A characterisation of bioaugmentation products for the treatment of waste fats, oils and grease (FOG)

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Ph.D.

2012

A characterisation of bioaugmentation products for the treatment of waste fats, oils and grease (FOG)

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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September 2012

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 I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, 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.

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I would like to dedicate this thesis to my parents for everything they have done for me, their great love and support.

"*I will lift up my eyes to the hills – where does my help come from?*
My help comes from the Lord, the Maker of heaven and earth".

Psalm 121:1-2

"*Rejoice in the Lord always. Again I will say, rejoice!*
Let your gentleness be known to all men. The Lord is at hand.
Be anxious for nothing, but in everything by prayer and supplication, with thanksgiving, let your requests be made known to God;
And the peace of God, which surpasses all understanding, will guard your hearts and minds through Christ Jesus".

Philippians 4:4-7

Acknowledgments

I wish to express my deepest gratitude to:

My supervisor Dr. Bríd Quilty, School of Biotechnology, Dublin City University, Ireland, for her guidance and encouragement throughout my studies. A special thanks for her understanding, support and assistance since coming to Ireland.

Prof. Seraphim Papanikolaou, School of Food Science and Technology, Agricultural University of Athens, Greece, for nominating me for this project. Special thanks for his support and encouragement in starting the project and the help and understanding I received from him and his lab during my period working there in January 2010.

Environmental Protection Agency (EPA) of Ireland for supporting the project financially.

Mr. Ciaran Gillen, BioFuture Ltd, Dublin, for supplying the commercial bioaugmentation products and for the help, advice and information on grease traps and the problems of FOG in Ireland.

The Head of the School of Biotechnology, all the academic staff, technicians and researchers in School of Biotechnology, DCU, for facilitating my work and for their advice and help.

Dr. Rosaleen Devery, School of Biotechnology, DCU, for facilitating my use of her lab equipment, as well as for her support and guidance. I would also like to thank Dr. Devery as my internal examiner, as well as Dr. Raffaella Villa, my external examiner, for their corrections and recommendations on this thesis.

Dr. Ann Parle-McDermott's lab and her staff, especially Stefano, School of Biotechnology, DCU, as well as Dr. Damien Keogh and his lab, School of Biotechnology, DCU, for facilitating my use of their equipment and for their help in the area of the molecular analysis. I would also like to thank Dr. Padraig Doolan, National Institute for Cellular Biotechnology, DCU, for the use of the nanodrop in his lab.

My appreciation goes to the Tsatsalis family from Minerva S.A, Edible Oils, Greece, for their support and supplying the olive oil used in my studies.

A special thanks to my lab colleagues, including our Merck (Germany) visitors and all the fourth year students for their good energy and help, especially with my piles of dirty glassware!

Special thanks to my sweet friend Aida and her family, mainly her husband Adi and her cute little daughter Aisyah, for their friendship, their motivation, great support, positive energy, smiles, love and of course for the delicious colourful cakes! Also to Ana, her family and Kamal for the encouragement, support and love and for the good memories, even though she was driving me mad most of the

time! I want to also thank Joanna, for her friendship, constant support and her advice regarding life and love. Thank you for believing in me.

Not to forget my friends in Greece, Katerina, Despoina, Kirki and especially Dimitris, who always listened to my personal and project problems! Also my flatmates and friends in Dublin, especially Yaller, my DCU friends and colleagues, mainly all my girlfriends for their encouragement, support, understanding during the stressful periods and for the good memories.

Special thank you to the congregation of Destiny Church in Dublin and my church in Greece for their support and prayers.

Special thanks to Damien Kelly for his continuous support, guidance, encouragement and advice throughout my studies. A very special thank you for his great understanding and for always listening to my non-stop talking! Thank you for always believing in me. I would also like to express my gratitude to his family; Mr. Jim, Brian, Darren, Jamila, Monica, Declan, Rachel and their girls for their support, company and for making me feel part of their family.

Lastly and most importantly, I want to thank my parents George and Anna, my sister Jenny, my brother-in-law Manolis and their children Anna-Maria and Grigoris. Thank you for your continuous and never ending support, understanding, guidance, encouragement and love. Not to forget the supply of Greek coffee and other Greek goodies that always helped to boost my energy and remind me of home.

Contents

Abstract

‘A characterisation of bioaugmentation products for the treatment of waste fats, oils and grease (FOG)’

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FOG is a by-product of food preparation activities, generated primarily by food service establishments (FSE). FOG is the cause of blockages in drainage systems and results in major expenditure by local authorities on emergency maintenance. Fats, oils and grease may be intercepted at source using grease traps, specially designed units which separate the FOG from the rest of the wastewater. The application of bioaugmentation technology to biodegrade fats, oils, and grease (FOG) in the grease trap will ensure that they do not enter the municipal sewer system thus avoiding blockages in sewers and the need for major expenditure on an annual basis by local authorities for emergency maintenance. This study was carried out to investigate a number of bioaugmentation products for their ability to degrade FOG. Laboratory based aerobic batch fermentation studies were carried out in Erlenmeyer flasks to investigate the biodegradation of butter (1% w/v) and oil (1% v/v). The experimental work was carried out at 30^{o}C and 150 rpm in a minimal medium and an enriched nutrient medium. Three commercial bioaugmentation products were evaluated – BFL, FF and Gnz. BFL contained 9 *Bacillus* spp., Gnz *5 Bacillus* spp. and FF 5 *Bacillus* spp., 2 *Pseudomonas* spp. and 1 Fungus. The bacteria were characterised and identified to species level using biochemical and molecular methods. The fungus was identified as *Mucor circinelloides.* While FF showed good degradative ability, this was found to be due to the fungus present. Gnz showed no ability to degrade butter or oil and BFL only degraded the fats under limited environmental conditions. When *Pseudomonas putida* CP1 was added to BFL (BFL-CP1), significant fat degradation was observed. BFL-CP1 showed greater than 80% degradation of both the butter and the oil in 7 days. Analysis of the fat metabolism by the mixed bacterial community suggested a cooperative activity between the *Bacillus* spp., capable of fat hydrolysis and the uptake of hydrolysed fats by the *Pseudomonas putida*. The findings emphasise the importance of the correct microbiological composition of bioaugmentation products.

List of Abbreviations

A	Absorbance
API	Analytical Profile Index
ATP	Adenine triphosphate
BOD	Biochemical Oxygen Demand
bp	Base pair
CARD	Catalysed reported deposition
cfu	Colony forming unit
CLMS	Confocal laser scanning microscopy
CLPP	Community level physiological profiling
CoA	Coenzyme A
COD	Chemical Oxygen Demand
CO_2	Carbon dioxide
DCC	Dublin City Council
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dH_2O	Distilled water
EDTA	Ethylenediaminetetraacetic acid
ENM	Enriched Nutrient Medium
EPS	Extracellular polymeric substances
FA	Fatty acid(s)
FAD	Flavinadenine dinucleotide
FAME	Fatty acid methyl ester
FFA	Free fatty acid(s)
FISH	Fluorescent *in situ* hybridization
FOG	Fats, Oils and Grease
g	Gram
GC	Gas chromatography
GFP	Green fluorescent protein
GRU	Grease removal unit
h	Hour

HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
ID	Identification
Kbp	Kilobase pair
kDa	Kilodalton
L	Liter
LCFA	Long chain fatty acids
LPS	Lipopolysaccharide
M	Molar
µl	Microlitre
mM	Millimolar
MM	Minimal Medium
ml	Millilitre
NAD	Nicotinamide adenine dinucleotide
OD	Optical density
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Retention time
SCO	Single cell oil
SCP	Single cell protein
SDS	Sodium Dodecyl sulphate
SDS-PAGE	Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis
TCA	Tricarboxylic acid
TLC	Thin layer chromatography
Tw20	Tween 20
Tw80	Tween 80
UV	Ultraviolet

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1 INTRODUCTION

1.1 Lipids

Lipids, characterized as oils, greases, fats and long-chain fatty acids, are important components of foods, many synthetic compounds and emulsions and also of municipal and industrial wastewater (Chipasa and Medrzycka, 2006). A significant characteristic is that lipids possess both hydrophobic and hydrophilic properties, therefore, they are called amphipathic molecules. These properties are mainly due to particular components, the fatty acids, which are carboxylic acids with a straight aliphatic chain. Lipids consist of fatty acids attached, as esters, to glycerol (Madigan and Martinko, 2006).

Simple lipids are called triglycerides or triacylglycerides because three fatty acids are linked to the glycerol molecule as illustrated in Figure 1.1. Triacylglycerols tend to be the most abundant lipid class in edible fats of natural origin but are generally absent from bacteria. The component fatty acids of edible fats and oils vary considerably. They differ in chain length consisting of 2 to 22 carbon atoms, may be saturated or unsaturated and contain an even number of carbon atoms (Wakelin and Forster, 1997; Berg *et al.*, 2002).

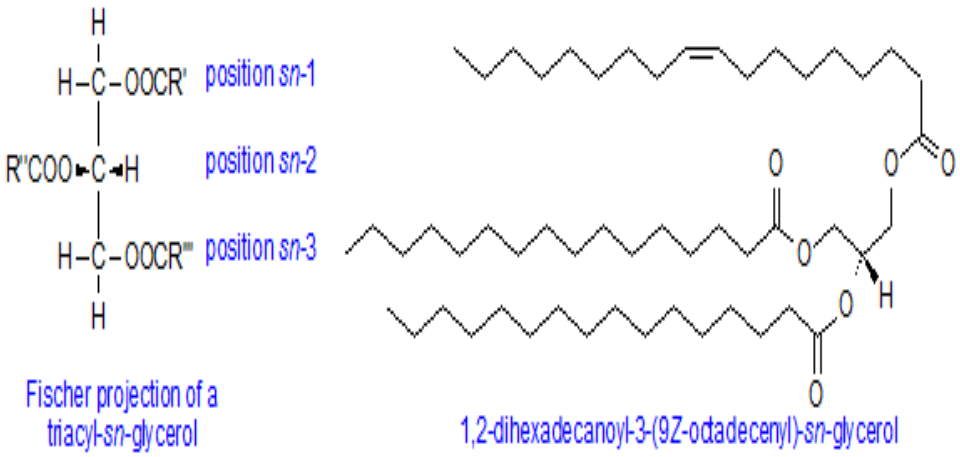


Figure 1.1 Structure of a triglyceride (http://lipidlibrary.aocs.org/)

The term “saturated” refers to hydrogen, meaning that the hydrocarbon tail has as many hydrogen atoms as possible, i.e. the last carbon contains 3 hydrogen atoms (CH_3-) and each carbon atom within the chain contains 2 hydrogen atoms (-CH_2-) (Fig. 1.2). Unsaturated fatty acids differ from the saturated ones by the presence of one or more double bond(s) within the carbon chain, with one alkenyl group (-CH:CH-) replacing a singly-bonded carbon atom (-CH_2-CH_2-). The configuration of the double bonds in most unsaturated fatty acids is separated by at least one methylene group (Berg *et al.*, 2002).

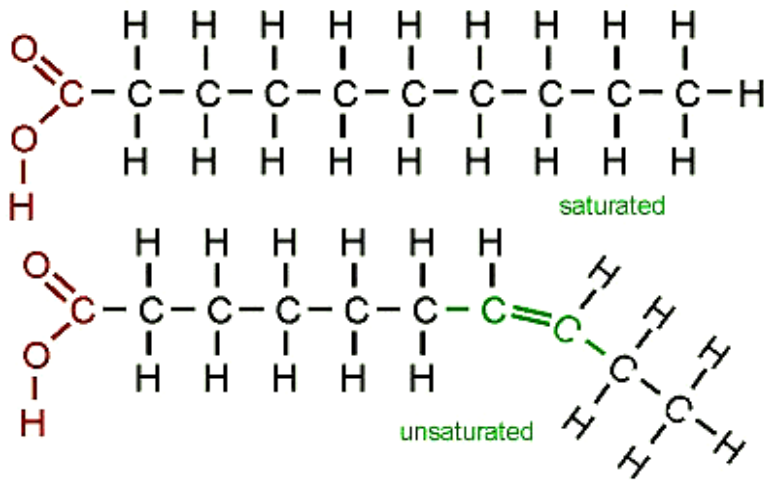


Figure 1.2 Saturated and unsaturated fatty acids (http://biology.clc.uc.edu)

Carboxylic acids as short as butyric acid (4 carbon atoms) and valeric acid (5 carbon atoms) are called short chain fatty acids. Carboxylic acids with 6 to 12 carbon atoms are normally referred to as medium chain fatty acids, while the ones with more than 12 carbon atoms are considered to be long chain fatty acids (LCFA) (Table 1.1). Most of the natural occurring fatty acids have an even number of carbon atoms because their biosynthesis involves acetyl-CoA, a coenzyme carrying a two-carbon atom group.

Table 1.1 List of some fatty acids

C-atoms: double bonds	Common name	Systematic name	Abbrev	Structural formula
4:0	Butyric	Butanoic	$C_{4:0}$	$CH_3(CH_2)_2COOH$
5:0	Valeric	Pentanoic	$C_{5:0}$	$CH_3(CH_2)_3COOH$
6:0	Caproic	Hexanoic	$C_{6:0}$	$CH_3(CH_2)_4COOH$
7:0	Enanthic	Heptanoic	$C_{7:0}$	$CH_3(CH_2)_5COOH$
8:0	Caprylic	Octanoic	$C_{8:0}$	$CH_3(CH_2)_6COOH$
9:0	Pelargonic	Nonanoic	$C_{9:0}$	$CH_3(CH_2)_7COOH$
10:0	Capric	Decanoic	$C_{10:0}$	$CH_3(CH_2)_8COOH$
12:0	Lauric	Dodecanoic	$C_{12:0}$	$CH_3(CH_2)_{10}COOH$
14:0	Myristic	Tetradecanoic	$C_{14:0}$	$CH_3(CH_2)_{12}COOH$
15:0	Valerenic	Pentadecanoic	$C_{15:0}$	$CH_3(CH_2)_{13}COOH$
16:0	Palmitic	Hexadecanoic	$C_{16:0}$	$CH_3(CH_2)_{14}COOH$
16:1	Palmitoleic	*cis*-9-hexadecenoic	$C_{16:1}$	$CH_3(CH_2)_5CH{:}CH(CH_2)_7COOH$
17:0	Margaric	Heptadecanoic	$C_{17:0}$	$CH_3(CH_2)_{15}COOH$
18:0	Stearic	Octadecenoic	$C_{18:0}$	$CH_3(CH_2)_{16}COOH$
18:1	Oleic	*cis*-9-octadecenoic	$C_{18:1}$	$CH_3(CH_2)_7CH{:}CH(CH_2)_7COOH$
18:2	Linoleic	*cis*-9,12-octadecadienoic	$C_{18:2}$	$CH_3(CH_2)_4(CH{:}CHCH_2)_2(CH_2)_6COOH$

The fatty acid components of microorganisms are often very different from those of edible fats of natural origin. In general, bacterial lipids tend to contain appreciable amounts of C_{14} to C_{18} straight-chain saturated and monoenoic fatty acids. The common C_{18} monoenoic acid is not oleic acid, however, but *cis*-vaccenic acid (18:1(*n*-7)). In addition, bacterial lipids can contain odd-chain, branched-chain, hydroxyl and/or cycloalkane (i.e. cyclopentane and cyclopropane) fatty acids which are only rarely synthesised by plants and animals (Sasser, 1990).

Apart from being major components of the triacylglycerols, fatty acids are also major components of most of the complex lipids present in biological membranes. These contain a complex mixture of lipids including phospholipids, glycosphingolipids and cholesterol. In general, phosphatidylcholine is the chief phospholipid found in membranes of animal cells while

phosphatidylethanolamine predominates in bacteria. Cholesterol and glycolipids (except for lipopolysacharides) are usually absent from bacterial membranes. The presence of a methyl branch or of a cyclopropane ring in the fatty acids in a bacterial membrane increases its fluidity in an analogous manner to that of double bonds in polyunsaturated fatty acids in the membranes of higher organisms (Berg *et al.,* 2002).

1.1.1 Biodegradation of Lipids

The biodegradation of lipids is difficult due to their low bioavailability (Cammarota and Freire, 2006). Biodegradation by microorganisms is generally a growth-associated process, in which the carbon in the substrate is used by the microbial populations. In this process, the energy required for the biosynthetic reactions is released and the by-products of the reactions are converted to cell constituents. Consequently, the microbial population increases in number and biomass (Martin, 1991). Aerobic degradation of lipids involves the following steps;

- Metabolic processes for optimising the contact between the microbial cell and the organic pollutants which require biosurfactant production and emulsification,
- Hydrolysis to glycerol and fatty acids by extracellular enzymes (lipases),
- Uptake of fatty acids: transportation into the microbial cell where oxygenases and peroxidases catalyze the activation of oxygen. Peripheral degradation pathways (β-oxidation) convert fatty acids to acetyl-CoA which enters the tricarboxylic acid (TCA) cycle (Fritsche and Hofrichter, 2006; Matsumiya *et al*., 2007; Chipasa and Medrycka, 2008).
- Glycerol is taken up by the cells and enters the glycolytic pathway (Figure 1.4).
- Finally, biosynthesis of cell biomass from the central precursor metabolites, e.g. acetyl-CoA, succinate, pyruvate, occurs.

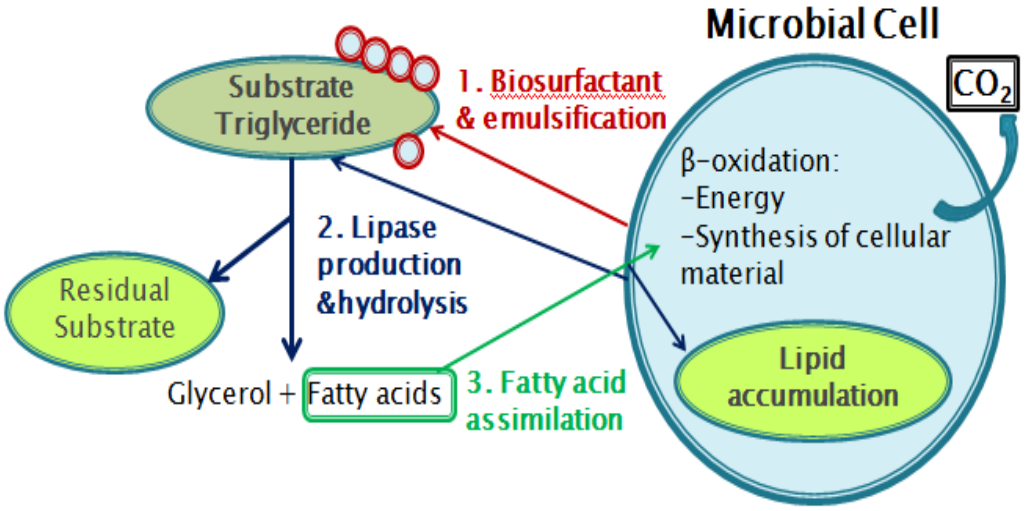


Figure 1.3 Main principles of aerobic degradation of triglycerides.

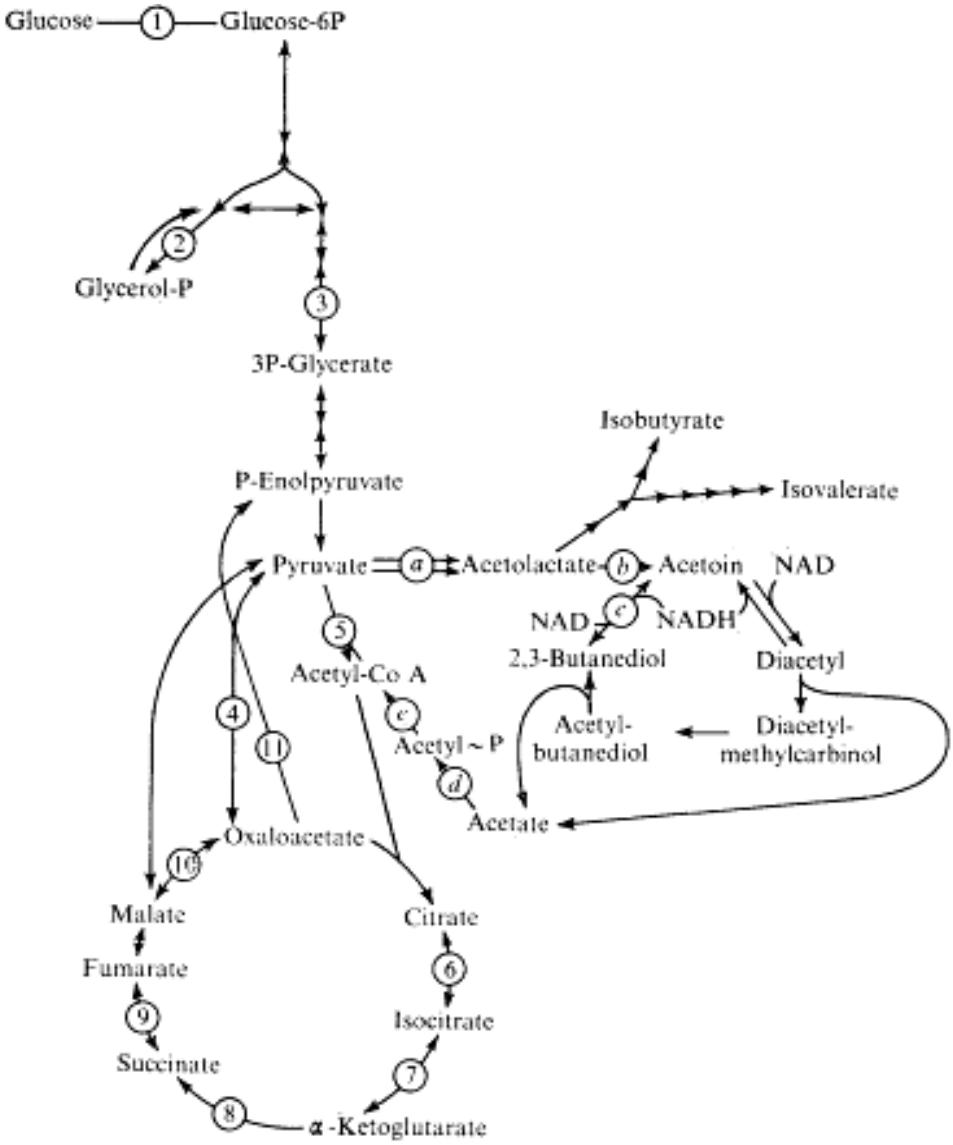


Figure 1.4 Biochemical pathways of relevant metabolic conversions, glycolysis and TCA (Speck and Freese, 1973)

1.1.1.1 Lipid Emulsification

A limitation of the biodegradation of lipids and grease is that these compounds are poorly accessible to bacteria like many other hydrocarbons due to their low solubility in aqueous systems compatible to microbial life. A possible way of enhancing the bioavailability of hydrophobic organic compounds is the application of surfactants. Some studies have indicated that surfactants enhance hydrocarbon degradation by microorganisms (Rashid and Imanaka, 2008).

Microorganisms synthesize a wide variety of surface-active agents (bio-surfactants) (Rosenberg and Ron, 1999; Ron and Rosenberg, 2002; Raza *et al*., 2007, Das *et al.*, 2009). They are often produced by bacteria capable of growing on hydrocarbons and have been shown to stimulate the growth of these bacteria and to accelerate bioremediation (Rosenberg and Ron, 1999; Jennings and Tanner, 2000). However, some biosurfactants have been also reported to be produced on water-soluble compounds such as glucose, sucrose, glycerol, or ethanol (Desai and Banat, 1997). They are amphipathic molecules consisting of hydrophilic and hydrophobic (generally hydrocarbon) domains (Kim *et al*., 1997; Desai and Banat, 1997; Jennings and Tanner, 2000). They adsorb to and alter the conditions prevailing at interfaces and reduce surface and interfacial tensions forming emulsions where lipids and water can be soluble. The emulsification of the hydrocarbons can intensify the contact between bacteria and water-insoluble hydrocarbons enhancing oil recovery (Figure 1.5) (Desai and Banat, 1997; Ron and Rosenberg, 2002).

Generally, their role is to i) increase the surface area of hydrophobic water-insoluble growth substrates, ii) increase the bioavailability of hydrophobic substrates by increasing their apparent solubility or desorbing them from surfaces, and iii) regulate the attachment and detachment of microorganisms to and from surfaces (Rosenberg and Ron, 1999).

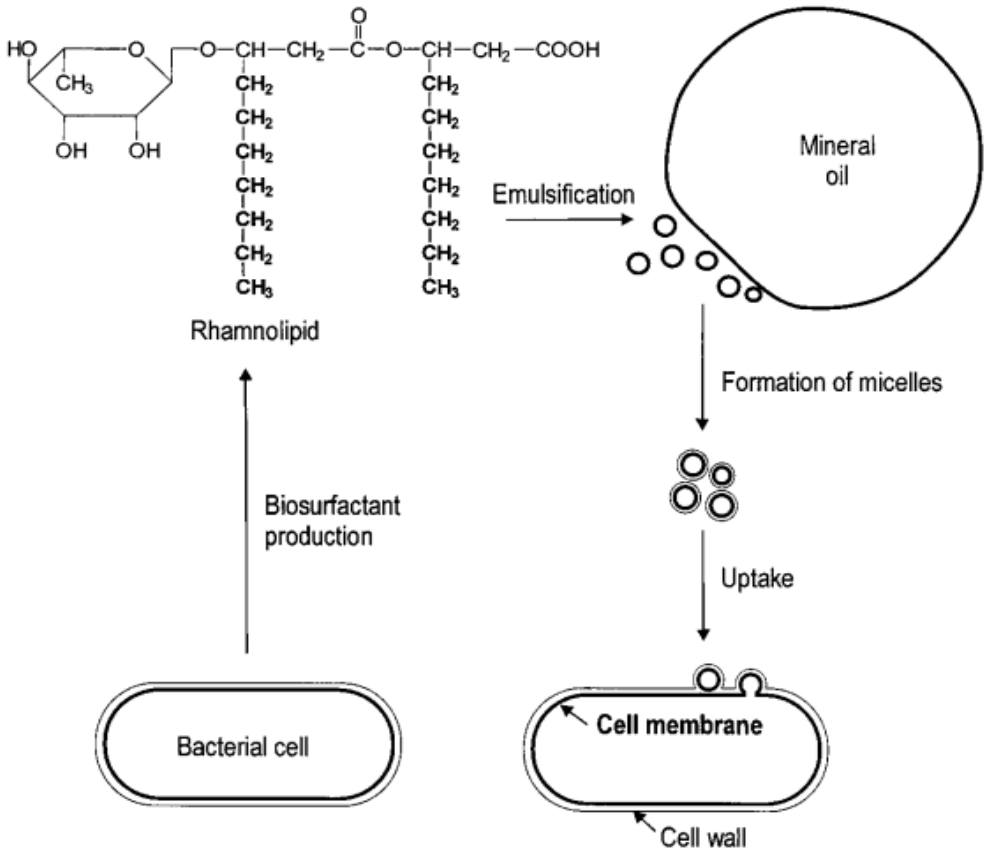


Figure 1.5 Biosurfactant production and lipid emulsification (Fritsche and Hofrichter, 2006)

Biosurfactants have important advantages and benefits relative to chemically synthesised surfactants. They have higher biodegradability, lower toxicity, better environmental compatibility and lower critical micelle concentration. They are also easier to produce, have the ability to be synthesised from renewable resources, have greater selectivity and greater specific activity at extreme temperatures, pH and salinity values (Kim *et al*., 1997; Raza *et al*., 2007; Nitschke and Costa, 2007; Das *et al*., 2009).

Biosurfactants are categorised mainly by their chemical composition and can be divided into two groups, the high- and low-molecular-mass bioemulsifiers. The low-molecular-mass bioemulsifiers lower surface and interfacial tensions and are generally glycolipids. Examples include trehalose lipids, sophorolipids and rhamnolipids, or lipopeptides, such as surfactin, gramicidin S and polymyxin. On the other hand, the high-molecular-mass bioemulsifiers bind tightly to surfaces, stabilising oil-in-water emulsions and contain amphipathic polysaccharides,

proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers (Rosenberg and Ron, 1999). Accordingly, the major classes of low-molecular-mass biosurfactants include glycolipids, which are synthesised by *Pseudomonas* species, lipopeptides and lipoproteins synthesised by many bacilli and other species, phospholipids and fatty acids synthesised by *Thiobacillus thiooxidans*, polymeric surfactants, and particulate surfactants (Desai and Banat, 1997; Youssef *et al*., 2004), polysaccharide-lipid complexes synthesised by *Acinetobacter* species (Youssef *et al.*, 2004). Among the most frequently occurring producers of surfactants or emulsifying polymers are members of the genera *Bacillus, Pseudomonas, Acinetobacter, Achromobacter, Arthrobacter, Brevibacterium, Corynebacterium, Candida* and *Rhodotorula* (Cameotra and Makkar, 1998).

For growth-associated biosurfactant production, parallel relationships exist between growth, substrate utilisation and biosurfactant production. Some examples are the production of rhamnolipid by *Pseudomonas* spp., glycoprotein AP-6 by *P. fluorescens*, surface active agent by *B. cereus* IAF 346 (Desai and Banat, 1997) biosurfactant production by *B. subtilis* C9 and *B. licheniformis* JF-2 (Kim *et al*., 1997) and biodispersan by *Bacillus* sp. strain IAF-343. The production of cell-free emulsan by *A. calcoaceticus* RAG-1 has been reported to be a mixed growth-associated and non-growth-associated type. Emulsan-like substances accumulate on the cell surfaces during the exponential phase of growth and are released into the medium when protein synthesis decreases (Desai and Banat, 1997).

Production under growth-limiting conditions is characterised by a sharp increase in the biosurfactant level as a result of limitation of one or more medium components. One example of high yield biosurfactant production under growth-limiting conditions is the rhamnolipid produced by *Pseudomonas aeruginosa* (Kim *et al*., 1997). A number of investigators have demonstrated an overproduction of biosurfactants by *Pseudomonas* spp. when the culture reaches the stationary phase of growth due to the limitation of nitrogen and iron. Production by resting or immobilised cells is a type of biosurfactant production in which there is no cell multiplication. The cells nevertheless continue to utilise the

carbon source for the synthesis of biosurfactants. Some examples are the production of rhamnolipid by *Pseudomonas* spp. and *P. aeruginosa* CFTR-6 (Desai and Banat, 1997). *Pseudomonas* species are well known for their ability to produce rhamnolipid biosurfactants on different carbon sources (Raza *et al*., 2007). Rhamnolipids produced by *P. aeruginosa* have been studied for many applications and this bacterium has been investigated and used in many studies for its surfactant production (Hori *et al.*, 2002; Whang *et al*., 2008,).

Species that belong to the genus *Bacillus* are one of the major producers of microbial surfactants (Das *et al*., 2008, 2009). *Bacillus subtilis* is the most effective known species which has been used extensively for the production of lipopeptide biosurfactants (Cooper and Goldenberg, 1987; Kim *et al*., 1997; Akpa *et al*., 2001; Youssef *et al*., 2007; Das *et al*., 2008; Yeh *et al.*, 2008; Das *et al.*, 2009). The organism was studied by Kim *et al*. (1997) using the oil film-collapsing assay. They determined that the biosurfactant C9-BS, produced by the bacterium, was a lipopeptide consisting of a C14-15 fatty acid tail linked to a peptide moiety composing of seven amino acid residues identical to the peptide moiety of surfactin.

Das *et al*. (2008, 2009) studied a marine strain of *Bacillus circulans* for its ability to increase the bioavailability and consequent degradation of a model polyaromatic hydrocarbon, anthracene. Although the organism could not utilise anthracene as the sole carbon source, the growth and biosurfactant production were better in an anthracene supplemented glycerol mineral salts medium compared to a normal glycerol mineral salts medium.

