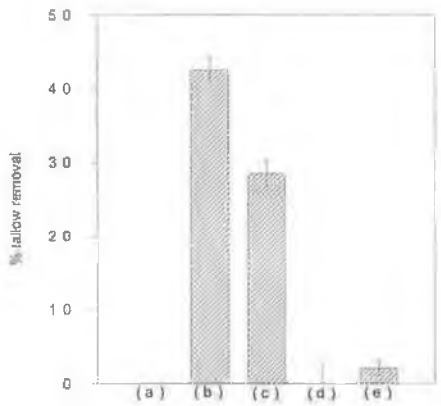

Figure 29: Tallow removal by *T. harzianum* Rifai RP1 in pH 6 buffered medium at (a) 4°C; (b) 25°C; (c) 30°C; (d) 37°C and (e) 55°C.

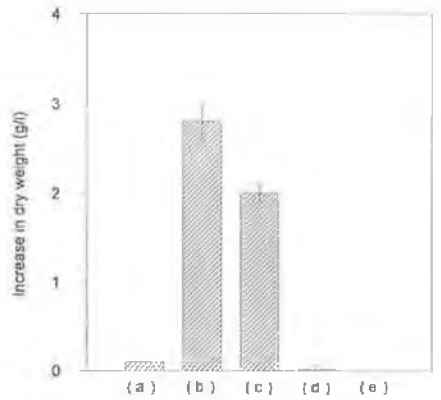

Figure 30: Dry weight increase by *T. harzianum* Rifai RP1 in pH 6 buffered medium at (a) 4°C; (b) 25°C; (c) 30°C; (d) 37°C and (e) 55°C.

3.2.2.5 The influence of other factors when grown at optimum pH and temperature

3.2.2.5.1 The influence of the inoculum size

The inoculum size used in the previous experiments was one 8mm diameter mycelial agar plug. It was found that the removal of tallow was directly related to the amount of RP1 biomass produced during incubation. The addition of larger inocula was examined to determine if it would result in higher production of biomass, or greater removal of tallow.

The inocula sizes were increased by adding additional mycelial agar discs to the culture media. Inocula sizes of 1, 5 and 10 mycelial agar discs were examined.

It was found that the removal of tallow decreased slightly with increasing inoculum size (Figure 31). With one mycelial agar plug as the inoculum, removal was 40%. When the number of plugs was increased to 5, tallow removal was 35%, and with 10 plugs, 30% removal was observed. There was no significant difference in dry weight increase between inoculum sizes (Figure 32). Increases of 2.6, 2.6 and 2.8g/l were observed in flasks with 1, 5 and 10 plug inocula respectively.

An inoculum of 1 agar plug was the most suitable for tallow utilisation experiments. The removal of tallow was slightly greater than with the larger inocula. Also, with the higher inoculum sizes, more malt extract agar was added to the flasks in the inoculum. The amount of carbohydrate added in the inocula was estimated using the Dubois assay. One 8mm plug of malt extract agar from a petri dish containing approximately 20ml of agar contained 3-5mg of carbohydrate. It was decided to use 1 agar plug of a 5-day old culture as the inoculum in subsequent studies.

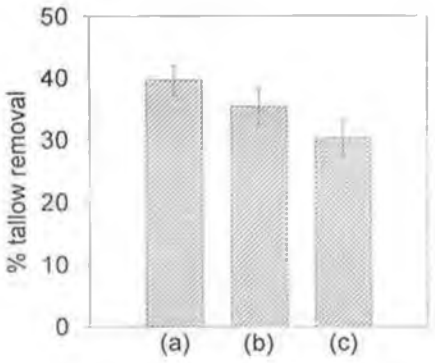

Figure 31: Tallow removal by *T. harzianum* Rifai RP1 with (a) 1, (b) 5 and (c) 10 mycelial agar plugs.

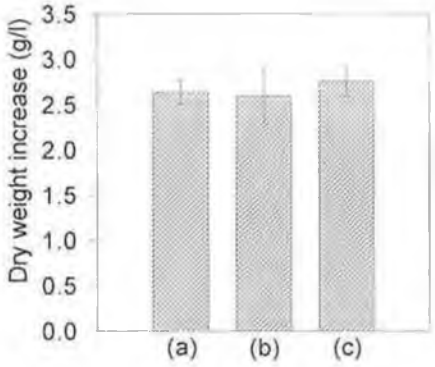

Figure 32: Dry weight increase of *T. harzianum* Rifai RP1 with (a) 1, (b) 5 and (c) 10 mycelial agar plugs.

3.2.2.5.2 The influence of the rate of agitation

The studies on tallow removal were carried out in shake flask cultures. During the isolation of the organisms, it was noted that all the filamentous fungi was found in shaking cultures, and not in non-shaking cultures. The level of aeration of the medium may have an effect on the growth, and hence the tallow removal, by *T. harzianum* Rifai RP1. The influence of varying the rate of agitation on cultures was therefore examined. Agitation rates of 0, 100, 130 and 200 rpm were studied.

It was found that the optimum agitation rate for removal of tallow was 130rpm (Figure 33). At this rate, removal was 40%. At 0, 100 and 200rpm, tallow removal was 7%, 17% and 24% respectively. The increase in dry weight followed the same trend (Figure 34). Increases of 1.9, 2.1, 2.6 and 2.3 g/l were observed at speeds of 0, 100, 130 and 200 rpm respectively. pH held to within 0.15 pH units of the original values. The optimum agitation rate was therefore deemed to be 130rpm, and was used for further studies.

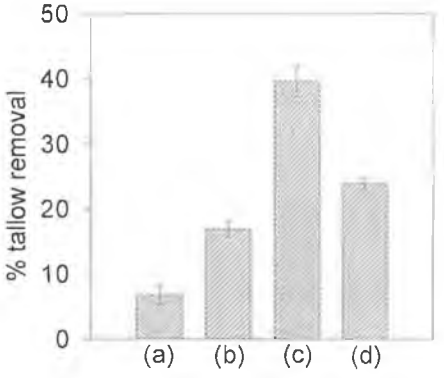

Figure 33: Tallow removal by *T. harzianum* Rifai RP1 at (a) 0 rpm; (b) 100 rpm; (c) 130 rpm and (d) 200 rpm.

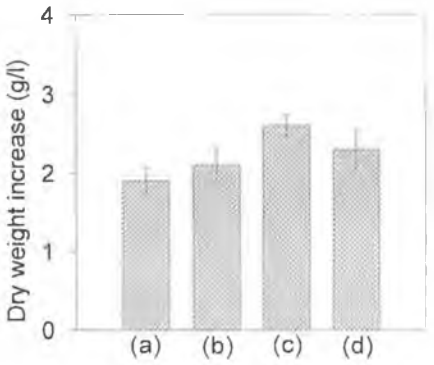

Figure 34: Dry weight increase of *T. harzianum* Rifai RP1 at (a) 0 rpm; (b) 100 rpm; (c) 130 rpm and (d) 200 rpm.

3.2.2.5.3 The influence of tallow concentration

In the previous experiments, a tallow concentration of 20g/l was added to the media. The removal of this tallow was approximately 40% under optimum environmental conditions. The reduction of the tallow concentration might improve the efficiency, if biomass production was not decreased by too high a degree. The influence of the concentration of tallow on growth and tallow removal rates was therefore examined.

The production of biomass was restricted with lower concentrations of the tallow substrate. Dry weight increase was greatest for an initial tallow concentration of 20g/l, with lower increases observed for 10g/l, 2g/l and 1g/l. Increases of 2.4g/l, 1.7g/l, 1.0g/l and 0.6g/l respectively were noted after 10 days (Figure 35).

However, the percentage of initial tallow removed increased as the concentration was decreased. The removal was greatest with 1g/l initial tallow, down to lowest percentage removal with 20g/l. Initial concentrations of 1, 2, 10 and 20g/l tallow were reduced by 80%, 74%, 45% and 33% respectively (Figure 36). Although the highest

proportion of extracellular fat was removed at the lower tallow concentrations, the specific removal dropped slightly as the initial concentrations were lowered (Figure 37). The variation of the concentrations of tallow has a significant effect on the ability of RP1 to biodegrade it. It was therefore decided to carry out the in-depth studies of tallow metabolism by RP1 at the four different concentrations under optimal environmental conditions, as described in Table 12.

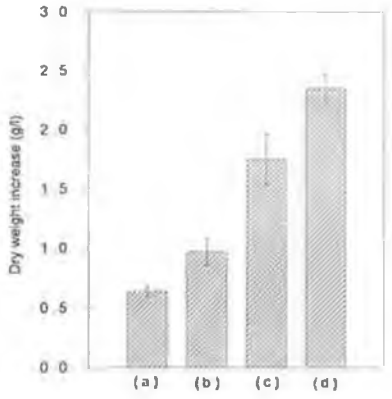

Figure 35: Dry weight increase of *T. harzianum* Rifai RP1 with (a) 1g/l, (b) 2g/l, (c) 10g/l and (d) 20g/l initial tallow.

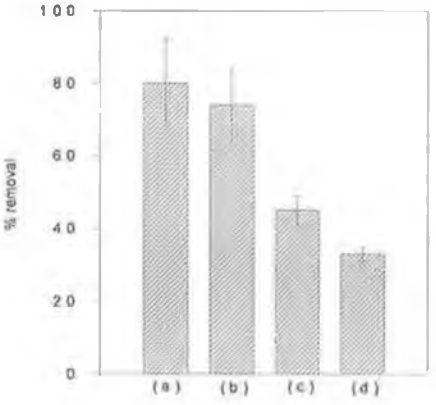

Figure 36: Tallow removal by *T. harzianum* Rifai RP1 (a) 1g/l, (b) 2g/l, (c) 10g/l and (d) 20g/l initial tallow.

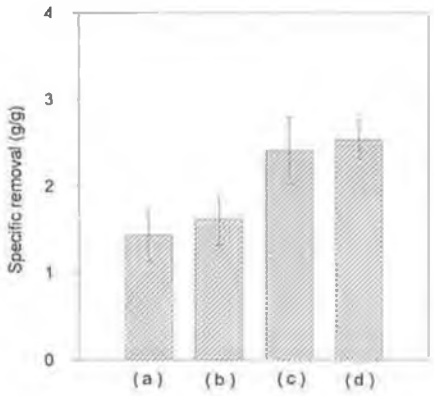

Figure 37: Tallow removed per unit dry weight increase (g/g) of *T. harzianum* Rifai RP1 with (a) 1g/l, (b) 2g/l, (c) 10g/l and (d) 20g/l initial tallow.

Table 12: Optimum environmental conditions for the culture of *T. harzianum* Rifai RP1 on tallow as the sole carbon source.

Parameter	**Optimum condition**
pH	6.0
Temperature	25°C
Agitation	130rpm
Inoculum	one 5-day old mycelial plug
Surfactant	none

3.3 An investigation of the biodegradation of tallow as the sole carbon source by *Trichoderma harzianum* Rifai RP1 under optimal environmental conditions.

In order to study the biodegradation of tallow at various concentrations, *T. harzianum* Rifai RP1 was set up in shake flask culture under optimum environmental conditions as determined previously. The substrate, tallow, was added at concentrations of 1, 2, 10 and 20g/l. Extracellular lipid, dry weight and intracellular lipid were monitored, as well as the glyceride composition of extracted lipid and fatty acid components in the glyceride fractions.

3.3.1 The determination of growth parameters

During growth of *T. harzianum* Rifai RP1 with tallow as the sole carbon source, the dry weight increased, while extracellular tallow disappeared from culture. The media were buffered to pH 6, so pH held to within 0.25 pH units of the original values.

Growth kinetics

The growth of RP1 on tallow was rapid over the first 2 days, after which it slowed, and the fungus reached stationary phase (Figure 38). After 10 days, the dry weight had increased by 0.7, 0.9, 1.5 and 2.0g/l for initial concentrations of 1, 2, 10 and 20g/l respectively.

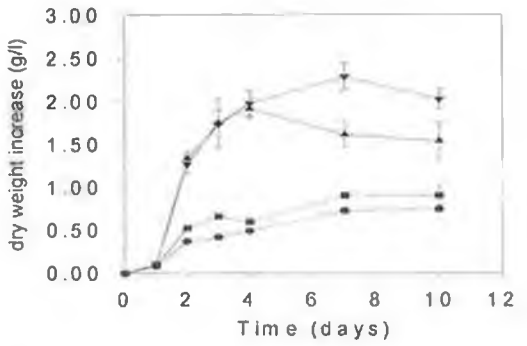

Figure 38: Growth of *T. harzianum* Rifai RP1 when grown on (●) 1g/l tallow, (■) 2g/l tallow, (▲) 10g/l tallow and (▼) 20g/l tallow.

The specific growth rates (μ) increased with increasing tallow concentrations (Table 13). Values of 0.827, 1.008, 1.392 and 1.364 day^{-1} were found for concentrations of 1, 2, 10 and 20g/l respectively.

Table 13: Specific growth rates of *T. harzianum* Rifai RP1 grown on tallow at 1, 2, 10 and 20g/l.

Initial tallow concentration (g/l)	Specific growth rate, μ (day^{-1})
1	0.827
2	1.008
10	1.392
20	1.364

The correlation between specific growth rates and substrate concentration followed Monod kinetics (Figure 39). Monod kinetics follows the equation:

$$\mu = \frac{\mu_{max} s}{s + k_s} \qquad \text{(Pirt, 1975)}$$

where:

μ, μ_{max} = Specific growth rate and maximum specific growth rate;

s = substrate concentration;

k_s = saturation constant.

A plot of $1/\mu$ against $1/s$ gives a straight line if Monod kinetics are followed, according to the equation:

$$\frac{1}{\mu} = \frac{k_s}{s\mu_{max}} + \frac{1}{\mu_{max}} \qquad \text{(Pirt, 1975)}$$

The double-reciprocal plot had a correlation coefficient of 0.986 (Figure 40) which confirmed the validity of the Monod relationship between specific growth rates and tallow concentrations. From this plot, the values for k_s and μ_{max} were found to be 0.758g/l and 1.438 day^{-1} respectively.

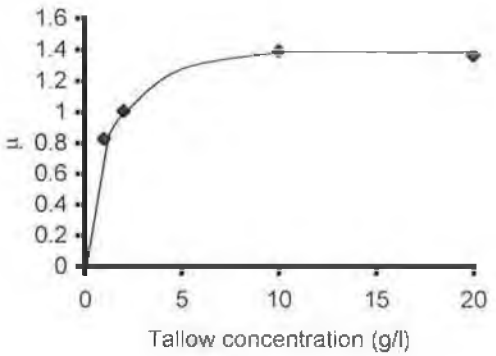

Figure 39: Specific growth rate of *T. harzianum* Rifai as a function of tallow concentration.

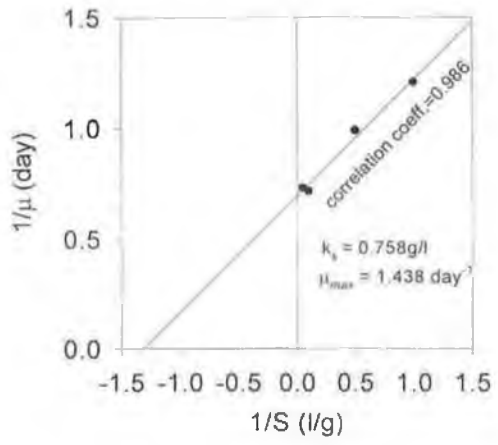

Figure 40: Double reciprocal plot for specific growth rate, μ, of cultures of *T. harzianum* Rifai RP1 with tallow as carbon source.

Substrate removal

During this growth of RP1, tallow was removed from cultures, showing a lag of 1 day at the different concentrations (Figure 41). With 1 and 2g/l tallow, following the lag, a period of rapid tallow removal was observed up to day 2, after which the rate of removal slowed. A more gradual removal pattern was seen with 10 and 20g/l. The proportion of the tallow removed by RP1 during the course of incubation varied between the different initial concentrations. It was high with 1 and 2g/l, with 83% and 79% respectively of the initial concentrations removed after 10 days. Smaller fractions were removed with 10 and 20g/l, final concentrations representing removals of 42% and 31% respectively.

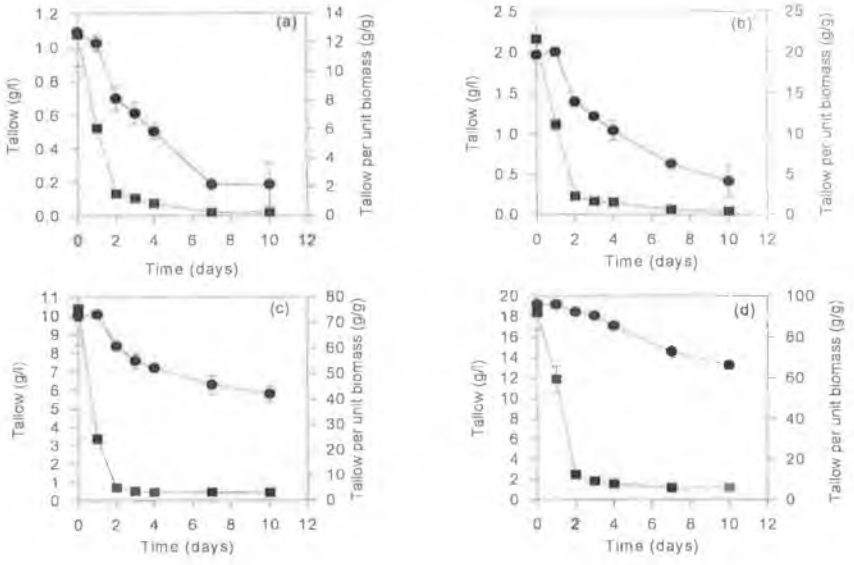

Figure 41: Tallow remaining (●, g/l) and tallow per unit biomass (■, g/g) when *T. harzianum* Rifai RP1 was grown on (a) 1g/l tallow, (b) 2g/l tallow, (c) 10g/l tallow, (d) 20g/l tallow.

Specific removals of tallow by RP1 also followed distinctive trends (Figure 41). They were fastest over the first 2 days, after which little specific removal was observed. The rate over the first two days increased with increasing tallow concentrations (Table 14). However, this trend in rates appeared to level at initial concentrations higher than 10g/l tallow. This suggested that the correlation between the specific tallow removal rate and the initial concentration of tallow may be described by Michaelis-Menten kinetics. Michaelis-Menten kinetics follow the relationship:

$$r = \frac{r_{\max} s}{s + K_m}$$ (Mathews and van Holde, 1990)

where:

r, r_{max} = reaction rate and maximum reaction rate;

K_m was the saturation constant and s = substrate concentration.

The double reciprocal plot had a correlation coefficient of 0.998, indicating that Michaelis-Menten kinetics was valid to describe the relationship between specific removal rates of tallow and initial tallow concentrations (Figure 42). The maximum theoretical specific removal rate of tallow could then be calculated, and was found to be 65.17$gg^{-1}day^{-1}$. The saturation constant, k_m, was found to be 10.94g/l.

Table 14: Specific tallow removal rates observed during growth of *T. harzianum* Rifai RP1 on tallow at 1, 2, 10 and 20g/l.

Tallow concentration (g/l)	Specific tallow removal rate ($gg^{-1}day^{-1}$)	
	days 0-2	days 2-10
1	5.51±1.09	0.17±0.03
2	9.68±1.43	0.22±0.03
10	35.24±7.22	0.17±0.06
20	39.73±4.21	0.72±0.78

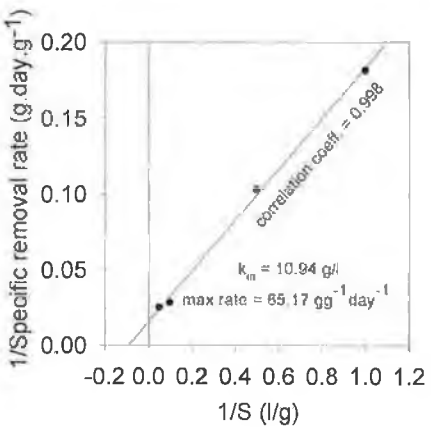

Figure 42: Double reciprocal plot for specific tallow removal rate by *T. harzianum* Rifai RP1 grown on tallow.

Intracellular lipid accumulation

It was found that during its growth on tallow, various quantities of lipid were accumulated intracellularly in the biomass of *T. harzianum* Rifai RP1 (Figure 43). The amount of intracellular lipid per unit biomass was similar at all the concentrations studied. Intracellular lipid accumulated from day 4 onwards, reaching concentrations of 0.45, 0.55, 0.38 and 0.33g/g for tallow concentrations of 1, 2, 10 and 20g/l respectively by the end of incubation.

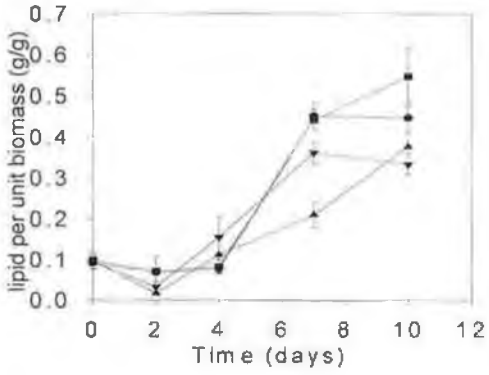

Figure 43: Intracellular lipid per unit biomass (g/g) of *T. harzianum* Rifai RP1 grown on (●) 1g/l tallow, (■) 2g/l tallow, (▲) 10g/l tallow and (▼) 20g/l tallow.