The potential commercial applications of bioemulsifiers include bioremediation of oil-polluted soil and water, enhanced oil recovery and replacement of chlorinated solvents used in clean-up of oil-contaminated pipes, vessels and machinery. They are also used in the detergent industry, formulations of herbicides and pesticides and in the formation of stable oil-in-water emulsions for the food and cosmetic industries (Rosenberg and Ron, 1999). In addition, biosurfactants have other uses in the petroleum industry, such as in enhanced oil recovery and transportation of crude oil. However, their production and recovery costs are high which limits

widespread application. Costs can be minimised by selecting microorganisms capable of producing biosurfactants in high yields and by optimising large-scale fermentation and recovery system conditions.

1.1.1.2 Lipid hydrolysis

Lipid hydrolysis is catalysed by specific enzymes called lipases. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyse, under natural conditions, the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, fatty acids and glycerol. They also catalyse the synthesis of long-chain acylglycerols (esterification) at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved (Sharma *et al*, 2002; Snellman and Colwell, 2004). They catalyse the exchange of ester bonds (transesterification) when present in non-aqueous media (Hasanuzzaman *et al*., 2004) and interesterification depending on the source of lipase and reaction conditions (Bhumibhamon *et al*., 2002).

Lipases are serine hydrolases which act at the lipid-water interface (Figure 1.6). Lipases have a catalytic triad composed of Serine-Histidine-Aspartate/Glutamate and usually also a consensus sequence (Glycine/Alanine-X-Serine-X-Glycine) at the active site serine called the 'nucleophilic elbow' (Gupta *et al.,* 2004; Bourlieu *et al.,* 2009). Structural investigations revealed that most lipases feature a lid which consists of an amphiphilic peptide loop which covers the active site of the enzyme in its inactive state. In the presence of hydrophobic substrate, the active site becomes accessible after a conformational change of this lid. This change of conformation is called 'interfacial activation' (Bourlieu *et al.,* 2009).

One property of lipase that has been observed is that lipase has an adsorption affinity with oil droplets. Shape and size of the oil droplets play an important role on the rate of production of the enzyme (Tamerler and Keshavarz, 2000).

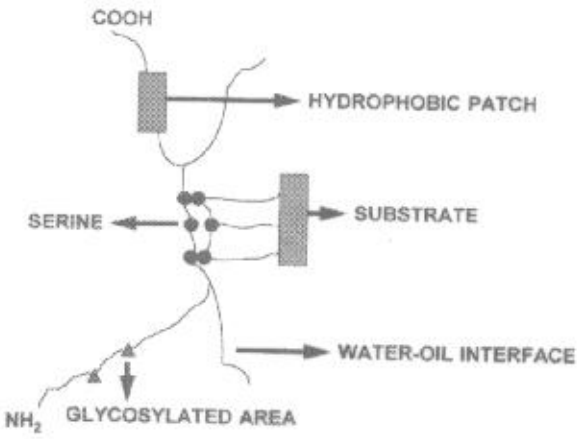


Figure 1.6 Lipase molecule showing its main features (Saxena *et al*, 1999)

Lipases display a high degree of specificity and enantioselectivity for esterification and transesterification reactions and they are highly specific as chemo-, regio- and enantioselective catalysts (Gupta *et al.*, 2004; Hasanuzzaman *et al.*, 2004). Microbial lipases may be divided into two groups. The first group contains the non-specific lipases, where the enzymes do not distinguish between the three positions of the glycerol esters bringing about a total hydrolysis of triglycerides to fatty acids and glycerol. The second group contains specific or regiospecific lipases (Sztajer and Zboinska, 1988; Thompson *et al*., 1999), where lipases hydrolyse esters in the 1 and 2 positions of glycerides, releasing free fatty acids and mixtures of mono- and di-glycerides. The 2-monoglycerides and the 1,2- or 2,3-diglycerides are unstable and therefore the enzymatic hydrolysis is followed by acyl group migrations, leading to 1-monoglycerides and 1,3-diglycerides. Therefore, the extension of the incubation time may result in a total splitting of triglycerides (Sztajer and Zboinska, 1988). Lipases may also display specificity depending on the type of the fatty acids and the length of the carbon chain. The Figure 1.7 illustrates the lipase specificity according to the region and structure.

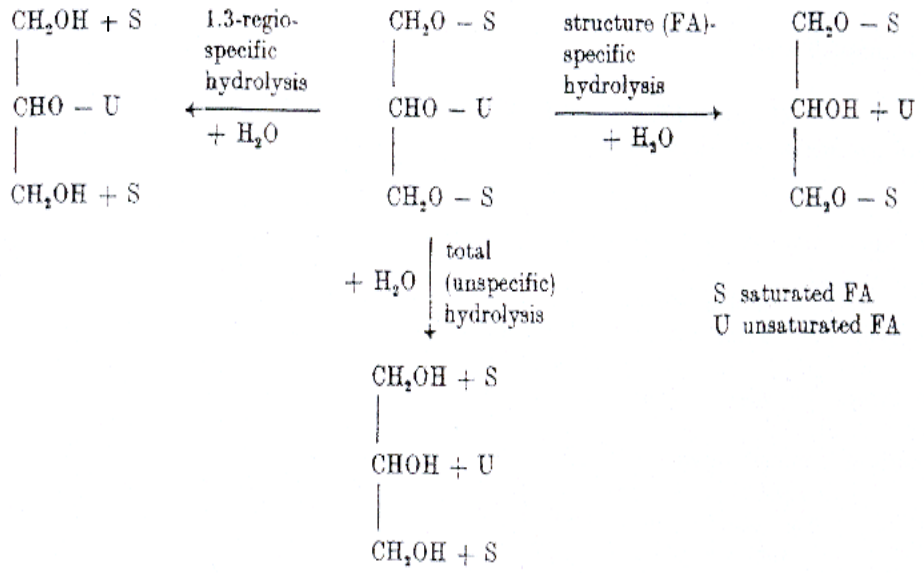


Figure 1.7 Examples of region and structure lipase specificity (Hadeball, 1991)

The lipase producing microorganisms are found in fat and oil contaminated sources. Under certain conditions, it is possible to isolate bacterial strains that are capable of degrading lipids by using a selective medium containing a source of lipid. These lipid-degrading bacteria often produce extracellular lipase enzymes, where these enzymes are generally inducible in the presence of different inducers such as olive oil, palm oil, oleic acid and Tween 20 (Shabtai 1991; Shabtai and Daya-Mishre 1992; Sigurgísladóttir *et al*. 1993). Lipases produced by different organisms have either hydrolytic or interesterification ability, depending on the source of lipase and the reaction conditions. For example, *Pseudomonas cepacia* (Dunhaupt *et al.*, 1992) and *Acinetobacter radioresistant* CMC1 (Hong and Chang, 1998) displayed hydrolytic activity while *P. fragi* CRDA323, *P. fluorescens* and *Pantoea aggomerans* dominated an interesterification reaction (Pabai *et al*., 1995). In the study of Bhumibhamon *et al*. (2002) hydrolysis reactions occurred but interesterification reactions were not determined.

Many important lipase-producing bacteria are members of the genera *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Chromobacterium*, *Bacillus*, *Pseudomonas* and *Burkholderia* (Gupta *et al.*, 2004). Of these, interest has been particularly focused on lipases from members of the genus *Pseudomonas*, such as *Pseudomonas aeruginosa* and *P. fragi*, which are especially interesting and

widely used for biotechnological applications (Arpigny and Jaeger, 1999; Gupta *et al.*, 2004). The use of lipases from *Pseudomonas* for the degradation of fats in the wastewater from restaurants has been widely reported (Bhumibhamon, 2002; Bhumibhamon and Phattayakorn, 2003; Cammarota and Freire, 2006).

Acinetobacter lipases have also been investigated (Arpigny and Jaeger 1999, Bhumibhamon, 2002; Bhumibhamon and Phattayakorn, 2003; Gupta *et al.*, 2004). Strains of *Acinetobacter* produce lipases of particular interest having high activity when grown on an array of carbon substrates. These extracellular enzymes share many biochemical properties with other bacterial lipases, such as the *Pseudomonas/Burkholderia* group lipases including lipases produced by *Pseudomonas aeruginosa, Pseudomonas fragi, Proteus vulgaris* and *Burkholderia cepacia* (Snellman and Colwell, 2004; Cammarota and Freire, 2006).

Bacillus lipases are a large and diverse family of enzymes. They can be easily produced and demonstrate versatile specificity and stability, great tolerance to solvents, salts and detergents. Therefore, *Bacillus* lipases can potentially be applied in the food industry, laundry formulations, the paper and leather industry and waste water treatment (Guncheva and Zhiryakova, 2011).

Lipases are significantly affected by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, agitation and dissolved oxygen concentration (Gupta *et al.*, 2004). The major factor, though, for the expression of lipase activity is always the carbon source. Lipase production is activated in the presence of a lipid source, such as oil, or other triacylglycerols, fatty acids, hydrolysable esters, tweens, bile salts, glycerol and is also affected by other carbon sources such as sugars, sugar alcohol, polysaccharides, whey, casamino acids and other complex sources. Lipase production from *P. aeruginosa* EF2 (Gilbert *et al.*, 1991) and *Acinetobacter calcoaceticus* (Mahler *et al*., 2000) was enhanced in the presence of long-chain fatty acids, such as oleic acid.

The type of nitrogen source in the medium, generally organic nitrogen, also influences lipase production. Peptone and yeast extract have been mainly used as nitrogen source for lipase production by various *Bacillus* and *Pseudomonas* species. Inorganic nitrogen sources such as ammonium chloride and diammonium hydrogen phosphate have also been reported to be effective in some microbes (Sharma *et al*., 2002).

Divalent cations in the medium are important stimulators or inhibitors of enzyme production. Rathi *et al.* (2001) detected stimulation in lipase production from *Burkholderia* sp. in the presence of Ca^{2+} and Mg^{2+}. The presence of calcium chloride has been also reported to stimulate lipase production from *Bacillus* sp RSJ1 (Sharma *et al*., 2002). However, most other metal ion salts inhibit lipase production, such as iron which was found to play a critical role in the production of lipase from *Pseudomonas* sp. G6 (Gupta *et al.,* 2004).

In addition to the nutritional factors, physiological parameters can also greatly influence the enzyme production by different microorganisms by modulating the bacterial growth. The initial pH of the growth medium is important for lipase production. Most bacteria, such as *Bacillus* sp., *Acinetobacter* sp. and *Burkholderia* sp. prefer pH around 7.0 for best growth and lipase production (Rathi *et al.*, 2001; Gupta *et al.*, 2004). However, maximum activity at a higher pH (>7.0) has also been observed in many cases (Sharma *et al*., 2002). Generally, *Bacillus* lipases are stable at pH values from 7 – 9. A number of enzymes have a broad range of pH stability including the acidic range (Guncheva and Zhiryakova, 2011).

The optimum temperature for lipase production is related to the growth temperature of the respective microorganism. Generally, it has been observed that lipases are produced in the temperature range 20 – 45^{o} C. The incubation period for maximum lipase production ranges from a few hours, such as 12 hours for *A. calcoaceticus* and *Bacillus* sp. RSJ1 (Mahler *et al*., 2000; Sharma *et al*., 2002; Gupta *et al.*, 2004) and 16 hours for *B. thermocatenulatus*, to several days, such as 72 and 96 hours for *Pseudomonas fragi* and *P. fluorescens* BW 96CC, respectively (Gupta *et al.*, 2004). Mohan *et al.* (2008) also reported maximum

lipase activity by *Bacillus* strains during 24 h of culture period. They studied the extracellular lipase production by *Bacillus* strains under different environmental conditions, such as varied pH (4-9), temperature (27, 37 and 47º C) and various substrate (coconut oil, sunflower oil, olive oil). Statistical analysis revealed that the variation in lipase production was more significant between bacterial strains than the independent influence of pH, substrate and medium temperature.

1.1.1.3 Fatty Acid Uptake

Once hydrolysis of the lipids is carried out, the released fatty acids are assimilated by the microbial cells. Fatty acid degradation and synthesis are relatively simple processes that are essentially the reverse of each other. The process of degradation converts the aliphatic compound into a set of activated acetyl units (acetyl CoA) that can be processed by the citric acid cycle (Berg *et al.*, 2002).

Fatty acids are oxidised by a process called *beta oxidation* (Figure 1.8), in which two carbons of the fatty acid are split off at a time. In eukaryotes, the enzymes are in the mitochondria, whereas in prokaryotes they are cytoplasmic. A fatty acid activated with CoA (coenzyme A) is oxidised to introduce a double bond, the double bond is hydrated to introduce an oxygen, the alcohol is oxidised to a ketone and, finally, the four carbon fragment is cleaved by coenzyme A to yield acetyl CoA and a fatty acid chain two carbons shorter. The process of beta oxidation is then repeated and another acetyl-CoA molecule is released. If the fatty acid has an even number of carbon atoms and is saturated, the process is simply repeated until the fatty acid is completely converted into acetyl CoA units (Berg *et al.*, 2002; Madigan and Martinko, 2006). Two separate dehydrogenation reactions occur in beta oxidation. In the first, electrons are transferred to flavinadenine dinucleotide (FAD), whereas in the second they are transferred to NAD+. Most fatty acids have an even number of carbon atoms, and complete oxidation yields acetyl-CoA. The acetyl-CoA formed is then oxidised by way of the citric acid cycle or is converted to hexose and other cell constituents via the glyoxylate cycle (Madigan and Martinko, 2006).

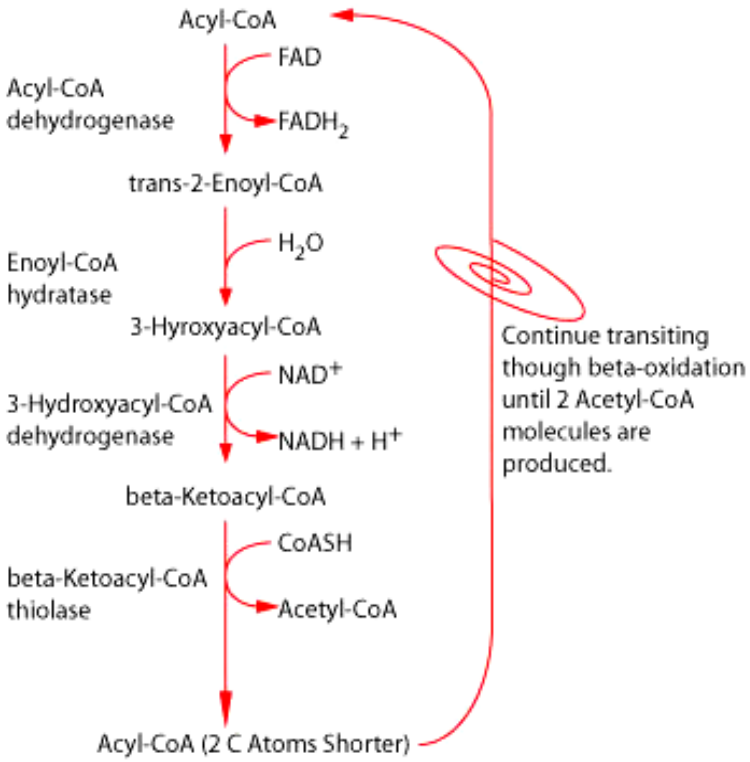


Figure 1.8 Beta oxidation process (http://flipper.diff.org/)

Fatty acids are good electron donors. For example, the oxidation of the 16-carbon fatty acid palmitic acid results in the synthesis of 129 ATP molecules. These include electron transport phosphorylation from electrons generated during the formation of acetyl-CoA from beta oxidations and from oxidation of the acetyl-CoA units themselves through the citric acid cycle (Madigan and Martinko, 2006). Novak and Kraus (1973) reported that the utilisation rate of fatty acids is different and depends on the length and degree of unsaturation of their carbon chains.

1.2 Waste Lipids – Fats, Oils and Grease (FOG)

Waste lipids, also described as fats, oils and grease (FOG) are derived from a variety of sources including dairies, slaughterhouses and food service establishments. Disposal of this waste through the sewerage system poses a considerable problem. Over time the grease can build up, accumulate in pipes causing many problems in terms of wastewater management, the production of

foul odours and the blockage of pipes and sewer lines (Bhumibhamon *et al*., 2002; Bhumibhamon and Phattayakorn 2003; Brooksbank *et al.*, 2007; Rashid and Imanaka, 2008). Blockages may cause raw sewage to overflow into parks, streets and premises with potentially detrimental environmental impacts (Brooksbank *et al*., 2007; Rashid and Imanaka, 2008). This can lead to expensive and unpleasant cleanup, potential contact with disease-causing organisms and an increase in operation and maintenance costs for local sewer departments which causes higher sewer bills for customers.

Chipasa and Mędrzycka (2006) estimated that the amount of lipids in municipal wastewater is approximately 30-40% of the total chemical oxygen demand. The activated sludge system is the most commonly used aerobic system for the treatment of municipal wastewater. The presence of lipid in the wastewater can lead to unpleasant consequences in the system by reducing the cell-aqueous phase transfer rates through the formation of a lipid coat around the biological floc. Further problems can arise resulting in interference with sedimentation due to the development of filamentous microorganisms leading to the production of sludge with poor activity (Cammarota and Freire, 2006). When FOG is not treated properly by sewage works it can enter rivers and oceans, thus spreading the pollution and causing serious environmental damage.

Anaerobic systems have been used for the treatment of lipid wastewater in food processing industries (Chipasa *et al*., 2006). However, FOG tends to accumulate in anaerobic units, including digesters and lagoons (Huban and Plowman, 1997). Anaerobic wastewater treatment is conducted in enclosed vessels and the major product of the biological degradation is an off-gas consisting of approx 80% methane and 20% carbon dioxide. The process has the advantage of producing lower levels of sludge than the aerobic process and being less energy intensive (Willey, 2001; Mendes *et al*, 2005). However, the presence of high amounts of lipid waste can be problematic, inhibiting the system causing formation of foam and floating sludge (Chipasa *et al*., 2006; Long *et al.*, 2012). Long chain fatty acids, produced during hydrolysis of fats and oils, are commonly present in FOG wastewaters (Cavaleiro *et al.*, 2010). Cirne *et al*. (2007) studied the effect lipid concentration has on anaerobic digestion and they found accumulation of long

chain fatty acids (LCFA) and reduced ability to degrade the lipids. The accumulation of LCFA can be toxic to the biomass, inhibiting not only their breakdown, but also the degradation of other nutrients in the waste. Methanogenic and acetogenic bacteria are particularly sensitive to fatty acid toxicity (Becker *et al*., 1999; Gonçalves et al., 2011; Long *et al.,* 2012). Oleic acid has been found to be the main fatty acid accumulated and at concentrations of 30-80 mg/L or higher was found to be toxic and responsible for the failure of the lipid treatment (Lalman and Bagely, 2001; Alves *et al*., 2001; Gonçalves *et al*., 2011). However, the cooperative activity between the ability of syntrophic bacteria to convert LCFA to acetate and hydrogen and the utilisation of these substrates by methanogenic archaea promotes complete LCFA degradation (Stams, 1997; Cavaleiro *et al*., 2010). Cavaleiro *et al*. (2010) studied the potential of LCFA conversion to methane by bioaugmenting a non-acclimated anaerobic granular sludge with *Syntrophomonas zehnderi*.

1.2.1 FOG bi-products

A number of approaches have been taken to divert lipid waste from the sewage system. Some lipids have been recovered to produce useful bi-products including single cell oil, single cell protein, organic acids, biosurfactants, lipases and biodiesel (Fickers *et al*., 2005; Papanikolaou *et al.,* 2007).

1.2.1.1 Single cell oil

Oleaginous microorganisms are microorganisms that have the ability to produce cellular oil grown on fatty materials. Oleaginous microorganisms have been investigated for many years because of their properties and their potential application in industry (Papanikolaou *et al.,* 2003) and wastewater treatment (De Felice *et al.,* 1997; Lanciotti *et al.,* 2005; Wu *et al.,* 2009). The microbes will either degrade the fat and consume the produced fatty acids for growth or will transform them changing the intracellular fatty acid concentration and producing new fatty acids. The incorporation of the fat substrate into the microbial cell and

the intracellular changes of fatty acids is defined by the enzymatic capabilities of the microorganisms and the produced oil is called single cell oil (SCO) (Aggelis and Komaitis, 1999; Papanikolaou *et al.*, 2002; Papanikolaou *et al.*, 2007).

SCO production is of particular interest due to the capacity of oleaginous microorganisms (mainly yeasts and moulds) to convert numerous raw materials, such as carbohydrates, and alcohols into value-added end products (fats and oils) (Ratledge, 1994; Papanikolaou and Aggelis, 2002). The advantages of using yeast as lipid producers are that 1) they produce lipids similar to vegetable oils and fats, 2) they grow well on cheap agro-industrial and food industrial wastes, 3) their lipids can be produced at a faster rate in bulk in large capacity reactors than the usual time-consuming agricultural practices and 4) most of the potential lipid producers and their products seem to be relatively non-toxic to humans (Papanikolaou and Aggelis, 2002).

Cocoa-butter-like oil has been identified as having commercial potential. SCO production by *Yarrowia lipolytica* cultivated on animal derived fats and glycerol as a potential cocoa butter substitute has been investigated (Papanikolaou *et al.*, 2001, 2002 and 2003). The accumulated lipid of *Y. lipolytica* cells comprised a high concentration of saturated fatty acids, such as stearic acid, which was comparable to the saturated fatty acid content of cocoa butter. The production of microbial polyunsaturated fatty acids, with high nutritional value such as eicosapentaenoic and arachidonic acids, has also been investigated (Zeng *et al.*, 2011; Liang *et al.*, 2012).

The ability of the oleaginous microorganisms to change the composition of waste fats and their properties with no need for chemical catalysts, which are difficult to remove, is called bioconversion and is the advantage of biological fat modification (Bednarski *et al.*, 1994; Aggelis *et al.*, 1997; Papanikolaou and Aggelis, 2010). Therefore, the production of value-added products is possible from cheap fatty substrates, such as lipid production from palm oil and stearin (Xu *et al.*, 2000) as well as from industrial lipids (Papanikolaou *et al.*, 2003) having similar composition to cacao-lipid. Bioconversion of animal fats rich in saturated fatty acids to lipids rich in unsaturated fatty acids has been also reported (Hou,

2009). In general, industrially important biological processes include hydrolysis, esterification, interesterification and transesterification (Koritala *et al*., 1987; Ratledge and Wynn, 2002; Kontkanen *et al.,* 2011). As an alternative to complete oxidation, the bioconversion of long chain fatty acids to more useful fatty acids has been investigated. *Mucor circinelloides* was employed to convert linoleic acid from sunflower oil to γ-linoleic acid (GLA), an acid of particular use in both pharmaceutical and cosmetic industries (Aggelis *et al*., 1991).

1.2.1.2 Single cell protein

Intensive research has been conducted to find and develop cheaper sources of protein to improve the worldwide problem concerning the supply of food protein (Anupama and Ravindra, 2000). The use of microorganisms for the production of protein also called single cell protein (SCP) has been of great interest. The consumption of microbial protein could be an important alternative to prevent protein energetic malnutrition in developing countries (Konlani *et al.,* 1996). However, animal feedstuffs comprise a high protein content and the potential of SCP as animal feed would increase protein availability for human consumption. Waslien and Steinkraus (1980) recommended feeding microbial cells to animals, thereby, releasing for human consumption the cereal grains and legumes. Testing of SCP products on pigs and chickens suggested that SCP could replace 10-20% of protein in foodstuffs (Giec and Skupin, 1988).

SCP is the manufacture of cell mass using microorganisms, typically fungi, by culturing on agricultural and industrial wastes. After fermentation, biomass is harvested and may be subjected to downstream processing steps like washing, cell disruption, protein extraction and purification. Considerations for commercial operation include culture conditions, pretreatment of substrates, nutrient supplementation and type of fermentation process. Protein content of yeast biomass ranges between 30-70% of the cell weight (Anupama and Ravindra, 2000). Cultivation of yeast on lipid waste and crude oil wastes produced SCP as a by-product of the waste treatment process at laboratory and pilot-scale.

1.2.1.3 Organic acids

Organic acids are important products in food and beverage technology. The average usage of the organic acid production is related to the food industry as an acidifier. Production of organic acids, such as citric acid, L-(+) iso-citric acid, α-ketoglutarate acid, acetic acid and gluconic acid, from yeasts grown on fats, lipids and many carbon sources was reported by Spencer and Spencer, 1990 and Papanikolaou *et al.,* 2002. Citric acid is the most important organic acid having many uses in the food, detergent and pharmaceutical industries. It has been most commonly used as flavouring, pH stabiliser, preservative and as an antioxidant.

1.2.1.4 Enzymes

Enzymes produced during incubation of microorganisms on carbon sources are of great biotechnological importance. Lipases are hydrolytic enzymes of great scientific and industrial interest due to their ability to catalyse not only the hydrolysis of fats, but also reactions associated with acyl groups, such as trans-esterification, related to alcoholysis and glycerolysis, and inter-esterification related to acidolysis and esterolysis (Figure 1.9).

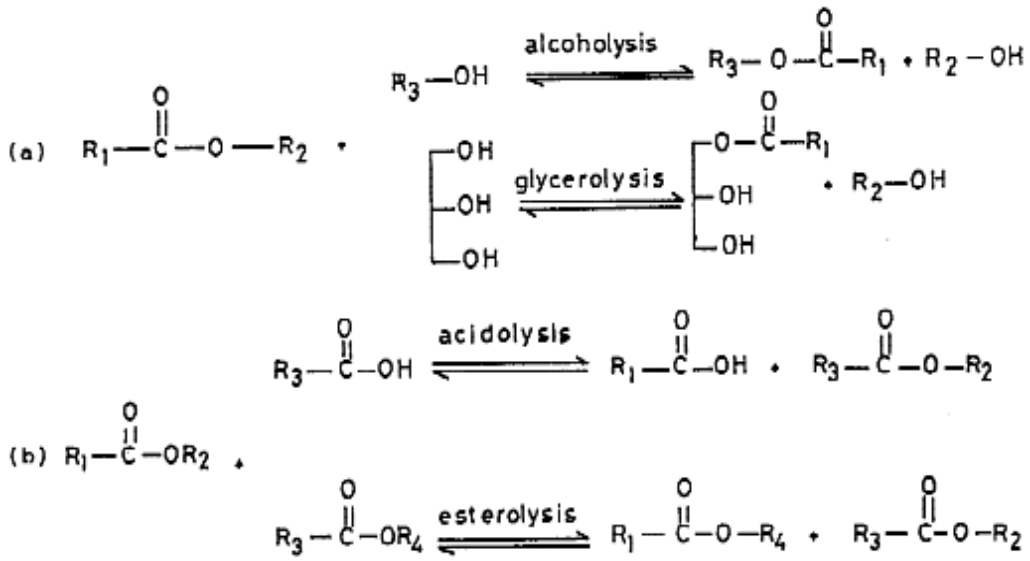


Figure 1.9 Industrially important reactions catalysed by lipases (a) Trans-esterification involves the transfer of an acyl group to an alcohol (alcoholysis) or glycerol (glycerolysis). (b) Inter-esterification described the transfer of an acyl group to a fatty acid (acidolysis) or a fatty acid ester (esterolysis) (Benjamin and Pandey, 1998).

Lipases find significant application in industry including the oleochemical industry, dairy industry, agricultural industry, cosmetics and the pharmaceutical industry. The application of lipases for the synthesis of new molecules has also been reported (Sharma *et al.,* 2001). Some of the examples of industrial applications of microbial lipases are shown in Table 1.2.

Table 1.2 Industrial applications of microbial lipases (Sharma *et al.,*2001)

Industry	**Action**	**Product or application**
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese and butter
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Food dressings	Quality improvement	Mayonnaise, dressings and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish products; fat removal
Fats and oils	Transesterification; hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono- and diglycerides
Chemicals	Enantioselectivity, synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification, hydrolysis	Specialty lipids, digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of fats

1.2.1.5 Biosurfactants

The interest in biosurfactants is mainly due to their environmentally friendly nature. Biosurfactants are biodegradable, have low toxicity and a unique structure which provides properties that chemical surfactants may lack (Nitschke and Costa, 2007). Biosurfactant applications have mainly focused on bioremediation of pollutants (Mulligan, 2005; Nitschke and Costa, 2007). However, biosurfactant use is of great interest in the pharmaceutical, cosmetics and food industries especially as emulsifiers, foaming and wetting agents, solubilisers, antiadhesives and antimicrobial agents (Hu and Ju, 2001). The replacement of artificial and chemically synthesised compounds by more natural food ingredients and additives is greatly desired by many consumers (Shepherd *et al.*, 1995; Nitschke and Costa, 2007). The economic limitation on commercial biosurfactant production can be overcome through the development of cheaper processes, the use of low-cost raw materials and increased product yields through the use of mutated strains and genetically engineered bacteria (Desai and Banat, 1997).

1.2.1.6 Biodiesel

Used cooking oil can be used either for conversion to bio-diesel or for incineration with energy recovery. Local authorities (Dublin City Council, 2012) backs the recovery of used cooking oil for use as a biofuel because this reduces the use of fossil fuels and thus carbon dioxide emissions. A growing number of companies offer commercial collection services for these purposes. Research has recently indicated that the lipids contained in municipal sewage sludge are a potential feedstock for biodiesel receiving increasing attention as an alternative, non-toxic, biodegradable renewable diesel fuel (Canakci, 2007; Kargbo 2010). Biodiesel is a mixture of fatty acid alkyl esters produced from vegetable oils, animal fats or waste cooking oils (Encinar *et al.*, 2011; Resitoglu *et al.*, 2012). Canakci (2007) and Encinar *et al.* (2011) conducted studies on the potential of restaurant waste lipids and animal fats as biodiesel feedstocks. They found that these wastes have a huge available potential for biodiesel production, however, as they contain a high level of free fatty acids transesterfication cannot be applied

directly. Therefore, it is necessary to reduce the level of FFA by using an acid catalyst process first. In many studies a two-step process was used to remove the high levels of FFA from the waste oils. The first step is acid-catalysed esterification to decrease the free fatty acid content to produce soap and water. The second step is alkaline-catalyzed transesterification where biodiesel and glycerol are produced (Figure 1.10) (Monterfrio *et al.*, 2010; Resitoglu *et al.*, 2012). In the case of Resitoglu *et al.* (2012), successful production of biodiesel was achieved from waste cooking oils from a grease trap following the two steps described previously. However, the FOG from grease traps was mixed with other food components and surfactants and it was necessary to be washed with pure water four times and dried prior to treatment. The glycerol product may be used in cosmetic and pharmaceutical industries (Dufreche *et al*., 2007). Though chemically achievable, it is likely however that the financial costs incurred from prior treatment of FOG and from converting it to biodiesel may render the process too expensive to compete with fossil diesel.

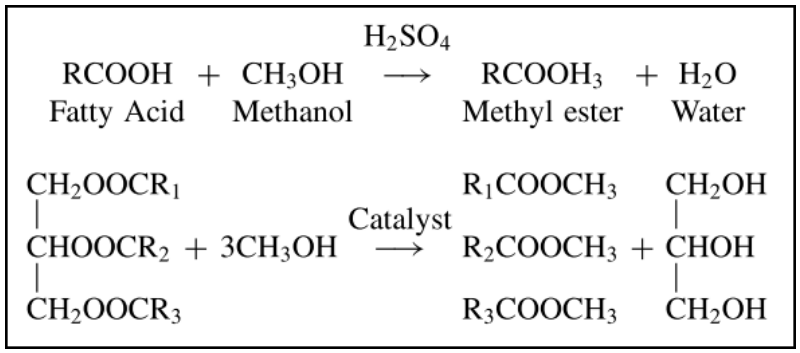


Figure 1.10 Biodiesel production (Resitoglu *et al.,* 2012)

1.2.2 Waste lipid from Food Service Establishments

Fats, oils and grease are by-products of washing up activities and cooking, such as meat fats, food scraps, lard, baking goods, cooking oil, sauces, shortening, butter and margarine, dairy products, food products such as mayonnaise, salad dressings, sour cream and others (Parjus *et al*., 2008). Too often, this grease is washed into the sewer system, usually through the kitchen sink. In Ireland, under the

provisions of the Water Services Act 2007, there is a duty of care on owners of premises not to allow anything which will cause blockages to enter the sewer.

Restaurants, cafeterias and fast-food establishments can spend tens of thousands of euro on plumbing emergencies each year to deal with grease blockages. An example of FOG removal from a blocked sewer in the Clontarf area of Dublin is shown in Figure 1.11 (Dublin City Council, 2012).

Under the provisions of the Local Government (Water Pollution) Act 1977 and Local Government (Water Pollution) (Amendment) Act 1990, local authorises in Ireland are responsible for issuing fats, oils and grease discharge licenses to food service establishments. The FOG discharge limit is set to <100mg/L. To aid in compliance with the provisions of this Act and to reduce the levels of lipids entering the sewage system whilst removing a large proportion of lipids from wastewater, a grease trap may be installed.

Figure 1.11 FOG removal from sewer in Clontarf area of Dublin (DCC, 2012)

1.2.2.1 Grease Traps

A grease trap can be a rectangular or circular vessel or tank that collects FOG preventing it from entering the drain. There are two distinct types of grease traps, passive grease traps and automatic grease traps or grease recovery units (GRU). Passive grease traps are governed by the Standard I.S. EN 1825:2002/2004, while

the GRU do not carry a recognised EU Standard but rather an industry guideline (PDI – G101) issued by the Plumbing and Drainage Institute of America (http://www.vfi.ie/files/FOG_Management_Submission_to_NSAI_June_09.pdf).

Wastewater passes under laminar-flow conditions through the grease trap and the fats, oils and greases gather on the surface before they reach the end of the trap. Passive grease traps capture the oil and grease from the flow of wastewater by slowing down the flow of warm greasy water through the grease trap and allowing it to cool. As it cools, the grease and oil separate out of the water and float to the top of the trap. The separation principle is based on Stokes' law relating rising velocity of a particle to its diameter and in theory separation efficiency is independent of depth (Willey, 2001). However, these facilities may fail to retain dissolved and emulsified fats allowing them to enter the water treatment system (Brooksbank *et al*., 2007).

Unlike the passive grease trap, automatic grease traps work on the basis of automatically recovering FOG by various means of skimming off or displacing the floating FOG (dependant on the model design) to an adjoining receptacle for eventual certifiable disposal. There are pumps/motors and elements in most GRU designs to aid the recovery process. Daily cleaning/maintenance is normally required on this system to ensure that the unit works to its optimum level of performance. Best practise requires that the grease traps be located externally, however, due to the requirement of electrical power for the operation of the automatic grease traps, those units are found under the sink (http://www.vfi.ie/files/FOG_Management_Submission_to_NSAI_June_09.pdf).

A third grease management product which is connected to grease traps is a device, BioAmp, that is automatically programmed for regular dosing of bacteria. BioAmp is a compact, computer controlled microbial fermentation unit that is installed on site and delivers a large amount of active, safe bacteria for the degradation of FOG in the grease traps (http://www.bioamp.co.uk/).

The types of grease traps including the BioAmp grease management unit are illustrated in (Figure 1.12). The most common type of grease trap found in food service facilities in Ireland is the passive grease trap. The disposal of waste fats, oils and grease (FOG) from grease traps is a significant environmental challenge and can be handled in different ways depending on the city, municipality or water district. The separated lipids may be incinerated or dumped in landfills (Matsumiya *et al*., 2007). In some areas, where there is available land, grease trap waste is delivered to a soil regeneration operation where oily waste and greases are bioremediated using microbes and nutrients (Parjus *et al*., 2008). However, incineration of lipids with heavy oils and landfill dumping cause several environmental problems (Matsumiya *et al.*, 2007).

a) Passive grease trap

b) Grease recovery unit

c) microbial fermentation unit

Figure 1.12 Types of grease management: a) passive grease trap, b) grease recovery unit, c) microbial fermentation unit

Proper cleaning and maintenance of the grease trap and drain line is essential to reduce the amount of solids and grease going into the public sewer system and minimize the likelihood of back-ups or blockages. Lack of effective treatment of grease trap wastes results in higher cost of wastewater treatment for municipalities and higher costs to the customers for servicing grease traps. The removal and disposal of waste from the grease trap is costly and requires a professional pumping service with labor and technical skills. Therefore, it is desirable to consider degrading the grease and oil in the grease traps. The use of biological systems has been investigated to convert grease to inert solids *in situ* (Parjus *et al*., 2008; Rashid and Imanaka, 2008).

1.3 Bioaugmentation

Two bioremediation approaches have been used to degrade fats and oils in grease traps. The first uses enzyme preparations, primarily lipases, which can break down fats and oils to fatty acids and glycerol. However, fatty acids tend to form micelles – colloidal particles that may aggregate and precipitate from solution as a result of environmental changes, such as changes in pH, temperature and salt concentration, causing clogging.

The second approach, biological augmentation or bioaugmentation, involves the addition of live microbial cells to the grease trap. The microbes not only break down fats and oils to fatty acids and glycerol but also metabolise them further (in the presence of oxygen) to carbon dioxide and water. This approach deals with the waste at source eliminating the needs for transport (Keenan and Sabelnikov, 2000; Chipasa and Medrzycka, 2006; Brooksbank *et al*., 2007).

Microorganisms have the ability to adapt to inhospitable environments (El-Fantroussi and Agathos, 2005). They can be protected from the environmental conditions by their cell envelopes and thus have a greater tolerance for extreme environmental changes (in pH, temperature and chemical composition) compared with enzyme preparations. In addition, microorganism preparations are less expensive and more stable than enzymes, and microbes can also reproduce

themselves at the waste sites. For these reasons, addition of selected microorganisms to wastewater treatment system is more advantageous than the use of enzymes (Keenan and Sabelnikov, 2000; Chipasa and Medrzycka, 2006). This natural biological treatment has been found to be the most efficient method for removing fats, oils and grease (FOG) (El-Masry *et al.*, 2004; El-Fantroussi and Agathos, 2005).

The application of bioaugmentation technology to biodegrade fats, oils, and grease in the grease trap will ensure that they do not enter the municipal sewer system thus avoiding blockages in sewers and the need for major expenditure on an annual basis by local authorities for emergency maintenance. It has also been reported that the added bacteria generate downstream benefits by removing FOG deposits on sewer walls and in pump sumps (Brooksbank *et al*., 2007).

1.3.1 Composition of bioaugmentation products

Bioaugmentation products generally comprise a mixture of microorganisms. Microorganisms in nature normally grow in mixtures and a variety of interactions have been identified in the mixed microbial communities including neutralism, commensalism/co-metabolism, synergism, mutualism, amensalism (antagonism) and parasitism (Table 1.3).

Neutralism is the phenomenon where no interaction is observed within two microbial populations or the interactions are of minimal importance. Neutralism can occur due to environmental conditions which do not allow microbial growth, for example when the two populations are out of their natural habitats. Commensalism is the interaction where one population benefits while the other remains unaffected. The unaffected population does not benefit from the second one, neither is it negatively affected as long as the two populations do not compete for the same substrate. In the commensalism relationship the unaffected population modifies the habitat making it suitable for the needs of the other population which benefits from the metabolic activities of the unaffected one (Atlas and Bartha, 1998).

Co-metabolism is a particular interaction within commensal relationships, where a primary organism oxidizes a substrate, while the oxidation products are available for use by the other microbial population as the primary one was not able to assimilate them (Alexander, 1994; Atlas and Bartha, 1998). Active populations derive no nutritional benefit from the substrates they co-metabolise, therefore, the microorganisms involved in co-metabolic transformation do not increase in numbers or biomass as a result of the degradation of the chemical of interest. This lack of growth is a reflection of the inability of the organisms to use the substrate for energy generation or biosynthetic purposes and it is in marked contrast to the increase in population size or biomass when a mineralisable substrate is introduced into the same sample. Thus, a separate growth substrate is usually provided to the organisms and the resulting enzymes catalyse the oxidation of co-metabolised substrates (Criddle, 1993). However, co-metabolic reactions have impacts in nature that are different from growth-linked biodegradations and when the transformations take place it is usually totally unclear whether the microorganisms do or do not have a second substrate available on which they are growing. The majority of co-metabolic studies have reported the use of a simpler substrate as the co-metabolite to achieve the degradation of a more complex compound (Atlas and Bartha, 1998). Because populations are usually small, a compound subject to co-metabolism is modified slowly and the rate of degradation does not increase with time. The product of co-metabolism will often be used by other microorganisms (Reineke, 2001).

In synergistic relationships two microbial populations benefit, but the association is not an obligatory one. It is difficult to determine whether or not both populations benefit and the relationship is or is not obligatory. Syntrophism is the interaction of two or more populations that supply each others nutritional needs. Example of a syntrophic interaction is the production of enzymes that are not produced by either population alone. Relationships of syntrophism are frequently based on the ability of one population to supply growth factors for another population. Mutualism is an obligatory association between two populations. Relationships of mutualism allow organisms to exist in habitats that could not be occupied by either population alone. This does not exclude the possibility that the populations may exist separately in other habitats. Metabolic activities and

physiological tolerances of the populations can also be different from those of either population separately. Amensalism is the interaction when one microbial population produces a substrate, toxin, antibiotic or bacteriocin that is inhibitory to the other population. Lastly, parasitism is the relationship where one population, the parasite, derives its nutritional requirements from the other population, the host, which is negatively affected (Atlas and Bartha, 1998).

Table 1.3 Types of interaction between microbial populations (Atlas and Bartha, 1998)

	Effect of interaction	
Name of interaction	Population A	Population B
Neutralism	0	0
Commensalism	0	+
Synergism (protocooperation)	+	+
Mutualism (symbiosis)	+	+
Competition	–	–
Amensalism	0 or +	–
Predation	+	–
Parasitism	+	–

0 = no effect
+ = positive effect
– = negative effect

Mixed microbial communities have the most powerful biodegradative potential, because the genetic information of more than one organism is necessary to degrade the complex mixtures of organic compounds present in contaminated areas. The genetic potential and certain environmental factors, such as temperature, pH and available nitrogen and phosphorus sources, therefore, seem to determine the rate and the extent of degradation.

There is increasing evidence from the literature that in order to avoid ecological barriers, microorganisms from the same ecological niche as the polluted area should be used (El Fantroussi and Agathos 2005; Thompson *et al*. 2005, Loperena *et al*. 2009). When investigating microbes for use in grease traps, the isolated

microorganisms should be studied for their efficiency to degrade food wastes and screened on the basis of their ability to produce many different enzymes in order to be able to degrade the varied range of the components of food waste (Huban and Plowman, 1997; Loperena *et al.*, 2009).

Wakelin and Forster (1997) investigated FOG removal from fast-food restaurant wastes using pure and mixed cultures. They found that while the mixed culture performed well, the pure culture an *Acinetobacter* sp. also performed well achieving 60-65% fat degradation from an initial concentration of 8 g/L fat. Similarly, Bhumibhamon *et al*., (2002) reported efficient degradation of palm oil by an *Acinetobacter* sp. Tano-Debrah *et al*. (1999) designed an inoculum containing a mixed-culture of 15 bacteria. They investigated a range of oils at a concentration of 20g in 100ml medium and found up to 73% degradation in seven days. Keenan and Sabelnikov (2000) reported good degradation of lipids by bacterial strains *Acinetobacter* sp., *Rhodococcus* sp. and *Caseobacter* sp. Mongkolthanaruk and Dharmsthiti (2002) evaluated a mixed population comprising *Pseudomonas aeruginosa* LP_{602}, *Acinetobacter calcoaceticus* LP_{009} and *Bacillus* sp B_{304} in order to lower the biochemical oxygen demand (BOD) and lipid content of lipid-rich wastewater. When *Bacillus* sp. B_{304} was combined with *Pseudomonas aeruginosa* LP_{602} it was shown that the *Bacillus* sp. B_{304} enhanced the wastewater treatment ability of *Pseudomonas aeruginosa* and better results were obtained than when the *Pseudomonas aeruginosa* LP_{602} was introduced as a single culture. The addition of the third strain *Acinetobacter calcoaceticus* LP_{009} promoted more effective fat removal.

The potential for Gram-negative bacteria to remove oil and grease from contaminated industrial effluents was investigated by El-Bestawy *et al*. (2005), where *Pseudomonas* sp. (L1) and *P. diminuta* (L2), *P. pseudoalcaligenes* (L3) and *Escherichia* sp. (L4) were investigated under different environmental conditions. Results revealed differences in their optimum conditions for maximum degradation of vegetable oil. All the tested bacteria were able to degrade palm oil completely and utilise the free fatty acids as a carbon source. The combination *Pseudomonas* sp. and *P. diminuta* produced the highest degradative activity, followed by *Pseudomonas* sp., *P. diminuta* and *P. pseudoalcaligenes*.

The fat-degrading microorganisms *Bacillus* sp., *Acinetobacter* sp. and *Pseudomonas* sp. isolated from dairy wastewaters and were also studied by Loperena *et al*. (2009) as a mixed population for their degradation ability on a laboratory scale. Studies showed that 93% of the protein and 75% of the fat present in the wastewater was removed by the bacteria.

Prasad and Manjunath (2011) evaluated FOG biodegradation by individual bacteria and a mixed culture consisting of *Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *Serratia marsescens*, *Pseudomonas aeruginos* and *Staphylococcus aureus*. The lipid degradative capacity of *P. aeruginosa* was high compared to the other bacteria. The formulated bacterial consortium was more effective than the single cultures in treatment of lipid-rich wastewater reducing the BOD value from 3200 mg/L to less than 40 mg/l (99% reduction), while the lipid content was reduced from 25,000 mg/L to less than 80 mg/L (99% degradation) within 12 days of incubation.

A number of investigators have studied the performance of commercial bioaugmentation products (Saravia *et al*., 2004; Loperena *et al*., 2006 and 2007). Brooksbank *et al.* (2007) tested a number of commercial oil-degrading supplements using 1ml of several fats and oils (lard, soya, sunflower, rapeseed). Among the several commercially available FOG-degrading microbial supplements containing single or multiple bacterial species that were tested, none of the single species, all whole cell preparations of *Bacillus subtilis*, were capable of significantly enhancing the degradation of any of the oils used. The F69 Organica commercial inoculum was the only one among the multi-species supplements examined that was capable of significantly degrading the fats.

1.3.2 Aerobic biological FOG removal by Fungi and Yeasts

Oleaginous microorganisms have been investigated for many years because of their properties and their potential application in industry (Papanikolaou *et al.*, 2003) and wastewater treatment (De Felice *et al.,* 1997; Lanciotti *et al.,* 2005; Wu *et al.,* 2009). Yeasts belong to the group of oleaginous microorganisms that are

able to degrade fats. The microbes will either degrade the fat and consume the produced fatty acids for growth or will transform them changing the intracellular fatty acid concentration and producing new fatty acids. The incorporation of the fat substrate into the microbial cells and the intracellular changes of fatty acids is defined by the enzymatic capabilities of the microorganisms. The yeast *Y. lipolytica* has the ability to produce extracellular and intracellular lipases and also lipases connected to the cell membrane. The organism can use triglycerol as a sole carbon source which it hydrolyses to fatty acids and glycerol. The released fatty acids are further metabolised through the β-oxidation pathway, while the glycerol enters the glycolysis pathway. The glycerol can be a satisfactory carbon source for the growth of the yeast. The ecological niche for *Y. lipolytica* encompasses lipid-rich food like margarine, olive oil and cheese and meat or shrimp products and it is also found in sewage and oil plants (Barth and Gaillardin, 1997; Casaregola *et al.*, 2000). *Yarrowia lipolytica* has been widely studied due to its considerable biochemical properties, its ability to produce several biotechnologically important metabolites, its dimorphism and amenability to molecular techniques.

Excellent removal of fat by *Y. lipolytica* was observed by Davin and Quilty (2001) who reported 75% beef tallow (1% w/v) removal by a newly isolated *Y. lipolytica* strain under optimal fermentation conditions. Likewise, biological treatment of salad oil and grease from food wastewater has been studied by *Yarrowia lipolytica* W29 in the report by Wu *et al*. (2009). The initial concentration of the oil was ~2 g/L and they observed 93% removal of salad oil and 85% removal of grease under optimum conditions when the incubation time was 50 hours. These findings together with the findings of others (Tan and Gill, 1985; Papanikolaou and Aggelis, 2010) suggest that the more highly saturated the fat, the greater the challenge for biodegradation.

Bednarski *et al.* (1994) studied the growth of the filamentous fungi, *Aspergillus niger*, *Geotrichum candidum* and *Mucor meihei*, on animal fats, tallow and poultry, using an initial fat concentration of 30 g/L. They reported 18±4% and 36±4% beef tallow and poultry fat removal, respectively, after 5 days. Tan and Gill (1985) reported 90% removal of 2.2 g/L beef tallow *Saccharomycopsis lipolytica* after just 8-12 hours.

Jeffery *et al.* (1999) reported the assimilation of sunflower oil by the fungus *Mucor circinelloides* f. *circinelloides*. The uptake was greatly enhanced by the added carbon source, sodium acetate. Aggelis and Sourdis (1997) also observed lipid degradation in the oleaginous fungus *Mucor circinelloides* growing on vegetable oil.

Fong *et al*. (2000) developed a microbial consortium isolated from a local wastewater treatment plant capable of decreasing BOD levels suggesting its potential for use in the biological treatment of food waste.

1.3.3 Considerations in bioaugmentation product development

When considering the production of microbial mixtures for use in bioaugmentation, a number of factors need to be considered. The additive supplements must not represent a human health hazard. In addition, they need to have a reasonable shelf life preferably not requiring refrigeration. Consequently, members of the genus *Bacillus* and closely related bacteria are used in many commercial supplements rather than the Gram-negative bacteria which are successful in many laboratory-based trials (Brooksbank *et al.*, 2007).

When microorganisms are introduced to an ecosystem, they may have difficulty in establishing and surviving in the native population. One approach which can be used to overcome this is to immobilise the microbial cells on a matrix which will anchor them in their new environment. Immobilisation of the cells can provide higher enzyme activity yields, better operational stability, greater resistance to environmental perturbations and lower effective operational cost (Hemachander and Puvanakrishnan, 2001; Loukidou and Zouboulis, 2001). The properties of the carbon source, hydrophobicity and toxicity, should be considered as a major factor when choosing a suitable carrier (Ławniczak *et al*., 2011). Some common carrier materials include alginate, agarose, polyurethane (Cavaleiro *et al.*, 2010) and rice bran (Bhumibhamon and Phattayakorn, 2003; Nisola *et al.*, 2009).

Obuekwe and Al-Muttawa (2001) used sawdust, styrofoam and wheat bran as carriers for two hydrocarbon-degrading bacteria and found that immobilised cells had a good utilisation of hydrocarbons in liquid medium. Xu and Lu (2010) used peanut hull powder as a bulking agent and carrier material because of its porous structure to improve oxygen diffusion and to immobilize a greater quantity of bacterial cells in order to investigate bioremediation of crude oil-contaminated soil.

Bhumibhamon and Phattayakorn (2003) used immobilised cells of *Pseudomonas* sp. to degrade food waste. The medium for the enrichment of *Pseudomonas* sp. was rice bran. A layer of biofilm was formed on the surface of plastic balls. Rashid and Imanaka (2008) also immobilised cells of isolates of the genus *Bacillus* for the degradation of grease trapped in a grease trap by growing them in a rich medium (LB) and applying them to porous rock. The pores or cavities worked as beds or compartments for the cells. The rock was put in the grease trap containing food waste and grease. The air was supplied in tanks through pipes. The microorganisms stuck in the pores and gradually degraded the waste food and grease. The results of analysis showed an efficient degradation of grease. These isolates collectively were able to decrease the suspended solid of the trapped grease from 102 to 40 mg/L. These microorganisms showed rapid growth and they were also capable of producing several extracellular enzymes, which together are important factors in organic biodegradation.

Nisola *et al* (2009) employed a newly isolated lipolytic strain, *Pseudomonas aeruginosa* D2D3 in a whole-cell immobilised FOG-trap system in order to investigate the feasibility of the cell immobilised trap system for FOG-containing wastewater treatment. Minimal medium was used to provide inorganic nutrients to *Pseudomonas aeruginosa* D2D3 and soybean oil as the sole carbon source. The matrices used were selected according to economic advantage, availability and mainly the adsorption capacity. The samples were allowed to equilibrate in simulated wastewater for 120 min and three materials with superior adsorption capacities (gram oil adsorbed/gram matrix) were chosen: rice bran, polyurethane and ceramic beads. In theory, both polyurethane and ceramic exhibit hydrophobic characteristics which can enhance cellular affinity to the substrate adsorbed into

the matrix, thereby improving the volumetric biodegradation rate of the substrates in a whole-cell immobilised system as compared with a suspended microbial system. Rice bran, an organic matrix, is rich in varied nutrient composition (13% protein, 13.2% FOG, 18.3% carbohydrate, 38.3% fiber, 7.8% vitamins B and E and trace amounts of lipase).

The property of many microorganisms to form biofilms or flocs has been reported as beneficial for oil and grease removal demonstrating matrices of naturally immobilised cells (Hamer, 1997; El-Marsy *et al*., 2004; Zheng *et al*., 2008; Ibrahim *et al*., 2009). The irreversible binding of bacteria to surfaces forming biofilms is the consequence of the production of extracellular polymeric substances (EPS) (Figure 1). EPS (free or cell-bound EPS) has been generally characterised as a highly hydrated gel in a biofilm consisting of proteins, nucleic acids, glycoproteins, polysaccharides, lipids and glycolipids that arise as a result of different cellular processes such as active secretion, cell lysis, shedding of cellular materials and absorption of matter from the environment (Omoike and Chorover, 2004; Zheng *et al*., 2008; Karunakaran and Biggs, 2010). The distribution of free and cell-bound EPS is illustrated in Figure 1.13.

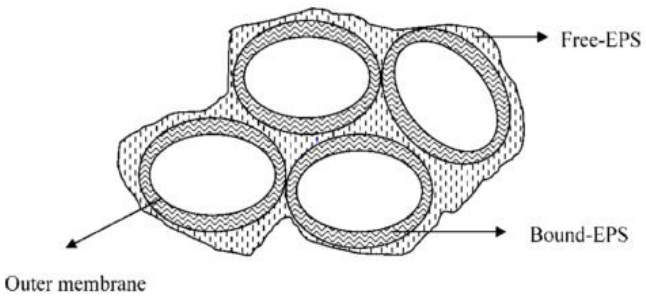


Figure 1.13 Bound and free EPS surrounding bacteria (Eboigbodin and Biggs, 2008)

McLaughlin *et al.* (2006) showed that when an aggregating form of *Pseudomonas putida* was added to activated sludge, the organism became associated with the sludge floc and enhanced the performance of the mixed microbial community. They proposed the benefit of including bacteria capable of aggregation in a bioaugmentation product.

1.4 Genus *Pseudomonas*

The genus *Pseudomonas* is a diverse group of bacteria that is well known for its ability to cause disease in plants and animals and for its role in biodegradation and bioremediation. Pseudomonads are also known for their metabolic diversity which allows them to grow under extreme nutrient limitation, as well as to produce commercially and environmentally important products.

The genus contains more than 140 species, most of which are saprophytic in soil or water where they play an important role in decomposition, biodegradation and the carbon and nitrogen cycles. The term Pseudomonad is commonly used to describe a rod-shape, strictly aerobic Gram-negative, nonsporulating, polarly flagellated bacterium. They are oxidase and catalase positive. The rods are generally straight but maybe slightly curved, 0.5 – 1 μm in diameter and 1.5 – 5 μm in length. These bacteria are generally motile. Most species fail to grow in acidic conditions (pH 4.5 or lower). The optimum growth temperature for most strains is 28° C but many are capable of growth between 4-45° C (Bergey's Manual of Systematic Bacteriology, 2001). The capacity of Pseudomonads for growth in very simple media and their widespread occurrence makes them appear as prime participants in the process of mineralization of organic matter in nature. Many strains of *Pseudomonas* were isolated from enrichment cultures by using a great variety of low molecular weight organic compounds as the only sources of carbon and energy (Palleroni, 2008 and 2010).

One of the most striking properties of these species is their remarkable nutritional versatility. Organic compounds, such as alcohols, aliphatic acids, amides, amines, amino acids, aromatic compounds, carbohydrates and hydrocarbons are readily used by *Pseudomonas* species as growth substrates (Todar, 2004). Members of the genus *Pseudomonas* demonstrate a great metabolic diversity and consequently are able to colonise a wide range of ecological niches.

The identity of the members of genus *Pseudomonas* has changed dramatically during the transition between artificial classification based on phenotypic properties and revisionist classification based on genotypic properties (Todar, 2004). In the past, *Pseudomonas* species were subdivided on the basis of rRNA homology into five similarity groups (Palleroni, 2010). There were about forty species. More recently only members of Group I were held in the genus *Pseudomonas*. Group I is the largest group, including fluorescent strains such as *P. aeruginosa, P. fluorescens* and *P. putida* and the plant pathogens *P. syringae* and *P. cichorii*. It also includes the nonfluorescent species *P. stutzeri* and *P. mendocina.* The members of groups II, III, IV, V were moved into new or previously existing genera such as *Burkholderia, Xanthomonas* and *Comamonas* based on 16S rRNA analysis (Bergey's Manual of Systematic Bacteriology, 2001).

Pseudomonas strains are often resistant to antibiotics, disinfectants, detergents and heavy metals and are able to develop resistance to organic solvents that can disrupt the cell membranes of unadapted bacteria (Ramos *et al*., 2002). Whereas chromosomal genes encode for all essential functions of cells, plasmids carry information for a wide range of biological functions that give the host cell a survival or growth advantage under particular environmental conditions. A number of pathways for degradation of novel compounds is known to be plasmid encoded. Plasmids encoding degradation of simple organics as well as hydrocarbons and synthetic compounds have been characterised in *Pseudomonas* species. Those degradative plasmids play a key role in the biodegradation of toxic or non toxic compounds in the environment and in the resistance of *Pseudomonas* species to many antibiotics (Boronin, 1992; Tsuda, 1999). *Pseudomonas* isolates often contain multiple transmissible plasmids and transposons which are readily exchanged among Pseudomonads and transmitted to other bacteria (Timmis, 2002).

Pseudomonas putida strains are non-pathogenic bacteria with a saprophytic lifestyle and have developed a remarkable metabolic versatility, as evidenced by the capacity of some strains to use more than 100 different carbon sources. *Pseudomonas putida* is a rapidly growing bacterium frequently isolated from soils

and waters, particularly polluted soils. It can recycle organic wastes in aerobic compartments of the environment, thus playing a key role in the maintenance of environmental quality. Its biochemistry and physiology, its rapid growth and ease of handling in the laboratory and its amenability to genetic analysis and manipulation have resulted in *P. putida* becoming a laboratory work tool for research (Timmis, 2002).

Its prominence has also been favoured by its frequent occurrence as the predominant organism found in selective enrichments in which an 'exotic' compound is offered as the sole source of carbon and energy, probably as a result of its rapid growth under the copiotrophic conditions of such selective enrichments (i.e. relatively high concentrations of substrates, non-limiting minerals, high aeration and incubation temperatures of 20–30° C). The fact that many of these phenotypes are based on plasmid-encoded pathways that channel such substrates to metabolites, supplying central metabolic pathways, has simplified their genetic and biochemical analysis. *Pseudomonas putida* CP1 degrades monochlorophenols by the modified *ortho*-cleavage pathway. It possesses a large 110 kb plasmid, having a gene for the key enzyme (chlorocatechol 1,2-dioxygenase) of the modified *ortho*-cleavage pathway (McLaughlin and Quilty, 2001).

P. putida strains also have chromosomally encoded pathways for the catabolism of a variety of organic compounds. The fact that *P. putida* possesses variety of degradative functions presumably reflects its extensive spectrum of 'housekeeping' catabolic pathways and enzymes, its tendency to freely acquire plasmids from other bacteria and its relaxed-specificity gene expression system, allowing the expression of genes derived from a wide variety of different bacteria (Jimenez *et al*., 2002; Nelson *et al*., 2002; Timmis, 2002). These functions along with the fact that *Pseudomonas putida* is listed among microorganisms most commonly found in various environments, make it a very important and interesting tool for bioremediation and biodegradation studies.

1.5 Genus *Bacillus*

Aerobic spore-forming bacteria represent a major microflora and play an important role in ecosystem development. The investigation of these micro-organisms is highly important for microbiologists dealing with ecological studies and environmental protection (Reva *et al*., 2001). The ubiquity and diversity of these bacteria in nature, the unusual resistance of their endospores to chemical and physical agents, the developmental cycle of endospore formation, the production of antibiotics, the toxicity of their spores and protein crystals for many insects, and the pathogen *Bacillus anthracis,* have attracted ongoing interest in these bacteria since Cohn and Koch's discoveries in the 1870s.

Bacteria that belong to genus *Bacillus* are aerobes or facultative anaerobes consisting of an unusually wide taxon characterised as Gram positive rods and under stressful environmental conditions the cells form endospores. Endospores are very resistant to heat, radiation and chemical disinfection, becoming metabolically active when a suitable substrate is made available. Thus, this genus plays an important role in the biological treatment of pollutants enriched with carbon and nitrogen. Sporulation can be enhanced by adding magnesium sulfate four-hydrate to the medium. The endospores of *Bacillus* spp., like those of the *Clostridium* spp., are more resistant than the vegetative cells to heat, drying, disinfectants, and other destructive agents, and thus may remain viable for centuries. With a few exceptions, strains of the genus *Bacillus* form catalase, which, in addition to the aerobic production of spores, distinguishes bacilli from clostridia. The production of catalase also differentiates bacilli from strains of *Sporolactobacillus*. The strains that produce no catalase or only trace amounts, are strains of *B. larvae, B. lentimorbus*, *B. popilliae,* and some strains of *B. stearothermophilus* (Laskin and Lechevalier, 1984; Fritze, 2008).

There is great diversity of physiology among the aerobic spore-formers. Their collective features include degradation of most all substrates derived from plant and animal sources, including cellulose, starch, pectin, proteins, agar, hydrocarbons, and others; antibiotic production; nitrification; denitrification;

nitrogen fixation; facultative lithotrophy; autotrophy; acidophily; alkaliphily; psychrophily; thermophily; and parasitism. Endospore formation, universally found in the group, is thought to be a strategy for survival in the soil environment, wherein these bacteria predominate. Aerial distribution of the dormant spores probably explains the occurrence of aerobic sporeformers in most habitats examined.

Some *Bacillus* species are strictly aerobic and others are facultatively anaerobic. Strains of *B. polymyxa* fix atmospheric nitrogen. Strains of some species grow well in a solution of glucose, ammonium, phosphate and a few mineral salts; others need additional growth factors as amino acids; still others have increasingly complex nutritional requirements. Strains of *B. fastidiosus* grow only when uric acid or allantoin is available. Although a pH of 7.0 is suitable for growth of most bacilli, a pH of 9.0 to 10.0 was described as a growth prerequisite for *B. alcalophilus*, and *B. acidocaldarius* was described as growing at pH values of 2.0 to 6.0, with optimal growth at pH 3.0 to 4.0. The bacilli also exhibit great variation in temperatures of growth; some thermophiles grow from a minimum temperature of 45° C to a maximum temperature of 75° C or higher, and some psychrophiles grow at temperatures from -5 to 25° C. All of the species can hydrolyse casein and ferment glucose. With the exception of the *B. pumilus*, the rest can also hydrolyze starch. Typical strains of *B. alvei*, as well as some strains of *B. circulans*, are actively motile and may form motile colonies on agar.