The determination of yields

The yields of *T. harzianum* Rifai RP1 biomass on tallow also varied at the different substrate concentrations. The Yield coefficients, $Y_{x/s}$ (the yield of total dry weight on the substrate) was calculated from the formula

$$Y_{x/s} = \frac{\Delta x}{\Delta s}$$ (Pirt, 1975)

where:

Δx = the amount of biomass formed;

Δs = the amount of substrate utilised.

The lower initial concentrations gave higher dry weight yield per unit fat removal (Table 15). Coefficients of 0.83, 0.58, 0.37 and 0.34 were observed at initial concentrations of 1, 2, 10 and 20g/l respectively.

Table 15: Yield coefficients ($Y_{x/s}$) observed following 10 days incubation of *T. harzianum* Rifai RP1 with tallow at 1, 2, 10 and 20g/l.

Initial Tallow concentration (g/l)	Yield coefficient ($Y_{x/s}$)
1	0.83±0.13
2	0.58±0.10
10	0.37±0.06
20	0.34±0.03

The accumulated lipid accounted for a proportion of the dry weight produced during incubation, which was used earlier to calculate biomass yields. Since some of the extracellular lipid removed was not metabolised, the calculated yield might not have given an accurate indication of biomass produced per unit substrate. The yield of fat-

free biomass on the metabolised lipid was expressed as the yield coefficient, $Y_{xf/sm}$. The metabolised lipid (sm) was the extracellular lipid removed, less any accumulation in the biomass:

$$sm = s_{rem} - s_{acc}$$

where:

s_{rem} = lipid removed from the supernatant;

s_{acc} = lipid accumulated in the biomass.

The fat-free biomass (xf) was the fungal cellular material of RP1, not including the stored lipid.

$$xf = x - s_{acc}$$

The yield coefficient, $Y_{xf/sm}$, was calculated from the formula:

$$Y_{xf/sm} = \frac{\Delta xf}{\Delta sm}$$

The calculated values for $Y_{xf/sm}$ were similar to those of $Y_{x/s}$ (Table 16). As seen with the yield coefficients, $Y_{x/s}$, the values with 1 and 2g/l initial tallow, 0.86 and 0.44 respectively, were higher than those with 10 and 20g/l initial tallow. At the higher concentrations, the Yield coefficients were 0.33 and 0.29 respectively. This indicated that accumulation of the lipid intracellularly did not alter the overall yield of biomass of RP1 on tallow.

Table 16 : Yield coefficients ($Y_{xf/sm}$) observed following 10 days incubation of *T. harzianum* Rifai RP1 with tallow at 1, 2, 10 and 20g/l.

Initial tallow concentration (g/l)	Fat free biomass (Δxf) (g/l)	Lipid metabolised (Δsm) (g/l)	Yield coefficient ($Y_{xf/sm}$)
1	0.463±0.051	0.541±0.133	0.86±0.22
2	0.453±0.120	1.021±0.200	0.44±0.14
10	1.156±0.235	3.504±0.411	0.33±0.08
20	1.495±0.124	5.230±0.386	0.29±0.03

3.3.2 The composition of extracellular lipid

Biodegradation of tallow occurs by hydrolysis of the glycerides, followed by assimilation of fatty acids into the biomass. Since the glycerides were composed of mixed fatty acids, the mode of breakdown may vary from organism to organism. The composition of the breakdown products of tallow biodegradation by *T. harzianum* Rifai RP1 was therefore of interest. In order to examine this, extracellular lipid extracts were analysed with respect to their glyceride and free fatty acid compositions.

3.3.2.1 Concentration patterns of the lipid constituents

Glyceride composition of tallow

Triglycerides were the main constituent of tallow at the beginning of incubation (Table 17). 1,3 diglycerides were the next major component, followed by free fatty acids. 1,2 diglycerides and monoglycerides were each present at less than 1% of the

tallow. Biodegradation of tallow occurs by the hydrolysis of triglycerides to diglycerides, which in turn are hydrolysed to monoglycerides, which are finally hydrolysed to release the last fatty acids. Each step of hydrolysis adds to the free fatty acids, which are assimilated by the microbial cell.

Table 17: Percentage of glycerides and free fatty acids in tallow used as the substrate for *T. harzianum* Rifai RP1.

Component	Percentage
Triglycerides	87.3±0.9%
1,3 diglycerides	8.2±2.0%
1,2 diglycerides	0.6±0.3%
monoglycerides	0.2±0.1%
free fatty acids	3.7±0.1%

Triglyceride concentrations

It was essential that the triglycerides were hydrolysed for effective removal of the tallow substrate from cultures. The concentrations of triglycerides were therefore followed to determine if triglyceride hydrolysis was occurring.

The hydrolysis of the triglycerides was evident at all the tallow concentrations (Figure 44). This hydrolysis mirrored the removal of total lipids. With 1 and 2g/l tallow, triglyceride concentrations decreased rapidly after day 1, the rate slowing after day 4. At the higher concentrations of 10 and 20g/l, hydrolysis was more gradual, with no rapid initial drop in concentrations.

With 1g/l initial tallow, triglycerides dropped from 0.95g/l to 0.06g/l over the 10 days of incubation. At 2g/l tallow, they reached 0.12g/l from 1.72g/l. These residual

concentrations indicated that triglycerides were hydrolysed by 94% and 93% respectively. With 10g/l, triglycerides decreased from 8.71g/l to 2.90g/l. When 20g/l tallow was added, triglycerides dropped from 16.77g/l to 7.25g/l. These final concentrations indicated lower levels of hydrolysis, with 67% and 57%, respectively, hydrolysed after 10 days.

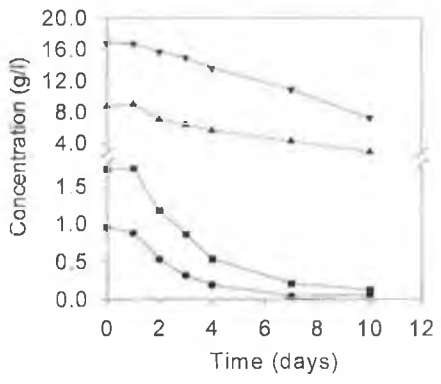

Figure 44: Extracellular triglyceride concentrations in cultures of *T. harzianum* Rifai RP1 grown on (●) 1g/l tallow, (■) 2g/l tallow, (▲) 10g/l tallow and (▼) 20g/l tallow.

Di- and Monoglyceride concentrations

Biodegradation of triglycerides to fatty acids and glycerol proceeds through di- and monoglyceride intermediates. Hydrolysis of the triglycerides did not present a problem for *T. harzianum* Rifai RP1. However, hydrolysis of the di- and monoglycerides must also occur for effective biodegradation of tallow. Their extracellular concentrations were therefore followed to determine if they were being broken down as they were produced by hydrolysis of the triglycerides.

The concentrations of diglycerides and monoglycerides remained relatively constant throughout the growth of RP1 on tallow (Figure 45). 1,2 diglycerides and monoglycerides were detected at very low levels, but 1,3 diglycerides contributed more significantly to the extracellular lipid. With 1 and 2g/l tallow, triglyceride levels

had dropped to similar concentrations as 1,3 diglycerides by day 10. With 10 and 20g/l tallow, both diglycerides and monoglycerides remained at concentrations significantly lower than triglycerides throughout incubation.

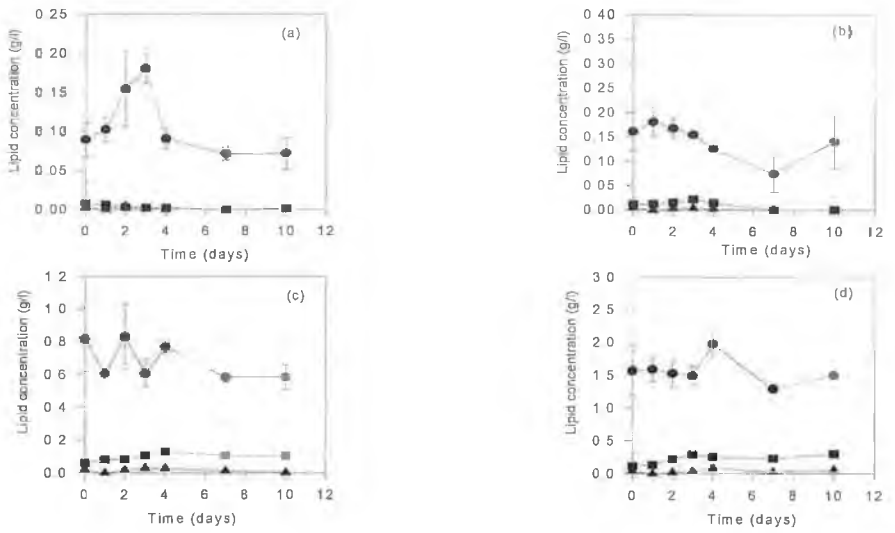

Figure 45: Extracellular diglyceride and monoglyceride concentrations during incubation of *T. harzianum* Rifai RP1 on (a) 1g/l tallow; (b) 2g/l tallow; (c) 10g/l tallow; (d) 20g/l tallow. Symbols: ● 1,3 diglycerides; ■ 1,2 diglycerides; ▲ monoglycerides.

Free fatty acid concentrations

The fatty acids released by hydrolysis of the tallow glycerides were available to *T. harzianum* Rifai RP1 as free fatty acids which could be assimilated by the biomass. The concentrations of extracellular free fatty acids were of interest to monitor if uptake was proceeding as quickly as production.

Extracellular free fatty acid concentrations fluctuated during incubation of RP1 on tallow (Figure 46). With initial tallow concentrations of 1 and 2g/l, their

concentrations increased sharply from day 2 to 4, after which they decreased. The peak concentrations observed on day 4 were 0.22 and 0.37g/l dropping to residual concentrations of 0.06 and 0.15g/l for 1 and 2g/l respectively. A peak was not seen with tallow concentrations of 10 and 20g/l. In these cases, free fatty acid concentrations increased gradually over the period studied. Their final concentrations were 2.19 and 4.14g/l respectively.

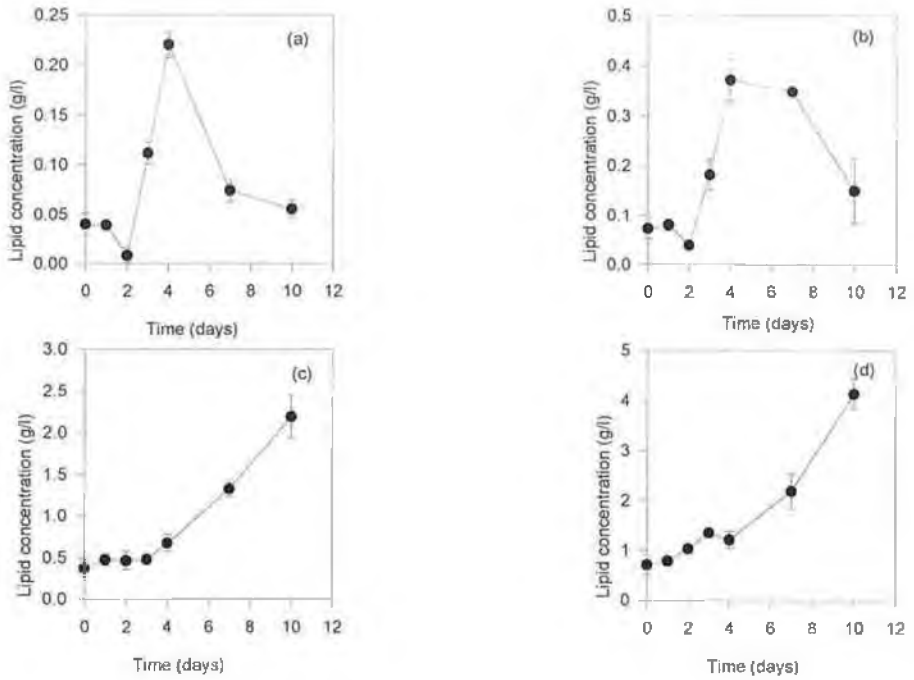

Figure 46: Extracellular free fatty acid concentrations during incubation of *T. harzianum* Rifai RP1 on (a) 1g/l tallow; (b) 2g/l tallow; (c) 10g/l tallow; (d) 20g/l tallow.

Fatty acids released by hydrolysis of glycerides

The hydrolysis of the glycerides resulted in the release of free fatty acids, which were then available for assimilation by RP1. By calculating the amount of these released fatty acids, the time course of hydrolytic activity could be followed. This was of interest to examine if the accumulation patterns of the extracellular free fatty acids could be explained by the patterns of hydrolytic activity during incubation.

The amount of free fatty acids released by glyceride hydrolysis was calculated by expressing the glyceride concentrations as millimoles and then using the formula:

$$F_{tot} = 3T + 2D + M$$

where:

F_{tot} = the number of millimoles of fatty acids produced by glyceride hydrolysis;

T = the decrease in the number of millimoles of triglycerides;

D = the decrease in the number of millimoles of diglycerides;

M = the decrease in the number of millimoles of monoglycerides.

The resulting values were then expressed in g/l. In cultures with initial tallow concentrations of 1 and 2g/l, the fatty acid release due to glyceride hydrolysis was rapid after the initial lag of 1 day, and slowed with little extra release after day 7 (Figure 47). At the end of incubation, 0.89 and 1.58g/l respectively of fatty acid had been released. At 10g/l, the rapid release was apparent initially also, and continued at a relatively constant rate to day 10, when 5.82g/l had been released. With 20g/l tallow, after the lag of 1 day, fatty acid release also continued at a relatively constant rate up to day 10. At day 10, 9.10g/l of fatty acid had been released due to glyceride hydrolysis.

The release per gram of biomass was similar for the different concentrations up to day 4 (Figure 48). After day 4, the release remained constant for tallow concentrations of 1 and 2g/l, but continued to increase for 10 and 20g/l. After 10

days, the free fatty acids released per gram of biomass had reached 1.1gg^{-1} and 1.6 gg^{-1} for 1 and 2g/l tallow. With 10 and 20g/l tallow, it had reached 3.1gg^{-1} and 4.1gg^{-1} respectively.

It can be seen that hydrolysis of glycerides had ceased after day 4 with 1 and 2g/l tallow. Hydrolysis continued with 10 and 20g/l to the end of incubation. Similar specific concentrations of fatty acid had been released at all concentrations of tallow up to day 4. After day 4, the specific concentrations were similar for 10 and 20g/l tallow, and only a small difference was observed between 1 and 2g/l .

It was seen that with 1 and 2g/l tallow, hydrolysis of glycerides ceased on day 4, the point at which extracellular accumulation of free fatty acids peaked and concentrations began to drop. With the higher concentrations, hydrolysis continued to the end of incubation, with accumulation of the free fatty acids continuing in parallel.

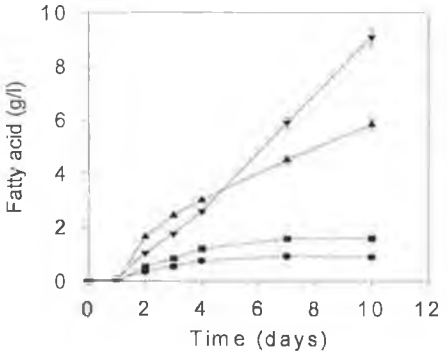

Figure 47: Fatty acid released by hydrolysis of extracellular glycerides in cultures of *T. harzianum* Rifai RP1 with (●) 1g/l initial tallow, (■) 2g/l initial tallow, (▲) 10g/l initial tallow and (▼) 20g/l initial tallow.

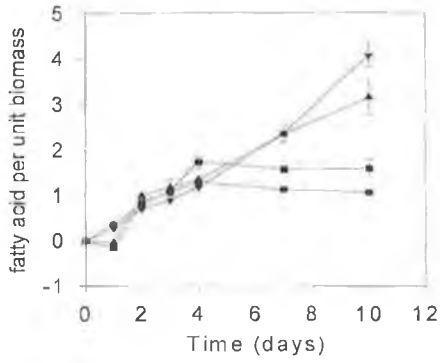

Figure 48: Fatty acids released by glyceride hydrolysis per unit biomass of *T. harzianum* Rifai RP1, with (●) 1g/l initial tallow, (■) 2g/l initial tallow, (▲) 10g/l initial tallow and (▼) 20g/l initial tallow.

3.3.2.2 Fatty acid composition of extracellular lipid components

Lipases may have fatty acid specificities, or may hydrolyse fatty acids from glycerides at different rates depending on chain length or degree of saturation. If preferential cleavage of any particular fatty acid from glycerides occurred, then the fatty acid composition of the glycerides would change over the course of incubation. The fatty acid compositions of the glycerides were therefore examined, to observe if any of its constituent fatty acids were resistant to hydrolysis from the glyceride backbone by RP1 lipases. This would give an indication of any fatty acid specificity by the lipases.

The main fatty acids detected in tallow were palmitic, stearic and oleic acids, with lower concentrations of myristic and linoleic acids (Table 18). Before incubation, oleic acid was at 41% and 46% of the fatty acids in tallow triglycerides and free fatty acids respectively. This was higher than the other main fatty acids, palmitic and stearic acid. Palmitic accounted for 24% and 25%, while stearic was at 28% and 25% respectively in the triglycerides and free fatty acids. However, in the diglycerides,

these fatty acids were present at similar levels, palmitic, stearic and oleic accounting for 34%, 31% and 32% of fatty acids respectively. Myristic and linoleic acids were present at significantly lower levels.

The fatty acid compositions after 10 days of incubation were similar to the initial values (Table 18). The glycerides and free fatty acids contained mainly palmitic, stearic and oleic acid. In the triglycerides, palmitic acid accounted for 27% to 30%, stearic acid was 28% to 35%, while oleic acid comprised 32% to 43% of the fatty acids. In the free fatty acids, palmitic was at 26% to 33%, stearic at 23% to 33%, and oleic at 35% to 45%. The diglycerides also consisted mainly of these fatty acids, with palmitic at 27% to 40%, stearic at 21% to 23% and oleic acid at 34% to 47%. In the glycerides and free fatty acids, myristic and linoleic acid were again present at significantly lower levels.

Since the compositions of the glycerides at the start and end of incubation did not show any significant changes, there was no fatty acid specificity evident during hydrolysis. Also, the free fatty acids were similar in composition, so no assimilation preference was evident by RP1 biomass.

The concentration of tallow substrate did not influence the fatty acid specificity of hydrolysis or free fatty acid assimilation. No composition pattern differences were noted between the concentrations examined.

Table 18: Fatty acid composition of extracellular triglycerides, diglycerides and free fatty acids in cultures of *T. harzianum* Rifai RP1 grown on 1, 2, 10 or 20g/l tallow for 10 days. (TG = triglycerides; DG = diglycerides; FFA = free fatty acids)

Fatty acid	Tallow conc. (g/l)	TG		DG		FFA	
		Initial %	Final %	Initial %	Final %	Initial %	Final %
Myristic	1	4.7±0.7	0±0	0.9±0.7	0±0	0.8±0.7	0±0
	2	4.7±0.7	0±0	0.9±0.7	0±0	0.8±0.7	0±0
	10	4.7±0.7	5.3±2.6	0.9±0.7	0±0	0.8±0.7	7.5±2.9
	20	4.7±0.7	0±0	0.9±0.7	0±0	0.8±0.7	0±0
Palmitic	1	23.5±0.8	30.3±1.6	33.9±0.8	40.1±1.6	25.3±0.8	29.0±1.6
	2	23.5±0.8	27.7±1.4	33.9±0.8	35.4±1.6	25.3±0.8	26.4±0.9
	10	23.5±0.8	26.6±1.1	33.9±0.8	36.9±2.3	25.3±0.8	27.6±1.3
	20	23.5±0.8	29.1±1.7	33.9±0.8	26.6±1.6	25.3±0.8	32.8±0.9
Stearic	1	28.1±0.9	27.8±1.3	31.0±0.9	22.0±1.3	24.9±0.9	32.5±1.3
	2	28.1±0.9	29.4±1.5	31.0±0.9	22.9±1.6	24.9±0.9	25.5±0.7
	10	28.1±0.9	34.5±2.3	31.0±0.9	20.8±0.9	24.9±0.9	23.0±0.9
	20	28.1±0.9	31.9±1.8	31.0±0.9	22.7±1.3	24.9±0.9	24.2±1.5
Oleic	1	41.1±0.8	39.3±1.6	32.1±0.8	35.4±1.6	45.6±0.8	35.4±1.6
	2	41.1±0.8	42.8±1.8	32.1±0.8	34.0±2.1	45.6±0.8	44.9±0.9
	10	41.1±0.8	31.5±1.7	32.1±0.8	39.6±0.9	45.6±0.8	38.8±1.2
	20	41.1±0.8	37.0±1.4	32.1±0.8	47.2±2.4	45.6±0.8	39.7±1.1
Linoleic	1	2.6±0.5	2.6±0.9	2.1±0.5	2.4±0.9	3.5±0.5	3.1±0.9
	2	2.6±0.5	0±0	2.1±0.5	7.7±1.6	3.5±0.5	3.2±0.9
	10	2.6±0.5	2.1±1.9	2.1±0.5	2.7±1.3	3.5±0.5	3.0±0.7
	20	2.6±0.5	2.1±0.9	2.1±0.5	3.5±0.8	3.5±0.5	3.4±1.0

3.3.2.3 Removal of the main fatty acid constituents

It was noted above that the concentrations of triglycerides decreased, diglycerides and monoglycerides remained relatively constant and free fatty acids accumulated transiently for 1 and 2g/l and permanently with 10 and 20g/l. In all these fractions, fatty acids were present. These were then hydrolysed from glycerides and taken up by RP1. The specific rates of removal of total extracellular myristic, palmitic, stearic, oleic and linoleic acids were of interest for comparison.

It was observed that specific removal of myristic acid was rapid over the first 2 days, with little variation in specific concentrations for the remainder of incubation (Figure 49). This pattern was apparent at all 4 concentrations of tallow. Similar patterns were observed for palmitic, stearic, oleic and linoleic acids, with a rapid decrease in specific concentrations, followed by a period of little change. To compare the relative rates at which individual fatty acids were assimilated, specific rates of removal from days 0 to 2 were calculated.

When the initial concentration of tallow was 1g/l, the greatest specific removal rate was of oleic acid (Tables 19). A rate of $1.99 gg^{-1}day^{-1}$ was noted. The removal rates of the next two most significant fatty acids, palmitic and stearic, were lower at 1.17 and $1.39 gg^{-1}day^{-1}$ respectively. The rates for myristic and linoleic acids were significantly lower, at 0.24 and $0.14 gg^{-1}day^{-1}$ respectively.

At an initial concentration of 2g/l tallow, a similar pattern was observed. Oleic had the highest specific removal rate, at $3.47 gg^{-1}day^{-1}$ over the first 2 days. Stearic followed, at $2.40 gg^{-1}day^{-1}$, while palmitic had a rate of $2.04 gg^{-1}day^{-1}$. Again, the lowest rates were for myristic and linoleic acids, at 0.39 and $0.23 gg^{-1}day^{-1}$ respectively.

At 10g/l initial tallow, oleic acid had again a significantly higher removal rate from days 0 to 2. Its rate of 12.72$gg^{-1}day^{-1}$ was greater than that of 8.84$gg^{-1}day^{-1}$ observed with stearic acid, and of 7.58$gg^{-1}day^{-1}$ with palmitic acid. Myristic and linoleic acid displayed lower rates of 1.39 and 0.95$gg^{-1}day^{-1}$ respectively.

With the highest tallow concentration of 20g/l, the trend was again repeated. Oleic acid had the highest specific removal rate of 14.34$gg^{-1}day^{-1}$ over the first two days, compared to 9.99$gg^{-1}day^{-1}$ with stearic and 8.41$gg^{-1}day^{-1}$ with palmitic acid. The lowest rates were again with myristic and linoleic acids, of 1.65 and 0.95$gg^{-1}day^{-1}$ respectively.

Although the specific removal rates of oleic acid were the highest, it was present in the highest concentration extracellularly. Stearic acid and palmitic acid were assimilated at lower rates, being present at lower concentrations. Therefore no definite inference of higher affinity for oleic acid could be drawn.

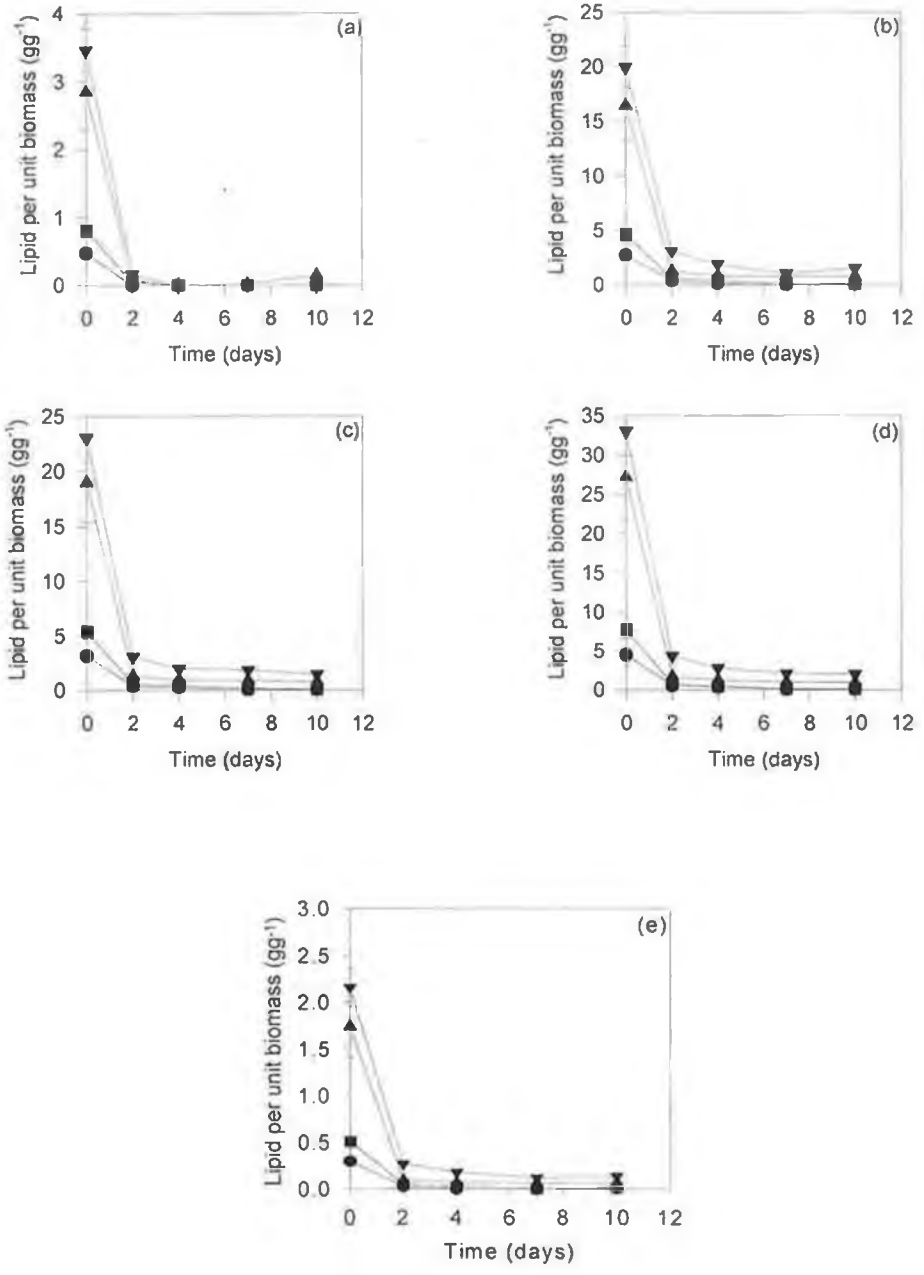

Figure 49: Total extracellular concentrations per unit biomass of (a) myristic acid, (b) palmitic acid, (c) stearic acid, (d) oleic acid and (e) linoleic acid in cultures of *T. harzianum* Rifai RP1 grown on (●) 1g/l initial tallow, (■) 2g/l initial tallow, (▲) 10g/l initial tallow and (▼) 20g/l initial tallow.

Table 19: Specific removal rates for total extracellular fatty acid contituents over the first two days of incubation, when *T. harzianum* Rifai RP1 was grown on 1, 2, 10 or 20g/l tallow.

Tallow conc. (g/l)	Specific removal rate ($gg^{-1}day^{-1}$)				
	Myristic	Palmitic	Stearic	Oleic	Linoleic
1	0.24±0.04	1.17±0.25	1.39±0.28	1.99±0.01	0.14±0.03
2	0.39±0.05	2.04±0.32	2.40±0.36	3.47±0.52	0.23±0.03
10	1.39±0.27	7.58±1.57	8.84±1.81	12.72±2.59	0.82±0.17
20	1.65±0.16	8.41±0.98	9.99±1.10	14.34±1.56	0.95±0.10

When the specific fatty acid removal rates at the various concentrations were compared, an increase in rates was observed up to 10g/l, after which the increase in rates levelled off (Table 19). This pattern was observed earlier for tallow and triglycerides, so the correlation again may follow Michelis-Menten kinetics.

To test this hypothesis, double reciprocal plots were constructed for each of the fatty acids (Figure 50). Good correlation coefficients were found with all the fatty acids (Table 20). For myristic, palmitic, stearic, oleic and linoleic acids, they were 0.992, 0.997, 0.997, 0.997 and 0.994 respectively. From the plots, the maximum theoretical specific removal rates were calculated for the fatty acids. The lowest rates were found with myristic and linoleic acids, of 2.30 and $1.36gg^{-1}day^{-1}$ respectively. In order of increasing maximum rates, palmitic, stearic and oleic acids had rates of 13.82, 15.97 and $23.29gg^{-1}day^{-1}$ respectively. The saturation constants (K_s) were lowest for myristic and linoleic acids, at 8.81 and 8.90g/l respectively. Palmitic, stearic and oleic acids had similar saturation constants, at 10.95, 10.63 and 10.83g/l respectively. The saturation constants for palmitic, stearic and oleic acids were similar to those observed for tallow and triglycerides.

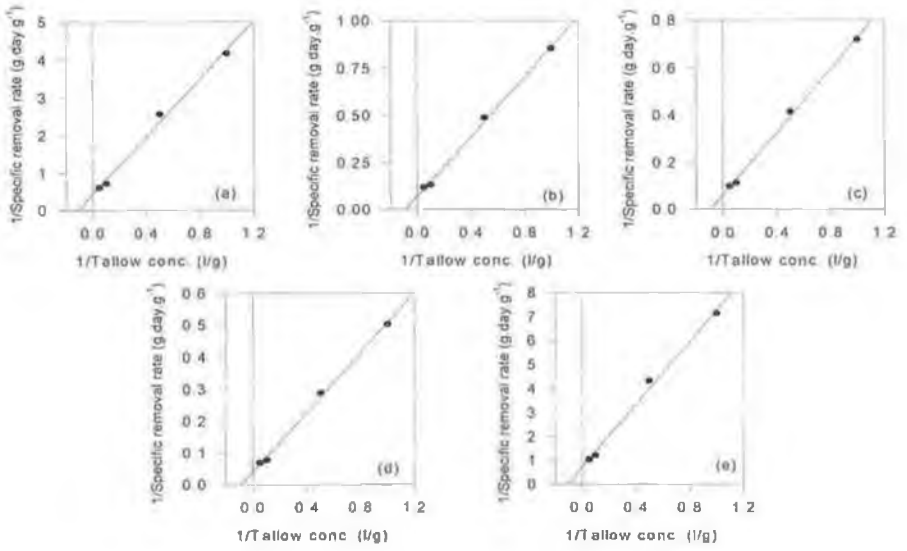

Figure 50: Double reciprocal plots for specific fatty acid removal rates by *T. harzianum* Rifai RP1 grown on tallow. Legend: (a) myristic acid; (b) palmitic acid; (c) stearic acid; (d) oleic acid; (e) linoleic acid.

Table 20: Maximum theoretical specific fatty acid removal rate ($gg^{-1}day^{-1}$), saturation constant, k_s (g/l) and correlation coefficient, r^2, of specific fatty acid removal rates by *T. harzianum* Rifai RP1 grown on tallow at 1, 2, 10 and 20g/l.

Fatty acid	Max. rate	K_s	r^2
Myristic acid	2.30	8.81	0.992
Palmitic acid	13.82	10.95	0.997
Stearic acid	15.97	10.63	0.997
Oleic acid	23.29	10.83	0.997
Linoleic acid	1.36	8.90	0.994

3.3.3 Composition of intracellular lipid

The intracellular lipid accumulated in the biomass was a product of fatty acids taken in from the medium. Mono-, di- and triglycerides may be re-synthesised in the fungal cells, and may be present in stored lipid. To determine if glycerides were re-assembled intracellularly, the glyceride composition of intracellular lipid was examined.

3.3.3.1 Intracellular glycerides and free fatty acids

The main components found in the intracellular lipid were triglycerides, diglycerides and free fatty acids (Figure 51). Monoglycerides were not detected. The specific concentrations of these components varied during incubation. When *T. harzianum* Rifai RP1 was grown on 1g/l tallow, free fatty acids were the dominant lipid class in intracellular lipid, rising from $0.04gg^{-1}$ on day 2 to $0.35gg^{-1}$ by day 10. Triglycerides rose from $0.02gg^{-1}$ on day 2 to a peak of $0.15gg^{-1}$ by day 7, with a final specific concentration of $0.10gg^{-1}$ on day 10. Diglycerides remained very low throughout, with levels remaining below $0.01gg^{-1}$.

With 2g/l tallow, the pattern was similar to that seen with 1g/l, with little accumulation from days 2 to 4. Overall, free fatty acids increased from $0.05gg^{-1}$ on day 2 to a final value of $0.44gg^{-1}$ over the 10 days. Triglycerides again peaked on day 7, reaching $0.15gg^{-1}$ from $0.02gg^{-1}$, ending on $0.10gg^{-1}$ by day 10. Diglycerides remained below $0.01gg^{-1}$.

When the tallow concentration was increased to 10g/l, free fatty acids increased throughout incubation, from a day 2 level of $0.01gg^{-1}$ to $0.28gg^{-1}$ on day 10. Triglycerides increased gradually, from $0.01gg^{-1}$ to $0.09gg^{-1}$ by day 10. Diglycerides again remained below $0.01gg^{-1}$.

At the highest tallow concentration of 20g/l, free fatty acids were present at lower concentrations than triglycerides for much of the incubation, increasing gradually from $0.02gg^{-1}$ to $0.17gg^{-1}$ by the end of incubation. Triglycerides were present at $0.01gg^{-1}$ on day 2, increased to a peak of $0.20gg^{-1}$ by day 7, with a residual concentration on day 10 of $0.14gg^{-1}$. As with the previous tallow concentrations, diglycerides remained at less than $0.01gg^{-1}$ throughout incubation.

At all the tallow concentrations, triglyceride re-synthesis was occurring intracellularly, as significant specific concentrations were present in the stored lipid. Free fatty acids, however, formed a very significant proportion of the intracellular lipid. The level of triglycerides became more significant at the higher concentrations of tallow, as the levels of free fatty acids decreased. The intermediates in triglyceride synthesis and degradation, the di- and monoglycerides, did not accumulate in significant quantities in the fungal biomass.

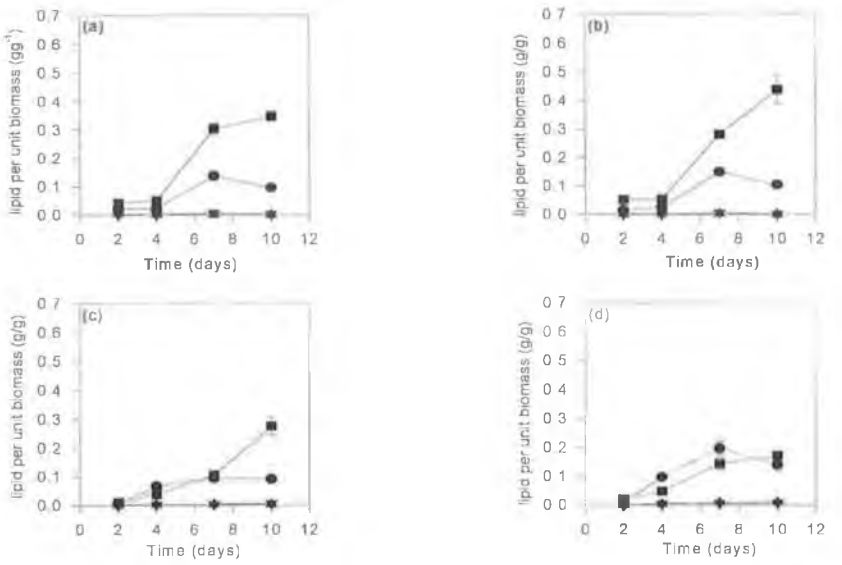

Figure 51: Intracellular glycerides and free fatty acids per unit biomass during incubation of *T. harzianum* Rifai RP1 on (a) 1g/l tallow, (b) 2g/l tallow, (c) 10g/l tallow, (d) 20g/l tallow. Symbols: ● triglycerides; ■ free fatty acids; ▲ 1,3 diglycerides; ▼ 1,2 diglycerides.

3.3.3.2 Fatty acid composition of intracellular lipid components

The component fatty acids in intracellular glycerides and free fatty acids may have been those assimilated from the extracellular medium, or synthesised *de novo*. The organism may show a preference for the accumulation of a particular fatty acid, because it is beneficial to it, or it cannot metabolise the particular fatty acid readily. It may also be the case that other fatty acids are more readily utilised for growth and energy needs. To determine if particular fatty acids are stored preferentially by *T. harzianum* Rifai RP1, the fatty acid compositions of intracellular lipid components were examined.

The main components of intracellular triglycerides were palmitic, stearic and oleic acid (Figure 52). Myristic acid was not detected, while linoleic acid was present in trace amounts. With the lower concentrations of 1 and 2g/l tallow, stearic acid was at the highest concentration in triglycerides. Palmitic and oleic acid were present at similar concentrations. Their concentrations mirrored the trend in triglyceride concentrations, increasing from day 4 to 7, before decreasing slightly to day 10. With the higher concentrations of tallow, the three fatty acids were present at similar levels throughout. Again, their concentrations mirrored the trends apparent in triglyceride concentrations. Although there were slightly higher concentrations stearic acid with 1 and 2g/l tallow, in general, no specificity was displayed for accumulation of any of the three main fatty acids in intracellular triglycerides.

Since diglycerides were present at very low concentrations, so too were their component fatty acids. The main fatty acids detected were palmitic, stearic and oleic acids (Figure 53). No significant preferential accumulation was evident at any of the tallow concentrations examined. Although oleic acid was present at higher concentrations with 10 and 20g/l tallow, these concentrations were very low, so no inference of specificity could be drawn from the data.

Intracellular free fatty acids were a significant component of intracellular lipid. The fatty acids making up the free fatty acids were therefore of importance when examining accumulation specificity. Again, the main component fatty acids were those making up the major part of tallow fatty acids. Palmitic, stearic and oleic acid were detected in similar amounts with 1, 2 and 10g/l tallow substrate (Figure 54). With 20g/l tallow, oleic and stearic acids were present in similar concentrations, with slightly lower quantities of palmitic acid. At all concentrations, myristic acid was undetected in intracellular free fatty acids, while linoleic acid was present in trace amounts. There was no significant specificity for accumulation of any of the three main fatty acids among the free fatty acids.

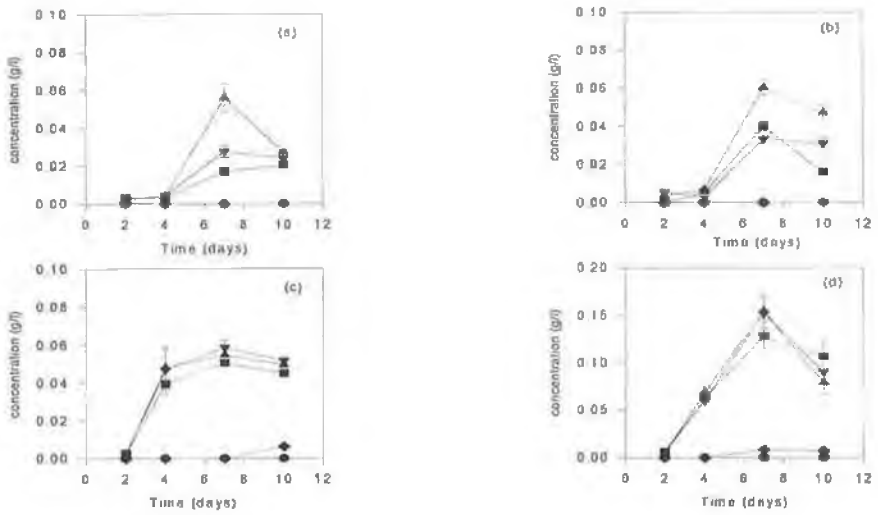

Figure 52: Fatty acid composition of intracellular triglycerides of *T. harzianum* Rifai RP1 grown on (a) 1g/l tallow, (b) 2g/l tallow, (c) 10g/l tallow and (d) 20g/l tallow. Symbols: ● myristic acid; ■ palmitic acid; ▲ stearic acid; ▼ oleic acid; ◆ linoleic acid.

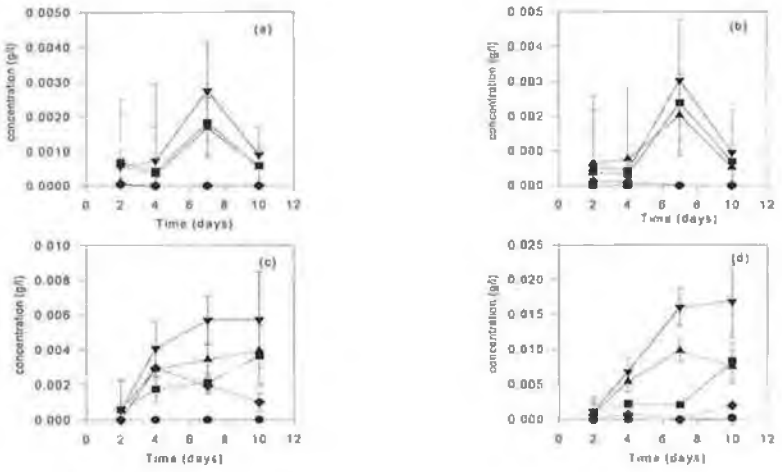

Figure 53: Fatty acid composition of intracellular diglycerides of *T. harzianum* Rifai RP1 grown on (a) 1g/l tallow, (b) 2g/l tallow, (c) 10g/l tallow and (d) 20g/l tallow. Symbols: ● myristic acid; ■ palmitic acid; ▲ stearic acid; ▼ oleic acid; ◆ linoleic acid.