The species are categorized in three groups according to shape of the spore and swelling of the sporangium by the spore; The first group is subdivided by diameter of the rod and appearance of its protoplasm. The cells *B. megaterium* and *B. cereus* are usually wider (1.0-1.5 μm and 2-5 μm, respectively) than the cells of *B. licheniformis, B. subtilis, B. pumilus, B. firmus*, and *B. coagulans* (0.6-1 μm and 1.5-5 μm, respectively). In addition, the cells of *B. megaterium* and *B. cereus*, when grown on glucose agar and lightly stained, are filled with unstained globules, whereas the cells of other species are not (Laskin and Lechevalier, 1984; Fritze, 2008).

Some properties to separate *B. megaterium* and *B. cereus* are anaerobic growth, Voges-Proskauer and egg yolk reactions, resistance to lysozyme, and acid production from mannitol. With the exception of the acid production from mannitol, *B. megaterium* has negative reaction to the rest. Other useful characteristics are growth in an inorganic ammonium basal solution with glucose, the methyl-red test, and production of urease and of acid from raffinose and inulin.

B. licheniformis, B. subtilis, B. pumilus, B. firmus and *B. coagulans* belong to a group that share the same characteristics as width and length of the rods (0.6-1 μm and 1.5-3 or 5 μm, respectively). *B. lichenifromis* and *B. coagulans* can grow under anaerobic conditions and for the minimum temperature of growth which is 15° C, in contrast with the rest that cannot grow without oxygen and have minimal temperature of growth 5° C. Growth in 7% NaCl can be used in order to distinguish *B. lichenifromis* and *B. coagulans. B. subtilis, B. pumilus, B. firmus* are obligate aerobes. Between them, *B. firmus* is negative to the VP reaction and there is no growth at pH 5.7. *B. pumilus* cannot hydrolyse starch and reduce NO_3 to NO_2. *Bacillus pasteurii* requires alkaline media to grow containing ammonia (approximately 1% ammonium chloride) or urea (1%).

Many bioaugmentation products have been seen to comprise members of this genus. Their application for the biodegradation of FOG is due to their ability to produce important lipases and biosurfactants. The most typical strains of *bacillus* species that are widely found and used in the industries and bioaugmentation technology are the follows: *Bacillus subtilis, Bacillus megaterium, Bacillus licheniformis, Bacillus polymyxa, Bacillus pumilus, Bacillus thurigiensis, Bacillus stearothermophilus* (Laskin and Lechevalier, 1984; Fritze, 2008).

1.6 Detection and identification of bacteria in the environment

Traditionally, microbial community dynamics have been studied using culturable techniques such as the plate count technique where direct counts of growing colonies indicate the viable number of cells. This method is fast, inexpensive and can provide information on the active, heterotrophic component of the population. Some limitations include the growth medium selections, growth conditions (temperature, pH, light) and the potential for colony-colony inhibition or of colony spreading (Kirk *et al*., 2004; Liu *et al*., 2006). In addition, plate growth favors culturable microorganisms with fast growth rates (Kirk *et al*., 2004). The community composition is then assessed by identifying the isolates from the dominant colonies that were cultured. This approach can often be costly and time-consuming as each isolate has to be further examined for its physiological, biochemical and ecological characteristics. A fundamental problem with many traditional physiological and biochemical methods has been their dependency on cultivation of the microorganisms and/or analysis of their phenotypic expression (e.g. respiration, enzymes and catabolic potential) (Liu *et al*., 2006). Community-level approaches based on direct extraction and analysis of biochemicals such as proteins, phospholipid fatty acids (PFLA), DNA, and RNA eliminate the bias associated with culturing microorganisms.

1.6.1 Community-level physiological profiling

Garland and Mills (1991) developed a rapid community level cultural approach, subsequently called, community-level physiological profiling (CLPP). This approach is used increasingly to characterise microbial communities and examples of this approach include the BIOLOG system (Figure 1.14). The BIOLOG system is an automated technology for rapid identification of microorganisms based on the differential utilisation of 95 carbon sources (De Paolis and Lippi, 2008). Community level physiological profiling is an adaptation of a technique developed by BIOLOG for the identification of pure bacterial cultures. The community-level substrate utilization test is based on direct

incubation of environmental samples in Biolog EcoPlates (Garland and Mills, 1991; Choi and Dobbs, 1999; Garland, 2000; Salomo *et al*., 2009).

The system is based on interpreting patterns of sole-carbon substrate utilisation indicated by color development in a 96-well microtiter plate. Whether of fresh or saltwater origin, bacterial communities utilised more than 95% of substrates (Choi and Dobbs, 1999). CLPP with Biolog ECO-plates was used by Grove *et al*. (2004) to assess the changes in functional diversity of the microbial community in a compost biofilter over time, providing a simple and rapid method to assess changes in community structure of biofiltration systems. Biolog EcoPlates™ have been specifically created for bacterial community analysis of environmental samples (Salomo *et al*., 2009) and consist of 3 replicates of 31 ecologically relevant carbon sources and one control well per replicate (Kirk *et al*., 2004; Salomo *et al*., 2009). There is evidence that the selection of the carbon substrates allows greater discrimination between communities (Grove *et al*., 2004).

Similar to the Biolog system is the API system. There is a number of API strips available with various carbon sources that can be used to measure functional diversity. In principle, Biolog and API systems provide a community level physiological profile (CLPP) or a metabolic profile of the bacterial or fungal community's ability to utilize specific carbon sources. CLPPs can differentiate between microbial communities, are relatively easy to use and produce a large amount of data reflecting metabolic characteristics of the communities.

Limitations of metabolic profiling are: the methods select for only culturable microorganisms capable of growing under the experimental conditions, it favours fast growing microorganisms, is sensitive to inoculum density and reflects the potential, and not the *in situ*, metabolic diversity (Kirk *et al*., 2004). For instance, fungi and slow-growing bacteria that represent only a minor fraction of the *in situ* population may have a competitive advantage with the Biolog well and have minimal influence on the microbial metabolic profile overestimating the contribution of these species. In addition, the Biolog sole C-source plates contain high concentrations of carbon sources, which may not be representative of those present in soil (Kirk *et al*., 2004; Liu *et al*., 2006). Moreover, the contents of the

plates have been adjusted to nearly neutral pH, which can be a limitation for those microorganisms that have adapted to acidic or alkaline soils. Disregarding those disadvantages, CLPP is useful when studying the functional diversity of soils and is a valuable tool especially when used in conjunction with other methods (Kirk *et al*., 2004).

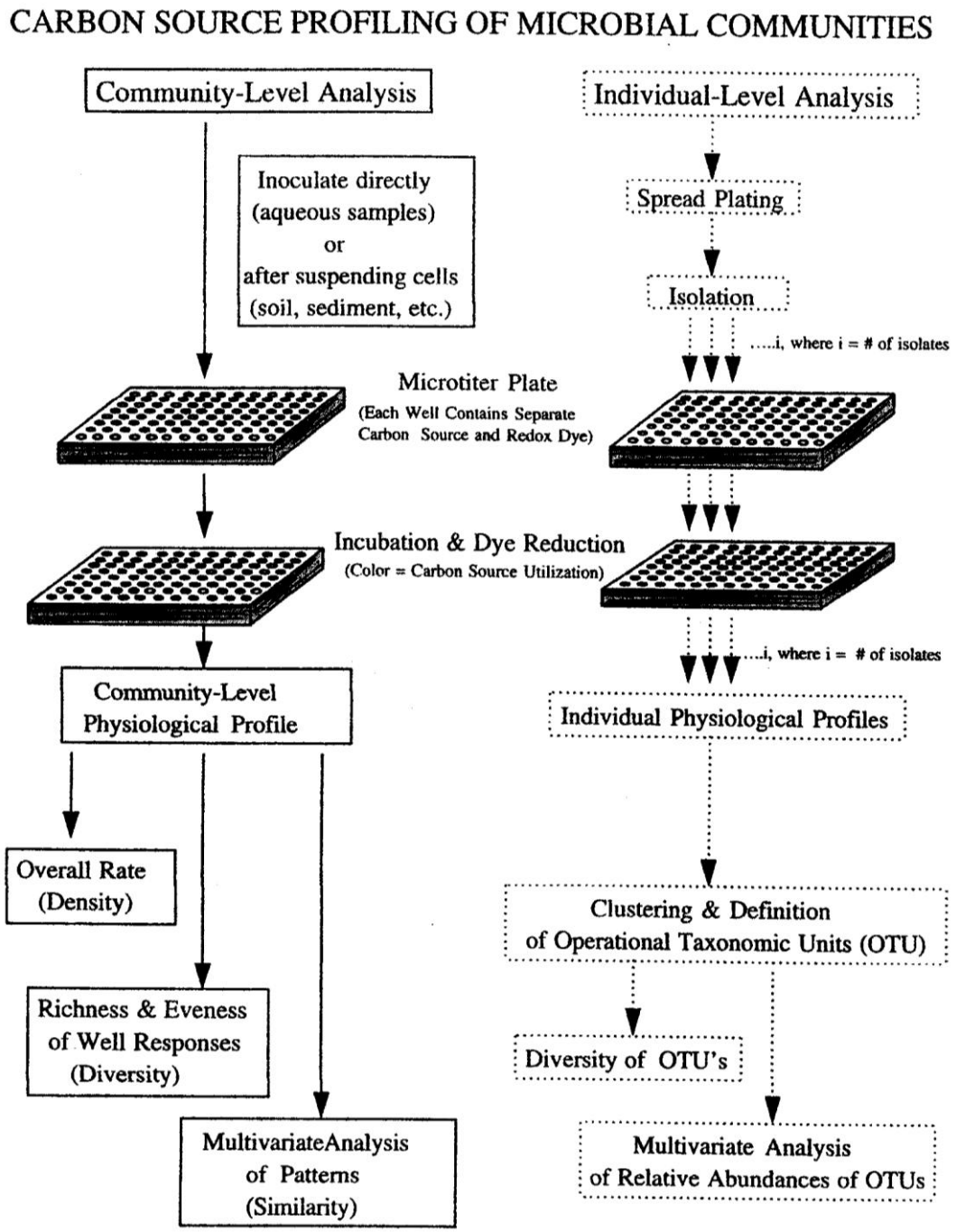


Figure 1.14 Comparison of isolate-based versus community level analytical approaches for carbon source profiling using Biolog microplates (Garland, 1997).

1.6.2 Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis

The Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) method is based on the classification of proteins according to their size. Individual polypeptide chains form a complex with negatively charged molecules of SDS and migrate as a negatively charged SDS-protein complex through a porous gel of polyacrylamide (Figure 1.15) (Alberts *et al*., 2002). SDS-PAGE of whole-cell proteins allows fast screening of large numbers of strains for comparative purposes (Pot *et al.*, 1993). Protein gel electrophoresis has been used for the separation and comparison of cellular proteins of strains belonging to the same species or subspecies (Kampfer, 1995) and is a useful tool for the characterisation of microbes based on their protein profiles.

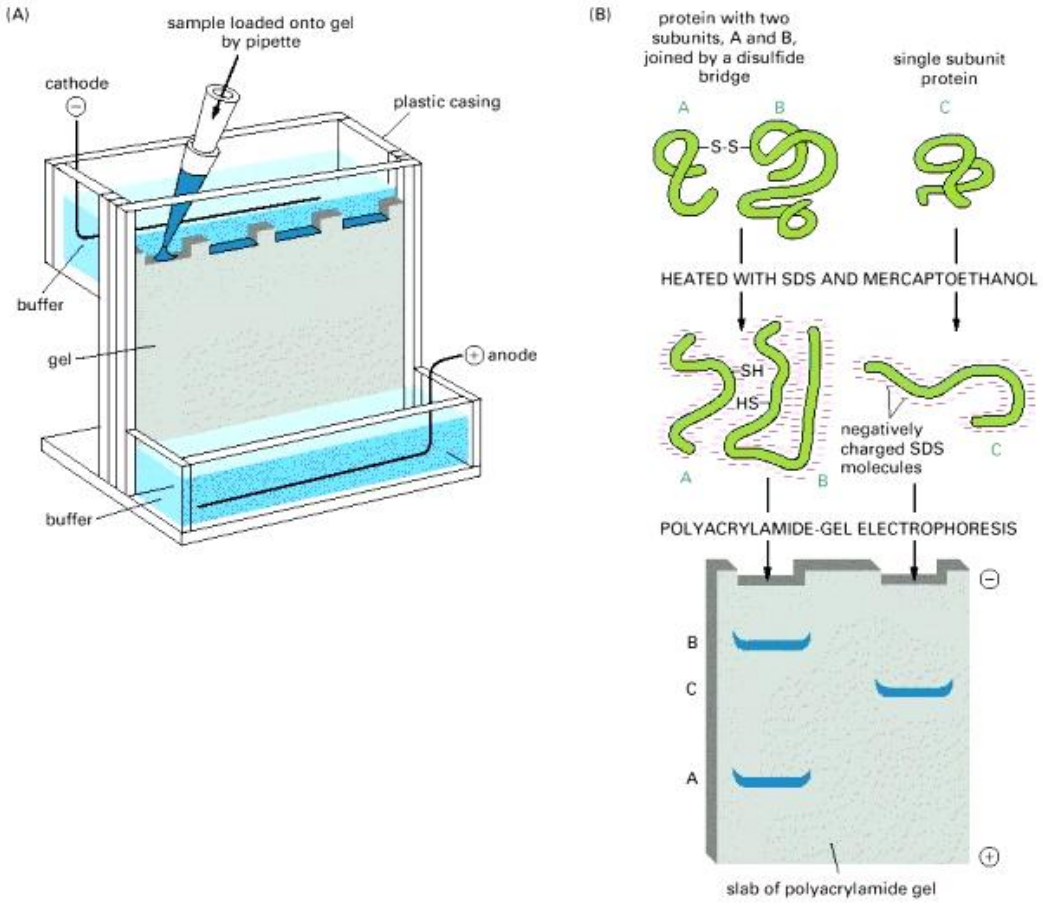


Figure 1.15 SDS-PAGE; (A) An electrophoresis apparatus, (B) Method (Alberts *et al.*, 2002)

1.6.3 Cellular Fatty Acid Methyl Esters (FAME)

Fatty acid methyl ester (FAME) analysis or phospholipid fatty acid (PLFA) analysis is a biochemical method that does not rely on culturing of microorganisms. This method provides information on the microbial community composition based on classifications of fatty acids (Kirk *et al*., 2004).

Bacterial fatty acids, in contrast to many other phenotypic characteristics, are genetically highly conserved, due to the cell structure and function (Dawyndt *et al*., 2006). Abel *et al*. (1963) introduced the use of cellular fatty acids for bacterial identification. More than 300 fatty acids are already found in bacteria. Differences in chain length, positions of double bonds and binding of functional groups make them very useful taxonomic markers (Dawyndt *et al*., 2006; Kunitsky *et al*., 2006).

The fatty acid composition of a particular strain is stable, given standardised culture conditions (Slabbinck *et al*., 2009). Analysis of the fatty acid methyl esters for individual bacterial species produces a fatty acid profile unique to that particular species (Liu *et al*., 2006). A full fatty acid profile is needed to identify a specific species. Individual fatty acids, which occur in more than one species, cannot be used to represent specific species (Kirk *et al*., 2004). It is possible to differentiate major taxonomic groups (Kirk *et al*., 2004; Nelson *et al*., 2010) and compare microbial community members without distinguishing individual strains based on physiological characteristics (Liu *et al*., 2006).

Whole-cell fatty acid methyl ester (FAME) analysis of bacteria using gas chromatography is an easy, cheap and fast-automated identification tool (Slabbinck *et al*., 2008, 2009 and 2010). With the advent of fused silica capillary columns (which allows recovery of hydroxyl acids and resolution of many isomers), it has become practical to use gas chromatography of whole cell fatty acid methyl esters to identify a wide range of organisms (Sasser, 1990). The process of fatty acid analysis is illustrated in Figure 1.16.

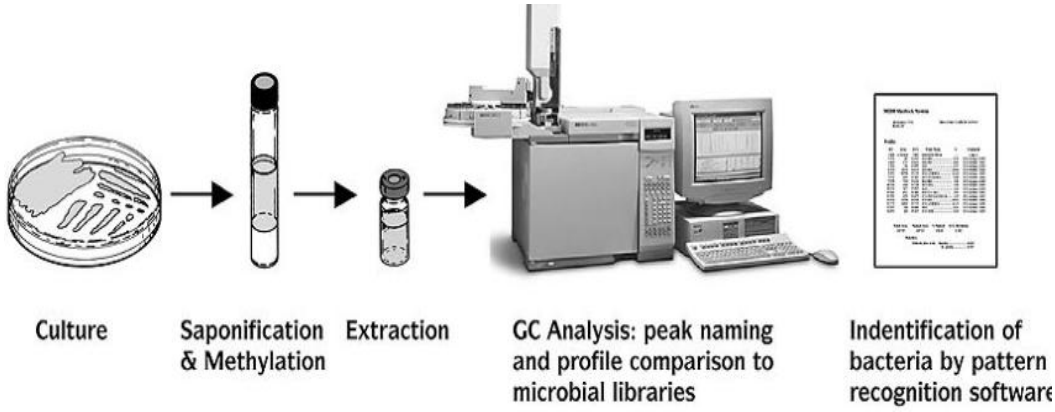


Figure 1.16 System of fatty acid analysis (Sasser, 1990)

Branched chain fatty acids (iso and anteiso acids) are common in many Gram-positive bacteria, while Gram-negative bacteria are composed of predominately straight chain fatty acids. The presence of lipopolysaccharide (LPS) in Gram-negative bacteria gives rise to the presence of hydroxy fatty acids in those genera (Figure 1.17). Thus, the presence of 10:0 3OH, 12:0 3OH, and/or 14:0 3OH fatty acids indicates that the organism is Gram-negative and conversely, the absence of the LPS and hydroxy fatty acids indicates that the organism is Gram-positive (Figure 1.18). As a result, it is not necessary to perform the traditional Gram stain prior to FAME analysis (Kunitsky *et al*., 2006).

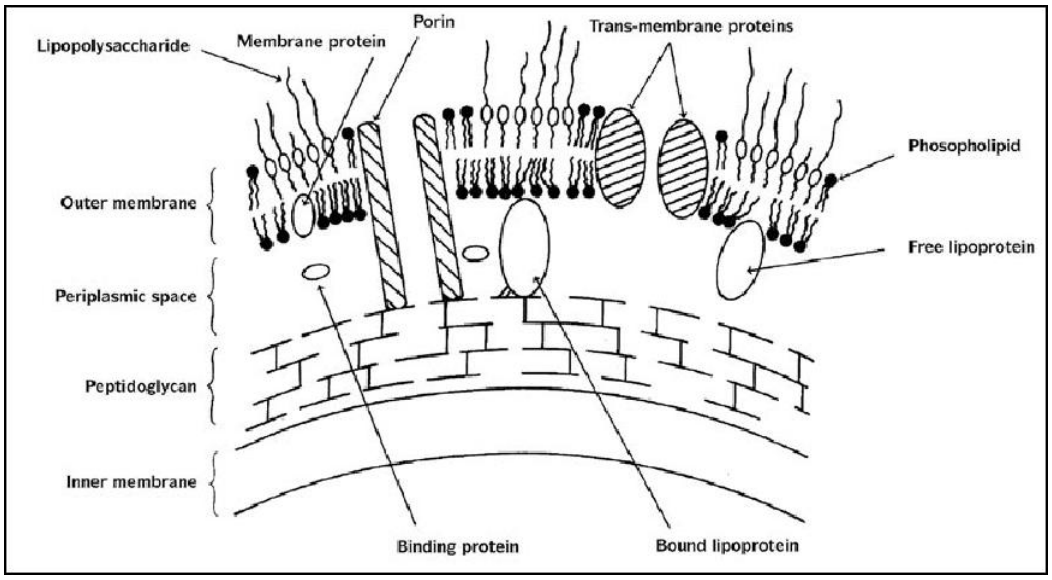


Figure 1.17 Gram-negative bacterial cell wall (Kunitsky *et al*., 2006).

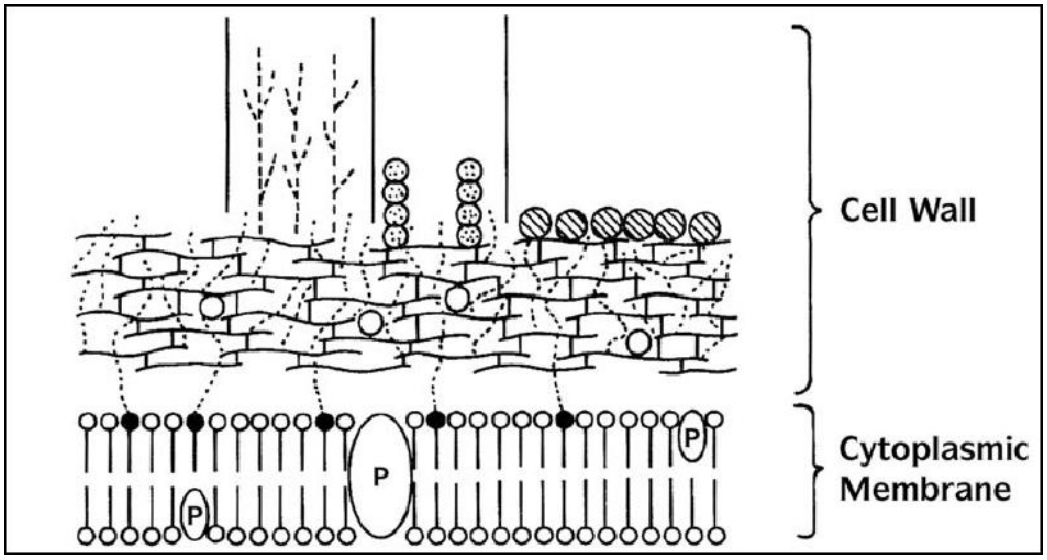


Figure 1.18 Gram-positive bacterial membrane (Kunitsky *et al*., 2006).

1.6.4 16S rRNA sequencing

A number of molecular approaches have been developed to study the microbial diversity in wastewater treatment without the need of isolation and cultivation (Bouchez *et al*., 2000) including DNA cloning and 16S rRNA sequencing (Kirk *et al.*, 2004; Gentry *et al.*, 2004; Sanz and Köchling, 2007). The use of 16S rRNA sequencing in the classification of bacterial species is well established. The 16S rRNA gene is present in all bacteria and it can be used to measure relationships between them (Harmsen and Karch, 2004; Clarridge III, 2004).

The ribosome is an organelle in cells that assembles proteins. It is composed of both ribosomal RNA and ribosomal proteins, known as the ribonucleoprotein. Ribosomes can be found floating freely in the cytoplasm or bound to the endoplasmic reticulum or the nuclear envelope and are usually found in large numbers in cells. Ribosomal RNA (rRNA) is the major proportion of cellular RNA and makes up about 65% of the bacterial ribosome (Rodnina *et al.*, 2007).

Ribosomal RNA sequences do not always coincide with characterisations based on classic taxonomic methods. Whereas genotypic classification is based on relatively stable and uniform molecular targets, phenotypic classification is

subject to variations in morphology, metabolic status and interpretation. When sequence data are included with other methods (API, BIOLOG) in a polyphasic approach, a comprehensive taxonomic and phylogenetic assessment can be obtained (Kolbert and Persing, 1999).

The 16S rRNA gene is highly conserved within a species and among species of the same genus. Nucleotide substitutions have occured within ribosomal nucleic acids at a steady rate throughout evolutionary history (Woese, 1987). Some regions of rRNA genes evolve at different rates resulting in regions of nucleotide conservation and variability. The degree of conservation is believed to result from the importance of the 16S rRNA as a critical component of the cell.

In 1980, molecular techniques such as the polymerase chain reaction (PCR) and DNA sequencing were developed for the classification of bacteria. The PCR application described by Fox *et al.* (1980) can be used to copy a DNA fragment into many identical copies (Figure 1.19). The conserved regions allow for the selection of universal primers for PCR amplification of almost all prokaryotes. Bacteria can be identified by amplifying the 16S rRNA gene, sequencing it and comparing it to other bacterial sequences in a database, such as GenBank, the largest database of nucleotide sequences.

DNA sequencing relies on the use of chain-terminating dideoxynucleoside triphosphates (ddATP, ddTTP, ddCTP and ddGTP) to produce a continuous series of fragments in reactions catalysed by polymerase. Dideoxynucleoside triphosphates resemble deoxynucleoside triphosphates except that they lack a 3′ hydroxyl group. They can add to a growing chain during polymerization but they cannot be added onto and therefore serve as chain terminators. This method developed by Sanger in 1977 is now completely automated: robotic devices mix the reagents and then load, run, and read the order of the nucleotide bases from the gel. This is facilitated by using chain-terminating nucleotides that are each labelled with a different colored fluorescent dye; in this case, all four synthesis reactions can be performed in the same tube, and the products can be separated in a single lane of a gel. A detector positioned near the bottom of the gel reads and records the colour of the fluorescent label on each band as it passes

through a laser beam (Figure 1.20) and a computer then reads and stores this nucleotide sequence (Alberts *et al*., 2002).

Figure 1.21 shows a tiny part of the data from an automated DNA-sequencing run as it appears on the computer screen. Each coloured peak represents a nucleotide in the DNA sequence—a clear stretch of nucleotide sequence can be read here between positions 173 and 194 from the start of the sequence. This particular example is taken from the international project that determined the complete nucleotide sequence of the genome of the plant *Arabidopsis* (Alberts *et al*., 2002).

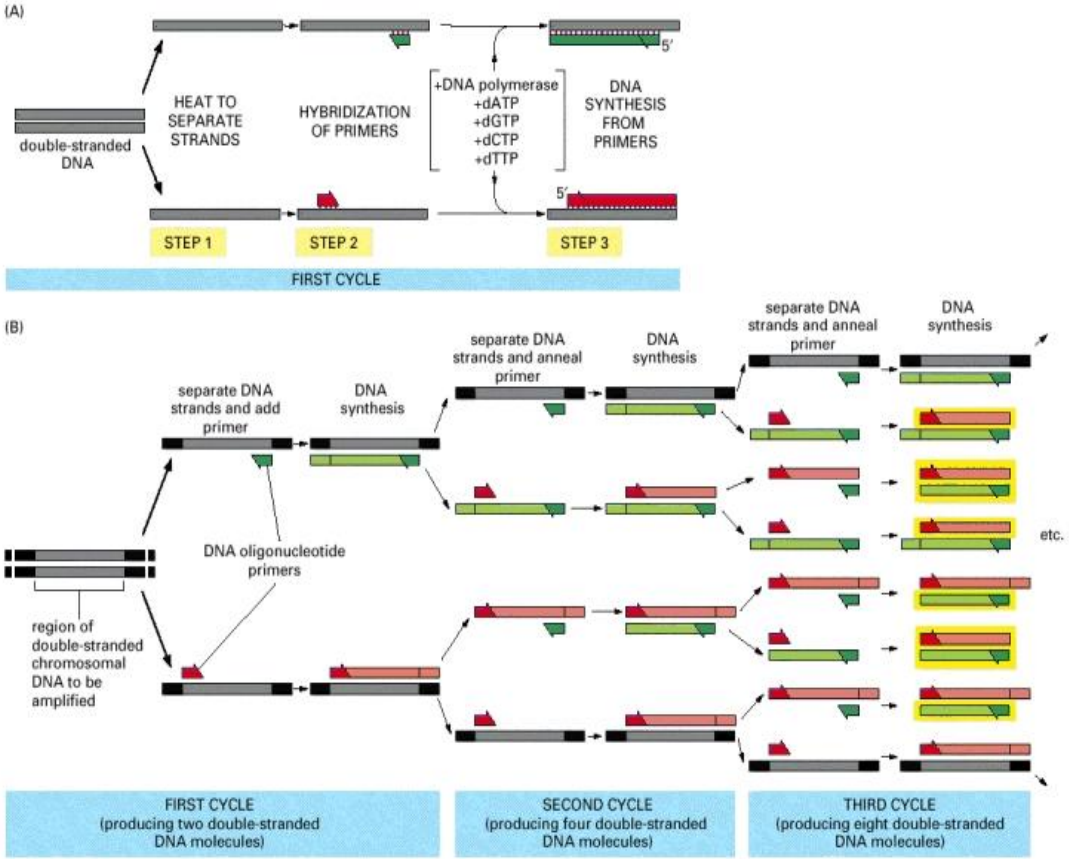


Figure 1.19 Amplification of DNA using the PCR technique. (A) The first cycle of PCR, and (B) the first three cycles, repetitions over and over again (Alberts et al. 2002).

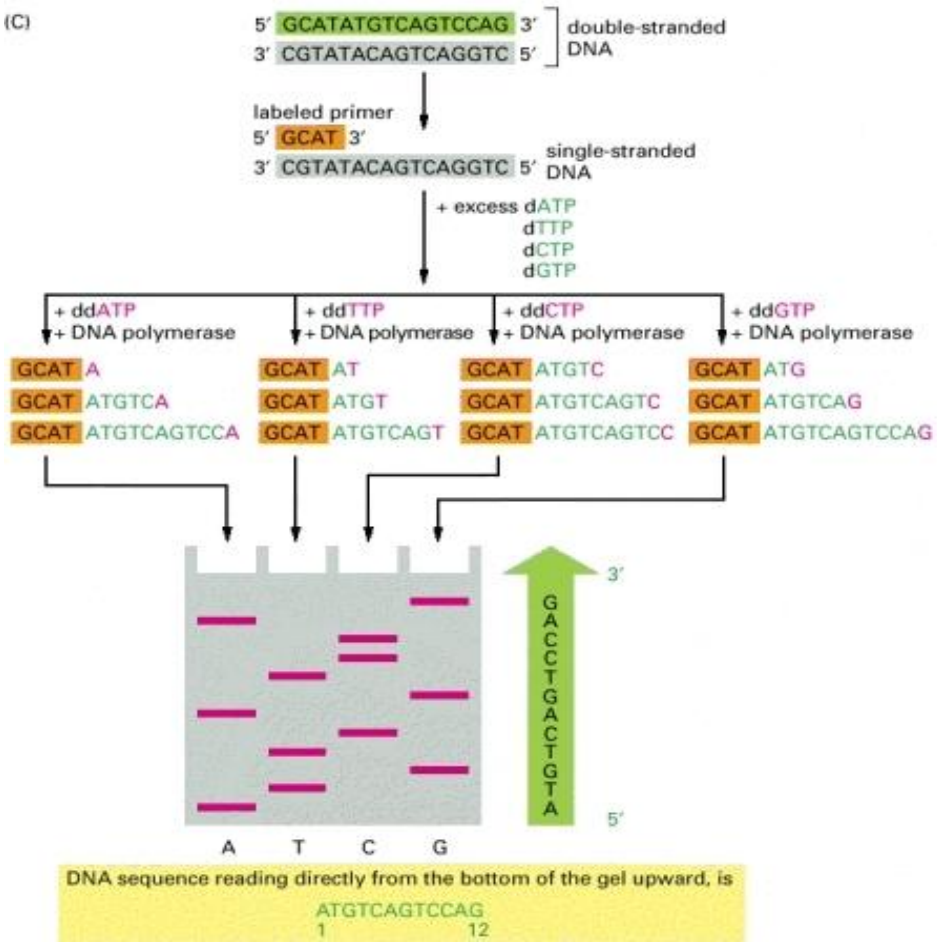


Figure 1.20 The enzymatic or dideoxy method of DNA sequencing (Alberts *et al*., 2002)

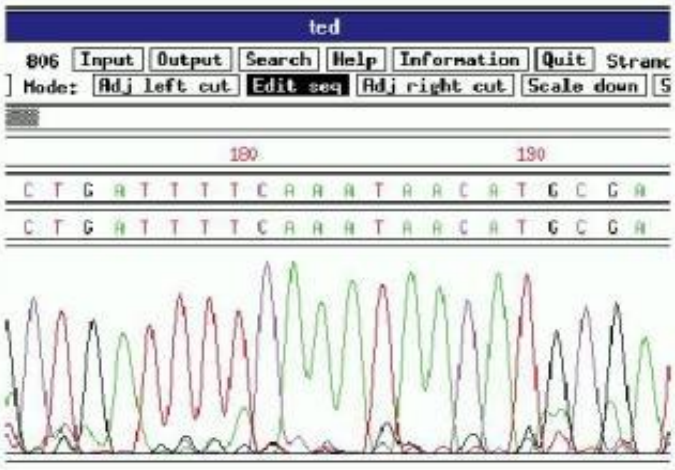


Figure 1.21 Automated DNA sequencing (Alberts et al., 2002)

Sometimes to distinguish between particular taxa or strains it is necessary to sequence the entire 1550 bp of the 16S rRNA gene. However, in many cases the first 500 bp sequence provides satisfactory differentiation for the identification of

strains and can actually show greater percentage difference between strains because the region shows slightly more diversity per kilobase sequenced (Clarridge III, 2004).