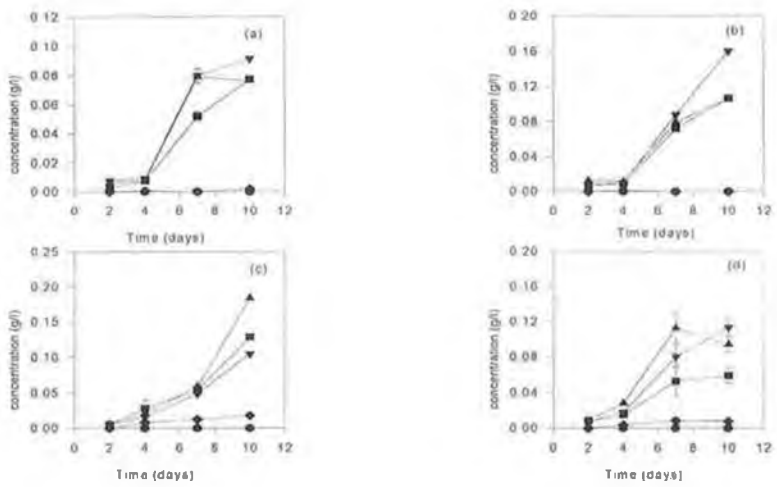

Figure 54: Fatty acid composition of intracellular free fatty acids of *T. harzianum* Rifai RP1 grown on (a) 1g/l tallow, (b) 2g/l tallow, (c) 10g/l tallow and (d) 20g/l tallow. Symbols: ● myristic acid; ■ palmitic acid; ▲ stearic acid; ▼ oleic acid; ◆ linoleic acid.

3.3.3.3 Total intracellular fatty acid accumulation

The fatty acids accumulated intracellularly were bound in tri- and di-glycerides, and also were present as free acids. The total percentage of any particular fatty acid intracellularly would give an indication if accumulation differences occurred.

On day 10, the percentage of each fatty acid was noted (Table 21). Myristic was not detected in intracellular fatty acids, while linoleic acid was present only in trace amounts. The main fatty acids, palmitic, stearic and oleic acid all accumulated intracellularly, neither one accumulating significantly more than others.

Table 21: Fatty acid composition of total intracellular lipid on day 10 of *T. harzianum* Rifai RP1 grown on 1, 2, 10 or 20g/l tallow.

Fatty acid	% fatty acid			
	1g/l tallow	2g/l tallow	10g/l tallow	20g/l tallow
Myristic acid	0%	0%	0%	0%
Palmitic acid	30.6%	26.3%	29.5%	33.3%
Stearic acid	32.5%	32.7%	41.9%	28.8%
Oleic acid	36.3%	40.9%	24.5%	35.2%
Linoleic acid	0.6%	0%	4.2%	2.7%

3.4 An investigation of the metabolism of a mixture of palmitic, stearic and oleic acids by *Trichoderma harzianum* Rifai RP1 under optimal environmental conditions.

In section 3.3, it was found that the main fatty acids in tallow were palmitic, stearic and oleic acid. All three fatty acids were assimilated by *T. harzianum* Rifai RP1, and no specificity was evident in this assimilation. Intracellularly, the three fatty acids accumulated in stored lipids, and again, no specificity was evident in the accumulation. However, oleic acid was present at higher concentrations in tallow than stearic or palmitic acid, which may have influenced uptake and accumulation rates.

The specific growth rates of RP1 on tallow followed Monod kinetics. With 10 and 20g/l tallow, the maximum growth rate was reached, suggesting that the growth was limited at these tallow concentrations. An extracellular accumulation of free fatty acids was also observed at these concentrations. It has been suggested also that fatty acids may be toxic to microorganisms, limiting growth and inhibiting their own metabolism as noted in section 1.

To examine the utilisation of palmitic, stearic and oleic acids by *T. harzianum* Rifai RP1, studies were carried out with these fatty acids as substrates. RP1 was set up in culture under the optimum conditions as described in section 3.2. A mixture of the fatty acids, palmitic, stearic and oleic acid were used as the carbon source. A range of concentrations were examined: 0.04, 0.12, 0.25 and 0.60g/l of each fatty acid, giving total fatty acid concentrations of 0.12, 0.36, 0.75 and 1.80g/l. Extracellular lipid, dry weight and intracellular lipid were monitored, as well as the glyceride composition and fatty acid components in the extracted lipid.

3.4.1 The determination of growth parameters

During incubation of *T. harzianum* Rifai RP1 with a mixture of palmitic, stearic and oleic acids as the sole carbon source, dry weight increased while extracellular fatty acid concentrations decreased. pH was monitored and it held to within 0.3 pH units of the original buffered value, 6.0.

Growth of *T. harzianum* Rifai RP1

After incubation for 10 days, the dry weight increase was greatest for the highest initial concentration of extracellular fatty acids (Figure 55). With 1.8g/l fatty acids, an increase of 0.47g/l was observed. At initial concentrations of 0.75, 0.36 and 0.12g/l, increases of 0.26, 0.22 and 0.09g/l were seen. After an initial lag of 1 day, rapid growth was observed. Growth then slowed, reaching stationary phase after 7 days.

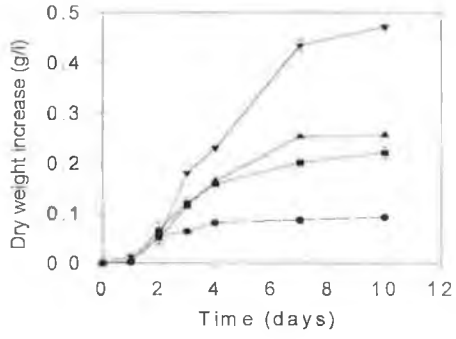

Figure 55: Growth of a culture of *T. harzianum* Rifai RP1 on a mixture of palmitic, stearic and oleic acids as carbon source. Symbols: ● 0.12g/l fatty acids; ■ 0.36g/l fatty acids; ▲ 0.75g/l fatty acids; ▼ 1.80g/l fatty acids.

An exponential increase in dry weight was observed at all initial concentrations of fatty acids. The specific growth rates (μ) were similar at all the fatty acid

concentrations (Table 22). Rates of 0.1029, 0.0906, 0.0963 and 0.1029 were noted for 0.12, 0.36, 0.75 and 1.80g/l respectively.

Table 22: Specific growth rates of *T. harzianum* Rifai RP1 grown on a mixture of palmitic, stearic and oleic acids at 0.12, 0.36, 0.75 or 1.80g/l.

Total fatty acid concentration (g/l)	Specific growth rate, μ (day^{-1})
0.12	0.1029±0.0091
0.36	0.0906±0.0110
0.75	0.0963±0.0038
1.80	0.1029±0.0022

Removal of the fatty acid mixture

During the growth of *T. harzianum* Rifai RP1 on the fatty acid mixture, fatty acids were removed from culture after an initial lag of 1 day (Figure 56). This lag was followed by a period of rapid removal, after which removal slowed. The residual concentrations on day 10 indicated that the percentage of total fatty acids removed decreased as their concentrations increased. Percentage removals of 97%, 73%, 61% and 43% were observed for 0.12, 0.36, 0.75 and 1.80g/l of fatty acids.

The specific rates of removal decreased with increasing fatty acid concentrations (Table 23). Rates of 0.798, 0.431, 0.227 and 0.218$gg^{-1}day^{-1}$ were noted for 0.12, 0.36, 0.75 and 1.80g/l fatty acids respectively.

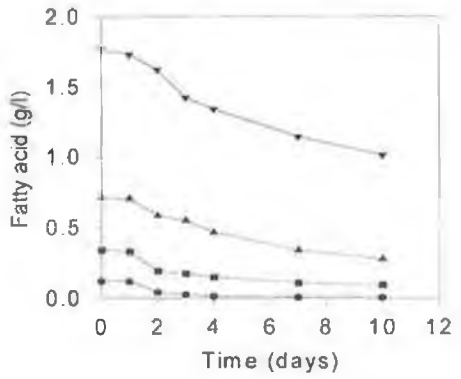

Figure 56: Total extracellular fatty acids concentrations in cultures of *T. harzianum* Rifai RP1 with (●) 0.12g/l, (■) 0.36g/l, (▲) 0.75g/l, (▼) 1.80g/l initially of a mixture of palmitic, stearic and oleic acids.

Table 23: Specific total fatty acid removal rate by *T. harzianum* Rifai RP1 grown on a mixture of palmitic, stearic and oleic acids at 0.12, 0.36, 0.75 or 1.80g/l.

Fatty acid concentrations (g/l)	Specific removal rate ($gg^{-1}day^{-1}$)
0.12	0.798±0.051
0.36	0.431±0.063
0.75	0.227±0.071
1.80	0.218±0.055

Accumulation of intracellular lipid

During growth of *T. harzianum* Rifai RP1 on a mixture of palmitic, stearic and oleic acids, various amounts of intracellular lipid accumulated in the biomass. The specific concentration of intracellular lipid increased with increasing concentration of fatty

acids (Figure 57). They reached specific concentrations of 0.466, 0.304, 0.220 and 0.093gg^{-1} for 1.80, 0.75, 0.36 and 0.12g/l fatty acid.

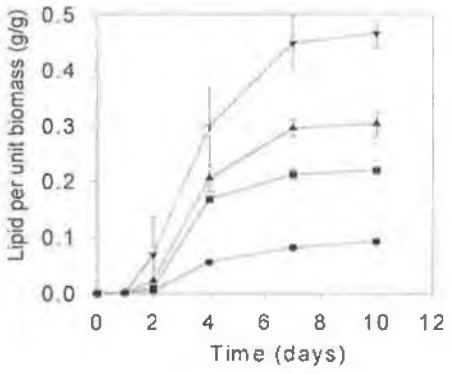

Figure 57: Intracellular lipid per unit biomass of *T. harzianum* Rifai RP1 grown on (●) 0.12g/l, (■) 0.36g/l, (▲) 0.75g/l and (▼) 1.80g/l of a mixture of palmitic, stearic and oleic acids.

The determination of yields

The yields of RP1 biomass on the fatty acid mixture also varied with the different concentrations of fatty acids. The yield coefficients ($Y_{x/s}$), the yields of total dry weight on the substrate after 10 days, were calculated as described in section 3.3. They were similar at all fatty acid concentrations (Table 24). Values of 0.80, 0.90, 0.59 and 0.63 were observed for 0.12, 0.36, 0.75 and 1.80g/l respectively.

Table 24: Yield coefficients, $Y_{x/s}$ (the yield of total dry weight on substrate), for *T. harzianum* Rifai RP1 grown on a mixture of palmitic, stearic and oleic acids at 0.12, 0.36, 0.75 or 1.80g/l.

Initial fatty acid concentrations (g/l)	Yield coefficient ($Y_{x/s}$)
0.12	0.80±0.07
0.36	0.90±0.06
0.75	0.59±0.03
1.80	0.63±0.05

Since intracellular lipid accumulation occurred, some of the substrate fatty acids were not being metabolised for growth and energy needs of *T. harzianum* Rifai RP1. As was noted in section 3.3 with the tallow substrate, the yield of biomass, without the stored lipid, may be different than total biomass yields. Therefore, yield coefficients at day 10 of fat free biomass on metabolised fatty acids ($Y_{xf/sm}$) were calculated (Table 25). The highest coefficient was with 0.36g/l initial fatty acids, at 0.73. The coefficient at 0.12g/l was slightly lower at 0.62, while at higher concentrations of 0.75g/l and 1.80g/l, the coefficients were significantly lower. At these concentrations, the coefficients were 0.14 and 0.07 respectively.

The yield coefficients, $Y_{(xf/sm)}$, were similar to $Y_{(x/s)}$ for 0.12 and 0.36g/l fatty acid substrate. However, $Y_{(xf/sm)}$ were significantly lower than $Y_{(x/s)}$ at 0.75 and 1.80g/l. The higher concentrations of fatty acids resulted in higher accumulation of intracellular lipid, and less production of fat-free biomass.

Table 25: Yield coefficient, $Y_{xf/sm}$ (yield of fat free biomass on metabolised fatty acids), for *T. harzianum* Rifai RP1 grown on a mixture of palmitic, stearic and oleic acids at 0.12, 0.36, 0.75 or 1.80g/l.

Fatty acid concentrations (g/l)	Fat free biomass (xf) (g/l)	Fatty acids metabolised (sm) (g/l)	Yield coefficient ($Y_{xf/sm}$)
0.12	0.038±0.008	0.061±0.005	0.62±0.13
0.36	0.064±0.012	0.088±0.011	0.73±0.16
0.75	0.029±0.019	0.207±0.029	0.14±0.09
1.80	0.023±0.025	0.304±0.065	0.07±0.08

3.4.2 The composition of extracellular lipid.

Since the substrate in these studies was a mixture of free fatty acids, it would be expected that no glycerides would be detected in the extracellular medium. However, lipase can have reverse as well as forward activity, so analysis was carried out to check for the presence of glycerides. It was found that the extracellular lipid composition was entirely free fatty acids throughout incubation. No mono-, di-, or triglycerides were detected at any stage.

3.4.2.1 The composition of extracellular free fatty acids

The composition of the extracellular free fatty acids was studied to determine if differences exist between the removal rates of the individual fatty acids present. In cultures with initial total fatty acid concentrations of 0.12g/l, the concentrations of each fatty acid dropped rapidly over the first 4 days of incubation (Figure 58). Oleic acid dropped from 0.0304g/l on day 1 to a residual concentration on day 10 of 0.0002g/l. A slight increase was noted in the concentration of stearic acid at the beginning of incubation. From an initial concentration of 0.039g/l, it rose to 0.043g/l

after 1 day, before dropping to a final concentration of 0.0019g/l on day 10. The concentration of palmitic acid decreased after a lag of 1 day to a residual concentration of 0.0016g/l at the end of incubation. These concentration decreases represent greater than 80% removal of each fatty acid after 4 days. By the end of incubation, the removal of oleic acid was the highest, with 99% removed, while stearic and palmitic acids had removal of 95% and 96% respectively.

Specific removal rates were highest for oleic acid, at $0.939 gg^{-1}day^{-1}$ (Table 26). Slightly lower rates were observed for palmitic and stearic acids, which had rates of 0.828 and $0.714 gg^{-1}day^{-1}$ respectively.

When the initial fatty acid concentration was increased to 0.36g/l, a slightly different pattern of removal was observed. Oleic acid decreased throughout incubation, but its concentration did not decrease as sharply as was seen with 0.12g/l (Figure 58). There was a gradual drop until day 4, after which the rate of removal decreased, leaving a residual concentration of 0.0064g/l on day 10. Again stearic acid showed a slight increase over the first day of incubation. It rose from 0.1223 to 0.1334g/l, before dropping during incubation to a final level of 0.0443g/l. Palmitic acid dropped after a 1 day lag from 0.1100g/l to a concentration on day 10 of 0.0414g/l. Oleic acid was the only fatty acid to reach greater than 90% removal in this case. After 10 days of incubation, 94% of it had been removed (Figure 58). In the cases of palmitic and stearic acids, the percentage removals were significantly lower. After 10 days, 63% and 64% respectively were removed from the culture medium.

The specific removal rates were lower than those observed with 0.12g/l fatty acids. However, they were similar for the three fatty acids (Table 26). The rate of removal for oleic acid was $0.644 gg^{-1}day^{-1}$, while those of palmitic and stearic aid were 0.656 and $0.653 gg^{-1}day^{-1}$ respectively.

As seen with 0.36g/l fatty acids, when 0.75g/l was added, there was a significant difference between the removal of the unsaturated oleic acid and the saturated fatty acids, stearic and palmitic acid. Oleic acid dropped gradually throughout incubation from 0.219g/l to a final concentration of 0.026g/l (Figure 58). Both saturated fatty acids showed a slight increase during the first day of incubation, palmitic acid increasing from 0.257 to 0.281g/l, and stearic acid from 0.232 to 0.244g/l. Both then decreased in concentration gradually, palmitic acid dropping to 0.131g/l, while stearic acid reached 0.117g/l. Oleic acid reached the highest percentage removal, with 88% removed by the end of incubation. Palmitic and stearic acids were significantly lower in removal, only reaching 49% and 50% respectively.

Specific removal rates were again lower than the previous concentrations. Again, there was not a significant difference noted between the different fatty acids (Table 26). Oleic had a removal rate of $0.249 gg^{-1} day^{-1}$, while those for palmitic and stearic acid were 0.171 and $0.207 gg^{-1} day^{-1}$ respectively.

At the highest initial concentration of 1.80g/l, a significant difference was again observed between the removal of the saturated and unsaturated fatty acids. Oleic acid decreased from 0.545g/l at the beginning of incubation to a residual concentration of 0.115g/l (Figure 58). Palmitic decreased after a 1 day lag from 0.647g/l to a final value of 0.464g/l. An increase was again observed in stearic acid from 0.579g/l initially to 0.639g/l after 2 days, then dropping to 0.434g/l by the end of incubation. The percentage removal of saturated fatty acids was significantly lower than with the previous fatty acid concentrations. Palmitic and stearic acids only reached 28% and 25% respectively after 10 days. However, oleic acid was removed at a higher rate, with 79% being removed by the end of incubation.

Again, the specific removal rates dropped as more fatty acid substrate was added. Also, as previously, the rates observed were similar for the three fatty acids (Table

26). Oleic acid was removed at 0.231$gg^{-1}day^{-1}$, while palmitic and stearic acid were removed at 0.148 and 0.171$gg^{-1}day^{-1}$ respectively.

It can be seen that as higher concentrations of fatty acids were added as substrate, the specific removal rates dropped. Also, RP1 assimilated the various fatty acids at similar rates, but over the 10 days studied, the percentage removal of oleic acid was higher than for the saturated acids.

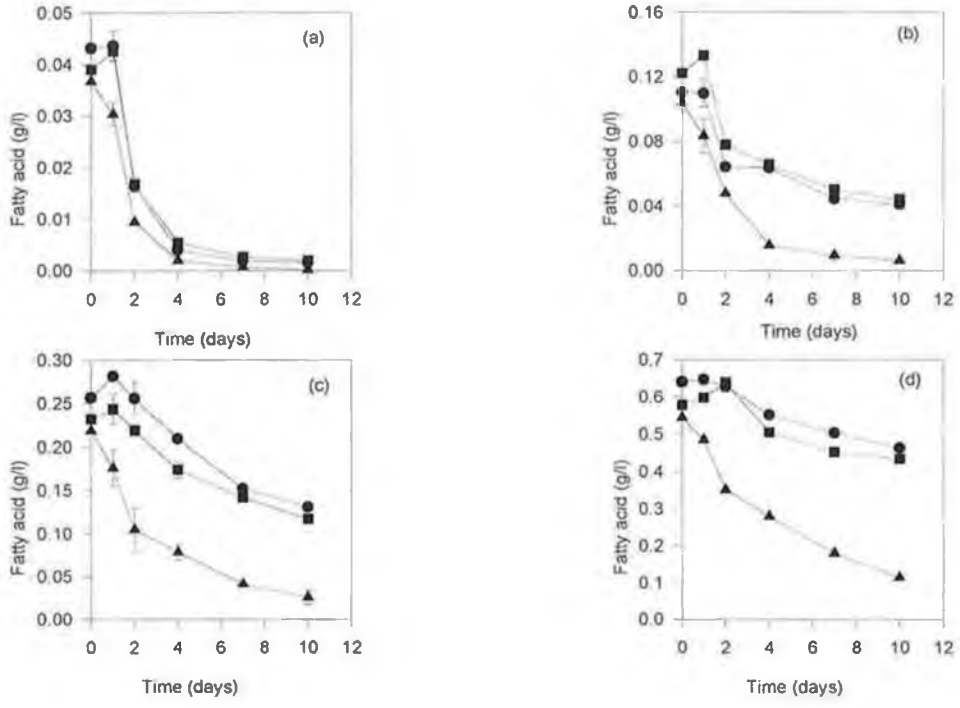

Figure 58: Concentrations of extracellular fatty acids in cultures of *T. harzianum* Rifai RP1, with an initial total extracellular fatty acid concentration of (a) 0.12g/l, (b) 0.36g/l, (c) 0.75g/l or (d) 1.80g/l. Symbols: ● Palmitic acid; ■ Stearic acid; ▲ Oleic acid.

Table 26: Specific removal rate of fatty acids in cultures of *T. harzianum* Rifai RP1 grown on a mixture of palmitic, stearic and oleic acids, at 0.12, 0.36, 0.75 and 1.80g/l total fatty acids.

Fatty acid concentration (g/l)	Specific removal rate ($gg^{-1}day^{-1}$)		
	Palmitic acid	Stearic acid	Oleic acid
0.12	0.828±0.037	0.714±0.030	0.939±0.027
0.36	0.656±0.031	0.653±0.021	0.644±0.049
0.75	0.171±0.008	0.207±0.031	0.249±0.057
1.80	0.148±0.006	0.171±0.008	0.231±0.008

3.4.3 Composition of intracellular lipid

The intracellular lipid was produced from the fatty acid substrates, with no glycerides present extracellularly. The composition of this lipid was of interest to determine if glyceride synthesis was occurring in the biomass of *T. harzianum* Rifai RP1, and if any differences were noted in the accumulation of particular fatty acids.

3.4.3.1 Intracellular glycerides and free fatty acids

The intracellular lipid was analysed with respect to its glyceride composition, and was found to consist of triglycerides and free fatty acids. No diglycerides or monoglycerides were detected (Table 27). After 10 days, the composition of the intracellular lipid was similar at the different fatty acid substrate concentrations.

Specific concentrations of intracellular free fatty acids increased in all cases from day 2 onwards (Figure 59). With 0.12g/l initial fatty acid substrate, it rose from a day 1 level of $0.0011gg^{-1}$ to $0.0876gg^{-1}$ by day 10. A similar pattern was observed with 0.36g/l, rising from $0.0011gg^{-1}$ to $0.2105gg^{-1}$ over the same period. With 0.75 and

1.80g/l, these rises were from 0.0002gg^{-1} in both cases to final levels of 0.2882 and 0.4225gg^{-1} respectively.

Specific concentrations of intracellular triglycerides were significantly lower. With 0.12g/l initial fatty acid, they rose from 0.0006gg^{-1} to a peak of 0.0099gg^{-1} on day 7, finishing with 0.0049gg^{-1} on day 10. A day 7 peak was also observed with 0.36g/l, rising from 0.0002 to 0.0282gg^{-1}, with a final value of 0.0097gg^{-1}. With 0.75 and 1.80g/l, the specific concentration rose throughout the 10 days, from 0.0002 and 0.0003gg^{-1}, to final values of 0.0161 and 0.0437gg^{-1} respectively.

Table 27: Composition of intracellular lipid of *T. harzianum* Rifai RP1 following 10 days incubation with a mixture of palmitic, stearic and oleic acid at 0.12, 0.36, 0.75 or 1.80g/l. (FFAs = Free Fatty Acids; ND = not detected).

Fatty acid concentration	% in intracellular lipid			
	Triglycerides	Diglycerides	Monoglycerides	FFAs
0.12g/l	5±1.4%	ND	ND	95±1.9%
0.36g/l	4±1.6%	ND	ND	96±2.6%
0.75g/l	5±1.5%	ND	ND	95±1.5%
1.80g/l	9±2.8%	ND	ND	91±2.9%

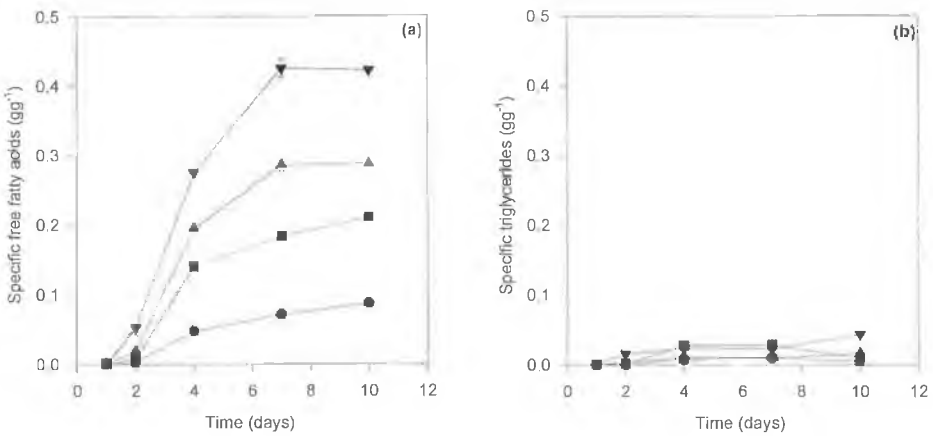

Figure 59: Specific concentration of (a) intracellular free fatty acids and (b) intracellular triglycerides in cultures of *T. harzianum* Rifai RP1, grown with a mixture of palmitic, stearic and oleic acids at total initial concentrations of ● 0.12g/l, ■ 0.36g/l, ▲ 0.75g/l and ▼ 1.80g/l.

3.4.3.2 Fatty acid composition of intracellular glycerides and free fatty acids

The triglyceride and free fatty acid fractions were analysed to determine their fatty acid composition. This would determine if accumulation of specific fatty acids was occurring in preference to others, or if any fatty acid was more easily metabolised. It would also give an indication if particular fatty acids were preferentially incorporated into the triglycerides.

Stearic acid was the only fatty acid detected in intracellular triglycerides at all concentrations (Table 28). However, in the intracellular free fatty acids, palmitic, stearic and oleic acid were detected. Their proportions were similar at all the substrate concentrations. Oleic acid made up the smallest proportion, at 19%, 16%, 20% and 23% with 0.12, 0.36, 0.75 and 1.80g/l fatty acid substrate respectively. Higher proportions were seen for palmitic acid, making up 38%, 36%, 36% and 36%

respectively. The highest percentages were of stearic acid, accounting for 44%, 48%, 44% and 40% respectively. In total in the intracellular lipid, stearic acid was accumulated to the greatest degree. It accounted for 46%, 50%, 47% and 46% of total intracellular fatty acids. Palmitic acid was at lower levels of 35%, 35%, 35% and 33% respectively. Oleic acid made up only 18%, 15%, 19% and 21% respectively of the intracellular lipids.

When grown on a mixture of palmitic, stearic and oleic acids, RP1 accumulated stearic acid intracellularly to a higher degree than both palmitic or oleic acids. Oleic acid was the least accumulated, being more readily metabolised than either of the saturated fatty acids.

Table 28: Fatty acid composition of intracellular free fatty acids, triglycerides and total intracellular lipid of *T. harzianum* Rifai RP1 grown on 0.12, 0.36, 0.75 or 1.80g/l of a mixture of palmitic, stearic and oleic acids. (FFA = Free Fatty Acids, TG = Triglycerides, Tot = Total intracellular lipid)

Substrate concentration		% palmitic acid	% stearic acid	% oleic acid
0.12g/l	FFA	38±3.2%	44±2.9%	19±2.9%
	TG	ND	100%	ND
	Tot	35±3.0%	46±3.2%	18±2.6%
0.36g/l	FFA	36±3.2%	48±2.9%	16±2.8%
	TG	ND	100%	ND
	Tot	35±3.1%	50±3.2%	15±2.7%
0.75g/l	FFA	36±5.2%	44±5.6%	20±3.7%
	TG	ND	100%	ND
	Tot	35±4.9%	47±5.5%	19±3.5%
1.80g/l	FFA	36±3.9%	40±1.8%	23±3.5%
	TG	ND	100%	ND
	Tot	33±3.6%	46±1.7%	21±3.2%

4. Discussion

In a bioreactor capable of removing fat from a waste stream, the organisms present must be capable of hydrolysing the lipid triglycerides and assimilating the released fatty acids. The assimilated fatty acids should then be preferably oxidised to TCA cycle intermediates, and not stored as intracellular lipid in the biomass. Storage of intracellular lipid can result in a high fat content sludge which could present subsequent disposal problems.

The finding of suitable organisms involves the choosing of sources with a high probability of containing such species, and the selective isolation of potential microbes from those sources. Activated sludge and wastewater from a rendering operation were chosen as good potential sources of fat-degrading organisms. The wastewater from the production of tallow and bonemeal contains high levels of tallow, which could promote the growth of tallow utilisers in the waste treatment system. Although the wastewater was treated by a Dissolved Air Flotation solids removal system, significant amounts of solid fat were observed entering the activated sludge stage of treatment. Therefore it was likely that a population of microorganisms would have been present which could metabolise hard fats. Tallow itself could also be a source of lipid-degraders. Its endogenous microbial population might include organisms which rely on tallow as their sole source of carbon and energy.

The isolation of fat degraders from a rendering factory wastewater treatment plant, for use in a bioreactor, had not previously been reported in the literature. Therefore, it was a novel source for study. Wastewater treatment plants from related industries have yielded lipolytic organisms. These plants were all likely to have received lipid waste. Shikoku-Chem (1994) isolated a *Pseudomonas* species and an *Acinetobacter* species capable of degrading fat from activated sludge from a food factory. Okuda *et al.* (1991) reported the isolation of several lipid degrading *Bacillus* species from wastewater treatment systems of meat processing plants. Sludges from various food

factories were used to isolate *Candida* yeasts by Chigusa *et al.* (1996). Restaurant grease traps have yielded mixed microbial communities capable of lipid utilisation (Wakelin and Forster, 1998, Tano-Debrah *et al.*, 1999), both studies incorporating the isolates into bioreactors for grease removal. A strain of *Torulopsis candida* was recovered from soil by Koh *et al.* (1983). This yeast was capable of effectively assimilating palm oil. Most other studies on fat biodegradation, however, utilised microorganisms from culture collections, frequently basing selection on lipolytic activity (eg. Bednarski *et al.*, 1993, Kajs and Vanderzant, 1981, Koritala *et al.*, 1987, Tan and Gill, 1984, 1985 & 1987).

Enrichment cultures are used to isolate microorganisms with specific characteristics from mixed cultures, where their populations may be low. In an enrichment culture, the environmental conditions are altered to increase the population size of organisms of interest. The conditions manipulated may include the use of selective substrates or inhibitors, and the chemical composition of the medium. The substrates are used at high concentrations, to induce enhanced growth of the populations of microorganisms which readily utilise them (Brown *et al.*, 1978).

In the present study, tallow was used as the carbon source in the enrichment culture, promoting the growth and survival of lipid metabolising microorganisms. Various concentrations of lipids had been used in published literature for isolation and growth studies. Tan and Gill (1985 and 1987) used tallow concentrations in the range 1 to 8g/l in their series of experiments. A concentration of 10g/l of olive oil was used by Okuda *et al.* (1991) for isolation of lipolytic bacteria, while Kajs and Vanderzant (1981) used 10g/l tallow in their study with yeasts. Koh *et al.* (1983) used 20g/l palm oil during screening of yeasts for growth on the oil. Bednarski *et al.* (1993) studied the growth of filamentous fungi on 30g/l tallow and poultry fat. A concentration of 20g/l was chosen as a sufficiently high concentration of tallow for enrichment in the present work. The minimal medium chosen was based on that described by Shikoku-

Chem (1994). This medium had previously been successfully used for the growth of lipolytic organisms isolated from a food-factory wastewater treatment plant.

As the wastewater and activated sludge were from a system receiving multiple waste streams, it was likely that other carbon sources were also present in lower concentrations. Although tallow was the main carbon source, the other carbon compounds may have been acting as substrates for species not capable of growth on lipid. Therefore, in order to select for lipid utilisers among the population, olive oil agar was used as the isolation medium on days 3 and 15. In the later stages of incubation, the main populations were most likely those capable of living or sustaining themselves on the lipid substrate, as the trace amounts of carbon added in the inocula would have been exhausted, and tallow would have been the carbon source. Therefore, on day 21, the isolation medium was nutrient agar, which allowed the recovery of slower-growing organisms which were potential tallow utilisers.

Although the behaviour of the enrichment cultures was not extensively studied, it was noted that the pH dropped, accompanied by an increase in biomass. This pH drop may have implications for the species surviving during the later stages of enrichment culture. It is likely that the main isolates from days 15 and 21 were those capable of tolerating acidic conditions. In a study by Wakelin and Forster (1998), a significant pH drop was also observed, producing a high acidity effluent from fed-batch cultures. The substrate in their study was restaurant grease, which is mainly composed of triglyceride lipids.

The accuracy of optical density measurements depends on the nature of the microbial growth and on interferences from medium components. In the enrichment cultures, aggregation of the microorganisms could have caused errors in the OD measurements. Also, small amounts of dispersed lipid caused slight clouding in some of the cultures. However, OD measurement did give an indication of biomass production. In shaking cultures, inoculated with activated sludge, more biomass

production was observed at ambient temperature than at 30°C. Under aerobic conditions, the predominant microbial populations may have had an optimum growth temperature of less than 30°C. This phenomenon was seen in both the laboratory and treatment plant situation. In the wastewater treatment plant from which they were isolated, it was observed that the efficiency of the system decreased significantly at temperatures of 28-30°C.

The production of biomass and the drop in pH were less marked in cultures with tallow serving as the only source of microorganisms, than in activated sludge or wastewater inoculated cultures. The microbial populations were much greater initially in inoculated cultures. With tallow only, the low populations of organisms on the tallow would have depended on it as a carbon and energy source. Tallow is a very challenging substrate, and the smaller initial populations would have resulted in less biomass production.

A wide range of both filamentous and non-filamentous organisms were isolated from the enrichment cultures. Activated sludge is a rich source of microorganisms containing bacteria, yeasts and filamentous fungi among others, depending on the waste stream characteristics. In all, 22 non-filamentous and 5 filamentous isolates were recovered from the activated sludge from the rendering plant. Wastewater yielded a slightly lower number of strains, with 15 non-filamentous species and 6 filamentous fungi isolated. Activated sludge, being a mixed microbial culture itself, would be expected to yield a higher number of lipid degraders. Filamentous fungi were not isolated from non-shaking cultures during enrichment. This suggested that they were highly aerobic in nature, and were not sustained with the lower levels of oxygenation present in stationary flasks.

Enrichment cultures with tallow as the only source of microorganisms yielded less organisms than inoculated cultures, yielding only 13 non-filamentous strains. These strains were isolated singly in most cases. Some had similar morphological

characteristics, and some were shown in the laboratory to be the same species. There was greater morphological diversity in wastewater and activated sludge inoculated enrichment cultures, indicating a greater range of microbiological species.

During the production of tallow, the rendering process operated at temperatures which kills all potential pathogens, so the resulting tallow was sterile. The organisms isolated from tallow were therefore from environmental sources. These possible sources would have been during handling or storage of the tallow. No fungi were isolated from it, indicating that the environmental conditions were not suitable for growth of lipid utilising fungi. It may also be the case that the low water environment of pure tallow was suitable only for a limited number of species. The study by Shabtai (1991) described a species surviving under such conditions. A strain of *Pseudomonas aeruginosa* capable of surviving in such an environment was studied. Although the organism did not grow efficiently, it had an 85% survival rate in a 99% triglyceride culture. Such organisms may survive in the low water environment until conditions are suitable for growth, as in the enrichment cultures.

Screening of the isolates in pure culture found that most of the organisms removing greater than 20% of the added tallow were isolated from activated sludge, with four non-filamentous and two filamentous strains. The two filamentous organisms were also found in wastewater, which also yielded two non-filamentous species. Of the isolates obtained from tallow, only one, NF23, was capable of removing more than 20%. The sources with the greater microbial diversity yielded more organisms with higher ability to remove tallow.

One of the strains, the filamentous fungus F2, removed the highest amount of tallow in pure batch cultures, and therefore was selected for further study. The study of a pure culture, as opposed to a mixed culture, would allow a more accurate understanding of the breakdown pathways and the effect of environmental conditions on this breakdown. In mixed cultures, interactions between species and potential

shifts in the population distribution of species would introduce variability in the systems.

Filamentous fungi are well known for their ability to secrete extracellular enzymes (Bennett and Faison, 1997), which would be particularly suited to a solid substrate such as tallow. The enzymes break down potential energy sources which are then absorbed into the fungal biomass. Bednarski *et al.* (1993) studied the growth of three filamentous fungi, *Aspergillus niger, Geotrichum candidum* and *Mucor meihei*, on tallow and poultry fat. They found that these fats can be digested to varying degrees by the species studied, and suggested that filamentous fungi may be potentially used for fat biodegradation in waste treatment. Also, as the majority of previous published works on fat utilisation by microorganisms were carried out on yeasts and bacteria, a filamentous fungus presented a novel subject for the present investigation.

The hyphae and reproductive structures of F2 were examined and their characteristics were noted. Using this information and identification keys, it was determined that F2 was *Trichoderma harzianum* Rifai, and was designated RP1 as it was isolated from a rendering plant waste treatment system. The identity was determined in several stages, based on keys by Onions *et al.* (1986) and Barnett and Hunter (1972). Starting with the hyphae, it was noted that they were septate, so RP1 belonged to the Ascomycetes, Basidiomycetes or Deuteromycetes (Onions *et al.*, 1986). It was necessary then to examine the reproductive structures for further identification. However, When *T. harzianum* Rifai RP1 was grown on malt extract agar, it was noted that although mycelial growth was rapid, sporulation was weak. Sporulation in *Trichoderma* fungi is sensitive to light (Betina and Farkas, 1998, Gressel and Hartmann, 1968, Gupta *et al.*, 1997) and starvation of the organism (Betina and Farkas, 1998). Sporulation by RP1 on malt extract agar was found only after incubation under daylight at ambient temperature after 21 days. This was, however, limited in area. It was also noted by the DSMZ that sporulation was induced when RP1 was grown on wood chips under light. Although the organism successfully

produced sporulating areas on wood shavings and paper in the laboratory in the present study, this was very limited and insufficient for use as inocula. Other work in the laboratory demonstrated that radial growth of colonies of a sporing form of *Trichoderma harzianum* on agar was reduced and sporulation was increased when the organism was exposed to visible and UV light. However, where they were present, the reproductive structures were identical regardless of the cultural conditions.

Upon examination of RP1, it was noted that no sexual reproduction was present. Both the Ascomycetes and Basidiomycetes reproduce sexually, by means of ascospores and basidiospores respectively (Onions *et al.*, 1986). Ascospores are contained in closed receptacles, generally in groups of eight spores, or multiples of eight. Ascomycetes can, however, also reproduce asexually, by the formation of conidia. Basidiomycetes include visible fungi such as mushrooms and toadstools, and bear basidiospores in specialised structures, the most complex being the visible mushrooms. RP1 therefore did not belong to the Ascomycetes or Basidiomycetes, but belonged to the Deuteromycetes.

As the mycelium was well-developed, and spores were produced by conidiophores, it was determined that F2 belonged to the Hyphomycetes (Onions *et al.*, 1986). Other asexual reproductive structures of the Deuteromycetes include pycnidia, pycnothria, stromata and acervuli. These are specialised structures made from masses of hyphae, bearing the spores in cavities, of which none were present in RP1. Following the key for families of the Hyphomycetes, it was noted that RP1 belonged to the Moniliales, as it did not produce spores in synnemata or sporodochia. These are compacted masses of hyphae, and were not present on RP1.

The key for the Moniliales by Barnett and Hunter (1972) was then followed. The conidia were not coiled or curved, but were globose. The pigmentation of the RP1 was questioned. Although the sporing areas of RP1 were green when examined macroscopically, under the microscope the pigmentation of individual conidia was

not dark. The hyphae were also not pigmented. This led to an examination of the nature of the conidia. They were present as single spores and were globose or spherical. Their shape was not variable, and not ovoid or egg-shaped.

The key then led to the structure of the conidiophore. The conidiophores were well developed and distinct. They were not underdeveloped to the extent that they were just phialides. Nor were they like small pointed sterigmata. Following this information, the possibility of the organism being a conidial state of powdery mildew was suggested. However, mildews are parasitic diseases of plants. They grow and reproduce solely on living plant tissue and so RP1 did not fall into this category (Crute, 1981).

The structure of the conidia and conidiophores was then again examined. The distinction of the conidia from the apical cells of the conidiophores was questioned. It was noted from observations that the phialides were skittle shaped. However, the conidia were separate and distinct from the apical cells of the conidiophores. This led to the further examination of characteristics of the conidiophores. It was obvious from microscopic examination that the conidiophores were extensively branched, and the phialides were in groups. Following the key further, the question of catenulate conidia arose. The conidia of RP1 were not catenulate (i.e. they were not in chains).

The presence or absence of aleuriospores was the next characteristic in the key. Aleuriospores are spores formed at the end of a conidiogenous cell or hypha by substantial expansion of the cell. They are sometimes surrounded by a thick pigmented wall. They are only shed by the rupture of the cell wall surrounding them. There were no large rough-walled aleuriospores in RP1.

The site of conidiogenesis was then determined. The conidia of RP1 were produced only at the apex of the phialides, and not at their sides. The next stage of examination was of the arrangement of the branches of the conidiophore. It was clear that the

branching was irregular, and not verticillate. Verticillate branching is where the branches are arranged regularly from a single point, like spokes. The key then questioned the source of the strain. RP1 was not isolated from an aquatic environment, on submerged dead leaves.

This led to four possible genera: *Botryotrichum, Trichoderma, Hansfordia* and *Nodulosporium.* A comparison was made to the reproductive structures of these four genera (Appendix D). They did not resemble the reproductive structures of *Botryotrichum, Hansfordia* or *Nodulosporium.* The identity was determined as *Trichoderma harzianum*, which was confirmed by Deutsche Sammlung von Mikroorganismen und Zellculturen GmbH (DSMZ) (Appendix C).

It has been noted that the main fatty acid produced by *Trichoderma* species are palmitic, stearic, oleic and linoleic acids (Sivasithamparam and Ghisalberti, 1998). The reported main fatty acids in tallow include these, palmitic, stearic and oleic being the most abundant (Kajs and Vanderzant, 1981). This indicated that these fatty acids were not new in the metabolic pathways of RP1. The enzymatic systems required for metabolism of substrates containing them should have been present in RP1 cells. However, nothing has been reported in the literature on the biodegradation of tallow, or any other lipid substrate, by a *Trichoderma* species.

In order to study the biodegradation of tallow by *T. harzianum* Rifai RP1, the optimum conditions for growth and tallow removal from culture were determined. Factors studied included the nature of the inoculum, the physical nature of the substrate, the influence of pH buffering, temperature, rate of agitation and substrate concentration.

As noted previously, sporulation of RP1 was limited and difficult to induce. The amount of spores produced as insufficient to use as inocula. Mycelial agar discs of RP1 were therefore used as the inocula in these studies. These inocula would ideally

be actively growing cultures to ensure their viability. The age of the cultures might have affected viability, so the influence of the age of the inoculum on growth and tallow removal over 10 days was examined. No significant difference was noted between cultures of 2, 5 or 8 days old, so the age of the inoculum in this range did not affect its viability. The percentage removal of tallow was similar regardless of the age of the inoculum, at 14-15%. Later studies were carried out on the size of the inoculum under optimum conditions of pH and temperature. The addition of a larger inoculum might have affected substrate removal over the 10 day incubation period. When the inoculum size of RP1 was increased, no improvement was observed. A slight decrease was even noted from 40% with one mycelial plug, to 30% with 10 mycelial agar plugs. The biomass production was not increased. With the increased inocula, however, another factor became significant. The amount of carbohydrate added in the malt extract agar increased, and would be utilised in preference to the tallow in the early stages of incubation. Since no improvement was found by increasing the inoculum size, the use of one agar plug was continued for further experimental work.