The reliability of DNA sequences generated in laboratories has been greatly improved by the introduction of automated sequencing systems and DNA alignment software. However, other factors, such as the purity of the DNA template and number of overlapping nucleotide fragments in the alignment contribute to the reliability of the final sequence (Sacchi *et al.*, 2002).

Using PCR and DNA sequencing, bacteria can be identified by amplifying the 16S rRNA gene, sequencing it and comparing it to other bacterial sequences in a database, such as GenBank, the largest database of nucleotide sequences (Figure 1.22). The phylogenetic structure of bacteria has been studied by comparing sequences of 16S rRNA genes thus distinguishing the different taxa (Zhang *et al.*, 2002).

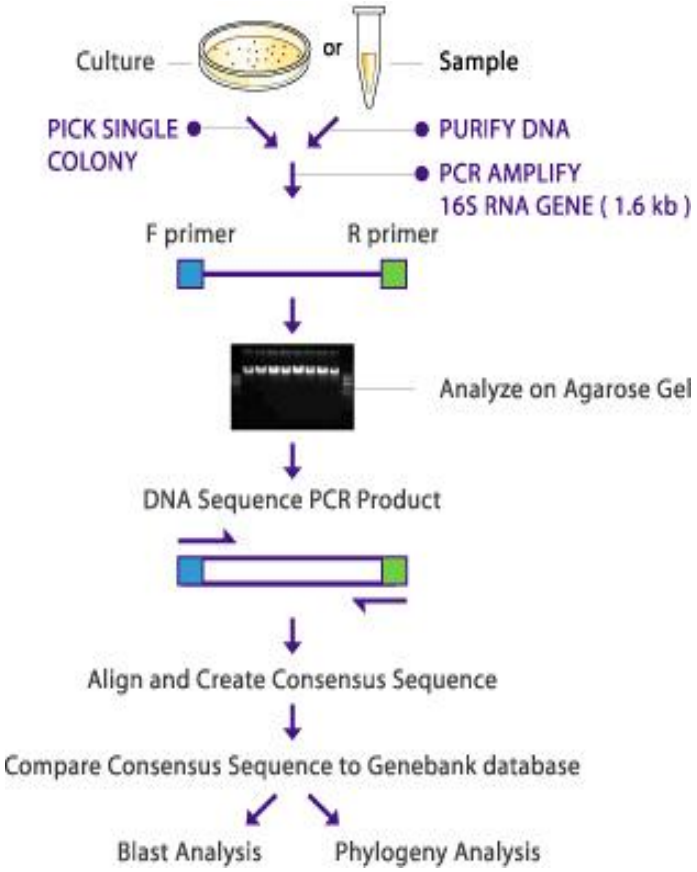


Figure 1.22 16S rRNA sequencing experimental approach (http://www.acgtinc.com)

A phylogenetic tree can be constructed which shows the bacterium's position in the evolutionary order based on base differences between species. This process is fast and very accurate and is aided by the large number of available programmes and databases. Databases are available that have thousands of 16S rRNA sequences from almost all known genera of bacteria (Zhang *et al.*, 2002). Advances in sequencing technology have also increased the speed with which sequence information can be obtained. 16S rRNA gene sequencing is now the gold standard of bacterial identification. It enables the identification of non-cultivable microorganisms and elucidates the relationship between unknown species and known ones (Woo *et al.*, 2000).

1.6.5 Fluorescent labelling techniques

Microbial diversity has been monitored *in situ* using fluorescent labelling such as fluorescent *in situ* hybridization (FISH) of targeted rRNA probes (Bouchez *et al*., 2000; Thompson *et al*., 2005; Sanz and Köchling, 2007) and labelled organisms using green fluorescent protein (GFP) (Ma *et al.*, 2011).

1.6.5.1 Fluorescence *in situ* hybridization (FISH)

Nucleic acid hybridization using specific fluorescent probes, which are short sequences of DNA labeled with a fluorescent dye (Thompson *et al*., 2005; Sanz and Köchling, 2007), is an important qualitative tool in molecular bacterial ecology (Liu *et al.*, 2006). This molecular method is widely used for the identification, quantification and, in combination with other techniques, characterisation of phylogenetically defined microbial populations in complex environments (Amann and Fuchs, 2008). It enables the direct monitoring of the response that the microbial communities perform *in situ* (Bouchez *et al*., 2000; Thompson *et al*., 2005) detecting not only culturable, but also unculturable microorganisms (Moter and Göbel, 2000). Therefore, FISH has been defined as a suitable method for bioaugmentation studies (Bouchez *et al*., 2000; Thompson *et al*., 2005).

These hybridisation techniques can be performed on extracted DNA and RNA, or *in situ* hybridisation can be conducted at the cellular level (Liu *et al*., 2006). The use of oligonucleotide probes targeting 16S rRNA sequences presents a revolution in microbial ecology (Figure 1.23). Within the area of wastewater treatment, hybridisation techniques are by far the most extensively used, so any one review may not refer to all techniques (Sanz and Köchling, 2007). A disadvantage of FISH is the lack of sensitivity unless sequences are in high copy number (Liu *et al*., 2006). The limitation of detecting slow-growing or starving cells because of low physiological activity being often correlated with low ribosome content per cell, has been overcome by using a tyramide signal amplification technique, called CARD-FISH (catalysed reported deposition-fluorescence *in situ* hybridization) (Figure 1.24) (Liu *et al*., 2006; Sanz and Köchling, 2007).

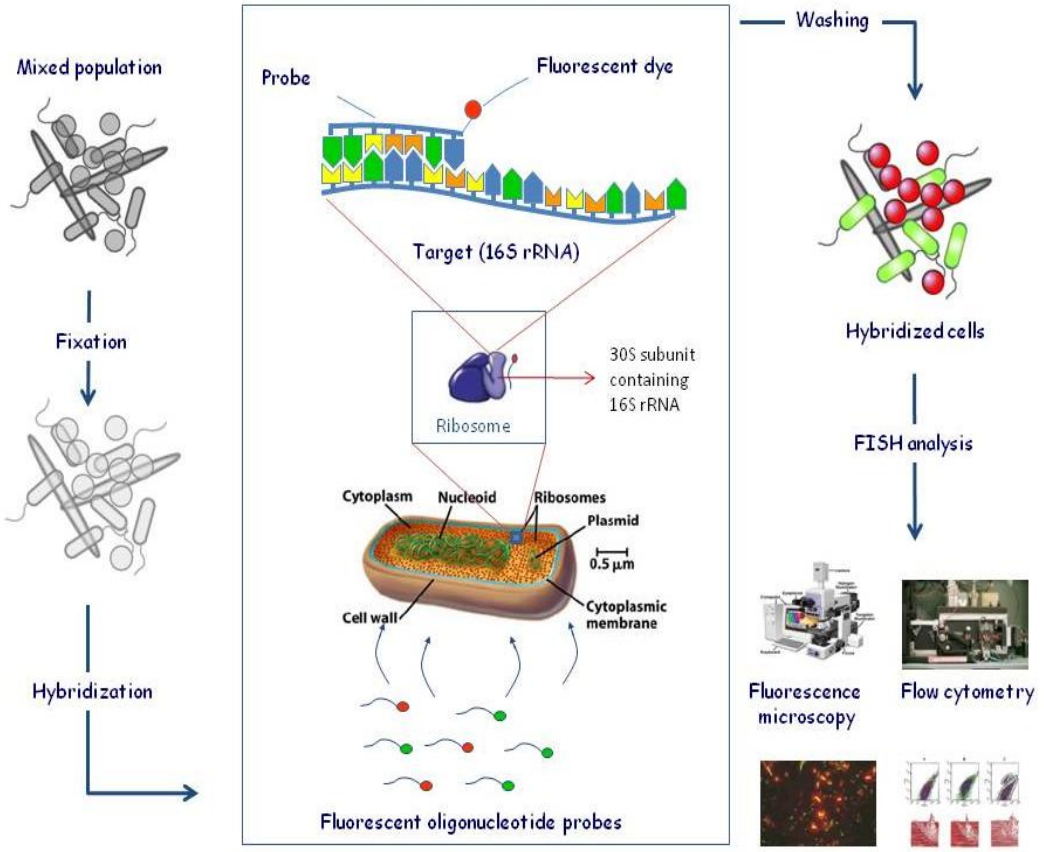


Figure 1.23 Fluorescence *in situ* hybridization (FISH) (http://www.biovisible.com)

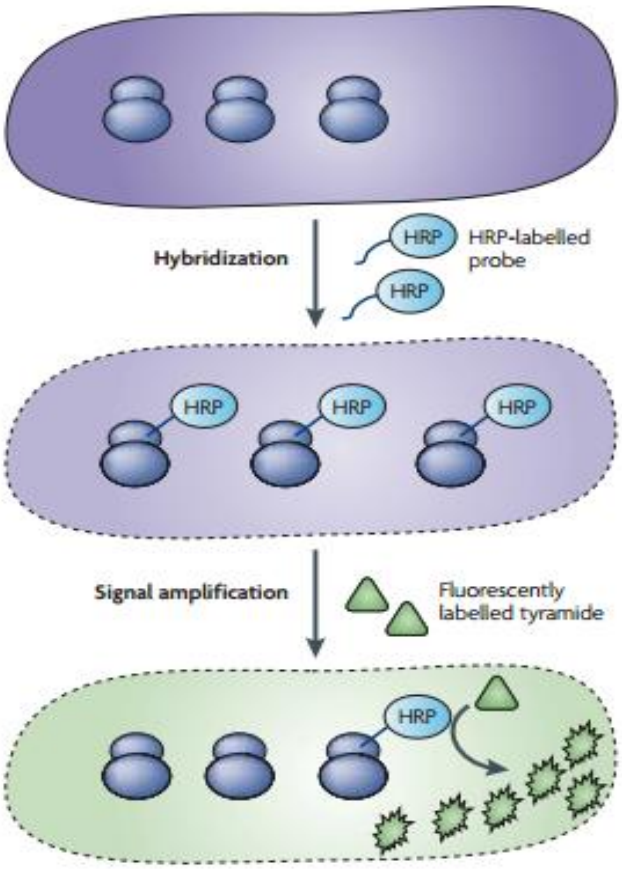


Figure 1.24 The principle of CARD-FISH (catalysed reported deposition-fluorescence in situ hybridization) (Amann and Fuchs, 2008)

Amann and Fuchs (2008) studied the improvement on the sensitivity of FISH techniques for single-cell identification in microbial communities using four mono-labelled oligonucleotides, multiple-labelled oligonucleotide probes, and horseradish peroxidise (HRP) labelled probes in combination with catalysed reported deposition (CARD). The hybridization involves a single oligonucleotide that is covalently crosslinked to the HRP label. Amplification of the signal relative to that achieved with probes that are labelled with a single fluorochrome is based on the radicalization of multiple tyramide molecules by a single horseradish peroxidase.

Combination of FISH technique with confocal laser scanning microscopy (CLSM) is a reliable means for visualising and monitoring bacterial communities in the environment, and the population dynamics of aggregated microbial ecosystems (Bouchez *et al*., 2000).

1.6.5.2 Green Fluorescent Protein (GFP)

The *gfp* gene, encoding the green fluorescent protein GFP from the jellyfish *Aequorea victoria*, is currently considered the best molecular tool for *in situ* studies using fluorescence microscopy or flow cytometry. The fluorescence from GFP is independent of substrate, although proper folding of the protein depends on oxygen (Normander et al., 1999; Koch *et al.,* 2001).

There is great interest in the *gfp* gene as a potential marker for tracking and visualizing bacteria in environmental samples (Lowder *et al*., 2000; Ma *et al.,* 2011). A number of studies involving GFP-labeled strains of bacteria have revealed that GFP expression does not alter the biochemical, morphological, or survival characteristics of the labeled bacteria (Ma *et al.,* 2011). Bastos *et al*. (2001), who studied phenol degradation by *gfp*-transformed cells and wild-type cells, confirmed that chromosomal insertion of *gfp* did not interfere with the phenol degradation capabilities of the cells.

Another advantage that has established the *gfp* as the most useful reporter gene and live-cell marker is its bright clear visualization under fluorescent microscope (Eberl *et al.,* 1997; Nancharaiah *et al*., 2005). Moreover, when microorganisms are chromosomally labeled with *gfp,* it ensures genetic stability and reduces the risk of gene transfer to indigenous microbial populations (Bastos *et al.,* 2001; Errampalli et al. 1999; Ma *et al.,* 2011;). The advantages of the GFP along with disadvantages are listed in Table 1.4.

GFP-labeled bacteria have been used in monitoring either single cells or a cell population in survival studies (Eberl *et al.,* 1997; Errampalli *et al*. 1999; Bastos *et al.,* 2001; McLaughlin *et al.,* 2006), for quantitative description of a model biofilm (Nancharaiah *et al.,* 2005), as an oxygen-sensing reporter for *in situ* studies of spatial and temporal variations in bioavailability of oxygen in natural habitats (Højberg *et al.*, 1999) and for detection of viability (Lowder *et al.,* 2000). Lowder *et al.* (2000) reported that gfp-tagged cells remained fluorescent following

starvation and entry into the viable but nonculturable state, but that fluorescence was lost when the cells died, presumably because membrane integrity was lost.

Table 1.4 Advantages and disadvantages of GFP marker in environmental applications (Errampalli *et al.,* 1999)

ADVANTAGES
Ease of detection
No exogenous substrate needed
No processing of cells required
Able to monitor single cells
No fixing or staining of samples/cells necessary; but detection of fluorescence still possible in formaldehyde fixed cells
Non-destructive; detection without disruption of microbial community
Possible to monitor on-line or in real time
Extremely stable-heat (65°C); pH (6-12); resistant to denaturants and proteases
GFP expressed in cytoplasm; should have minimal effect on cell-surface dynamics
Continually synthesised; minimises fluorescence signal dilution during bacterial replication
Allows analysis of living cells; repeated readings under various conditions for the same cell is possible
No GFP background in indigenous bacterial populations
Dual detection possible with different coloured markers
DISADVANTAGES
Variability of GFP expression in different species unknown
Plasmids may be unstable - use chromosomal insertion
Influence of environmental conditions on GFP expression in unknown
Interference by other fluorescent particles or bacteria
Extended lifetime of fluorescence once cell had died or lysed
GFP may not work under anaerobic conditions

1.7 Aims of the project

Grease traps are commonly utilised by food service establishments (FSE) to limit the discharge of fats, oils and grease (FOG) into the wastewater network. The effectiveness of a grease trap is dependent on the regularity of its maintenance and/or the application of bio-augmentation. The overall aim of this study was to characterise three commercial bioaugmentation products for their ability to degrade fats and for their constituent microorganisms.

The specific objectives of the project were:

- To evaluate the ability of three commercial bioaugmentation products to degrade a soft and a hard fat;
- To isolate, identify and characterise the microorganisms present in the three products using physiological, biochemical and molecular methods;
- To investigate the role of the microbial isolates in the products and
- To optimise the microbial composition to inform optimal product design.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Microorganisms

Three commercial bioaugmentation products designed to degrade fats, oils and grease comprising mixed microbial cultures were used (Table 2.1).

Table 2.1 Three bioaugmentation products used in degradation studies at different forms

Bioaugmentation Product	Form type	
BFL	Powder form	
FF	Tablet form	
Gnz	Liquid form	

Pseudomonas putida CP1 and *Pseudomonas putida* CP1::Tn7-gfp were obtained from the culture collection of the Microbial Ecology Group, School of Biotechnology, DCU.

Bacillus subtilis B-14596, *Bacillus amyloliquefaciens* NRS-762 and *Bacillus licheniformis* B-14368 were obtained from the Agricultural Research Service (ARS) culture collection (NRRL) (http://nrrl.ncaur.usda.gov/).

The bacterial isolates were maintained on plate count agar (Oxoid) at 4°C, after routine sub-culturing for 48 h at 30° C. Stocks of bacterial isolates were maintained a) on slopes of plate count agar and b) using 500µl of overnight culture in 500µl of 80% (v/v) glycerol stored at -80° C.

A fungal isolate was obtained from the bioaugmentation product FF and it was maintained on malt extract agar (Oxoid) after sub-culturing for 5 days at 22° C.

An 8mm mycelial agar plug of a 5-day old culture was used for the sub-culturing of the fungus.

2.1.2 Media

2.1.2.1 Minimal Medium

Degradation studies were carried out using minimal medium as described by Loperena *et al*. (2006). The components were dissolved in distilled water.

Ingredient	Concentration (g/L)
NH_4Cl	0.57
KH_2PO_4	0.43
K_2HPO_4	1.09
Na_2HPO_4	1.33
$MgSO_4 \times 7H_2O$	0.023
$CaCl_2$	0.028
$FeCl_3 \times 6H_2O$	0.025

2.1.2.2 Enriched Nutrient Medium

The enriched nutrient medium was adapted from Brooksbank *et al*. (2007).

Ingredient	Concentration (g/L)
yeast extract	0.2
glucose	0.1
KNO_2	1.0
$MgSO_4 \times 7H_2O$	0.2
NaH_2PO_4	0.1
$CaCl_2 \times 2H_2O$	0.001
$MnSO_4 \times H_2O$	0.01
ferric ammonium citrate	0.005

2.1.2.3 Nutrient Broth

The nutrient broth was prepared in accordance with the manufacturer's instructions. 10ml aliquots of the broth were dispensed into glass universals prior to sterilization by autoclaving at 121° C for 15 min.

2.1.2.4 Tween 20 or Tween 80 agar

The Tween 20 or Tween 80 agar medium were prepared as described by Paparaskeyas *et al*. (1992). The ingredients were dissolved in distilled water and the pH was adjust to 7.5.

Ingredient	Concentration (g/L)
Peptone	10
NaCl	5
$CaCl_2$ x $2H_2O$	0.1
Agar	20
Tween20 or Tween80	1%

2.1.2.5 Agars

Plate count agar, MacConkey agar, nutrient agar and malt extract agar were obtained from Oxoid. All the media were prepared in distilled water according to the manufacturer's instructions and autoclaved at 121^{o} C for 15 min.

2.1.2.6 Media for detecting enzyme activity

Nutrient agar containing 1% (w/v) starch, casein, xylan, and carboxymethylcellulose sodium salt and 1% (v/v) tributyrin (Sigma) was used for detection of amylase, protease, xylanase, cellulase and lipase respectively.

2.1.2.7 SDS-PAGE resolving gel (12% w/v)

The resolving gel used in SDS-PAGE was prepared by adding 2.5 ml 1.5 M Tris HCl (pH 8.8), 50 µl SDS (20% w/v), 4 ml acrylamide bisacrylamide (30%/0.8% w/v) and 50 µl ammonium persulphate (10% w/v) to 3.39 ml distilled water. Ten µl of TEMED was mixed with the solution to set the gel. The ammonium persulphate was freshly prepared on the day of use.

2.1.2.8 SDS-PAGE stacking gel (4% w/v)

The stacking gel used in SDS-PAGE was prepared by adding 1.25 ml 0.5 M Tris-HCl (pH 6.8), 25 µl SDS (20% w/v), 0.67 ml acrylamide bisacrylamide (30%/0.8% w/v) and 25 µl ammonium persulphate (10% w/v) to 3.02 ml distilled water. 10 µl Temed was mixed with the solution to set the gel. The ammonium persulphate was freshly prepared on the day of use.

2.1.2.9 Preparation of gel agarose

Agarose gel, concentration 1% (w/v), was prepared by the addition of 0.3 g agarose (Sigma) to 30 ml of 1xTBE buffer where 0.3 g of agarose. The mixture was boiled for 2 min until the agarose was sufficiently dissolved. The solution was allowed to cool down and then 2 µl (10 mg/ml) ethidium bromide (final concentration 0.4 µg/µl) was added.

2.1.3 Buffers

2.1.3.1 Ringer's Solution

Ringer's solution (Oxoid) was prepared by adding one tablet to 500 ml of distilled water and autoclaved at 121° C for 15 minutes.

2.1.3.2 Phosphate Buffered Saline

Phosphate Buffered Saline (Oxoid) was prepared by dissolving one tablet into 100 ml distilled water (pH 7) and autoclaved at 121° C for 15 minutes.

2.1.3.3 TE Buffer

Tris-acetate buffer was prepared by dissolving 10 mM Tris-HCl and 1 mM EDTA in distilled water and adjusting the pH to 8.0 with HCl. The buffer was stored at room temperature.

2.1.3.4 STET buffer

STET buffer was prepared by dissolving 8% sucrose, 5% Triton X-100, 50 mM Tris-HCl and 50 mM EDTA in distilled water (pH 8.0).

2.1.3.5 Lysis buffer

a) *For the Gram positive cells*:

Lysis buffer was prepared by dissolving 50 mg lysozyme in 1ml TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8) and stored at -20° C.

b) *For the Gram negative cells*:

Lysis buffer was prepared by dissolving 40mM TE buffer (pH 7.8), 20mM sodium acetate, 1mM EDTA and 1% (w/v) SDS in distilled water.

2.1.3.6 10xTBE Buffer

Dilute 108 g Tris Base, 55 g Boric acid and 9.3 g EDTA to 800 ml distilled water and adjust volume to 1L with additional distilled water.

2.1.3.7 1xTBE Buffer

Dilute 100 ml of 10xTBE to 1L with 900 ml of distilled water.

2.1.3.8 Acetate Buffer (0.2 M)

For initial pH 4.8 in the culture medium 430ml of 0.2 M acetic acid were placed in a 1000ml volumetric flask and was made up to the mark with 0.2 M sodium acetate.

2.1.3.9 Sample buffer

Sample buffer was prepared by adding 1.25 ml 0.5 M Tris-HCl (pH 6.8), 5 ml glycerol, 2 ml SDS (10% w/v), 0.5 ml β-mercaptoethanol and 0.5 mg bromophenol blue to 1 ml of distilled water. All samples were diluted 1:4 (v/v) and heated to 95° C for 5 minutes prior to loading SDS-PAGE gel.

2.1.3.10 Staining solution

The staining solution for staining SDS-PAGE gels was prepared by adding 100ml acetic acid, 450 ml methanol and 0.25 g coomassie blue to 450 ml distilled water.

2.1.3.11 Destaining solution

Destaining solution used for destaining SDS-PAGE gels was made by adding 100 ml acetic acid and 450 ml methanol to 450 ml distilled water.

2.1.4 Source of chemicals and materials

Chemicals were obtained from Fluka, Lennox and Sigma-Aldrich. Butter was obtained from Irish Dairy Board, Kerrygold, Dublin, Ireland. Extra virgin olive oil was obtained from Minerva S.A, Edible Oils, Thessaloniki, Greece.

2.2 Methods

2.2.1 Culture Conditions in Batch Fermentation Studies

Inoculum Preparation

1. BFL: Concentration approximately $3x10^8$ cfu/g.
   10 g of powder were added to 100 ml tap water and 10 ml of the mixture were then transferred into the flask containing 100 ml culture medium, (inoculum size approximately $3x10^6$ cfu/ml in 100 ml medium)

2. Gnz: Concentration $4x10^{10}$ cfu/ml.
   a) Dilution of 1 ml product in 100 ml quarter-strength Ringer solution was prepared and 1 ml from the Ringer was transferred to 100 ml culture medium adjusting the inoculum size to approx. $4x10^6$ cfu/ml.
   b) Different volumes of the liquid product (40 µl, 800 µl and 4 ml) were added in 100 ml culture medium adjusting the inoculum size to approx. 1 x 10^7, 10^8 and 10^9 cfu/ml, respectively,

3. FF: Concentration $6x10^6$ cfu/tablet or $2x10^7$ cfu/g. The inoculum size increased to 10^{10} cfu/g after 24 h at 30° C, 150 rpm.
   a) Dilutions were prepared after 24 h incubation adjusting the inoculum size to $10^6 – 10^7$ cfu/ml,
   b) one tablet was transferred into 100 ml medium.

The inoculum was transferred into a 250 ml Erlenmeyer flask containing 100 ml of the sterile culture medium, minimal medium or enriched nutrient medium, with butter (7.5 g/L) or olive oil (8 g/L). Controls were prepared in the same manner but without the addition of the bacterial population. The flasks were incubated for up to 14 days at 30^o C shaking at 150 rpm and sampled periodically for analysis.

When the BFL bioaugmentation product was combined with the Gram negative strain, *Pseudomonas putida* CP1 or *Pseudomonas putida* CP1::Tn7-gfp, the

bioaugmentation product BFL was prepared as before. *Pseudomonas putida* CP1, incubated overnight in nutrient broth at 30° C shaking at 150 rpm, achieving an OD=0.9-1 (~10^9 cfu/ml), was added in the culture medium at 1% (v/v) final concentration.

Pure cultures were grown overnight in nutrient broth at 30° C shaking at 150 rpm. The cultures were washed with PBS and the OD was adjusted to 0.7. A 5% (v/v) concentration of the culture was added to the culture media to investigate fat biodegration and biosurfactant production.

The fungal isolate was grown on malt extract agar at 22° C. An 8mm mycelial agar plug of a 5-day old culture was added to each flask containing 100ml medium.

2.2.2 Monitoring microbial populations

2.2.2.1 Pour plate technique

The pour plate technique was used by preparing serial dilutions in Ringer's solution and plating onto plate count agar. The enumeration of the total numbers of the bacteria present was carried out using a colony counter and were expressed as cells/ml. The plates were inoculated at 30^o C for 48 hours.

2.2.2.2 Measurement of biomass using dry weight

The biomass collection took place using centrifugation at 4000 rev/min for 15 min. Following the first centrifugation, the pellet was washed once with ethanol and once with hexane applying centrifugation (4000 rev/min for 15 min) each time as described by Papanikolaou, *et al*. (2001). The supernatants were collected for further analysis. The biomass was collected in pre-weighted McCartney universals and placed into a drying kiln at 85^o C for 24 hours. The dry biomass was weighed and expressed as g/L.

Growth rate

Growth rate (h^{-1}), μ, was calculated based on the number of cells produced per hour during exponential growth as:

$$\mu = \frac{LnN_2 - LnN_1}{t}$$

where N_2= cell density (dry weight) at time 2 (at the end of growth period)

N_1 = cell density (dry weight) at time 1 (at the beginning of growth period)

Ln = natural logarithm

t = time interval (time 2 – time 1) in hours

2.2.2.3 Determination of spore-formers

A sample of culture was heated at 80° C for 20 minutes to kill all vegetative cells. Serial dilutions in Ringer's solution were prepared and plated onto plate count agar as described previously. The plates were incubated at 30^{o} C for 48 hours. Total numbers of the spore-formers was expressed as cells/ml.

2.2.2.4 Monitoring of labelled green fluorescent protein

A green fluorescent protein labelled-*Pseudomonas putida* CP1 (*Pseudomonas putida* CP1::Tn7-gfp) (Figure 2.1) was introduced to the BFL product the same way as it was described before for the *Pseudomonas putida* CP1 and it was used to monitor the population. The *Pseudomonas putida* CP1::Tn7-gfp was visualized with an epifluorescent microscope, Nikon T*i*-E at 100 x magnification (Figure 2.1). Fluorescent colonies were observed at 4x magnification. Images were captured using the Nikon DS-U2 camera attached to the microscope

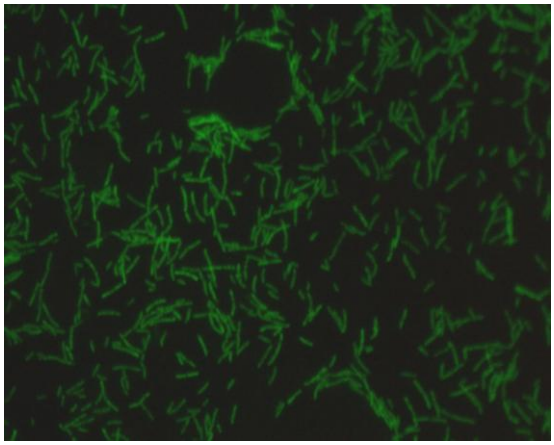


Figure 2.1 Green fluorescent protein labeled *Pseudomonas putida* CP1 (*P. putida* CP1::Tn7-gfp) under the microscope

2.2.3 Measurements of pH

pH was measured by using an Orion 420A pH meter.

2.2.4 Substrate utilization

2.2.4.1 Appearance of flasks

The appearance of the fats and the colour of the culture medium were observed throughout the fermentation studies and emulsification was noted. Changes in the colour of the culture medium indicated hydrolysis and uptake of the substrate.

2.2.4.2 Total fat determination by gravimetric analysis

For the determination of total fat a modified method from Shikoku-Chem (1994) and Brooksbank *et al*. (2007) was used. The pH was measured by using an Orion 420A pH meter. The samples were first acidified to pH 2 or lower with 1M HCl. Lipids were extracted by transferring the contents of the flasks into separating funnel and adding 30-40 ml n-hexane (high purity, 97%). The funnel was shaken vigorously and was left to stand to allow the layers to separate. The aqueous phase was drained off and the organic phase transferred to a conical flask. The aqueous phase was re-extracted again twice more with hexane and one time with chloroform (high purity, 97%). If a clear solvent layer cannot be obtained and an

emulsion of more than about 5 mL exists, emulsion and solvent layers were drained into a glass centrifuge tube and centrifuged for 5 min at 4000 rpm. The aqueous layers and any remaining emulsion were recombined in the separatory funnel. The centrifugation step was repeated whenever emulsion persisted in subsequent extraction steps. Finally, the organic phase was transferred to a vial and anhydrous sodium sulphate was added (approx. 3g) and then filtered through Whatman number 1 filter paper into a pre-weighed round flask. At the end the filter was rinsed with an additional 10 to 20 ml solvent. The flasks were then put in a warm water bath and the solvent was evaporated under oxygen-free nitrogen. The flasks were weighed again and the remaining fat was determined by subtracting the weight of the flask from the total weight of the flask and fat. The degradation and removal of the fat was calculated according to the following equation:

$$\text{Fat removal (\%)} = \frac{\text{Initial fat level (g/L)} - \text{Fat level after treatment (g/L)}}{\text{Initial fat in the medium (g/L)}} \times 100$$

Rate of substrate removal

Rates of substrate removal were calculated following the lag period and were expressed as g/L substrate removed per unit time. The expression of the remaining substrate in a plot against time developed a slope with equation:

$$y = ax + b,$$

where “a” represents the rate of fat removal after the lag period.

Rate of specific substrate removal

Rates of specific substrate removal were calculated following the lag period and were expressed as g/L substrate removed per g/L dry weight per time (g/g/h).

Yield coefficient

The determination of the yield coefficient, $Y_{X/S}$ was based on the degree of cell dry weight produced (X) per substrate consumed (S) [(g dry weight)/g substrate consumed)].

2.2.4.3 Fat degradation analysis by Thin Layer Chromatography (TLC)

The extracted fat was analysed for its hydrolyzed products using thin layer chromatography (TLC). TLC was performed using glass silica gel plates with a mobile phase of hexane, diethyl ether and acetic acid (70:28:2, v/v/v) as described by Cipinyte *et al*. (2009). The spots of oil and hydrolysis products were visualized by saturated iodine steam.