Tallow was generally present as solid balls in culture, reducing the surface area available for enzymatic attack. Increasing this surface area would improve its bioavailability and increase its biodegradation. The elevation of the temperature of incubation increased the surface area by melting the tallow. Tallow liquefaction takes place over the range 40°C to 50°C. However, it was found that although the bioavailability may have been increased, RP1 did not grow at the high temperatures. Other studies have used elevated temperatures to liquify solid lipid substrates. In the anaerobic system studied by Broughton *et al.* (1998), raising the temperature to 50°C to melt sheep tallow resulted in lower removal compared to that seen at 35°C. Becker *et al.* (1999) found that melting of the substrate did result in effective removal of wool grease. However, the organism used was *Bacillus thermoleovorans*, a thermophilic organism, which grew well at the temperature studied, 65°C. The successful application of a thermophilic mixed culture was reported by Nakona and Matsumura (2001). Their study focussed on a solid state system operated at 60°C,

with the culture supported on a solid matrix, and the lipid added in its liquid state. These reported studies demonstrated that although bioavailability could be improved, the biodegradation depended on the ability of the microbial populations to be metabolically active at the elevated temperatures. The temperature of incubation can affect the survival, growth and reproduction of an organism.

During the biodegradation of tallow, lipase activity, as well as the growth of the organism, influence the extent of the fat degraded. Lipases have also been shown to have specific temperature requirements. A survey of the lipases reported by White and White (1997) revealed that temperature optima are as diverse as the sources from which they are obtained. Those from fungal sources listed had activity peaks ranging from 20°C to 50°C and above. Examples of lipases from the literature included mesophilic enzymes, operating at 30-40°C (Catoni *et al.*, 1997, Destain *et al.*, 1997) and those with thermophilic optima (Somkuti *et al.*, 1969, Kwon and Rhee, 1984, Bailie *et al.*, 1995).

Trichoderma species have been shown to be adaptive to temperature dependency (Kubicek-Pranz, 1998). Their optimum temperature for growth may be influenced by the prevailing temperature in the environment from which they are isolated. As noted earlier, the growth of *Trichoderma harzianum* Rifai RP1 was greatly affected by the temperature of incubation. It appeared to be mesophilic, based on observations during identification and during studies of the physical nature of the substrate. It grew profusely at 25°C, but did not grow at 4°C, 37°C or 55°C. The temperatures reported in some of the literature for *T. harzianum* tended to be in the range 25°C to 30°C (Elad *et al.*, 1982, Gupta *et al.*, 1997, Krantz-Rülcker *et al.*, 1994, Muñoz *et al.*, 1995, Vikineswary *et al.*, 1997).

It was noted that RP1 grew optimally at 25°C in unbuffered cultures. The effect of temperature was examined in buffered culture to determine if buffering influenced the temperature optimum for growth and tallow removal by RP1. The optimum was

again found at 25°C, with 43% of the added tallow removed from culture. This temperature was used for further studies. At other temperatures, growth was reduced or absent, with none observed at 37°C or 55°C.

The optimum temperature for RP1 grown on tallow was similar to reported values in other lipid removal studies. As examples of these, several researchers used temperatures in the range 20-30°C, while examining the utilisation of lipid substrates. Okuda *et al.* (1991) reported a temperature of 30°C while growing *Bacillus* species on fat. Also using a fat substrate, yeast and bacterial species were grown at 30°C by Tan and Gill (1985 & 1987). Yeasts were also grown on fat by Kajs and Vanderzant (1981), at a temperature of 25°C. Filamentous fungi were examined on fats by Bednarski *et al.* (1993) at 28°C. A range of oils and fats were the substrate for a mixed bacterial culture by Tano-Debrah *et al.* (1999), using a temperature range of 20-25°C. Microorganisms have also been grown on oils at mesophilic temperatures. Koh *et al.* (1983) assimilated palm oil with a yeast at 30°C. A range of bacteria, yeasts and filamentous fungi were grown on soyabean oil at 28°C by Koritala *et al.* (1987). Mesophilic temperatures have been generally used for studies on the assimilation of lipid substrates. Although the elevation of the temperature would liquefy the fat substrate, the use of a thermophilic organisms for the biodegradation of tallow would also have other disadvantages. The maintenance of a higher temperature in a bioreactor would require the use of an insulated vessel resulting in higher capital costs. The energy requirements would also be greater for such a system.

Another method of increasing the bioavailability of the tallow is by the addition of a surfactant. Tween 80 was selected as a model surfactant to examine if dispersal of the fat in culture might enhance its assimilation. Although dispersal was observed, 2%(w/v) surfactant was required to achieve this. Despite this apparent increased bioavailability, the removal of extracellular lipid was halved from 20% to 11% in the presence of the surfactant. It was observed that the fungus could grow on the Tween, utilising it as a carbon source in the absence of tallow. Most studies on the

biodegradation of triglyceride lipids in the literature did not use an additional surfactant. It has been noted that the use of surfactants can lead to inhibition as well as enhancement in certain cases, and that many carbon based surfactants act as preferential substrates instead of the insoluble carbon compound being studied (Rouse *et al.*, 1995). Also, tallow is relatively resistant to dispersion, and the concentrations of surfactant required may be inhibitory in some cases (Van der Meeren and Verstraete, 1996). Koh *et al.* (1983) found a range of surfactants to be inhibitory to yeast growth on palm oil. Petruy and Lettinga (1997) noted that anionic surfactants can be inhibitory to anaerobic biomass at concentrations as low as 10ppm. They also suggested that surfactants could increase the toxicity of lipids due to dispersion. On the other hand Hedgecock (1970) reported that toxicity of free fatty acids to *Mycobacterium tuberculosis* was reduced by the addition of a nonionic surfactant. It was decided therefore that the normal pathway of tallow biodegradation by *T. harzianum* Rifai RP1 may have been affected by the addition of a surfactant, and further studies would be carried out without one.

It was observed that a large pH drop accompanied growth of RP1 on tallow. This was also noted in the enrichment cultures. Many microorganisms have a limited pH range in which they utilise substrates optimally. Lipase is a key enzyme in the biodegradation of triglyceride lipids, and in several publications, fungal lipases have been shown to have relatively narrow optimum pH ranges (Catoni *et al.*, 1997, Destain *et al.*, 1997, Kwon and Rhee, 1984, Somkuti *et al.*, 1969, White and White, 1997). Growth of *Trichoderma* species is also sensitive to pH, the optimum range being quoted as pH 4 to 6.5 (Kubicek-Pranz, 1998). The pH drop may therefore have affected the ability of RP1 to metabolise tallow. Buffering of the culture medium was examined to determine if the control of pH may enhance the removal of extracellular lipid.

Citrate buffer, at 0.1M, was chosen as it has a wide effective buffering range. The optimum tallow removal with this buffer, of 34%, was observed at pH 6. However,

RP1 could utilise the buffer as a carbon substrate. This affected the ability of the buffer to maintain a constant pH at pH 4 and 5. Also, RP1 was growing with citrate as a carbon source, so tallow was no longer the sole carbon source. Confirmation of pH 6 as the optimum pH was achieved using phosphate and phthalate buffers, which did not act as substrate for the fungus.

Trichoderma harzianum has previously been used for the treatment of an industrial waste, although with low levels of lipid. Controlling the pH to 4 was by the continuous monitoring of the pH and the addition of acid or base as required. This was shown to enhance biomass production, although COD removal was not improved (Vikineswary *et al.*, 1997). Utilising fats as substrate for fungal species, Bednarski *et al.* (1993) used a pH of 4.5, automatically controlling it in fermentor studies. Yeasts have been the subject of several studies, using a variety of pH values. Kajs and Vanderzant (1981) adjusted their media to pH 4.8, and maintained it at pH 4.4-4.8 by continuous monitoring and adjustment. Tan and Gill (1984) found pH 7.0 to be suitable for growth of *Saccharomycopsis lipolytica* with an olive oil substrate. They controlled the pH by continuous monitoring and manual adjustment. The studies above used continuous monitoring and acid-base adjustment to control the pH. However, they were carried out in fermentors. In the current study, batch cultures were used. Buffers were chosen for the pH control, because of ease of use and the reduced risk of contamination which might be introduced on pH probes and in added acids or bases.

The fact that the optimum pH for removal of tallow by RP1 was pH 6 would be due to numerous factors. The processes involved would include the activity of lipase enzymes, the assimilation of fatty acids from the medium, the metabolism of the assimilated fatty acids and the growth of the organism itself. Therefore, the optimum pH may not, for example, be the optimum for assimilation of fatty acids. It represented the value at which the combination of the processes involved worked together most efficiently.

As observed during enrichment, the agitation of cultures with tallow as the substrate can have a significant influence on the amount of growth occurring. *T. harzianum* Rifai RP1 was isolated from agitated cultures, suggesting that it was sensitive to the level of oxygenation in the culture. None of the filamentous fungi isolated during enrichment were found in non-agitated cultures. The influence of the level of agitation under conditions of optimum pH and temperature was examined. It was found to have an effect on the removal of tallow, but a lesser effect on growth of RP1. Tallow tended to remain in smaller pieces in non-agitated medium. In agitated systems, it formed aggregates quickly, presenting less surface area for microbial attack. Despite this apparent reduction in bioavailability, the agitated cultures tended to be more efficient, those at 130rpm being slightly more so than the other speeds. At the lower speeds, the reduced oxygenation may have been limiting. At 200rpm, the tallow formed larger aggregates, which would have reduced its bioavailability compared to the slower speeds. Agitation at 130rpm was the speed where the aeration and bioavailability were at levels to maximise removal of the tallow.

In the literature, as noted earlier, several concentrations of fat were used by different researchers during assimilation studies. The concentration of tallow might affect the growth and its own removal by RP1. With levels too low, substrate limitation might occur, but if it was too high, inhibition might be the dominant factor. To determine if any difference existed if lower concentrations were used, studies were carried out with 1, 2 and 10g/l, as well as 20g/l. The percentage removal was higher at the lower initial tallow concentrations. This indicated substrate overloading was occurring at the higher concentrations, reducing its rate of removal. Biomass production was, however, higher with higher tallow concentrations. At the lower concentrations of tallow substrate, the growth of RP1 was limited. Since opposing influences were affecting the growth and tallow removal by RP1, an in-depth study was required to examine how these influences were manifested.

The glyceride and free fatty acid compositions in cultures of *T. harzianum* Rifai RP1 grown on tallow was also examined, to determine if the removal of any of the lipid components was rate limiting in the removal of tallow. The phenomenon of intracellular lipid accumulation was examined, to determine if metabolism of the tallow was complete, and to examine if any particular component of the tallow was resistant to complete breakdown.

During the detailed studies on tallow utilisation by *T. harzianum* Rifai RP1, the majority of biomass production occurred during the early stages of incubation. After day 4, it slowed, with no significant increase after day 7 with any of the tallow concentrations. Vikineswary *et al.* (1997) noted that the exponential phase of growth of *T. harzianum* on palm oil sludge lasted 1 day, after which the fungus rapidly entered stationary phase. The cultures were incubated at 30°C, with an initial medium pH of 4.0.

Other studies have also reported reaching the stationary phase rapidly, while studying fungi on lipid substrates. Jeffery *et al.* (1999) found that *Mucor circinelloides* f. *circinelloides* reached stationary phase in approximately 3 days, when grown on sunflower oil with acetate. With sunflower oil alone at 30g/l, growth was gradual, with no evident exponential or stationary phase. The incubation temperature was 30°C, with an initial culture pH of 5.8. In a different study, *Mortierella elongata* reached stationary phase on 30g/l linseed oil after 2 days (Bajpai *et al.*, 1992). The temperature in this case was 25°C, with an initial pH of 6.1. While the onset of the stationary phase of RP1 grown on tallow was comparable to that observed in other studies, growth is governed by the environmental conditions, the variation of which can change the length of the exponential phase.

No report was found in the literature of Monod kinetics being applied to the growth of a microorganism on a lipid substrate. In the present study, the maximum specific growth rate (μ_{max}) was reached at the concentrations of 10 and 20g/l tallow substrate.

The addition of tallow at greater than 10g/l did not increase the specific growth rate of RP1. The specific growth rates would have been limited by the assimilation of free fatty acids into the biomass, which were later shown to accumulate extracellularly, demonstrating their restricted uptake rate into RP1 biomass. The concentration of extracellular lipid was not limiting. The μ_{max} determined for RP1 grown on tallow was $1.438day^{-1}$ or $0.06h^{-1}$, while the saturation constant (k_s) was 0.76g/l. Other researchers determined the μ_{max} and k_s of filamentous fungi on other substrates.

Resende *et al.*(2002) described mathematically the growth of *Penicillium chrysogenum* in bioreactors for the production of penicillin. The medium contained a mixture of carbon sources, in the form of glycerol, glucose and corn steep liquor. Their μ_{max} was $0.11h^{-1}$, higher than that noted for RP1 on tallow. They also reported a k_s of $1X10^{-5}$g/l, which was significantly lower than that calculated for RP1 on tallow. This means that *P. chrysogenum* required a much smaller concentration of the mixed carbon substrate to reach its μ_{max} than RP1 required to reach its μ_{max} on tallow.

García García *et al.* (1997) studied the biodegradation of phenolic compounds in wastewater from a sugar factory by *Aspergillus terreus* and *Geotrichum candidum*. Applying Monod kinetics, they found that the μ_{max} for *A. terreus* on the phenolic compounds in the wastewater was $0.06h^{-1}$, the same as noted for RP1 on tallow. The second organism, *G. candidum* had a lower μ_{max} of $0.047h^{-1}$. The k_s for *A. terreus* and *G. candidum* were 13.5g/l and 4.6g/l respectively. These were very high compared to that of RP1 on tallow. These organisms had a higher affinity for the phenolic compounds than RP1 had for tallow.

García García *et al.* (2000) used four fungi, *Phanerochaete chrysosporium*, *Aspergillus niger*, *Aspergillus terreus* and *Geotrichum candidum* on another wastewater containing phenolic compounds. In this case the wastewater was from an olive oil mill. While no Monod constants were calculated for *G. candidum*, as it did

not degrade the phenolic compounds in this case, they were for the other three organisms. The μ_{max} for *P. chrysosporium* on the phenolic compounds was the same as for RP1 on tallow, at $0.06h^{-1}$, while those for *A. niger* and *A. terreus* were slightly higher, each at $0.09h^{-1}$. The k_s values were significantly higher than that of RP1, at 2.0, 7.5 and 4.0g/l for *P. chrysosporium, A. niger* and *A. terreus* respectively, based on total organic carbon. As noted above, this meant that these organisms had a higher affinity for the phenolic compounds than RP1 had for tallow.

Most of the removal of tallow also occurred in the early stages of incubation for concentrations of 1, 2 and 10g/l. With 20g/l, this period of rapid removal was not observed. However, specific removal rate was not reduced with 20g/l tallow. Over the first 2 days of incubation, the maximum specific rate of removal was observed with all four concentrations. This rate increased with increasing concentration, but levelled at 10 and 20g/l. The relationship between specific removal rates and tallow concentrations was described by Michaelis-Menten kinetics. The saturation constant for specific tallow removal rate was 10.94g/l. At this point, the removal rate was at half the maximum theoretical rate. Therefore, the rates theoretically could increase with the addition of considerably more substrate. The rate observed with 20g/l tallow was $40gg^{-1}day^{-1}$, which was lower than the maximum theoretical rate of $65gg^{-1}day^{-1}$. The rates were favourable when compared to specific removal of $0.5gg^{-1}day^{-1}$ of soyabean oil achieved by a mixed yeast population (Chigusa *et al.*, 1996). This was the only lipid removal rate by microorganisms found in the literature.

On uptake into the biomass, fatty acids underwent a number of possible fates. They could have been metabolised via β-oxidation, being utilised for biomass growth and maintenance. Alternatively, they could have been incorporated, changed or unchanged, into structural lipids of the cell, or they could have been stored as reserve lipids. *T. harzianum* Rifai RP1 accumulated significant quantities of intracellular lipid in its biomass. The specific accumulation of intracellular lipid appeared to be independent of extracellular tallow concentration. Specific concentrations of between

0.33 and 0.55gg^{-1} were recorded in RP1. These are higher levels than previously recorded for *Trichoderma* fungi. Serrano-Carreon *et al.* (1992) recorded lipid accumulation of 0.25gg^{-1} for *T. harzianum*, when grown with sucrose as the carbon source. The same study reported 0.32gg^{-1} for *T. viride*, grown on a glucose based medium. *T. reesei* was the subject of studies by Brown *et al.* (1988) and Brown *et al.* (1990). Both studies observed 0.16gg^{-1} lipid accumulation on glucose based media.

Although intracellular lipid accumulation studies by *Trichoderma* fungi on lipid substrates have not been reported in the literature, other filamentous fungi have been the subject of such experiments. With tallow as the carbon source, Bednarski *et al.* (1993) recorded specific accumulation of between 0.36gg^{-1} for *Geotrichum candidum* to 0.50gg^{-1} for *Mucor miehei*. Interestingly, on poultry fat, these organisms accumulated 0.32 and 0.39gg^{-1} respectively.

Lipid accumulation by *Mucor* species was reported by several researchers. The amounts ranged from 0.26gg^{-1} in *M. hiemalis* (Akhtar *et al.*, 1983), 0.37gg^{-1} in *M. circinelloides* (Aggelis *et al.*, 1995a), to a high of 0.62gg^{-1} in *M. mucedo* (Čertík *et al.*, 1997), all on oil substrates. Čertík *et al.* also studied a range of other filamentous fungi, with accumulation ranging from 0.33 to 0.66gg^{-1} recorded. On soyabean oil, Koritala *et al.* (1987) found that *Aspergillus flavus* accumulated up to 0.57gg^{-1}. A range of oils were examined by Kendrick and Ratledge (1996), with *Entomophtora exitalis* showing both the lowest and highest levels of lipid of the study. On safflower oil, 0.25gg^{-1} was accumulated, while on mortierella oil, 0.48gg^{-1} was found. *Mortierella elongata* accumulated approximately 0.40gg^{-1} when grown on 30g/l linseed oil (Bajpai *et al.*, 1992).

Aggelis *et al.* (1995a) suggested that intracellular lipid accumulation was linked to the extracellular lipid concentration and the amount of fat free biomass. This was based on growth of the filamentous fungus, *Mucor circinelloides*, on sunflower oil, and a mathematical model was developed. It was proposed that intracellular lipid

build-up was linked to assimilation of extracellular oil. The oil was taken in at a rate in excess of requirements for growth and maintenance of the fungus. When extracellular lipid was exhausted, the levels of intracellular lipid decreased, as the organism utilised it for growth and survival. This research group later published a more advanced mathematical model based on the same data (Aggelis and Sourdis, 1997). However, in the case of a fat substrate, the model was not deemed to be satisfactory in predicting the accumulation of intracellular lipid. An attempt to apply the models to the growth of *Yarrowia lipolytica* on animal fat presented some problems. These problems were due to the discrimination against stearic acid by *Y. lipolytica*, and further models are currently being investigated which consider this discrimination (Aggelis, *personal communication*, 2002). Akhtar *et al.* (1983) also noted that intracellular accumulation occurred during active growth in *Mucor hiemalis* grown on olive oil triglycerides. When the growth phase was over, the concentration of intracellular lipids reduced, as they were utilised by the fungus.

The yields of dry weight on the tallow substrate ($Y_{x/s}$) decreased with increasing tallow concentration. The yields of fat-free biomass on metabolised lipid ($Y_{xf/sm}$) were also examined. Fat free biomass was the amount of RP1 biomass produced less the intracellular lipid accumulated. It represented the actual growth of the organism, while eliminating the effect of the lipid accumulation. As with $Y_{x/s}$, the values for $Y_{xf/sm}$ decreased with increasing concentrations. At the higher concentrations of tallow the growth of new biomass by RP1 was limited. This inhibition could have been caused by the toxic effects of extracellular free fatty acids, which later were found to accumulate in the culture medium. The values for $Y_{x/s}$ and $Y_{xf/sm}$ were similar at all the tallow concentrations. Therefore the yields of RP1 were not affected by the accumulation of intracellular lipid.

The limited growth of biomass at higher concentrations of substrate was also reported by Wakelin and Forster (1997). Concentrations of greater than 8g/l of corn oil were found to reduce yields of an *Acinetobacter* sp. after 3 days. They also observed that

yields varied greatly between lipid substrate types and species. The yields of RP1 on tallow, of 0.34 to 0.83gg^{-1}, compared favourably with those found by Wakelin and Forster, who reported yields on oil substrates ranging from 0.15 to 0.67gg^{-1}. Other researchers did not report yields on lipid substrates. An example of the yields of *Trichoderma* fungus, *T. viride*, grown on glucose was noted by Roels (1983). The yields of RP1 were comparable to the values of 0.62 and 0.77 quoted by Roels.

From a waste treatment perspective, the percentage of the tallow removed from culture should be as high as possible. High levels of removal were achieved with 1 and 2g/l tallow at 83% and 79% respectively after 10 days. The percentage dropped for 10 and 20g/l removing 42% and 31% respectively. In the waste treatment system of the rendering plant from which RP1 was isolated, the retention time for influent wastewater was approximately three days. After three days, RP1 had removed at best 44% of the tallow from batch cultures, which was added at 1g/l.

Bednarski *et al.* (1993) were the only researchers who reported the growth of filamentous fungi on animal fats. They found up to 23% removal of tallow from shake flask cultures by the filamentous fungus *Geotrichum candidum*. These researchers used an initial concentration of 30g/l, monitoring the removal after 5 days. Although the highest tallow concentration with RP1 was 20g/l, the removal was approximately 16% after 5 days, slightly lower than that reported for *G. candidum*. However, the percentage removal achieved by RP1 was 31% after 10 days. Bednarski also used a strain of *Mucor miehei* under the same conditions, resulting in a removal rate of 19%, similar to the rate by RP1 over 5 days.

No other example of filamentous fungal growth on tallow has been found in the literature. The degrees of utilisation of other lipids by moulds have been reported. Soyabean at a concentration of 40ml/l was completely assimilated by *Aspergillus flavus* after 5 days (Koritala *et al.*, 1987). Other fungi in this study did not remove the oil as efficiently. Fungi from the Deuteromycetes, to which *T. harzianum* belongs,

removed from 1% with *Cladosporium resinae* to 38% with *Alternaria solani*. Sunflower oil at 10g/l was completely assimilated by *Mucor circinelloides* (Aggelis *et al.*, 1995a). Also with sunflower oil, at 30g/l, *Mucor circinelloides* f. *circinelloides* removed 25% of the lipid as sole carbon source (Jeffery *et al.*, 1999).

Bacteria and yeasts have also been studied with respect to assimilation of tallow. These have shown in cases a greater removal ability than moulds. Tan and Gill (1985) claimed 90% removal of tallow over approximately 12 hours, with the yeast *Saccharomycopsis lipolytica*. However, they used a high concentration inoculum (12%v/v) of a culture actively growing on olive oil. The removal quoted was based on dispersed tallow, with an initial concentration of 1.1g/l. Okuda *et al.* (1991) achieved 80% removal of 5g/l tallow over 24 hours using a *Bacillus* species.

Various levels of performance were noted with mixed cultures . The anaerobic reactor operated by Petruy and Lettinga (1997) removed 22% of 6g/l milk fat after 22 days. Restaurant grease was removed at greater than 90% efficiency over 33 days in the system studied by Wakelin and Forster (1998). The mixed bacterial consortium developed by Tano-Debrah *et al.* (1999) had degraded oils and fats in the range 24% to 73% after 7 days on a range of oils and fats at 100g/l.

The composition of the extracellular lipid was of interest to examine if any of the lipid components were resistant to biodegradation by *T. harzianum* Rifai RP1 biomass. It was found to be composed of mainly triglycerides, with varying levels of mono- and diglycerides and free fatty acids.

Hydrolysis of the tallow triglycerides was efficiently carried out by *T. harzianum* Rifai RP1 lipases. Triglyceride concentrations dropped in parallel with extracellular lipid levels throughout incubation. Hydrolysis of the intermediates of triglyceride breakdown, the mono- and diglycerides, was also efficiently carried out by RP1 lipases. No build-up occurred of any of the glycerides. Akhtar *et al.* (1983) noted an

increase in partial glycerides in extracellular lipid, when *Mucor hiemalis* was grown on olive oil triglycerides. The triglycerides were added at 10g/l, and although its lipases were effective in triglyceride hydrolysis, partial glycerides were not efficiently degraded. On a range of organisms studied by Koritala *et al.* (1987), soyabean oil was metabolised to form free fatty acids at varying concentrations, and trace amounts of mono- and diglycerides.

The extracellular hydrolysis of glycerides in cultures of RP1 was not growth linked, as it continued after growth had ceased. It may, however, have been dependent on substrate concentrations. No change was noticed in the concentration of fatty acids released by hydrolysis for 1 and 2g/l tallow after 4 days. At this stage, triglycerides were hydrolysed by greater than 90%. At the higher concentrations of 10 and 20g/l tallow, the triglyceride concentration remained sufficiently high for hydrolysis to continue, reaching only 67% and 57% respectively by day 10.

The free fatty acids produced by this hydrolysis were not assimilated by RP1 biomass as quickly as they were produced so a build-up occurred. With 1 and 2g/l initial tallow, the build-up was transient, levels dropping when hydrolysis of the glycerides ceased. With 10 and 20g/l, build-up occurred throughout incubation, the rate increasing after 4 days, when the fungus had entered stationary phase. This indicated that assimilation of fatty acids was rate limiting, rather than the hydrolysis of the glycerides. It was noted earlier also that the stationery phase was reached after 7 days with 10 and 20g/l tallow, although the substrate was not exhausted. The buildup of free fatty acids extracellularly could have had an inhibitory effect on the growth of RP1.

The extracellular accumulation of free fatty acids was not unique to RP1 grown on tallow. This phenomenon has been reported by several researchers. A toxic effect of fatty acids was suggested by Wakelin and Forster (1997), who also noticed a free fatty acid accumulation extracellularly. Their substrates were oil at 8g/l, studying

several filamentous bacteria over a period of 8 days. Free fatty acid accumulation was also observed in a range of fungi, sometimes accounting for more than 90% of extracellular lipid by Koritala *et al.* (1987). Jeffery *et al.* (1999) observed the accumulation of extracellular fatty acids, with *Mucor circinelloides* f. *circinelloides* grown on 30g/l sunflower oil. Concentrations increased rapidly over the first 3 days, remaining high to the end of incubation after 7 days. They suggested that growth was limited by metabolism of free fatty acids rather than hydrolysis of glycerides. Several yeast species accumulated free fatty acids extracellularly on a 20g/l soybean substrate over 7 days, in a study by Chigusa *et al.* (1996).

The accumulation of long chain free fatty acids was also noted in anaerobic batch digestors with sheep tallow as the carbon source (Broughton *et al.*, 1998). The tallow was presented at concentrations of 5, 10 and 20g/l. The removal of free fatty acids from the waste stream presented a significant problem in the digestors. For 5g/l, accumulation persisted for approximately 10 days at mesophilic, and 60 days at thermophilic temperatures. At 10g/l, approximately 30 days at mesophilic and 70 days at thermophilic temperatures were required for free fatty acid removal. At 20g/l tallow, long chain fatty acids accumulated to higher concentrations, and for longer periods. At mesophilic temperatures, approximately 50 days were required to degrade the fatty acids, while at thermophilic temperatures, they did not degrade and persisted for the 100 days of incubation. In the anaerobic experiments carried out by Hanaki *et al.* (1981), the hydrolysis of milk fat readily occurred, but the resulting free fatty acids inhibited the biological activity in the biomass.

Becker *et al.* (1999) noted a build-up of fatty acids when the concentration of olive oil in a bioreactor was increased above 4g/l. The biomass of the organism, *Bacillus thermoleovorans*, experienced a toxic reaction, and cell numbers decreased dramatically. Further investigation revealed inhibition of growth in the presence of 0.1 to 0.15g/l oleic acid.

On the other hand, Aggelis *et al.* (1995a) did not note any accumulation of free fatty acids extracellularly during the growth of *Mucor circinelloides* on sunflower oil. In their study, 10g/l of the oil was completely utilised by the fungus within 5 days.

In the present work, the accumulation of free fatty acids was problematic during the biodegradation of lipids, as their assimilation was rate limiting. The component fatty acids might be a factor in this limitation, with some fatty acids being more resistant to assimilation than others. Therefore, the fatty acid compositions of the extracellular glycerides and free fatty acids were studied.

The main fatty acids in mono-, di- and triglycerides and in free fatty acids during growth of RP1 on tallow were palmitic, stearic and oleic acids. Myristic and linoleic acids were present, but only at very low, and sometimes undetectable, concentrations. None of the fatty acids present displayed resistance to hydrolysis from the glycerides. Also, assimilation of the free fatty acids was non-specific. The accumulation of free fatty acids extracellularly therefore did not appear to be due to the resistance of a particular fatty acid or type of fatty acid to assimilation by RP1 biomass.

The overall specific removal rates indicated that oleic acid was assimilated at a higher rate than palmitic or stearic acids. However, this may be due to oleic acid being present at higher concentrations in extracellular lipid. It accounted for approximately 40% of fatty acids in tallow, while stearic was 28% and palmitic was 24%. Free fatty acids were assimilated by the biomass, having been produced by glyceride hydrolysis. However, the substrate being assimilated, the free fatty acids, was being produced during incubation by glyceride hydrolysis, making rate comparisons less accurate. Also, it has been reported that no discrimination is shown by fungi during assimilation of free fatty acids (Jeffery *et al.*, 1999, Koritala *et al.*, 1987). On the other hand, Aggelis *et al.* (1997) found that a range of microorganisms preferentially assimilated unsaturated fatty acids from evening primrose oil. Due to the unequal

concentrations of the main fatty acids, further studies on the relative uptake of palmitic, stearic and oleic acids by RP1 were desirable.

Michaelis-Menten kinetics were again applied to the relationship between the specific removal rates and the tallow concentrations. The saturation constants for palmitic, stearic and oleic acids were in the range of 10.6 – 11.0g/l, which was similar to that for tallow. Those for myristic and linoleic acids were 8.8 – 8.9g/l. The affinity of RP1 for tallow was determined by its affinity for the main fatty acids present in the tallow.

Free fatty acids were the predominant component of intracellular lipids with 1 and 2g/l tallow. Triglycerides made a contribution, but were more significant with 10 and 20g/l tallow. Triglyceride synthesis ceased after 7 days, which may indicate that it was dependent on growth of RP1, which would have been reduced in the stationary phase of growth. Similar intracellular composition was noted by Koritala *et al.* (1987). The fungus *Amylomyces rouxii* accumulated mostly free fatty acids intracellularly (>80%) when grown on soybean oil.

The main fatty acids accumulated in the intracellular lipid were palmitic, stearic and oleic acids, the main components of the tallow substrate. At the lower concentrations of 1 and 2g/l tallow, stearic acid was incorporated into the triglycerides at slightly higher concentrations than oleic or palmitic acids. There was no preference in the composition of free fatty acids. At the higher concentrations, the preference for stearic in triglyceride fatty acids was not as apparent. Also, no preference was evident in free fatty acid components at either concentration.

Overall, the specific fatty acid concentrations indicated that no preference for the accumulation of a particular fatty acid occurred. However, oleic acid was present at higher concentrations in the tallow, but was not accumulated in greater concentrations. Also, no fatty acids were detected intracellularly that were not present

in tallow. Therefore, significant modification of assimilated fatty acids was not occurring.

This was also noted by other researchers. Kendrick and Ratledge (1996) found that the intracellular fatty acid composition in several filamentous fungi closely resembled the substrate oils. They hypothesised that fatty acids were accumulated directly into the intracellular lipids without any changes. No preference for the utilisation of a particular fatty acid was noted. Modification of the fatty acids was also not observed. They postulated that fatty acid elongases and desaturases were suppressed by the fatty acids taken into the fungal cells, resulting in the intracellular lipid resembling the substrate lipid.

Koritala *et al.* (1987) found that with *Aspergillus flavus*, the intracellular lipid composition changed from that of the substrate, when grown on soybean oil. However, the composition of the free fatty acid fraction intracellularly resembled that of extracellular free fatty acids. Since no preference is shown in absorbing free fatty acids, this was not unexpected.

In tallow, oleic acid was present in higher concentrations than the saturated acids, palmitic and stearic acid. As noted above, oleic accounted for 40% of fatty acids in tallow, while stearic and palmitic acids were at 28% and 24% respectively. Also, extracellular free fatty acids available to the fungus were produced during incubation of RP1 on tallow by glyceride hydrolysis. The differences in metabolism of fatty acids may therefore not have been apparent during biodegradation of tallow. In order to determine if differences existed between the main fatty acids of tallow, RP1 was grown with a mixture of the three fatty acids as the carbon source. The fatty acids were added in approximately equal quantities, so differences in assimilation rates, accumulation levels and metabolism would be clearer.

As was the case with tallow as the substrate, there have been no published reports of *T. harzianum* grown with fatty acid substrates. However, *Trichoderma viride* was isolated from soil samples enriched with oleic acid as a sole source of carbon and energy, but not saturated fatty acids. A different *Trichoderma* species, *T. koningii* was isolated from enrichments with myristic and palmitic acids as the sole carbon sources, but not with oleic acid. Further studies on the utilisation of fatty acids by these species was not carried out, as other fungi, including several *Aspergillus, Fusarium* and *Paecilomyces* species, were found to have better growth and were selected for detailed examination. These other fungi were examined with respect to their growth on myristic, palmitic, stearic and oleic acids, and the composition of intracellular lipid (Radwan and Soliman, 1988).

In another study, a *Trichoderma* species was grown on glucose in the presence of fatty acid methyl esters, demonstrating bioconversion of the methyl ester of palmitoleic acid (C16:1) to 9,12 hexadecadienoic acid (C16:2). This study also was examining the composition of intracellular lipid, with limited data on growth or utilisation of the carbon sources (Shirasaka *et al.*, 1998).

The growth of *T. harzianum* Rifai RP1 on the fatty acid mixture was rapid after a lag of 1 day. Stationary phase was reached with 0.12g/l after 4 days, while with the other concentrations, it was not reached until after 7 days. This was due to the limitation of the fatty acid substrates with 0.12g/l initially, where concentrations rapidly dropped over the first 4 days of incubation. At the higher concentrations, substrate concentrations did not drop to critically low concentrations during incubation.

The specific growth rates of RP1 were similar for all the concentrations studied, at $0.1day^{-1}$. These rates were, however, significantly lower than those observed on the tallow substrate, where it ranged from 0.8 to $1.4day^{-1}$. The C:N ratios differed in the two systems, which would give different growth rates. With the tallow substrate, the

C:N ratio was in the range of 1.9:1 with 1g/l to 38:1 with 20g/l. With the fatty acid mixture as the substrate, these ratios were 0.06:1 for 0.12g/l to 0.9:1 for 1.80g/l.

Also, more biomass was apparently produced with the higher substrate concentrations. However, it was noted that lipid accumulation was more significant with the higher concentrations, with intracellular lipid accounting for up to 47% of dry weight with 1.80g/l fatty acids. Although the yields of total biomass on substrate removed ($Y_{x/s}$) were similar, the yield of fat free biomass on metabolised lipid ($Y_{xf/sm}$) revealed that the production of fat free biomass from the substrate was actually very low with 0.75 and 1.80g/l fatty acids. Increasing the fatty acid concentration reduced the production of fat free biomass in favour of accumulation of lipid intracellularly. A toxic effect of intracellular free fatty acids may have inhibited biomass growth, inducing stationary phase prior to exhaustion of the substrate. This inhibition was less with the tallow substrate, where the values for $Y_{x/s}$ were similar to those of $Y_{xf/sm}$. The buildup of intracellular free fatty acids in RP1 was not as rapid on tallow, which would have reduced the toxic effect in the early stages of incubation, allowing growth to occur. The free fatty acids in the tallow would have had reduced bioavailability, due to the solid nature of the tallow and its tendency to form aggregates.

The percentage removal of the fatty acid mixture was very high at a concentration of 0.12g/l, with almost complete removal (97%). As the concentration increased, the percentage removal decreased, with only 73%, 61% and 43% achieved with 0.36, 0.75 and 1.80g/l respectively. At 0.12g/l, the percentage removal of the individual fatty acids was greater than 95% in all cases. At the higher concentrations, differences emerged between the removals of oleic acid and the saturated acids, palmitic and stearic acid. The percentage removal of oleic acid was higher than the others, the difference becoming greater as the concentration increased. At the highest concentration of 1.80g/l, oleic acid was removed by 79% after 10 days, while palmitic and stearic were only removed by 28% and 25% respectively.

However, all three fatty acids were removed at similar specific removal rates. While oleic acid was removed from the beginning of incubation at all the concentrations, a lag was more evident for the saturated acids as the concentrations increased. A slight rise in their concentrations was also noted in some cases. This may have been due to desaturation of oleic acid to stearic acid. The difference in lag then resulted in the different percentage removal between oleic acid and the saturated acids during incubation.

Although no differences were apparent in the removal of the main fatty acids in tallow, oleic acid was present at higher concentrations, accounting for 40% of tallow fatty acids. From the data obtained in this experiment, it can be seen that oleic acid was more easily assimilated by RP1, which was not obvious during growth on tallow.

The ease of assimilation of oleic acid was also noted by other researchers. Papanikolaou *et al.* (2001), observed that oleic acid was significantly more rapidly removed than palmitic or stearic acids by *Yarrowia lipolytica*. The organism was grown on a mixture of the fatty acids at 10g/l. Tan and Gill (1985) found that the yeast *Saccharomycopsis lipolytica* could assimilate and grow on palmitic and oleic acids, but could not utilise stearic acid. Iwahori *et al.* (1995) noted that certain Actinomycetes could not utilise palmitic acid, and were even inhibited from growing on glucose in its presence.

Activated sludge removed fatty acids by varying degrees in the study by Leohr and Roth (1968). With saturated fatty acids, they found that longer chain lengths were more resistant to removal. Unsaturated fatty acids were also more easily assimilated than saturated fatty acids.

biomass of RP1 when grown on the fatty acid mixture. The level of accumulation increased with increasing free fatty acid concentration, reaching a maximum of

$0.47gg^{-1}$ with 1.80g/l on day 10. This was similar to the values observed with the tallow substrate. However, at the lower concentrations of fatty acids, the levels reached were significantly lower. With 0.12g/l, only $0.09gg^{-1}$ was reached. The greater accumulation with the tallow substrate would have been due to significantly higher uptake of fatty acids. The free fatty acids would have been continually fed by glyceride hydrolysis. With the fatty acids mixture as the substrate, the concentration was continually dropping, with no substrate 'feed'.

As was found with the tallow substrate, free fatty acids were the predominant component of intracellular lipid. Triglycerides were the only glyceride class observed, but they were present at significantly lower levels. No mono- or diglycerides were detected. With tallow as the substrate, triglycerides were a more significant component of intracellular lipid. The inhibition of growth by the free fatty acids may have limited the synthesis of triglycerides intracellularly due to reduced metabolic activity.

Papanikolaou *et al.* (2001) found that *Yarrowia lipolytica* grown on a fatty acid mixture at 10g/l, accumulated mostly triglycerides, with significant but lower levels of free fatty acids. An inhibition of biomass production and triglyceride synthesis did not seem to be present in their study. Radwan and Soliman (1988) also observed that several fungal species, grown on fatty acids as the sole carbon source, accumulated mostly free fatty acids intracellularly.

Stearic acid was the only fatty acid detected in the triglycerides. A slight preference for stearic acid incorporation into intracellular triglycerides was also noted with the tallow substrate. In free fatty acids, there was a slight preference for both saturated fatty acids, with oleic acid present at lower levels. With the tallow substrate, no preference was evident. However, with the tallow, oleic acid was present in the substrate at higher levels than palmitic or stearic, which may have 'masked' the lower tendency to store it in RP1 biomass. Overall, stearic acid was the most significantly

accumulated fatty acid intracellularly, followed by palmitic acid, with oleic acid being the least accumulated of the three.

This preference for saturated fatty acid accumulation was also noted by other researchers. Stearic acid was accumulated at significantly higher concentrations than palmitic or oleic acids in the biomass of *Yarrowia lipolytica* grown on a mixture of the fatty acids at 10g/l (Papanikolaou *et al.*, 2001). They were mostly incorporated into triglycerides and free fatty acids, the predominant components of intracellular lipid. However, they found that oleic and linoleic acids were the main fatty acids in triglycerides, while stearic and palmitic acids were highest in the free fatty acids. They also noted that lipid accumulation was lower when the organism was grown on a fatty acid mixture rich in oleic acid. The presence of saturated acids may have stimulated the accumulation of the lipids. They suggested that unsaturated fatty acids such as oleic acid are rapidly incorporated and used for growth, but saturated fatty acids are more slowly taken up, and tended to accumulate intracellularly.

Lalman and Bagley (2001) noted that palmitic acid accumulated as a metabolic intermediate of oleic acid biodegradation in an anaerobic mixed culture. In the intracellular lipid of RP1, the saturated acids accumulated, and possibly may have been added to by biodegradation of the oleic acid. Lalman and Bagley also found that almost 30 days was required for the removal of 0.1g/l oleic acid from culture. This is significantly longer than that required with RP1, where greater than 95% removal of 0.12g/l was observed within 7 days. With 0.1g/l stearic acid, 50% still remained in the anaerobic culture after 30 days.

Overall, *T. harzianum* Rifai RP1 did not have any difficulty hydrolysing the extracellular glycerides of the tallow substrate. The rate limiting step was the assimilation of free fatty acids into the biomass, as evidenced by the extracellular accumulation of free fatty acids. When fatty acids were assimilated into the cell, triglycerides were synthesised. With free fatty acids as the sole carbon source, higher

concentrations had an inhibitory affect, with less fat free biomass produced and more accumulation. This was also accompanied by less triglyceride synthesis, which may have been due to the limited biomass, and hence limited metabolic activity. RP1 may have been protected from the toxic effect of fatty acids with tallow as the substrate by their reduced bioavailability in the fat masses.

5. Conclusions

1. The wastewater treatment system of a commercial rendering plant yielded 58 isolates, of which nine were capable of growth on tallow as the sole carbon source, removing greater than 20% of it from culture.

2. The filamentous isolate, F2, was identified as *Trichoderma harzianum* Rifai RP1. It removed tallow from batch cultures optimally at 25°C, shaking at 130rpm, with the pH controlled to pH 6.0. Its growth followed Monod kinetics, with μ_{max} and k_s of 1.438day^{-1} and 0.758g/l respectively.

3. The glycerides of tallow were effectively hydrolysed by *T. harzianum* Rifai RP1, with no fatty acid specificity. Extracellular free fatty acids accumulated while glyceride hydrolysis continued, their assimilation being the rate-limiting step of extracellular lipid removal. No fatty acid specificity was noted in this accumulation.

4. Complete metabolism of assimilated lipid did not occur during growth of *T. harzianum* Rifai RP1 on tallow. Lipid accumulation occurred intracellularly, which was composed of free fatty acids and triglycerides. No fatty acid specificity was apparent in accumulated lipid.