2.2.4.4 Fatty acid analysis by Gas Chromatography (GC)

Derivatization of the fatty acids

Using a modified method as described by Metcalfe and Schmiz (1961) and Brooksbank *et al* (2007), the extracted lipids, approx. 0.23 g or less, were transferred to a (5 ml) Teflon-lined screw cap vial with 4 ml of boron trifluoride-methanol (Sigma) complex in a nitrogen-free atmosphere. After heating to 100°C for 1 h, 3 ml of water and 6 ml of pentane were added to the derivatized samples and gently shaken to extract and clean the fatty acid methyl esters. The pentane layer was transferred to a new vial and the extraction process was repeated using a further 6 ml of pentane. The pentane was then evaporated under an oxygen free nitrogen flow and the fatty acids dissolved in 2ml hexane.

Gas Chromatography

Methyl esters of the fatty acids were analysed by Gas Chromatography, where 1µl aliquots of the samples was injected onto a Varian CP3800 Gas Chromatograph equipped with a flame ionization detector. The column was a Varian Select™ column Select FAME 100 m x 0.25 mm with a film thickness of 0.25 µm. The injector and the detector temperature were maintained at 260° C. The oven temperature was programmed according to the method described by Brooksbank *et al*. (2007) and was as follows; isothermal at 80° C for 2 min, 40° C/min to 160°

C, 0.5° C/min to 170° C, 10° C/min to 250° C and isothermal at 250° C for 10min. Chromatograms were recorded on a Carlo Erba Mega Series integrator.

A calibration was performed using a standard mixture of 19 fatty acid methyl esters (Grain Fatty Acid Methyl Ester Mix – Sigma) and the fatty acids of the extracted lipids were identified by comparison of the relative retention times of their methyl esters with those of the known standards. Eleven of the nineteen fatty acids of the standard mix were mainly used for the identification of the unknown fatty acids as being the most common. The concentration of each fatty acid contained in the standard mix, the retention time and the peak area of a typical injection of the FAME mixture analyzed in the Varian CP3800 GC are presented in Table 2.2 and traces are shown in Figure 2.2.

Table 2.2 Notation, chemical name, quantity present, retention time (RT) and peak area of a typical injection of the standard fatty acid mixture.

Notation	Chemical name	Amount mg/ml	RT (min)	Peak Area
Solvent	Hexane		9.615	90.0353
C8:0	Caprylic acid	0.15	12.181	0.1077
C10:0	Capric acid	0.28	13.814	0.1977
C12:0	Lauric acid	0.6	16.221	0.4151
C14:0	Myristic acid	0.3	20.101	0.2132
C14:1n9c	Myristoleic acid	0.18	21.808	0.1246
C15:0	Valerenic acid	0.18	22.942	0.1228
C16:0	Palmitic Acid	1.23	26.009	0.7996
C16:1n9c	Palmitoleic Acid	0.60	27.300	0.3845
C17:0	Heptadecanoic Acid	0.30	28.336	0.1899
C18:0	Stearic Acid	0.60	30.266	0.3953
C18:1n9t	Elaidic Acid	0.25	30.771	0.1610
C18:1n9c	Oleic Acid	2.10	31.066	1.3676
C18:2n6c	Linoleic Acid	1.55	32.219	1.0089
C20:0	Arachidic Acid	0.20	33.335	0.1296
C20:1	cis-11-Eicosenoic Acid	0.69	33.482	0.4473
C18:3n3	Linolenic Acid	0.19	33.998	0.1218
C22:0	Behenic Acid	0.18	35.904	0.1086

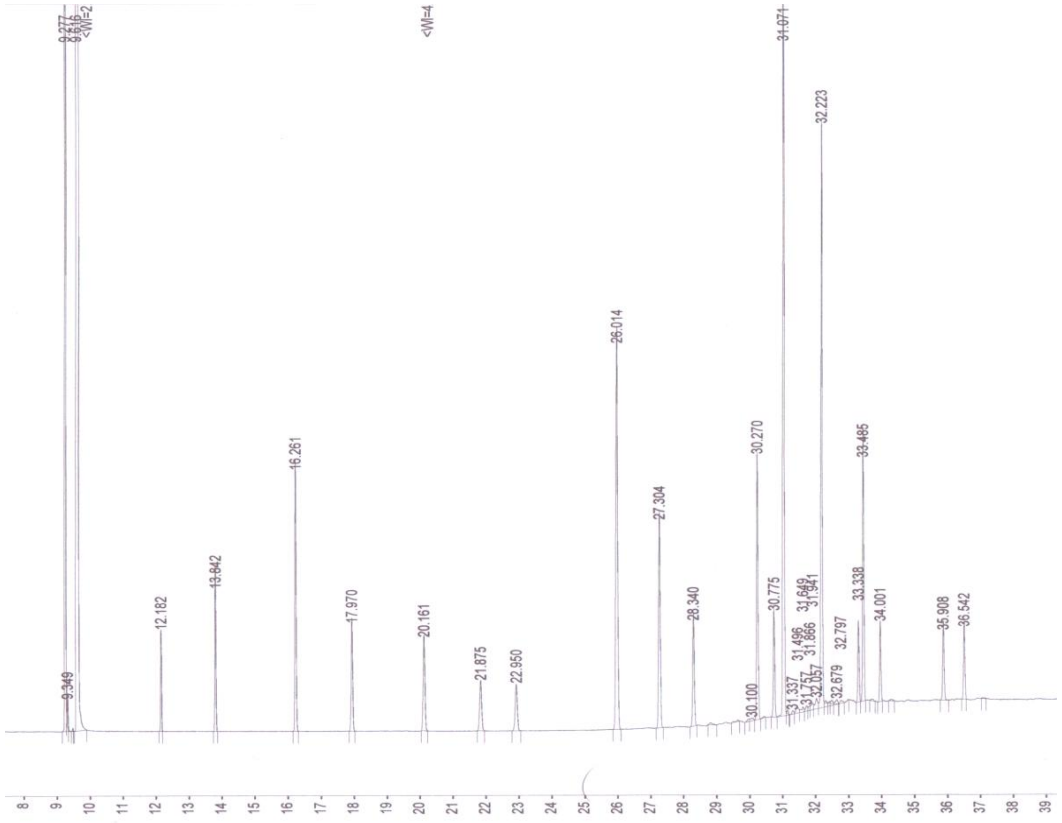


Figure 2.2 Traces of the standard FAME mixture using a Varian Select^{TM} column Select FAME (100 m x 0.25 mm, 0.25 µm film thickness)

The percentages of each fatty acid were calculated by dividing the area of its peak with the sum of the areas of all the peaks of interest:

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

where,

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

A_i = area of peak corresponding to component *i*

$\sum A$ = sum of the areas of peaks of components of interest

Quantification of Lipid FAME

The concentration in mg/ml of each fatty acid or [x] present in the extracted lipids can be calculated using the following equation,

$$[x] = A_x \text{ sample } \frac{[x \text{ Standard}]}{A_x \text{ Standard}}$$

where A_x sample, Ax Standard and [x Standard] refer to the peak area of the fatty acid of interest, the peak area and the concentration of the standard of the particular fatty acid in question, x.

2.2.4.5 Determination of the intracellular lipid

Total cellular lipid was extracted from dry biomass with a chloroform-methanol mixture (30 ml, 2:1 v/v). The samples were filtered through Whatman number 1 filter paper in a pre-weighted flask and the solvent was evaporated under oxygen free nitrogen. The intracellular lipid was determined gravimetrically and was expressed as g/L (Papanikolaou *et al.*, 2001).

2.2.5 Tests used in the identification of the bacteria

2.2.5.1 Colony morphology

The colony morphology was examined for shape, margin, surface, texture and color of the colony following growth on standard plate count agar at 30° C for 24 to 48 hours.

2.2.5.2 Gram staining

The Gram stain was carried out on 24 hour cultures according to the method described by Harley and Prescott (1990). Gram positive cells appeared purple and Gram negative cells red.

Controls: Positive – *Bacillus subtilis*
Negative – *Pseudomonas putida*

2.2.5.3 Cell morphology

The bacterial cells were observed microscopically using x100 oil immersion lens (Nikon). The cells were characterized for their shape and cellular arrangement.

Controls: Rods – *Pseudomonas putida*
Cocci – *Staphylococcus aureus*

2.2.5.4 Spore formation

A smear of a 48 hour culture was prepared and the spore stain was carried out as described by Harley and Prescott (1990). Endospores were detected microscopically using x100 oil immersion lens. The stained spore appeared green and the vegetative cell red.

Controls: Positive – *Bacillus subtilis*
Negative – *Pseudomonas putida*

2.2.5.5 Motility

A 24 h culture was examined microscopically in "hanging drop" preparations, using a x100 oil immersion. A "hanging drop" slide was prepared by placing a loopful of the bacterial suspension onto the centre of a coverslip. A depression slide onto which a ring of Vaseline had been spread around the concavity was lowered onto the coverslip, with the concavity facing down over the drop. When a seal had formed, the hanging drop slide was turned over and examined under the microscope.

Controls: Motile: *Pseudomonas putida*
Non-motile: *Enterococcus faecalis*

2.2.5.6 Oxidase activity

Oxidase strips (Oxoid) impregnated with NNN'N' tetramethyl-p-phenylene-diamine dihydrochloride were used for the detection of bacterial cytochrome oxidase enzyme. The formation of a purple colour within 5-10 seconds indicated oxidase positive results.

Controls: Positive – *Pseudomonas putida*
Negative – *Escherichia coli*

2.2.5.7 Catalase

A loopful of 24h culture was transferred into a drop of 3% (v/v) hydrogen peroxide. Effervescence, caused by the liberation of free oxygen as gas bubbles, indicated a positive result.

Controls: Positive – *Bacillus subtilis*
Negative – *Streptococcus pyogenes*

2.2.5.8 Extracellular Enzymes

The hydrolytic activity of the isolates was detected on nutrient agar containing the relevant substrate following incubations at 30° C.

- *Lipase production*

Lipase production was investigated using tributyrin, Tween 20 and Tween 80 agars. Enzyme production was indicated by a clearing surrounding the colonies. In the Tween 20 and Tween 80 agar plates, fatty acids produced as a result of Tween20 (or 80) degradation react with $CaCl_2$, forming opaque zones around the colony (Sierra, 1957; Paparaskeyas *et al*., 1992; Sanchez-Porro, *et al*., 2003).

Controls: Positive – *Bacillus subtilis*
Negative – *Pseudomonas putida*

- *Protease production*

Protease production was investigated by growing the organisms on casein agar. Enzyme production was indicated by a clearing of the agar on the casein plates surrounding the colony.

Controls: Positive – *Bacillus subtilis*
Negative – *Pseudomonas putida*

- *Amylase production*

Amylase production was investigated by growing the organisms on starch agar. Enzyme production was detected by staining the starch agar plates with iodine vapors. Unstained zones surrounding the amylase-producing colonies indicated amylase production (Rashid and Imanaka, 2008).

Controls: Positive – *Bacillus subtilis*
Negative – *Pseudomonas putida*

- *Xylanase and cellulase production*

Xylanase and cellulase production was investigated by growing the organisms on xylan and carboxymethylcellulose sodium salt agar plates. Enzyme production was detected by staining the xylan and carboxymethylcellulose sodium salt plates with 2% congo red for 30 minutes and washing with NaCl and distilled water for 30 min each (Rashid and Imanaka, 2008). A second method used for confirmation involved the addition of a few drops of 2% (w/v) congo red to the agar medium before autoclaving. The plates were observed for clear zones surrounding the colonies.

- *Lactose Fermentation*

MacConkey agar plates (Oxoid) were used for detecting enteric lactose fermenting bacteria. A 24h old culture was inoculated onto MacConkey plates. Incubations were carried out at 30° C for 48h. The change of the red colour around the colony to yellow indicated a positive reaction.

2.2.5.9 Biosurfactant Production

Biosurfactant production was investigated using a modification of the drop-collapse technique adapted from the method described by Bodour and Miller-Maier (1998) and Youssef *et al*. (2004). The drop-collapse was performed in a polystyrene lid of a 24-well plate. Before use each lid was rinsed three times each with hot water, ethanol and distilled water, and dried. After preparation, 100µl of mineral oil were added to each well. The lid was equilibrated for 1h at room temperature. Then 20µl of culture supernatant were added to the surface of the oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant producing cultures giving flat drops were scored as positive, with scoring system ranging from '+' to '++++' corresponding to partial to complete spreading on the oil surface. Those cultures that gave round drops were scored as "-" indicative as the lack of biosurfactant production (Youssef *et al.*, 2004).

For the quantitative test, a standard curve was prepared for SDS surfactant by adding drops containing varied surfactant concentrations to each well (Figure 2.3). After 1min the diameter of each drop was measured and droplets were examined at a standard time (1min) to ensure consistent results. Standard curves were prepared by plotting the surface concentration versus the drop diameter and these were used to determine surfactant concentrations in unknown samples (Bodour and Miller-Maier, 1998).

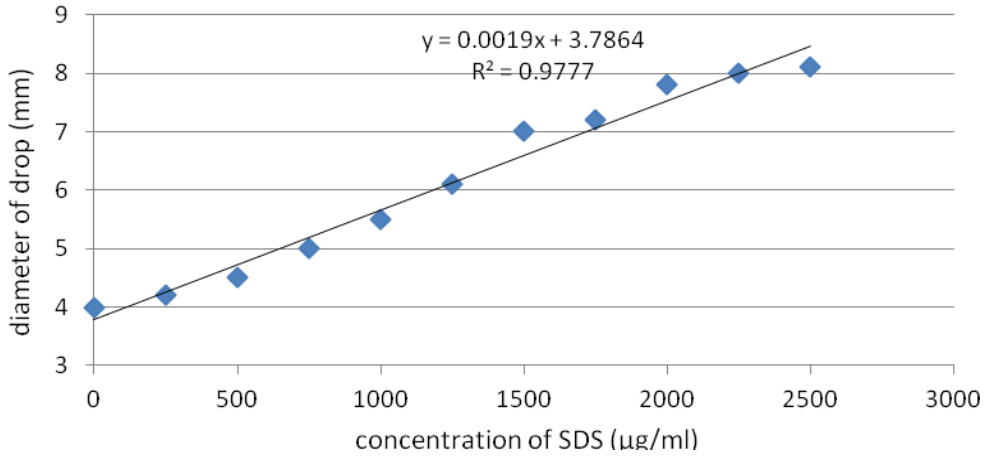


Figure 2.3. Standard curve for drop collapse quantification

2.2.5.10 Biosurfactant Extraction

Biosurfactant produced by the fungal isolate was extracted according to the method described by Das *et al.* (2008). The cultures were centrifuged to remove cells and acid precipitation of the supernatant was performed by adding concentrated hydrochloric acid (HCl) to lower the pH to 2. The acidified supernatant was kept at 4° C for complete precipitation of the biosurfactant. The precipitate thus obtained was centrifuged at 4000 rpm for 20 min to get the crude biosurfactant as a pellet. This pellet was resuspended in distilled water (dH_2O) followed by pH adjustement to 7.0-7.5. The samples were then lyophilized using a freeze-drying machine LABCONCO.

2.2.6 API System

2.2.6.1 API 20NE system for Gram negative Bacteria

The API identification system API20NE (BioMerieux, Marcy-l' Etoile, France, http://www.biomerieux.com) for non-enteric Gram-negative rods was used according to the manufacturer's instructions. An overnight nutrient broth culture (10 ml) was harvested and washed twice with sterile phosphate buffer solution (4000 rpm for 15 minutes). The pellet was resuspended in 0.85% (w/v) NaCl (10 ml). The suspension was then used to inoculate a portion of the tests. For assimilation tests, 200 µl of this suspension was used to inoculate auxiliary medium supplied by the manufacturer and this was then used to inoculate the remaining tests. The strips were read and interpreted after incubation at 30° C for 24 hours. Identification was obtained using the Analytical Profile Index: the pattern of the reactions obtained was coded into a numerical profile. On a results sheet the test were separated into groups of three and a number 1, 2 or 4 was indicated for each. By adding the numbers corresponding to positive reactions within each group, a 7-digit number was obtained which constituted the numerical profile. Identification was then obtained using the identification software (API 20NE V6.0 database, apiweb.biomerieux.com) by manually entering the 7-digit numerical profile. The profile was listed along with the percentage of

identification – an estimate of how closely the profile corresponded to the taxa relative to all the other taxon in the database and the T index – an estimate of how closely the profile corresponded to the most typical set of reactions for each taxon. The appearance of the positive and negative reactions are shown in Figure 2.4. *Pseudomonas putida* CP1 was used as control.

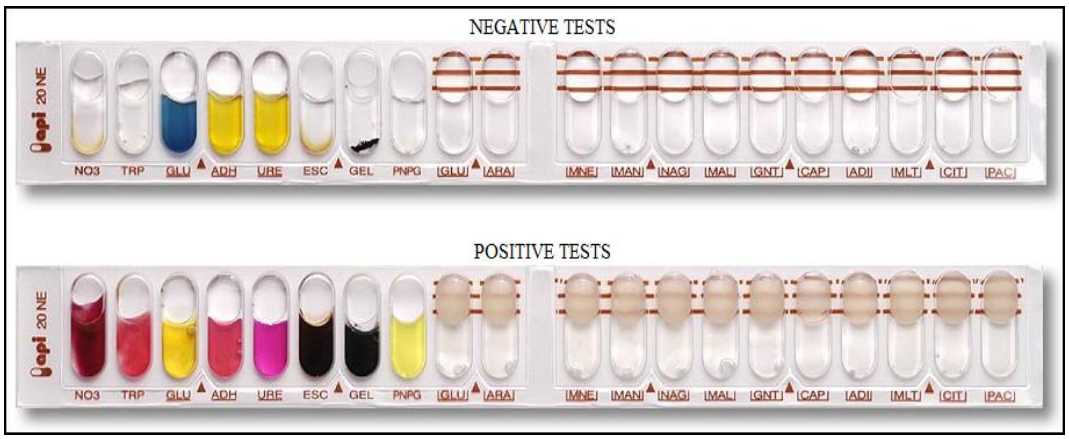


Figure 2.4 Negative and positive reactions on API 20NE

2.2.6.2 API 50CHB and API 20E for Gram positive spore-forming bacteria

Identification of *Bacillus* isolates was performed using a matrix of results from tests in the API 20E and API 50CHB strips, 12 tests in the API 20E strip and 49 tests in the API 50CHB strips (Figure 2.5), as indicated by Logan and Berkeley (1984). The latter contain carbohydrate substrates for the detection of assimilation or acid production (according to the suspension medium used). The inocula were prepared as previously described. For each strain two suspensions were further prepared, one for each API strip: (i) for API 20E strip in 4 ml sterile normal saline (0.85% (w/v) NaCl), and (ii) for API 50CHB strips in 10 ml of API 50CHEB (*Enterobacteriaceae*/*Bacillus*) medium. Only the first twelve tests of the API 20E strip were inoculated, the last eight being carbohydrate tests duplicated in the API 50CHB strips. Strips were incubated at 30° C for 48 h and read at 24 and 48 h. Results were scored according to the manufacturer's instructions. A test scoring positive at either reading time was considered positive. *Bacillus subtilis, Bacillus amyloliquefaciens* and *Bacillus licheniformis* were used as controls.

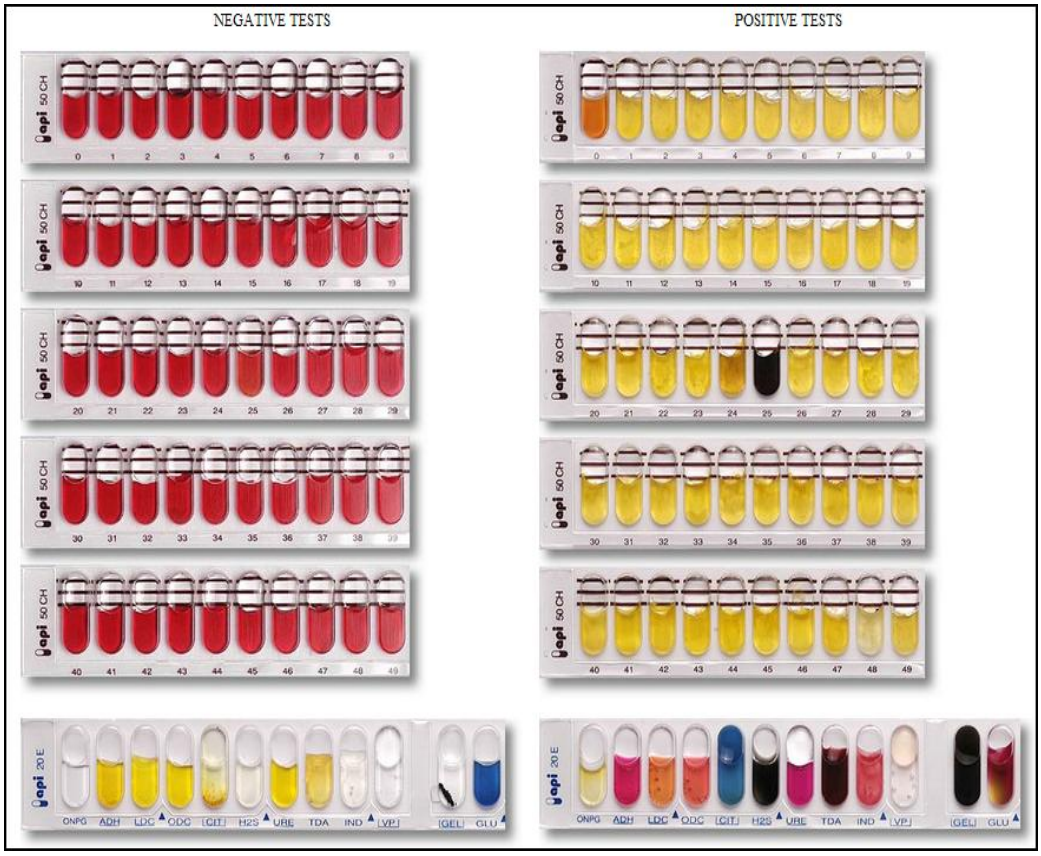


Figure 2.5 Negative and positive reactions on API 50CHB and API 20E

2.2.7 Genomic Identification using 16S Sequencing

2.2.7.1 DNA extraction

- *Alkaline Extraction*

The alkaline extraction method was used as described by Masco *et al*. (2007). According to the method, unpurified DNA is extracted after lysis of the cells. The cells were placed into eppendorf tubes and 20 µl of reagent (2.5 ml 10% SDS, 5.0 ml 1N NaOH and 92.5 ml MilliQ water) were added for lysis of the cells. The samples were incubated at 95^{o} C for 15 min followed by centrifugation at 13000 rpm. After the addition of 180 µl MilliQ water, the samples were centrifuged at 13000 rpm for 5 min and stored at -20^{o} C.

- *DNA extraction from Gram positive bacteria*

The bacterial genomic DNA was extracted from the cells in the mid-log phase (OD600 of 0.5 – 1) grown overnight in 10 ml nutrient broth at 30° C, 150 rpm using a modification of the method described by Gevers (2001). 1 ml of the culture was centrifuged (4000 rpm, 10 min, 4° C) and the cells were washed in 1ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The pellet was suspended in 300 µl STET buffer (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl, 50 mM EDTA, pH 8.0). 25 µl of lysis buffer were added for each sample (50 mg lysozyme/ml TE buffer) and were incubated at 37^{o} C for 1 h. After addition of 40 µl preheated (37° C) 20% SDS in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), cells were vortexed and incubated at 37^{o} C for 1 h. One hundred µl of TE buffer was added and the lysate was extracted with 1 volume phenol/chloroform/isoamylalcohol (25:24:1 v/v). The samples were mixed gently and phases were separated by centrifugation at 13000 rpm for 5 min. The supernatant was removed to a fresh tube and equal volume of phenol/chloroform/isoamylalcohol was added. The tube was mixed gently and centrifuged again. The aqueous phase was transferred into a fresh tube and carefully mixed with 70 µl 5 M NaCl and 1 ml cold 95% ethanol. DNA precipitated on ice (-20 ^{o} C) for 15 min to 1 h. DNA was collected by centrifugation for 30 min at 13000 rpm (4^{o} C) and the pellet washed with 500 µl ice-cold 70% ethanol (5 min at 13,000 rpm). The supernatant was removed and the samples were let to dry. The pellet was suspend in 100 µl TE for increasing the solubility of the DNA. 1 µl RNase was added and the solution was incubated at 37^{o} C for one hour and stored at -20^{o} C until their next use.

- *DNA extraction from Gram negative bacteria*

This method was modified from that described by Chen and Kuo (1993) and used to prepare genomic DNA. 1 ml of an overnight culture grown in 10 ml nutrient broth was centrifuged (4000 rpm, 10 min, 4° C) and the cells were washed in 1ml TE buffer. The pellet was resuspended in 200 µl of lysis buffer (2.1.3.5-b) and lysed by vigorous pipetting. Then 66 µl of a 5M NaCl solution was added, the

tube was mixed by inversion and the viscous mixture was centrifuged at 13,000 rpm for 10 min at 4° C. The supernatant was transferred into a fresh tube and an equal volume of phenol/chloroform/isoamylalcohol (25:24:1 v/v) was added and mixed gently by inversion 50 times. After centrifugation at 13,000 rpm for 5 min, the supernatant was removed to a fresh tube and equal volume of phenol/chloroform/isoamylalcohol was added. The tube was again mixed gently and centrifuged at 13,000 for 5 min. The extracted supernatant was transferred to a fresh tube and carefully mixed with 70 µl 5 M NaCl and 1 ml cold 95% ethanol. DNA precipitated on ice (-20^{o} C) for 15 min to 1 h. DNA was collected by centrifugation for 30 min at 13000 rpm (4^{o} C) and the pellet washed with 500 µl ice-cold 70% ethanol (5 min at 13,000 rmp). The supernatant was removed and the samples were let to dry. The pellet was suspended in 100 µl TE for increasing the solubility of the DNA. DNA precipitation and washing were carried out as described previously for the Gram positive cells. 1 µl RNase was added and the solution was incubated at 37^{o} C for 1 h and stored at -20^{o} C until their next use.

2.2.7.2 Nanodrop method

The concentration and quality of the isolated DNA were verified by measuring the absorbance ratio at 260/280 nm using a NanoDrop system as illustrated in Figure 2.6. The appropriate level of OD260/280 and OD260/230 is 1.8-2.2 (Masco *et al.*, 2007). Higher values of this ratio indicates presence of RNA, lower values indicate excessive amounts of protein.

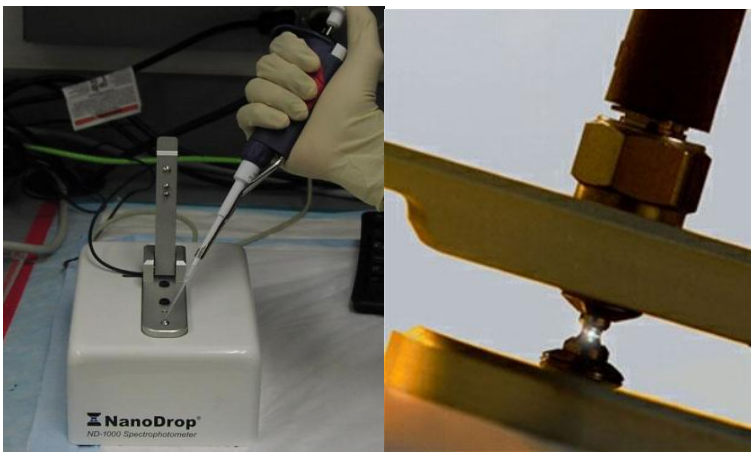


Figure 2.6. NanoDrop instrument

2.2.7.3 Gel agarose electrophoresis

The gel, prepared as described in 2.1.2.9, was poured into a electrophoresis apparatus and a comb was inserted to make the wells. When the gel was solidified, the comb was removed and the gel was placed in the device and immersed in 1xTBE buffer. Five µl of a DNA sample were mixed with 2µl loading dye (MyBio Ltd., www.mybio.ie) and the total 7µl sample was transferred into the wells. Two microliters of marker, 10 kb smartladder (MyBio Ltd., www.mybio.ie), were placed in the first well for the comparison of molecular weights. The electrophoresis device, Hybaid Electrophoresis system, configured to 90 V for 45 minutes. The bands on the gel became visible under UV light and the gel was photographed using the Imagemaster VDS image analysis system. The appearance of the extracted DNA is shown in Figures 2.7.

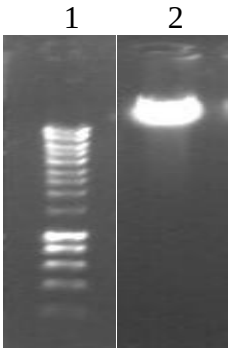


Figure 2.7 Appearance and quantification of DNA in gel agarose. Lane 1:DNA ladder, Lane 2:DNA sample

2.2.7.4 Amplification of the 16S rRNA gene

The DNA concentration of all the samples was adjusted to 40ng prior to the amplification step, ensuring the same DNA concentration in all the samples (200ng). The amplification of the 16S rRNA gene was conducted with a total volume 50µl containing 25µl of MyTaq Red mix Polymerase (MyBio), 1µl of each primer (10µM), 5µl DNA and 18µl MilliQ water for each reaction. The universal primers (Sigma) in Table 2.3 were to amplify the 16S rRNA gene from the bacterial isolates. The specificity of the primers was confirmed using gel agarose electrophoresis. The PCR product yielded an amplicon sized around 1.5 kbp. as shown in Figure 2.8. The amplified samples were stored at -20^{o} C.

Table 2.3 PCR amplification primers for 16S rRNA

Primer name	**Primer sequence**	**Reference**
pA (forward)	5'-AGA GTT TGA TCC TGG CTC AG-3'	Vardhan *et al.*, 2011
pH (reverse)	5'-AAG GAG GTG ATC CAG CCG CA-3'	Vardhan *et al.*, 2011

All the reactions were amplified in a Px2 Thermal Cycler (Thermo Electron Corporation, MA, USA) OR The PCR program was held in a thermal cycle GeneAmp PCR systems 9600 as described by Gomaa and Momtaz (2007):

- *Denaturation*: 95^{o} C for 5min
- *Annealing*: 95^{o} C for 1 min, 55^{o} C for 1 min and 72^{o} C for 2 min (30 cycles)
- *Final extension*: 7 min at 72^{o} C

The PCR products (50 µl) transferred to an agarose gel (2.1.2.9) produced a band as described in Figure 2.8. The agarose gel containing the relevant 16S rRNA fragment was excised from the gel with a scalpel while viewing the band on a UV transilluminator (Vilber Lourmat, Tourcy, France). The fragment was then recovered from the gel using a purification kit (Illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) following the manufacturer's instructions. The recovered fragments were re-amplified as previously described (2.2.7.4) to increase the concentration and the PCR products (50 µl) were purified again using the commercial kit. After the addition of the elution buffer the samples were dried using a vacuum drier, Savant DNA Speed Vac DNA110. The dried samples were diluted in sterile MilliQ water.

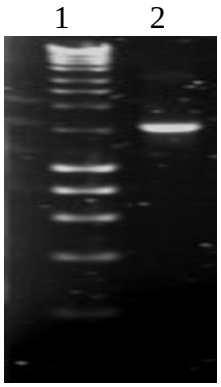


Figure 2.8 16S rRNA PCR amplified product (1.5kp) in agarose gel. Lane 1: DNA ladder, Lane 2: 16S rRNA PCR product

2.2.7.5 16S rRNA Sequencing

The 16S rRNA gene sequencing was performed by Eurofins MWG Operon sequencing service (Germany) (http://www.eurofinsdna.com) using the pA primer (Table 2.3).

2.2.7.6 Bioinformatic sequence analysis

Sequences were verified using the online BLAST searches on the NCBI website (www.ncbi/nlm/nih/gov). Nucleic acid alignments, as well as phylogenetic tree were performed using the ClustalW program on the European Bioinformatics Institute website (www.ebi.ac.uk).

2.2.8 SDS-PAGE

Proteins were resolved by polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), which was performed using 12% (w/v) gradient gels overlaid with a 4% (w/v) stacking gel. The gel plates were cleaned prior to use with hot soapy water, rinsed with distilled water, then rinsed with ethanol and allowed to dry. Seals were placed between the gel plates and the plates were clamped together. The components for both the separating gels and stacking gels were mixed. The resolving gel was poured first, about three-quarters of the way up the gel. This gel was overlaid with ethanol (to prevent air bubbles) and allowed to set for 30 minutes. The ethanol was then removed and the stacking gel was poured above the resolving gel. A comb was then inserted into the stacking gel and the gel was allowed to set for 60 min.

Total cell protein was extracted as described by Kaynar and Beyatli (2008). The isolates were incubated at 30° C for 48h in Nutrient Broth (Oxoid). The cells were washed twice with phosphate buffer saline (pH 7) and centrifuged at 4000 rpm for 15 min. The pellets were resuspended in 1ml sterile distilled water and homogenized using ultrasonic treatment (70% power, for 5 min performing 30 sec cool every 1 min) with a Branson digital sonifier model 102c. Samples were transferred to the sample buffer (1:4 dilution) (2.1.3.9) before the electrophoresis

was carried out. The samples were boiled for 5 min and 20 µl were transferred to each well of the gel. The samples were electrophoresed at 15 mA until the tracking dye entered the resolving gel, at which time the mA was increased to 30 mA for 3-4 hours.

Coomassie brilliant blue staining solution (2.1.3.10) was used to stain the SDS-PAGE gels. The gels were stained for 3-4 hours at room temperature with gentle shaking. The gels were rinsed in milli-Q water and transferred into destaining solution (2.1.3.11), then gently shaken at room temperature overnight, until blue bands and a clear background were obtained. Fresh destaining solution was added if required. The gels were kept in milli-Q water after destaining. The gels were observed visually for banding and compared with a wide range molecular weight standard (6,500-205,000 Da) was used as a marker. The gels were imaged using a regular camera.

2.2.9 FAME

Cultures were inoculated in 10 ml nutrient broth and incubated overnight at 30° C, at 150 rpm. Cells were spun down at 4000 rpm for 15 min and washed twice with PBS. Whole-cell fatty acids were extracted from the pellet according to the method described by Heipieper and Bont (1994). The pellet was resuspended in chloroform/methanol (2:1, v/v) and stored overnight. One volume of water and one volume of chloroform were added and mixed well. The phases were separated with centrifuging. The chloroform layer which contains the lipids was filtered and evaporated under oxygen-free nitrogen flow. The fatty acids were dissolved in 3ml boron trifluoride methanol solution (14%) and heated at 95° C for 15 min. After the samples were cooled down, 3 ml of distilled water and 6ml of hexane were added to extract and clean the fatty acid methyl esters. The mixture was shaken and the organic phase was collected. The fatty acids were extracted two more times. The hexane was evaporated under an oxygen-free nitrogen flow and the methyl ester fatty acids were dissolved in 2 ml hexane and stored at -20° C. The derivatized fatty acids were run in a GC and the fatty acids were identified as described previously (2.2.4.4).

2.2.10 Extracellular polymeric substances (EPS) extraction and analysis

2.2.10.1 EPS Extraction

The extracellular polymeric substances (EPS) were extracted using a modification of the method of Eboigbodin and Biggs (2008). 10 ml of cells were harvested by centrifugation at 4000 rpm for 15 min at 4° C. The cell pellets were used for extraction of the bound EPS, and the supernatant was used for the free EPS extraction. Both bound and free EPS were stored at -20° C until needed for further analysis.

- *Bound EPS Extraction*

The cell pellets were washed twice with 0.9% NaCl to remove any traces of the media. The washed cells were resuspended in 1:1 volume of solution 0.9% NaCl and 2% EDTA then incubated for 60 min at 4° C. The supernatant was then harvested by centrifugation at 4000 rpm at 4° C for 30 min and then filtered through 0.45 µm membrane (Pall).

- *Free EPS Extraction*

After the initial harvesting by centrifuge, the supernatant collected were recentrifuged at 4000 rpm for 30 min at 4° C to remove residual cells, and then the supernatant containing free EPS was precipitated with 1:3 volume ethanol and stored at -20° C for 18 h. Free EPS were then removed by centrifugation at 4000rpm for 14min at 4° C. The extract was resuspended in ultrapure water and dialyzed against ultrapure water to removed ethanol.

2.2.10.2 EPS Biochemical Analysis

- *Carbohydrate determination*

The test was conducted as described by Dubois *et al.*, (1956). 2 ml of sugar solution was added to a universal and 0.5 ml of 5% (w/v) phenol solution were added. Then 2.5 ml of concentrated sulphuric acid were added. The universals

were allowed to stand for 10 min and later placed in a waterbath at 30^0C for about 20 min. The concentration of polysaccharides in the solution was determined according to a calibration curve with glucose as the standard (Figure 2.9). The absorbance was measured at 490 nm.

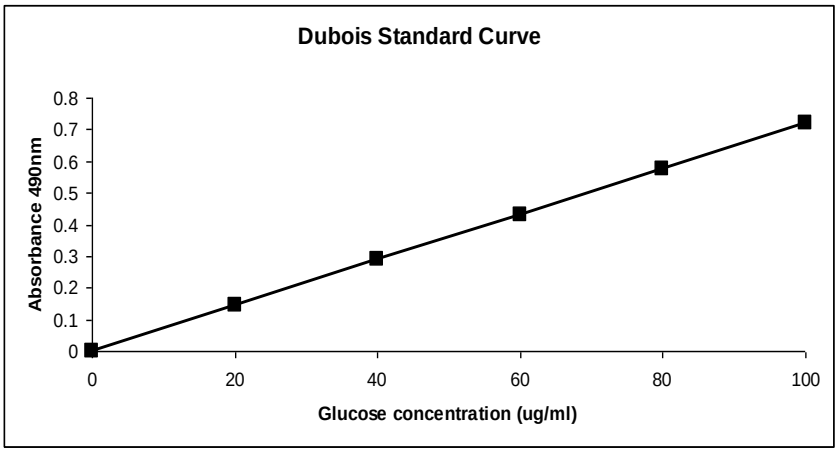


Figure 2.9 Calibration curve for Dubois assay

- *DNA determination*

The test to determine the DNA content of the sample was conducted accordingly to Burton *et al*. (1968). Diphenylamine reagent was prepared by adding diphenylamine to acetic acid and sulphuric acid. On the day of usage, 100 µl acetaldehydes (0.16% v/v) was added to 20 ml of reagent. 2 ml of diphenylamine reagent was added to 1 ml of sample and left to incubate at room temperature overnight. The absorbance was measured at 600 nm. The concentration was determined by comparison to a standard curve using high polymerised calf thymus DNA (Sigma) as a standard (Figure 2.10).

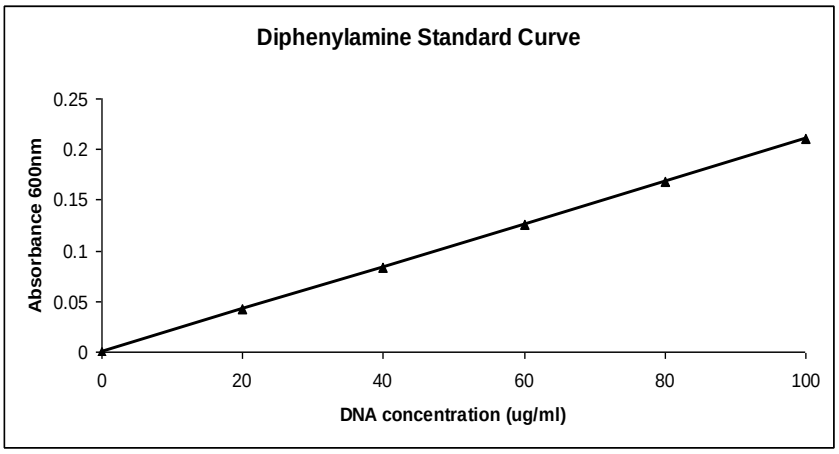


Figure 2.10 Calibration curve for Diphenylamine assay

- *Protein determination*

The assay was performed as written according to Bradford (1976). 1 ml of Bradford reagent (Sigma) was added to 1 ml of sample and immediately vortexed. The mixture was left at room temperature for 5 min, and the colour was measured at 595 nm. The concentration of the protein was determined by comparison to a standard curve with bovine albumin serum (BSA) from Sigma (Figure 2.11).

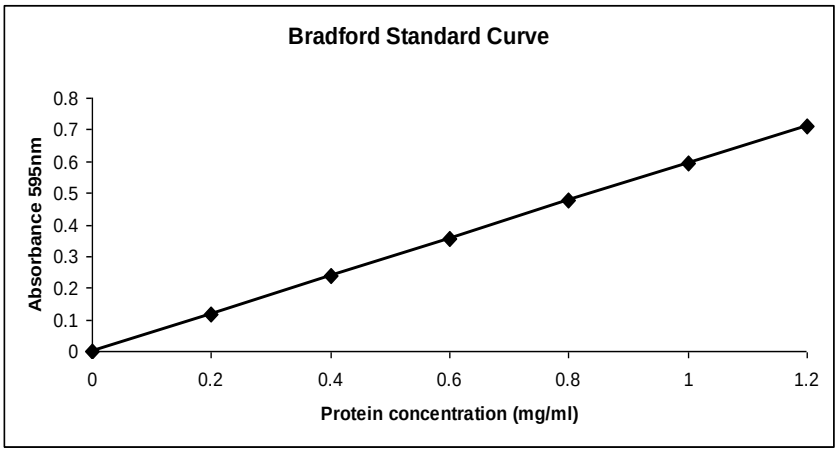


Figure 2.11 Calibration curve for Bradford assay

2.2.11 Characterization of the fungal isolate

The fungus was grown on malt extract agar at 22° C. Colour of the mycelium was noted, growth and sporulation were also examined. The fungus was examined microscopically using Nikon T*i*-E at 40x and 100 x magnification. Images were captured using the Nikon DS-U2 camera attached to the microscope. Observations were carried out for the presence of septae and spore-producing structures. Identification of the fungus was confirmed by DSMZ Identification Service (Germany, http://www.dsmz.de).

2.2.12 Data analysis

Experiments and analyses were performed in triplicate. Microsoft Office Excel Version 2007 was used for all statistical analysis of the experimental data including mean values, standard error means and equation of the line of the best fit.

3 RESULTS

3.1 An investigation of the biodegradation of fats and oils by three commercial bioaugmentation products

The biodegradation of butter (1% w/v) and olive oil (1% v/v) by three commercially available bioaugmentation products was investigated. The three products were coded Gnz, FF and BFL. The degradation was investigated in aerobic batch culture using two media, minimal medium and enriched nutrient medium, at 30° C, shaking at 150 rpm for up to 14 days. Control flasks which did not contain the bacterial population were also incubated under the same conditions. The ability of the mixed populations to degrade the fats was evaluated by determining the level of total fat present at the end of the cultivation period.

3.1.1 Biodegradation of Butter

When the mixed microbial populations were grown on butter (7.5 g total fat/L), the presence of the butter in the medium was obvious in solid form at the beginning of the study. During the incubation period, the butter was emulsified in the presence of BFL and FF but not in the presence of Gnz (Figure 3.1). In the case of BFL the emulsion was homogeneous, however in the case of FF distinct balls of culture and fat were observed. When the fat removal was determined, no fat removal was observed for Gnz following 14 days incubation using inocula sizes ranging from 4 $\pm$ 0.8 x 10^6 - 1 $\pm$ 0.1 x 10^9 cells/ml (Table 3.1). This finding was in keeping with the absence of any fat emulsification by the product. No fat removal was observed for BFL after 7 or 13 days of incubation although fat emulsification had taken place suggesting that while fat hydrolysis had taken place, no metabolism of the fat had occurred. The best result for butter degradation was obtained with FF but only in the enriched medium and not in the minimal medium. 28% of the fat was removed following 8 days of incubation and nearly all of the fat, 94%, was removed following 14 days incubation. Fat metabolism was accompanied by a rise in pH from 7.2 to 7.5 after 8 days and to 8.4 after 14 days.

A) Control Butter B) BFL C) FF in ENM D) Gnz

Figure 3.1 Bioaugmentation products grown on butter following 3 days of incubation A) control, B) BFL, C) FF in ENM, D) Gnz.

3.1.2 Biodegradation of Olive Oil

Biodegradation of the extra virgin olive oil (8 g fat /L, pH=7.2) by the three commercial mixed populations Gnz, FF and BFL was also evaluated. The appearance of the oil following 3 days of incubation is illustrated in Figure 3.2. Again no emulsification of the fat was observed for Gnz, however the oil was clearly emulsified when incubated with BFL and FF. As in the case of the butter, no fat removal was observed when Gnz was incubated with the oil even when the inoculum size was increased to 10^9 cells/ml and the incubation time was extended to 14 days. No significant fat removal was observed for BFL when minimal medium was used or when the organisms were incubated in the enriched medium for 7 days. However, good fat removal, 94%, was obtained in the enriched medium when the incubation time was extended to 13 days. Using a similar inoculum size, FF showed no fat removal in the minimal medium but 61% of the fat was removed in the enriched medium. When a smaller inoculum was used and the incubation time was increased to 14 days, 43% of the fat was removed in the minimal medium and 92% in the enriched medium where the pH increased to 8.2. The findings indicated that Gnz could not degrade hard or soft fats however FF and BFL showed good degradative ability under certain environmental conditions.

A) Control Olive oil B) BFL C) FF D) Gnz

Figure 3.2. Oil during incubation A) control, B) BFL, C) FF, D) Gnz

Table 3.1. Degradation of butter and olive oil by three bioaugmentation products

			%fat removed following incubation			
			TREATMENT			
Product	**Inoculum size (**no. bacteria/ml**)**	**Incubation Period** (Days)	**Butter (7.5 g/L)**		**Olive oil (8g/L)**	
			MM	ENM	MM	ENM
Gnz	• $(4 \pm 0.8) \times 10^6$	14	–	–	–	–
	• $(2 \pm 0.1) \times 10^7$		–	–	–	–
	• $(2 \pm 0.09) \times 10^8$		–	–	–	–
	• $(1 \pm 0.1) \times 10^9$		–	–	–	–
FF	• $(10 \pm 0.1) \times 10^6$	8	–	28%	–	61%
	• 4×10^4	13-14	–	94%	43%	92%
BFL	• 6×10^6	7	–	–	–	–
		13	–	–	34%	94%

MM = Minimal medium
ENM = Enriched nutrient medium
– = No fat removal

3.2 Characterization and identification of the microbial populations present in the three bioaugmentation products

The three commercial bioaugmentation products were examined in order to identify the microbial species present. The numbers and types of organisms present were distinguished initially on the basis of colony and cell morphology. In total 21 different bacterial species were isolated and a fungus was also present in the FF bioaugmentation product. The bacterial isolates were identified to species level using the API system and 16S rRNA sequencing. SDS-PAGE and FAME analyses were used to further distinguish between selected bacterial species. The fungus was characterized using morphological and biochemical characteristics.

3.2.1 Colony and cell morphology of the bacterial species present in the three commercial bioaugmentation products

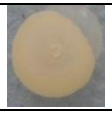
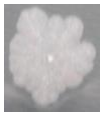
The colony colour, texture, surface appearance, elevation and margin were assessed for all the bacteria isolated from the three commercial mixed populations, 9 species from BFL (BFL 1 – 9), 7 from FF (FF A – G) and 5 from Gnz (Gnz I – V). Observations were also carried out on cell shape, Gram staining, catalase test, oxidase, motility and spore formation (Table 3.2). All the bacterial isolates were aerobic motile rods. They all produced catalase and oxidase. All of them were Gram positive rod shaped spore-formers indicating that they were members of the genus *Bacillus*, with the exception of two isolates from FF which were Gram negative rod shaped non spore-formers.

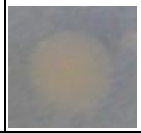
Enzyme and biosurfactant activity of the isolated strains

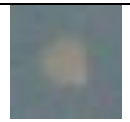
The 21 isolates were tested for their ability to produce hydrolytic enzymes (Table 3.3). The production of lipase was investigated by growing the organisms on tributyrin agar and Tween 20 agar and Tween 80 agars at pH 5.5 and pH 7.5. In

general, the detection of lipase production was better when the organisms were grown on tributyrin agar where all the isolates were seen to produce lipase except FFF and FFG which produced no lipase. Lipase production by these two isolates was detected on Tween agar but only at pH 5.5 and not at pH 7.5. FFF and FFG did not produce any other hydrolytic enzyme except in the case of xylanase where low levels were detected for FFG. In general all the other bacterial isolates produced protease, amylase, xylanase, and cellulose. The production of hydrolytic enzymes was however better for the isolates from BFL than for the isolates from FF or Gnz. Lactose fermentation was positive for the majority of the isolates. However, no biosurfactant was detected for any bacterial isolate tested.

Table 3.2. Characterization of the bacterial isolates present in the bioaugmentation products – BFL, FF and Gnz.

BFL	Colony morphology		Gram	spore	Cell shape	Catalase	Oxidase	Motility
1	Irregular shape, white color, dry texture around, clateriform elevation, wavy margin		+	+	Rods	+	+	+
2	Round shape, agar color, smooth texture		+	+	Rods Big thick	+	+	+
3	Rough surface, irregular, lobate margin, dry texture, white to brown color, flat elevation		+	+	Rods, thin chains	+	+	+
4	Irregular shaped, lobate margin, agar color		+	+	Rods, thin chains	+	+	+
5	Lobate-wavy margin, umbonate elevation, opaque color, smooth texture (colony showing viscous/sticky growth-carbohydrate capsule)		+	+	Rods pairs/chains	+	+	+
6	Rough, irregular dull surface with wrinkles, agar color, dry texture		+	+	Rods	+	+	++
7	Smooth surface, white colour, raised elevation.		+	+	Rods	+	+	+
8	Protuberances on surface, cloudy color.		+	+	Rods	+	+	++
9	Smooth surface, a bit irregular, white colour, more translucent around, opaque in the middle		+	+	Rods, thick pairs	+	+	+

FF	Colony morphology		Gram	spore	Cell shape	Catalase	Oxidase	Motility
A	smooth in middle, around dry texture, white color		+	+	Rods, thick big	+	+	+
B	Irregular elevation, raised, lobate margin,		+	+	Rods, group	+	+	+
C	Small irregular. Lobate margin agar color, umbonate		+	+	Rods, thin chains	+	+	++
D	Round, slightly clateriform elevation		+	+	Rods, small big chains	+	+	+
E	many protuberances, cloudy		+	+	Rods, thin chains	+	+	–
F	Shiny, round, entire, agar-yellow colour		–	–	Rods, small pairs/alone	+	+	++
G	Small, round, entire margin, agar colour turned into green colour		–	–	Rods, small pairs/alone	+	+	+

Gnz	Colony morphology		Gram	spore	Cell shape	Catalase	Oxidase	Motility
I	Irregular, dry around, lobate margin, raised in the middle		+	+	Rods, chains	+	+	+
II	Smooth surface, raised elevation, entire margin		+	+	Rods, small, pairs	+	+	+
III	Small irregular, in the agar Lobate margin agar color, umbonate		+	+	Rods, thin, chains	+	+	+
IV	Rough texutre, irregular surface, wrinkles, agar color		+	+	Rods, chains	+	+	+
V	many protuberances, cloudy color, irregular, wavy margin		+	+	Rods, thin chains	+	+	+

– negative
+ positive

Table 3.3 Enzymatic activity, lactose fermentation and biosurfactant production by the isolates of the three bioaugmentation products

BFL	**Lipase**					**Amylase**	**Protease**	**Cellulase**	**Xylanase**	**L. ferm.***	**Bios.****
	Tributyrin	**Tw20 (pH 5.5)**	**Tw20 (pH 7.5)**	**Tw80 (pH 5.5)**	**Tw80 (pH 7.5)**						
1	++++	–	+	–	–	+++	++	++	++	++	–
2	++	–	+	–	–	+++	++	++	++	++	–
3	++++	–	+++	–	+	+++	++	++	++	++	–
4	+++	–	+++	–	+	+++	++	+	++	++	–
5	++++	–	+	–	–	++++	+++	++	++	++	–
6	++	–	+	–	–	+	+	++	++	++	–
7	+++	–	+	–	–	+++	++	–	+++	–	–
8	++	–	+	–	–	+	+	++	–	++	–
9	+++	–	+	–	–	–	++	–	–	++	–
FF											
A	++++	++	++	–	–	+	++	++	++	–	–
B	++	++	++		–	–	++	–	–	++	–
C	+	–	++	–	–	+	+	++	++	++	–
D	++++	++	+++	–	–	+	+	–	++	–	–
E	+	–	+	–	–	+	+	++	+	+++	–
F	–	+++	–	+	–	–	–		–	+++	–
G	–	+++	–	+	–	–	–		+	+++	–
Gnz											
I	+++	++++	++	–	–	+	++	–	+++	–	–
II	++++	++++	++	–	–	++	++	–	++	–	–
III	+	++	+++	–	–	++	+	+	–	++	–
IV	+	–	++	–	–	+	+	+	+	++	–
V	+	–	++	–	–	+	+	+	+	++	–

Halo dimensions:

– negative	+ < 0.5	0.6 < ++ < 1.1	1.2 < +++ < 1.7	++++ > 1.7

*L.ferm.=Lactose fermentation,
**Bios. = Biosurfactant production

3.2.2 Identification of the bacterial isolates using the API system

The 21 different bacterial isolates were identified to species level using the API system. The API 50 CH strips using API 50 CHB medium combined with API 20E strips were used for the identification of members of the genus *Bacillus* and the API 20NE system was used for the identification of the Gram negative rods.

Bacterial isolates from BFL

The nine bacterial isolates from the BFL commercial bioaugmentation product were members of the genus *Bacillus*. The results obtained using the API system and the percentage similarities for each isolate are presented in Table 3.4. Four of the nine bacteria present BFL3, BFL4, BFL5 and BFL7, were identified as *Bacillus subtilis/amyloliquefaciens* (99.5%, 97%, 99.8% and 99.9% similarity, respectively). BFL6 and BFL8 were identified as *Bacillus licheniformis* (99.9%) and BFL1, BFL2 and BFL9 were identified as *Bacillus circulans* (98.6%), *Bacillus megaterium* (96.6%) and *Brevibacillus laterosporous* (99.9%), respectively.

Table 3.4. Identification of bacterial isolates of BFL product using API 50CHB and 20E.

Substrate in API strip	BFL bioaugmentation product								
	BFL 1	BFL 2	BFL 3	BFL 4	BFL 5	BFL 6	BFL 7	BFL 8	BFL 9
GLYCEROL	+	+	+	+	+	+	+	+	?
ERYTHRITOL	-	-	-	-	-	-	-	-	-
D-ARABINOSE	-	-	-	-	-	-	-	-	-
L-ARABINOSE	+	+	+	+	-	+	+	+	-
RIBOSE	+	+	+	+	+	+	+	+	+
D-XYLOSE	+	+	+	+	+	+	+	+	+
L-XYLOSE	-	-	-	-	-	-	-	-	-
ADONITOL	-	-	-	-	-	-	-	-	-
METHYL-XYLOSIDE	-	-	-	-	-	-	-	-	-
GALACTOSE	-	+	-	-	-	-	-	+	-
D-GLUCOSE	+	+	+	+	+	+	+	+	+
D ~ FRUCTOSE	+	+	+	+	+	+	+	+	+
D-MANNOSE	+	+	+	+	+	+	+	+	+
L-SORBOSE	-	-	-	-	-	-	-	-	-
RHAMNOSE	+	-	-	-	-	-	-	-	-
DULCITOL	-	-	-	-	-	-	-	-	-
INOSITOL	+	+	+	+	+	+	+	+	-
MANNITOL	+	+	+	+	+	+	+	+	-
SORBITOL	-	-	+	+	-	+	+	+	-
METHYL-D-MNNOSIDE	-	-	-	-	-	-	-	-	-
o< METHYL-D-GLUCOSIDE	+	-	+	+	+	+	+	+	-
N-ACETYL GLUCOSAMINE	-	(+)	-	-	-	-	?	+	+
AMYGDALIN	-	+	+	+	-	+	-	+	-
ARBUTIN	+	+	+	+	+	+	+	+	?
ESCULIN	+	+	+	+	+	+	+	+	+
SAUCIN	+	+	+	+	+	+	+	+	?
CELLOBIOSE	+	+	+	+	+	+	+	+	?
MALTOSE	+	+	+	+	+	+	+	+	+
LACTOSE	+	+	-	+	+	-	-	-	-
MELIBIOSE	+	+	+	+	+	-	+	-	-
SUCROSE	+	+	+	+	+	+	+	+	-
TREHALOSE	+	+	+	+	+	+	+	+	+
INULIN	-	+	+	-	-	-	+	(+)	-
MELEZITOSE	-	-	-	-	-	-	-	-	-
D-RAFFINOSE	+	+	+	+	+	-	+	+	-
STARCH	+	+	+	+	+	+	+	+	-

GLYCOGEN	+	+	+	+	+	+	+	+	-
XYLITOL	-	(+)	-	-	-	-	-	-	-
GENTIOBIOSE	-	+	+	-	-	+	-	(+)	-
D-TURANOSE	+	+	+	+	+	-	+	-	-
D- LYXOSE	-	-	-	-	-	-	-	-	-
O-TAGATOSE	-	-	-	-	-	+	-	+	-
D-FUCOSE	+	-	-	-	-	-	-	-	-
L- FUCOSE	+	-	-	-	-	-	-	-	-
D-ARABITOL	+	-	-	-	-	-	-	-	-
L-ARABITOL	+	-	-	-	-	-	-	-	-
GLUCONATE	-	-	-	-	-	-	-	-	-
2 KETO-GLUCONATE	-	-	-	-	-	-	-	-	-
2-nitrophenyl-ßD-galactopyranoside	-	-	-	-	-	-	-	-	-
β-galactosidase (Ortho NitroPhe)	+	+	+	-	-	+	+	+	-
L-ARGININE	-	-	-	-	-	+	-	+	-
L-LYSINE	-	-	-	-	-	-	-	-	-
L-ORNITHINE	-	-	-	-	-	-	-	-	-
TRISODIUM CITRATE	+	-	+	+	-	+	+	+	+
SODIUM THIOSULFATE	-	-	-	-	-	-	-	-	-
UREA	-	-	-	-	-	-	-	-	-
L-TRYPTOPHANE for Tryptophane DeAminase	-	-	+	-	(-)	-	-	-	-
L-TRYPTOPHANE for indole production	-	-	-	-	-	-	-	-	-
SODIUM PYRUVATE	+	-	+	+	+	+	+	+	+
GELATIN (bovine origin)	+	+	+	+	+	+	+	+	+
POTASSIUM NITRATE	(-)	(+)	+	-	-	+	?	-	-

– negative reaction
+ positive reaction

Table 3.5 ID and % similarities of BFL bacterial isolates according to API system

Code	ID	% similarity
BFL1	*Bacillus circulans*	98.6%
BFL2	*Bacillus megaterium*	96.6%
BFL3	*Bacillus subtilis/amyloliquefaciens*	99.5%
BFL4	*Bacillus subtilis/amyloliquefaciens*	97%
BFL5	*Bacillus subtilis/amyloliquefaciens*	99.8%
BFL6	*Bacillus licheniformis*	99.9%
BFL7	*Bacillus subtilis/amyloliquefaciens*	99.9%
BFL8	*Bacillus licheniformis*	99.9%
BFL9	*Brevibacillus laterosporus*	99.9%

Bacterial isolates from FF

Seven bacteria were isolated from the commercial bioaugmentation product FF. Five isolates were Gram positive (FFA, FFB, FFC, FFD and FFE) and two isolates were Gram negative (FFF and FFG). The results of the reactions in the API system for the Gram positive and Gram negative bacteria are presented in Table 3.6 and Table 3.7, respectively. The percentage similarities for all the FF isolates are shown in Table 3.8. Isolates FFA, FFB, FFC and FFD, were identified as *Bacillus subtilis/amyloliquefanciens* (99.9% for FFA, FFB, 91.3% for FFC and 99.4% for FFD) and FFE, as *Bacillus licheniformis* (99.9%). The two Gram negative isolates, FFF and FFG were identified using the API 20NE kit. FFF was identified as *Pseudomonas putida* with 99.9% homology, while FFG was identified as *Aeromonas hydrophila* with 99.9% similarity.

Table 3.6 Identification of the Gram positive FF isolates using API 50CHB and 20E

Substrate in API strip	FF bioaugmentation product				
	FFA	FFB	FFC	FFD	FFE
GLYCEROL	+	+	+	+	+
ERYTHRITOL	-	-	-	-	-
D-ARABINOSE	-	-	-	-	-
L - ARABINOSE	+	+	+	+	+
RIBOSE	+	+	+	+	+
D-XYLOSE	+	+	+	-	+
L - XYLOSE	-	-	-	-	-
ADONITOL	-	-	-	-	-
METHYL- XYLOSIDE	-	-	-	-	-
GALACTOSE	-	-	-	-	-
D-GLUCOSE	+	+	+	+	+
D ~ FRUCTOSE	+	+	+	+	+
D-MANNOSE	+	+	+	+	+
L-SORBOSE	-	-	-	-	-
RHAMNOSE	-	-	-	-	-
DULCITOL	-	-	-	-	-
INOSITOL	+	+	+	+	+
MANNITOL	+	+	+	+	+
SORBITOL	+	+	+	+	+
~< METHYL-D-M ~.NNOSIDE	-	-	-	-	-
o< METHYL-D-GLUCOSIDE	+	+	-	+	+
N-ACETYL GLUCOSAMINE	-	-	-	-	-
AMYGDALIN	+	-	+	-	+
ARBUTIN	+	(+)	+	-	+
ESCULIN	+	+	+	+	+
SAUCIN	+	+	+	-	+
CELLOBIOSE	+	+	+	-	+
MALTOSE	+	+	+	+	+
LACTOSE	+	+	+	-	-
MELIBIOSE	+	+	+	+	-
SUCROSE	+	+	+	+	+
TREHALOSE	+	+	+	+	+
INULIN	+	-	+	+	-
MELEZITOSE	-	-	-	-	-
D-RAFFINOSE	-	+	+	+	-
STARCH	+	+	+	+	+
GLYCOGEN	+	+	+	+	+
XYLITOL	+	-	-	-	-
GENTIOBIOSE	-	+	+	-	+
D - TURANOSE	+	-	+	+	-
D- LYXOSE	-	-	-	-	-

O-TAGATOSE	-	-	-	-	+
D-FUCOSE	-	-	-	-	-
L- FUCOSE	-	-	-	-	-
D-ARABITOL	-	-	-	-	-
L-ARABITOL	-	-	-	-	-
GLUCONATE	-	-	-	-	-
2 KETO- GLUCONATE	-	-	-	-	-
2-nitrophenyl-ßD-galactopyranoside	-	-	-	-	-
β-galactosidase	-	+	+	+	+
L-ARGININE	-	-	-	-	-
L-LYSINE	-	-	-	-	-
L-ORNITHINE	-	-	-	-	-
TRISODIUM CITRATE	-	+	+	+	+
SODIUM THIOSULFATE	-	-	-	-	-
UREA	-	-	-	-	-
L-TRYPTOPHANE for Tryptophane DeAminase	-	-	-	-	+
L-TRYPTOPHANE for indole production	-	-	-	-	-
SODIUM PYRUVATE	+	+	+	+	+
GELATIN (bovine origin)	+	+	+	+	+
POTASSIUM NITRATE	-	+	-	(+)	+

– negative reaction
+ positive reaction

Table 3.7 Identification of the Gram negative isolates of FF product using API 20NE

API 20NE substrates	FFF	FFG
Reduction of nitrates	-	+
Indole production	-	+
Glucose acidification	+	+
Arginine dihydrolase	+	+
Urease	-	-
Esculin hydrolysis	-	+
Gelatin hydrolysis	-	+
Â-galactosidase	-	+
Glucose assimilation	+	+
Arabinose assimilation	-	+
Mannose assimilation	+	+
Mannitol assimilation	-	+
N-acetyl-glucosamine assimilation	-	+
Maltose assimilation	-	-
Gluconate assimilation	+	+
Caprate assimilation	+	+
Adipate assimilation	-	-
Malate assimilation	+	+
Citrate assimilation	+	+
Phenyl-acetate assimilation	+	+
Cytochrome oxidase	+	+

Table 3.8 ID and % similarities of FF bacterial isolates according to API system

Code	ID	%Similarity
FFA	*B. subtilis/ amyloliquefaciens*	99.9%
FFB	*B. subtilis/amyloliquefaciens*	99.9%
FFC	*B. subtilis/amyloliquefaciens*	91.3%
FFD	*B. subtilis/amyloliquefaciens*	99.4%
FFE	*B. licheniformis*	99.9%
FFF	*Pseudomonas putida*	99.9%
FFG	*Aeromonas hydrophila*	99.9%

Bacterial isolates from the Gnz

Five strains were isolated from the commercial bioaugmentation product Gnz and all of them belonged to the genus *Bacillus*. The results of the API tests and the percentage similarities are presented in Table 3.9 and Table 3.10, respectively. The results showed that the product was a mixture of three *Bacillus subtilis/amyloliquefanciens* strains (Gnz-I, Gnz-II and Gnz-III, 99.9% similarity) and two *Bacillus licheniformis* strains (Gnz-IV and Gnz-V, 99.9% similarity).