5. When grown on a mixture of palmitic, stearic and oleic acids, RP1 preferentially assimilated oleic acid in preference to palmitic or stearic acid. With 0.75 and 1.80g/l of fatty acid substrate, biomass production was inhibited at the expense of intracellular lipid accumulation. This lipid consisted mainly of free fatty acids, with low levels of triglycerides. Stearic acid was accumulated in intracellular lipid to a greater extent than palmitic or oleic acids.

Further study

- The study of tallow biodegradation by *Trichoderma harzianum* Rifai RP1 in larger scale fermentors would provide valuable information about its performance in commercial bioreactors. Since RP1 was studied in batch culture, more studies would be needed to assess its performance in continuous culture. Waste treatment bioreactors most commonly operate in continuous culture mode.

- The performance of *Trichoderma harzianum* Rifai RP1 in mixed cultures would be of interest. Combining RP1 with other microorganisms capable of assimilating free fatty acids could result in a microbial consortium which readily removes and metabolises tallow and other hard fats. In a bioreactor, the use of a consortium would be more practical, and could be formulated to give the ability to biodegrade a range of lipid substrates.

- The investigation of the intracellular accumulation of modified lipids by RP1 on various lipid substrates could result in the production of useful by-products in a cost effective manner. Valuable lipids and lipid derivatives have been shown to accumulate in other species, and RP1 has been found in this study to accumulate significant levels of intracellular lipids. These could be extracted if they were found to be of commercial value

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Appendix A

Keys for the identification classes and families of Deuteromycetes

Key to the classes of Deuteromycetes from Onions *et al.* (1986).

Key to the classes of Deuteromycotina (following Ainsworth, 1973)		
1	Budding (yeast or yeast-like) cells with or without pseudomycelium characteristic, true mycelium lacking or not well-developed	*Blastomycetes*
1'.	Mycelium well developed, assimilative budding cells absent	2
2.	Mycelium sterile or bearing spores directly on special branches (conidiophores) which may be variously aggregated but not in pycnidia, pycnothria, acervuli or stromata	*Hyphomycetes*
2'.	Spores in pycnidia, acervuli, pycnothyria or stromata	*Coelomycetes*

Key to families in the class Hyphomycetes from Onions *et al.* (1986).

Key to the Hyphomycetes (following Saccardo)		
1.	Mycelium sterile	*Agonomycetes* or *Mycelia sterilia*
1'.	Mycelium bearing spores	2
2.	Conidiophores separate	*Moniliales* 3
2'.	Conidiophores in synnemata	*Stilbellaceae*
2''.	Conidiophores in sporodochia	*Tuberculariaceae*
3.	Mycelium and conidia hyaline	*Moniliaceae*
3'.	Mycelium and/or conidia dark coloured	*Dematiaceae*

Appendix B

Key for identification of genera of the family Moniliales (Barnett and Hunter, 1972).

MONILIALES

1a	Conidia more or less coiled or spirally coiled, hyaline or dark (parts of Moniliaceae, Dematiaceae and Tuberculariaceae)		2
1b	Conidia not coiled		12
2a	Conidiophores forming a sporodochium		3
2b	Conidiophores single or in loose clusters		5
3a	Conidial coil flat		4
3b	Conidial coil in a loose spiral	*Hobsonia*	152
4a	Sporodochia stalked; conidia septate	*Ivellaria*	152
4b	Sporodochia flat or cushion-shaped; conidia 1-celled	*Drepanocomis*	146
5a	Spore coil more or less flattened, not aquatic		6
5b	Spore coil spiral; not aquatic		11
5c	Conidia more or less globoid, having branched separate coils; aquatic	*Spirosphaera*	134
6a	Conidia thick in proportion to length		7
6b	Conidia filaments slender		10
7a	Conidia hyaline or dark, with transverse septa only		8
7b	Conidia dark, with transverse and oblique septa	*Actinosporium*	132
8a	Conidia in simple coil; not beaked		9
8b	Conidia racquet-shaped, with a beak	*Circinotrichum*	134
9a	Parasitic on higher plants	*Helicomina*	132
9b	Saprophytic on wood or bark	*Helicoma*	132
10a	Conidiophores hyaline, short	*Helicomyces*	132
10b	Conidiophores dark, tall	*Helicosporium*	132
11a	Conidia borne singly	*Helicoon*	132
11b	Conidia catenulate	*Helicoendendron*	132
12a	Both conidia and conidiophores (if present) hyaline or brightly colored; conidiophores single or in loose clusters (Moniliaceae)		13
12b	Either conidia or conidiophores (or both) with distinct dark pigment; conidiophores single or in loose clusters (Dematiaceae)		106
12c	Conidiophores compacted into sporodochia or synnemata		204
13a	Conidia typically 1-celled, globose to cylindrical		14
13b	Conidia typically 2-celled, mostly ovoid to cylindrical		67
13c	Conidia typically 3- to several-celled, shape variable		75

14a	Conidiophores absent or reduced to phialides or peglike sterigmata		15
14b	Conidiophores distinct, although sometimes short		24
15a	Pathogenic to humans		16
15b	Saprophytic, mostly in soil or on plant parts		17
16a	Filamentous in culture at 25 C	*Blastomyces* *Histoplasma*	76 76
16b	Both filaments and yeastlike cells at 25 C	*Candida*	64
17a	Conidia (arthrospores) formed by segmentation of mycelium		18
17b	Conidia not arthrospores		19
18a	Forming small compact colonies on agar	*Streptomyces*	62
18b	Mycelium spreading on agar	*Geotrichum*	62
19a	Setae absent		20
19b	Setae present, branched or coiled	*Circinotrichum* *Gyoerffyella*	86 86
20a	Mycelium with clamp connections		21
20b	Mycelium without clamp connections		22
21a	Spores not forcibly discharged; attacking microscopic animals	*Nematoctonus*	68
21b	Spores forcibly discharged; not attacking microscopic animals	*Tubercularia*	64
22a	Some spores budding or producing secondary spores		23
22b	Conidia not forming secondary spores	*Harposporium*	81
23a	Spores borne on sterigmata and forcibly discharged	*Sporobolomyces*	64
23b	Spores borne on sides of mycelium, not forcibly discharged	*Candida*	64
24a	Conidial state of powdery mildew, conidia in chains	*Oidium*	64
24b	Conidial state of powdery mildew, conidia not in chains	*Ovulariopsis*	64
24c	Not conidial states of powdery mildews		25
25a	Conidiophore cells distinct from conidia		26
25b	Conidia not differing greatly from apical cells of conidiophores	*Monilia*	66
26a	Conidiophores (or phialides) simple or sparingly branched; phialides, if present, not tightly clustered		27
26b	Conidiophores mostly branched, sometimes simple; phialides, if present, in groups or heads		41
27a	Conidia catenulate		28
27b	Conidia not catenulate		34

42a	Phialides in heads on simple conidiophores		43
42b	Phialides not in heads; conidiophores usually branched		44
43a	Conidia dry, no slime present	*Aspergillus*	90
43b	Conidia in head of slime, subtended by sterile branches	*Gliocephalotrichum*	90
43c	Conidia in heads of slime, without sterile branches	*Gliocephalis*	90
44a	Conidia in basipetal chains		45
44b	Conidial (arthrospores) chains formed by segmentation		47
45a	Conidiophores not in layer or column		46
45b	Conidiophores grouped into loose layer or column	*Metarrhizium* *Myrothecium*	90 146
46a	Conidia phialospores; phialides divergent, loose	*Paecilomyces*	90
46b	Conidia phialospores, phialides upright, brushlike	*Penicillium*	90
46c	Conidia annellospores	*Scopulariopsis*	94
47a	Arthrospores barrel-shaped, separated by short slender cell	*Amblyosporium*	62
47b	Arthrospores rod-shaped, not separated by cells	*Oidiodendron*	62
48a	Large rough-walled aleuriospores present		49
48b	Large rough-walled aleuriospores absent		50
49a	Aleuriospores 1-celled, with or without hyaline outgrowths	*Stephanoma* *Sepedonium*	76 76
49b	Aleuriospores, with attached colorless basal cell	*Mycogone* *Chlamydomyces*	76 76
50a	Conidia produced at or near apex of phialides or branches of conidiophores		51
50b	Conidia attached both at apex and side of conidiophore or its branches		60
51a	Larger conidiophores (at least) verticillate		52
51b	Branches of conidiophore irregular, not verticillate		53
52a	Phialospores in mucilaginous clusters	*Verticillium*	88
52b	Sympodulospores in dry clusters	*Calcarisporium*	98
53a	Not aquatic		54
53b	Aquatic on submerged dead leaves	*Dimorphospora*	66
54a	Conidia not aggregated in slime drops		55
54b	Conidia held in heads by slime drops		57
55a	Conidia abundant, borne on inflated apical cells		56
55b	Conidia single or in small clusters, not on inflated cells		58

56a Conidiophores tall with central axis and several equal, lateral branches. *Botryosporium* 70

56b Conidiophores tall with dichotomous or irregular branches . . . . . *Botrytis* 70

56c Conidiophores short with few branches . . . . . *Phymatotrichum* 72

57a Conidiophore branches brushlike, similar to *Penicillium* . . . . . *Gliocladium* 88

57b Conidiophore branches spreading, not brushlike . . . . . *Trichoderma* 88

58a Conidiophore branches loose; conidia usually present 59

58b Conidiophore branches compacted into globose or pyramidal head; conidia often absent . . . . . *Cristulariella* 68

59a Conidia sympodulospores, borne on short denticles . . . . . *Hansfordia* 94
Nodulosporium 96

59b Conidia aleuriospores, not on denticles . . . . . *Botryotrichum* 78

60a Fertile portion of conidiophore zigzag, rachislike 61

60b Fertile portion of conidiophore not zigzag or rachislike 63

61a Conidiophores simple or verticillately branched 62

61b Conidiophores irregularly branched . . . . . *Geniculosporium* 98
Nodulosporium 96

62a Conidiophores bulbous at base; parasitic on insects . . . . . *Beauveria* 96

62b Conidiophores not bulbous at base; not on insects . . . . . *Tritirachium* 96

63a Conidia borne on denticles (sharp or blunt) 64

63b Conidia not borne on denticles . . . . . *Botryoderma* 80

64a Conidiophores slender 65

64b Conidiophores stout 66

65a Conidiophores producing forked fertile branches near apex . . . . . *Ostracoderma* 74

65b Conidiophores with slender branches from main axis . . . . . *Calcarisporium* 98

66a Conidia on short denticles . . . . . *Rhinotrichum* 70

66b Conidia on slender elongated denticles . . . . . *Oliptrichum* 68

67a Conidiophores well developed, branched 68

67b Conidiophores distinct, simple or sparingly branched 71

67c Conidiophores none, reduced to cells of stroma . . . . . *Rhynchosporium* 104

68a Conidia (sympodulospores) ovoid to oblong 69

68b Conidia (sympodulospores) obovoid . . . . . *Geniculatia* 106

68c Conidia (phialospores) slender, cylindrical . . . . . *Cylindrocladium* 104

Appendix C

Identification report from Deutsche Sammlung von Mokroorganismen und Zellkulturen GmbH for filamentous fungi F2 and F9

Strains 'F2' and 'F3' in the report were two samples of filamentous fungus F2.

Strain 'F1' in the report was filamentous fungus F9

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Identification of Fungus cultures

Sent by: Dublin City University Dr. J. Fleming

Strain designation: F1, F2, F3

Substrate: activated sludge

Colony habit:

Colony on malteextract-agar at 25 C filling the Petri-dish within 3 days, mycelium hyaline, sporulating areas tufted, green. Colony reverse colour unchanged. No odour. No growth at 37 C.

Morphology

Conidiophores tree-like, branched at right angles, length of branches increasing to the basis, no sterile appendages. Phialides flask-shaped, straight or bent, arranged mostly in groups of three at the end of branches. Conidia ellipsoidal to sphaerical, smooth-walled, 3-3.5 x 3 um

Identity: *Trichoderma harzianum* Rifai

DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Braunschweig, 3.11.98

Remarks: The sporulating abilities of isolates F2 and F3 are strongly reduced. It is possible that this is due to incubating cultures in the dark. Sporulating areas on wood chips show the same conidiophore pattern as strain F1.

Appendix D

Reproductive structures of the genera *Trichoderma, Botryotrichum, Hansfordia* and *Nodulosporium.*

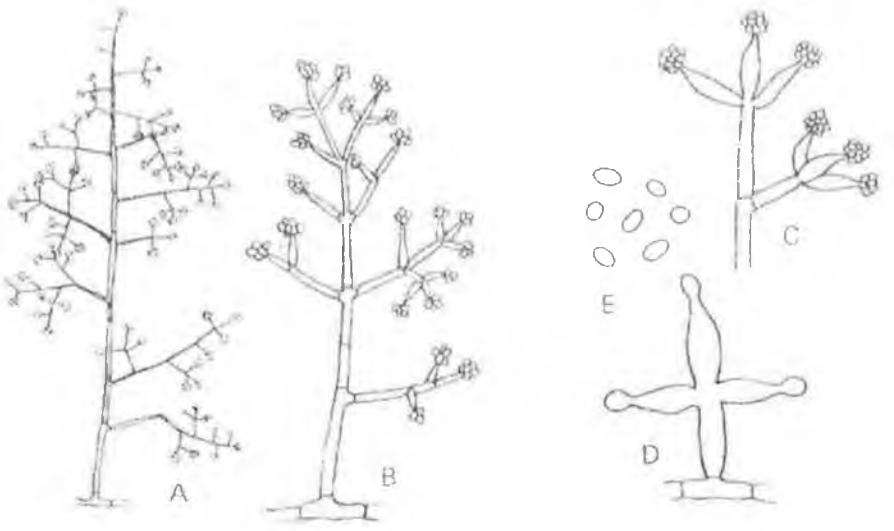

Figure 60: Reproductive structures of the genus *Trichoderma.* (A) and (B) large conidiophores showing extensive branching; (C) and (D) phialides showing production of conidia; (E) conidia (Barnett and Hunter, 1972)

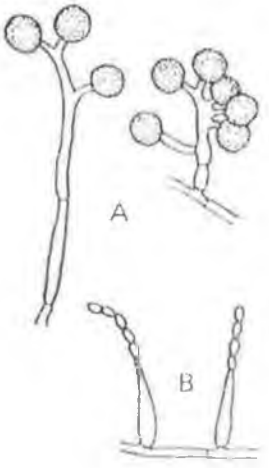

Figure 61: Reproductive structures of the genus *Botryotrichum.* (A) conidiophores with aleurispores; (B) phialides with phialospores (Barnett and Hunter, 1972)

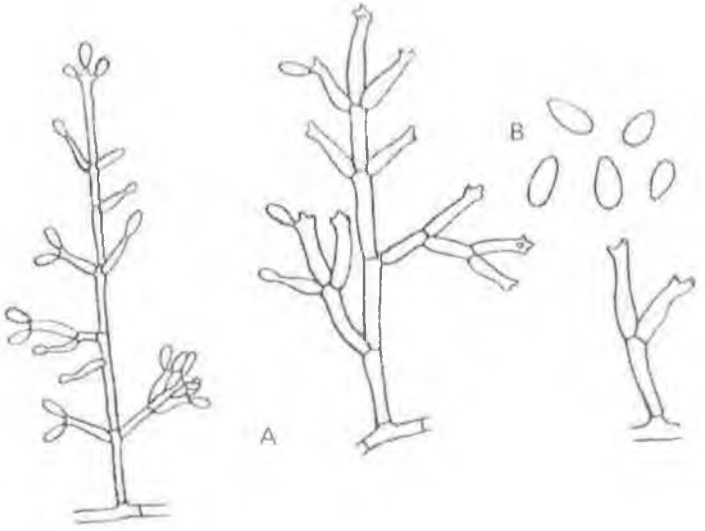

Figure 62: Reproductive structures of the genus *Hansfordia*. (A) conidiophores bearing conidia; (B) conidia (Barnett and Hunter, 1972)

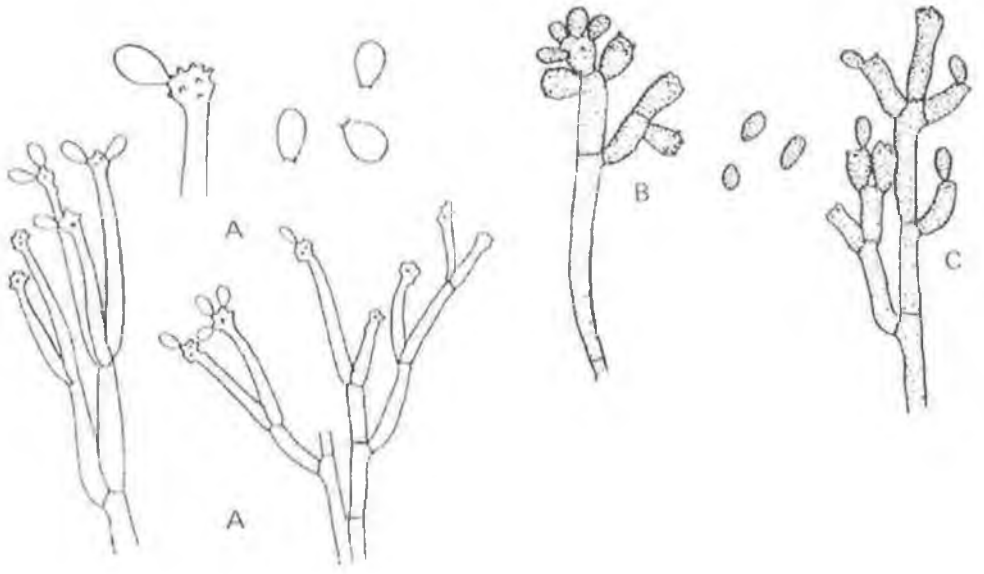

Figure 63: Reproductive structures of the genus *Nodulisporium*. (A) conidiophores and conidia of *Hypoxylon* sp.; (B) and (C) conidiophores and conidia of *Hypoxylon atropunctatum* (Barnett and Hunter, 1972)

Appendix E

Personal communication from Professor George Aggelis

From: George Aggelis <George.Aggelis@aua.gr>
To: john.fleming3@mail.dcu.ie
Cc:
Subject: modelling microbial growth on oils etc
Sent: Thu, 07 Feb 2002 10:32:55 +0200

Dear John Fleming,
Thank you for your e-mail concerning our work in Grasas. This was our first attempt to model growth of oleaginous microorganisms on vegetable oils.
1. How are the constants calculated for vegetable oil? Are they calculated using data from the full 10 days of incubation, or just using data from the first stage of growth?
They are calculated directly from eq. 10 (non linear regression) and also from eq. 7 (the k1) and eq. 9 (the k2)
2. Is it correct to calculate k1 from a plot of XL + L against ln(x) ? ln(x0)?
Yes because k1 is the degradation constant of the fat and therefore is related to x (see eq. 7).
3. Is it correct to calculate k2 from a plot of ln(L) against time?
Eq 8 is an empirical equation.
4. In your opinion, could it be possible to apply your model to the growth of a Trichoderma species on animal fat?
You can try with this model (and better with the advanced version published in Antonie, 1997, see below). However in our lab we had some problems to apply these models on Yarrowia lipolytica growing on animal fat. This is because Yarrowia lipolytica discriminate against stearic acid, and for this reason we currently work with a modified version which considers this metabolic discrimination.

5. You can find related works of our team on this subject:
1. On the production of SCO (mainly PUFA) - regulation
1.1. Using glucose, citric acid, glycerol as substrates

G.AGGELIS, M.PINA, R.RATOMAHENINA, A.ARNAUD, J.GRAILLE, P.GALZY, P.MARTIN
et J.PERAUD (1987) Production d'huiles riches en acide gamma linolenique par diverses souches de Phycomycetes. Oleagineux, 42, 379-386.

G.AGGELIS, R.RATOMAHENINA, A.ARNAUD, P.GALZY, P.MARTIN, J.PERAUD, M.PINA et

J.GRAILLE (1988) Etude de l'influence des conditions de culture sur la teneur en acide gamma linolenique de souches de Mucor. Oleagineux, 43, 311-317.

G.AGGELIS, M.PINA et J.GRAILLE (1990) Localisation de l'acide gamma linolenique dans les mycelium et dans les spores chez deux mucorales. Oleagineux, 45, 229-232.

G.AGGELIS (1996) Two altermative pathways for substrate assimilation by Mucor circinelloides. Folia microbiol., 41, 254-256.

G.AGGELIS, D.STATHAS, N.TAVOULARIS & M.KOMAITIS (1996) Composition of
lipids produced by some strains of Candida species. Production of single cell oil in a chemostat culture. Folia microbiol., 41, 299-302.

G.AGGELIS & M.KOMAITIS (1999) Enhancement of single cell oil production by Yarrowia lipolytica growing in the presence of Teucrium polium L. aqueous extract. Biotechnol. Lett., 21(9), 747-749.

A.KAVADIA, M.KOMAITIS, I.CHEVALOT, F.BLANCHARD, I.MARC, G.AGGELIS (2001)
Lipid and gamma linolenic acid accumulation in strains of Zygomycetes growing on glucose. J. Am. Oil Chem. Soc., 78(4), 341-346.

S.PAPANIKOLAOU & G.AGGELIS (2002) Lipid production by Yarrowia lipolytica growing on industrial glycerol in a single-stage continuous culture. Bioresource Technol., 82(1), 43-49.

H.GEMA, A.KAVADIA, D.DIMOU, V.TSAGOU, M.KOMAITIS & G.AGGELIS (2002)
Production of ă-linolenic acid by Cunninghamella echinulata cultivated on glucose and orange peels. Appl. Microbiol. Biotechnol. (in press).

1.2. Using vegetable oil as substrate

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Yours sincerely,
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Thank God it's over!