Table 3.9 Identification of bacterial isolates of Gnz product using API 50CHB and 20E

Substrate in API strip	Gnz bioaugmentation product				
	Gnz-I	Gnz-II	Gnz-III	Gnz-IV	Gnz-V
GLYCEROL	+	+	+	+	+
ERYTHRITOL	-	-	-	-	-
D-ARABINOSE	-	-	-	-	-
L-ARABINOSE	+	+	+	-	+
RIBOSE	+	+	+	+	+
D-XYLOSE	+	+	+	+	+
L-XYLOSE	-	-	-	-	-
ADONITOL	-	-	-	-	-
METHYL-XYLOSIDE	-	-	-	-	-
GALACTOSE	-	-	-	-	+
D-GLUCOSE	+	+	+	+	+
D~FRUCTOSE	+	+	+	+	+
D-MANNOSE	+	+	+	+	+
L-SORBOSE	-	-	-	-	-
RHAMNOSE	-	-	-	-	+
DULCITOL	-	-	-	-	-
INOSITOL	+	+	+	+	+
MANNITOL	+	+	+	+	+
SORBITOL	+	+	+	+	+
METHYL-D-M~.NNOSIDE	-	-	-	(+)	-
METHYL-D-GLUCOSIDE	+	+	+	+	+
N-ACETYL GLUCOSAMINE	-	-	-	+	-
AMYGDALIN	-	+	+	+	+
ARBUTIN	+	+	+	+	+
ESCULIN	+	+	+	+	+
SAUCIN	+	+	+	+	+
CELLOBIOSE	+	+	+	+	+

M A L T O S E	+	+	+	+	+
L A C T O S E	+	+	+	-	?
MELIBIOSE	+	+	+	-	+
S U C R O S E	+	+	+	+	+
T R E H A L O S E	+	+	+	+	+
INULIN	+	-	-	-	+
M E L E Z I T O S E	-	-	-	-	-
D-RAFFINOSE	-	+	+	-	+
S T A R C H	+	+	+	+	+
G L Y C O G E N	+	+	+	+	+
XYLITOL	+	-	-	-	-
GENTIOBIOSE	-	+	+	+	-
D - T U R A N O S E	-	-	+	+	+
D- LYXOSE	-	-	-	-	-
O-TAGATOSE	-	-	-	+	+
D-FUCOSE	-	-	-	-	-
L- FUCOSE	-	-	-	-	-
D-ARABITOL	-	-	-	-	-
L-ARABITOL	-	-	-	-	-
GLUCONATE	-	-	-	-	-
2 KETO- GLUCONATE	-	-	-	-	-
2-nitrophenyl-ßD-galactopyranoside	-	-	-	-	-
β-galactosidase	+	-	+	+	+
L-ARGININE	-	-	-	+	-
L-LYSINE	-	-	-	-	-
L-ORNITHINE	-	-	-	-	-
TRISODIUM CITRATE	+	+	+	+	+
SODIUM THIOSULFATE	-	-	-	-	-
UREA	-	-	-	-	-
L-TRYPTOPHANE for Tryptophane DeAminase	-	+	-	+	+
L-TRYPTOPHANE for indole production	-	-	-	-	-
SODIUM PYRUVATE	+	+	+	+	+
GELATIN (bovine origin)	+	+	+	-	-
POTASSIUM NITRATE	-	+	-	+	+

– negative reaction
+ positive reaction

Table 3.10 ID and % similarities of Gnz bacterial isolates according to API system

Code	ID	%Similarity
Gnz-I	*B. subtilis/amyloliquefaciens*	99.9%
Gnz-II	*B. subtilis/amyloliquefaciens*	99.9%
Gnz-III	*B. subtilis/amyloliquefaciens*	99.9%
Gnz-IV	*B. licheniformis*	99.9%
Gnz-V	*B. licheniformis*	99.9%

The control cultures used for the API system included *Bacillus subtilis* (B-14596), *Bacillus amyloliquefaciens* (NRS-762) and *Bacillus licheniformis* (B-14368) for the *Bacillus* species (Table 3.11) together with *Pseudomonas putida* CP1 for the Gram negative bacteria (Table 3.12). The results obtained from the API system for the bacterial isolates showed poor resolution between *Bacillus subtilis* and *Bacillus amyloliquefaciens.* This finding was substantiated when pure cultures of these two strains were tested. In total 19 of the bacterial species were identified as members of the genus Bacillus and while identification was similar in some cases when the API system was used, observations of cell and colony morphology together with enzyme production showed that all the isolates were distinct. 16S rRNA sequencing was investigated to determine if the isolates could be more clearly distinguished.

Table 3.11 Results of API 50CHB for the control strains *Bacillus subtilis* (B-14596), *Bacillus amyloliquefaciens* (NRS-762) and *Bacillus licheniformis* (B-14368)

Substrate in API strip	*B. amyloliquefaciens* NRS-762	*B. subtilis* B-14596	*B. licheniformis* B-14368
GLYCEROL	+	(+)	+
ERYTHRITOL	-	-	-
D-ARABINOSE	-	-	-
L-ARABINOSE	-	+	+
RIBOSE	+	+	+
D-XYLOSE	-	-	+
L-XYLOSE	-	-	-
ADONITOL	-	-	-
METHYL-XYLOSIDE	-	-	-
GALACTOSE	-	-	-

D-GLUCOSE	+	+	+
D ~ FRUCTOSE	+	+	+
D-MANNOSE	+	+	+
L-SORBOSE	-	-	-
RHAMNOSE	-	-	-
DULCITOL	-	-	-
INOSITOL	+	+	+
M A N N I T O L	-	+	+
SORBITOL	+	+	+
~< METHYL-D-M ~.NNOSIDE	-	-	-
o< METHYL-D-GLUCOSIDE	+	-	+
N-ACETYL GLUCOSAMINE	-	-	-
A M Y G D A L I N	+	+	+
ARBUTIN	+	+	+
ESCULIN	+	+	+
SAUCIN	+	+	+
CELLOBIOSE	+	+	+
M A L T O S E	+	+	+
L A C T O S E	-	-	?
MELIBIOSE	-	-	-
S U C R O S E	+	+	+
T R E H A L O S E	+	-	+
INULIN	-	+	-
M E L E Z l T O S E	-	-	-
D-RAFFINOSE	-	-	-
S T A R C H	-	-	+
G L Y C O G E N	-	-	+
XYLITOL	-	-	-
GENTIOBIOSE	-	-	-
D - T U R A N O S E	-	-	+
D- LYXOSE	-	-	-
O-TAGATOSE	-	-	+
D-FUCOSE	-	-	-
L- FUCOSE	-	-	-
D-ARABITOL	-	-	-
L-ARABITOL	-	-	-
GLUCONATE	-	-	-
2 KETO- GLUCONATE	-	-	-
2-nitrophenyl-ßD-galactopyranoside	-	-	-
β-galactosidase (Ortho NitroPhe)	+	+	+
L-ARGININE	-	-	+
L-LYSINE	-	-	-
L-ORNITHINE	-	-	-

TRISODIUM CITRATE	-	-	+
SODIUM THIOSULFATE	-	-	-
UREA	-	-	-
L-TRYPTOPHANE for Tryptophane DeAminase	-	-	+
L-TRYPTOPHANE for indole production	-	-	-
SODIUM PYRUVATE	+	-	+
GELATIN (bovine origin)	+	+	-
POTASSIUM NITRATE	-	+	+
ID	99.9% *Bacillus subtilis/ amyloliquefaciens*	99.9% *B. subtilis/ amyloliquefaciens*	99.9% *Bacillus licheniformis*

Table 3.12 Results of API 20NE for the control strain *Pseudomonas putida* CP1

API 20NE substrates	***Pseudomonas putida* CP1**
Reduction of nitrates	-
Indole production	-
Glucose acidification	-
Arginine dihydrolase	+
Urease	-
Esculin hydrolysis	-
Gelatin hydrolysis	-
Â-galactosidase	-
Glucose assimilation	+
Arabinose assimilation	+
Mannose assimilation	-
Mannitol assimilation	-
N-acetyl-glucosamine assimilation	-
Maltose assimilation	-
Gluconate assimilation	+
Caprate assimilation	-
Adipate assimilation	-
Malate assimilation	+
Citrate assimilation	+
Phenyl-acetate assimilation	+
Cytochrome oxidase	+
ID	*Pseudomonas putida* 99.8%

3.2.3 Identification of the bacterial isolates using 16S rRNA Sequencing

All the 21 bacterial isolates from the three bioaugmentation products were identified to species level using 16S rRNA sequencing. The genomic DNA for each isolate and the four control isolates (*Bacillus subtilis* B-14596, *Bacillus amyloliquefaciens* NRS-762, *Bacillus licheniformis* B-14368 and *Pseudomonas putida* CP1) was extracted as described in 2.2.11. The 16S gene was amplified using the primers pA and pH as described in 2.2.15. Agarose gel electrophoresis of the PCR product showed that one predicted fragment (approx. 1500 bp) was amplified in all the strains (see Fig. 2.4). The PCR products were purified as described in 2.2.16 and were sequenced by Eurofins MWG Operon sequencing service (Germany, http://www.eurofinsdna.com). The obtained sequences are listed in Appendix A.

BLASTN search analysis was used to compare the obtained sequence data with other genomic sequences available in the NCBI database (www.ncbi.nlm.nih.gov/BLAST). The identification and percentage similarities according to the partial sequencing of the 16S rRNA gene are described in Table 3.13 and compared with the results obtained for the API system. The identification of the isolates gave similar results for both systems except for BFL9 and FFG. Eleven isolates, BFL3, BFL4, BFL5, BFL7, FFA, FFB, FFC, FFD, GnzI, GnzII and GnzIII had all been identified as *B. subtilis/amyloliquefaciens* using the API system. The use of 16S sequencing identified the organisms more clearly as *B. subtilis or Bacillus amyloliquefaciens*.

Multiple sequence alignment using the CLUSTALW software was carried out. Analyses of the aligned sequences is described in Table 3.14. Highlighted regions show how minor differences in the nucleotide sequences of similar species distinguish the strains such as BFL6, BFL8 and FFE which had been identified as *B. licheniformis* and FFF and FFG which were identified as *Pseudomonas putida* and *Aeromonas hydrophila*. The 11 isolates identified as *Bacillus subtilis/amyloliquefaciens* using the API system were more clearly resolved using 16S sequencing except for BFL3, BFL4 and BFL7.

Table 3.13 Sequence similarities (%) using API system and 16S sequencing for all the isolates from the 3 bioaugmentation products and for the controls.

BFL product	**16S rRNA Sequencing - ID%**	**API – ID% similarity**
BFL1	99% *B. circulans*	98.6% *B. circulans*
BFL2	99% *B. megaterium*	96.6% *B. megaterium*
BFL3	100% *B. subtilis*	99.5% *B. subtilis/amyloliquefaciens*
BFL4	99% *B. subtilis*	97% *B. subtilis/amyloliquefaciens*
BFL5	99% *B. amyloliquefaciens*	99.8% *B. subtilis/amyloliquefaciens*
BFL6	99% *B. licheniformis*	99.9% *Bacillus licheniformis*
BFL7	99% *B. subtilis*	99.9% *B. subtilis/amyloliquefaciens*
BFL8	100% *B. licheniformis*	99.9% *Bacillus licheniformis*
BFL9	99% *B. cereus*	99.9% *Brevibacillus laterosporus*
FF product	**16S rRNA Sequencing - ID%**	**API – ID% similarity**
FFA	99% *B. subtilis*	99.9% *B. subtilis/ amyloliquefaciens*
FFB	100% *B. amyloliquefaciens*	99.9% *B. subtilis/amyloliquefaciens*
FFC	99% *B. subtilis*	91.3% *B.subtilis/ amyloliquefaciens*
FFD	100% *B. subtilis*	99.4% *B.subtilis/ amyloliquefaciens*
FFE	100% *B. licheniformis*	99.9% *Bacillus liceniformis*
FFF	99% *P. putida*	99.9% *Pseudomonas putida*
FFG	99% *P. putida*	99.9% *Aeromonas hydrophila*
Gnz product	**16S rRNA Sequencing - ID%**	**API – ID% similarity**
GnzI	99% *B. amyloliquefaciens*	99.9% *B.subtilis/amyloliquefaciens*
GnzII	99% *B. amyloliquefaciens*	99.9% *B.subtilis/amyloliquefaciens*
GnzIII	99% *B. subtilis*	99.9% *B.subtilis/amyloliquefaciens*
GnzIV	99% *B. licheniformis*	99.9% *Bacillus licheniformis*
GnzV	99% *B. licheniformis*	99.9% *Bacillus licheniformis*
Controls	**16S rRNA Sequencing - ID%**	**API – ID% similarity**
B. subtilis	99% *B. subtilis*	99% *B. subtilis/amyloliquefaciens*
B. amyloliquefaciens	99% *B. amyloliquefaciens*	99% *B. subtilis/amyloliquefaciens*
B. licheniformis	99% *B. licheniformis*	99% *B. licheniformis*
P. putida CP1	99% *P. putida*	99.8% *Pseudomonas putida*

Table 3.14 ClustalW alignments comparing the nucleotide sequences of the 16S rRNA genes

```
Name                                            Position                                   n
                                           20                  40                  60
FFG                  ---TACACATGC-AGTCGA--GCGGAT-GAC-GGGAGCTTGCTCCT-TGAT-TCAGCGGC 50
CP1                  ---TACACATGC-AGTCGA--GCGGAT-GAC-GGGAGCTTGCTCCT-TGAT-TCAGCGGC 50
FFF                  --CTACACATGC-AGTCGA--GCGGAT-GAC-GGGAGCTTGCTCCT-TGAT-TCAGCGGC 51
GnzV                 ----ATACATGCTAGTCGA--GCGGACCGAC-GGGAGCTTGCTCCC-TTAGGTCAGCGGC 52
GnzIV                --CTATACATGC-AGTCGA--GCGGACCGAC-GGGAGCTTGCTCCC-TTAGGTCAGCGGC 53
B.licheniformis      --CTATACATGC-AGTCGA--GCGGACCGAC-GGGAGCTTGCTCCC-TTAGGTCAGCGGC 53
BFL6                 ---TATACATGC-AGTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 52
BFL8                 TGCTATACATGCAAGTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 56
FFE                  ----------------------------------------------TTATGTTAGCGGC 13
GnzI                 -GCTATACATGC-AGTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 54
GnzII                -GCTATACATGC-AGTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 54
FFB                  --CTATACATGC-AGTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 53
B.amyloliquefaciens  ----ATACATGCTAGTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 52
BFL5                 --CTATACATGC-AGTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 53
B.subtilis           --CTATACATGCAAGTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 54
FFD                  ---TATACATGCAAGTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 53
GnzIII               --CTATACATGCA-GTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 53
BFL3                 -----TACATGCA-GTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 50
BFL7                 -GCTATACATGCA-GTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 54
FFC                  --CTATACATGCA-GTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 53
FFA                  -GCTATACATGCA-GTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 54
BFL4                 -GCTATACATGCA-GTCGA--GCGGACAGAT-GGGAGCTTGCTCCC-TGATGTTAGCGGC 54
BFL2                 --CTATACATGC-AGTCGA--GCGAACTGATTAGAAGCTTGCTTCTATGACGTTAGCGGC 55
BFL9                 -GCTATACATGC-AGTCGA--GCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGC 56
BFL1                 ---CACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGG----------AACACCAGTGGC 47
                                                                 *    ** ***
                                            80                  100                 120
FFG                  GGACGGGTGAGTAATGCCTAGG-AATCTGCCTGGTAGTGGGGGACAACGTTTCGA--AAG 107
CP1                  GGACGGGTGAGTAATGCCTAGG-AATCTGCCTGGTAGTGGGGGACAACGTTTCGA--AAG 107
FFF                  GGACGGGTGAGTAATGCCTAGG-AATCTGCCTGGTAGTGGGGGACAACGTTTCGA--AAG 108
GnzV                 GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA--AAC 110
GnzIV                GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA--AAC 111
B.licheniformis      GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA--AAC 111
BFL6                 GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA--AAC 110
BFL8                 GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA--AAC 114
FFE                  GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA--AAC 71
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B.licheniformis      CGGGGCTA-ATACC---GGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCT 167
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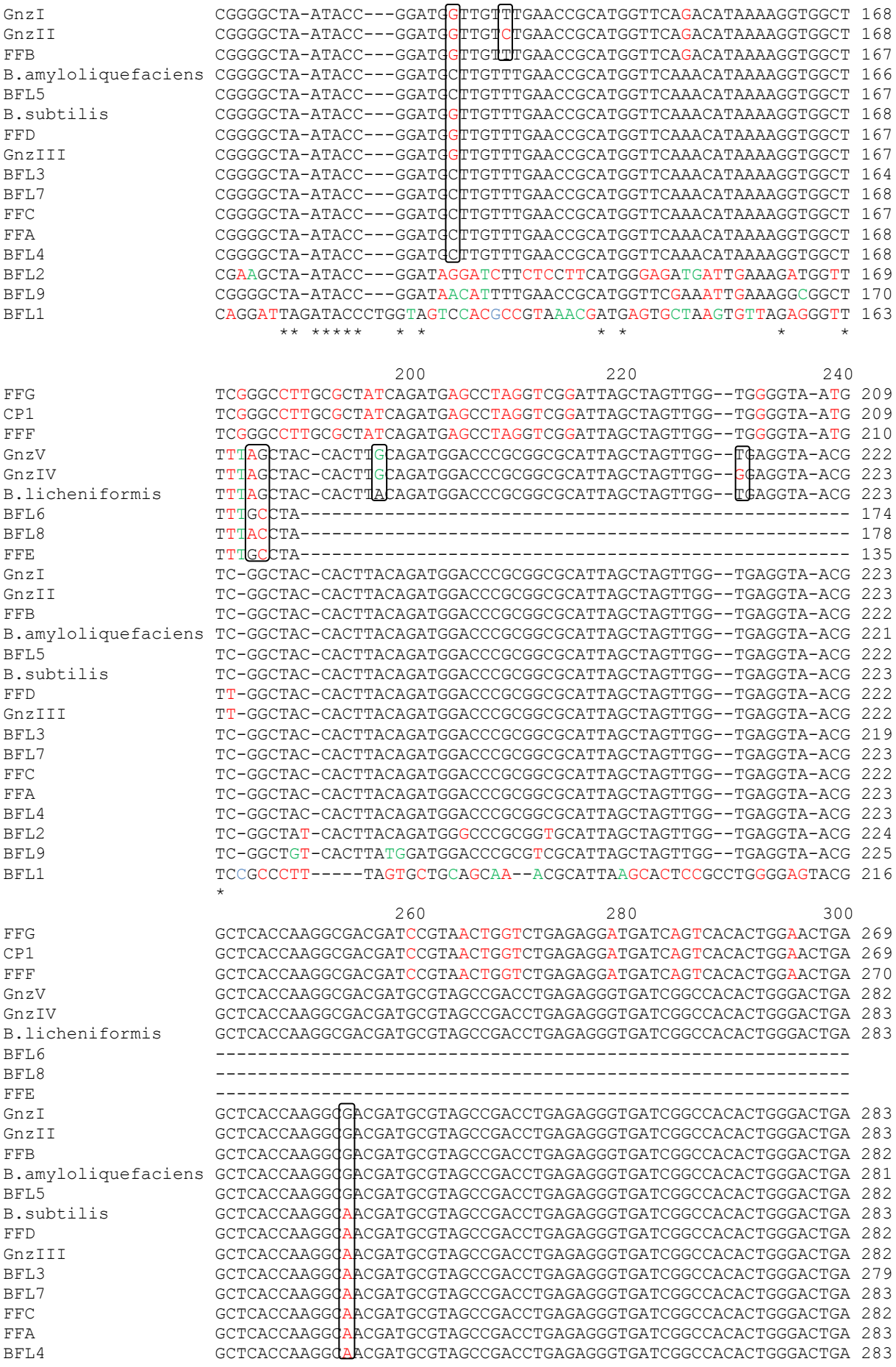
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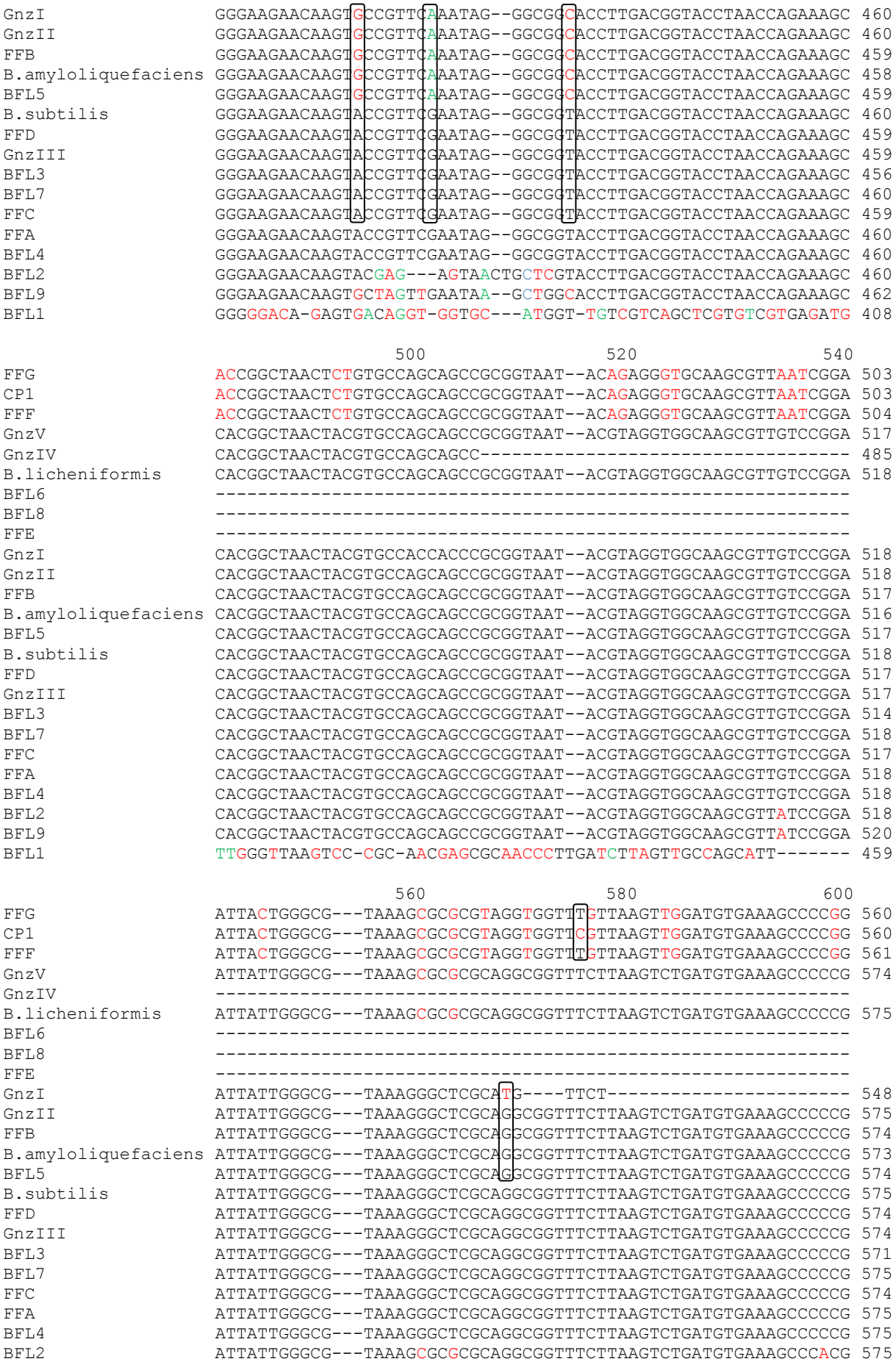
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CP1                  AATTTCCTGTGTAGCGG-TGAAATGCGTA-AT------------------------------ 649
FFF                  AATTTCCTGTGTAGCGG-TGAAATGCGTAGATATAGGAAGGAACACCAGTGG-CGAAGGC 677
GnzV                 ------------------------------------------------------------
GnzIV                ------------------------------------------------------------
B.licheniformis      AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 691
BFL6                 ------------------------------------------------------------
BFL8                 ------------------------------------------------------------
FFE                  ------------------------------------------------------------
GnzI                 ------------------------------------------------------------
GnzII                AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 691
FFB                  AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 690
B.amyloliquefaciens  AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 689
BFL5                 AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 690
B.subtilis           AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGGGCGAAGGC 692
FFD                  AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 690
GnzIII               AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 690
BFL3                 AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 687
BFL7                 AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 691
FFC                  AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 690
FFA                  AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 691
BFL4                 AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 691
BFL2                 AATTCCACGTGTAGCGG-TGAAATGCGTAGAGATGTGGAGGAACACCAGTGG-CGAAGGC 691
BFL9                 AATTCCATGTGTAGCGG-TGAAATGCGTAGAGATATGGAGGAACACCAGTGG-CGAAGGC 693
BFL1                 TA--CAAAGGGCAGCAA---AACCGCG-----ACGTCGAGCAAATCCCAT-----AAAAC 614

                                      740                 760                 780
FFG                  GACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA 738
CP1                  ------------------------------------------------------------
FFF                  GACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA 737
GnzV                 ------------------------------------------------------------
GnzIV                ------------------------------------------------------------
B.licheniformis      GACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGA 751
BFL6                 ------------------------------------------------------------
BFL8                 ------------------------------------------------------------
FFE                  ------------------------------------------------------------
GnzI                 ------------------------------------------------------------
```
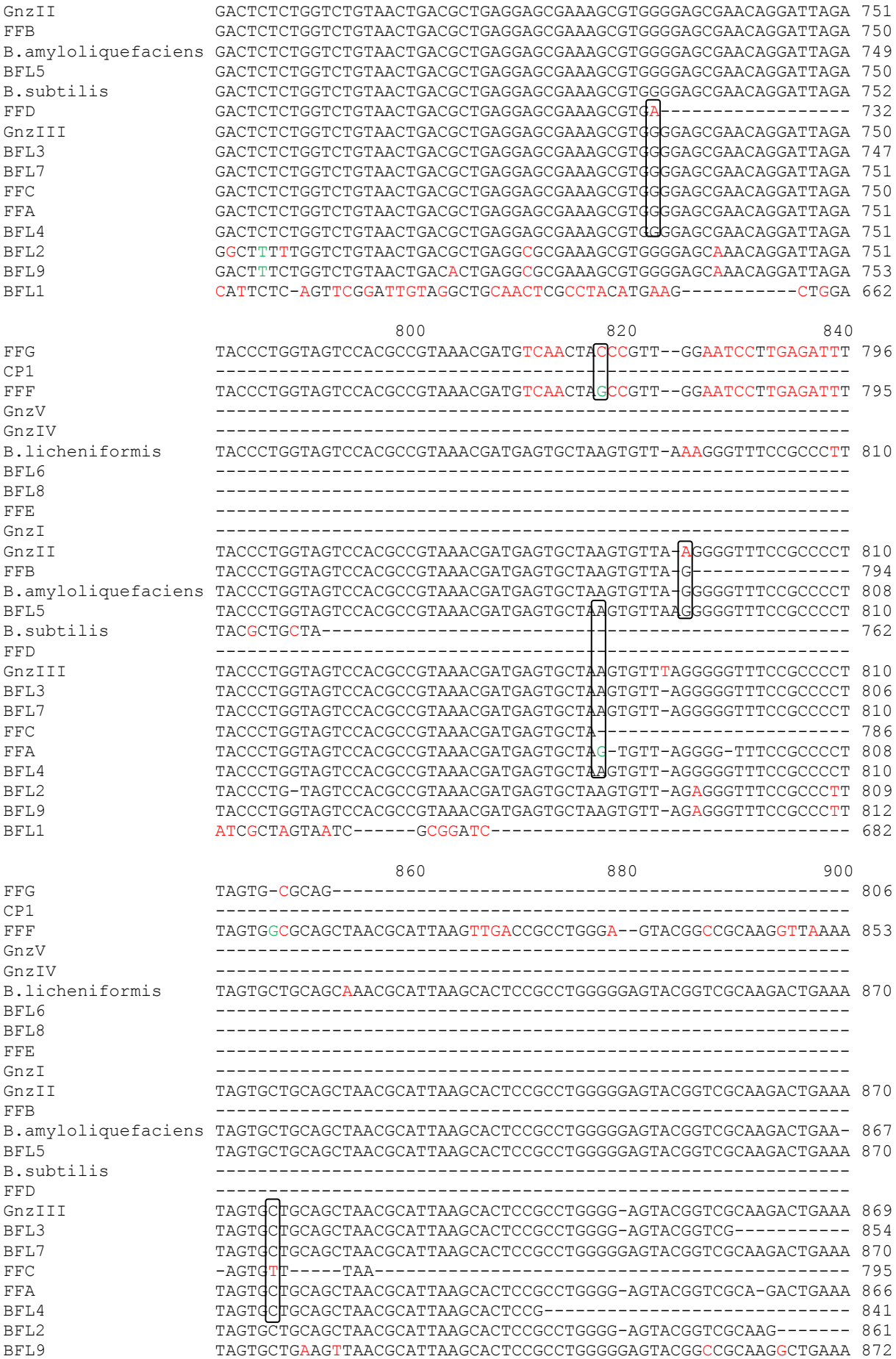


GnzII GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 751
FFB GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 750
B.amyloliquefaciens GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 749
BFL5 GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 750
B.subtilis GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 752
FFD GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGA---------- 732
GnzIII GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 750
BFL3 GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 747
BFL7 GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 751
FFC GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 750
FFA GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 751
BFL4 GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA 751
BFL2 GGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGA 751
BFL9 GACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGA 753
BFL1 CATTCTC-AGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAG----------CTGGA 662
800 820 840
FFG TACCCTGGTAGTCCACGCCGTAAACGATGTCAACTACCCGTT--GGAATCCTTGAGATTT 796
CP1 ----------
FFF TACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTT--GGAATCCTTGAGATTT 795
GnzV ----------
GnzIV ----------
B.licheniformis TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT-AAAGGGTTTCCGCCCTT 810
BFL6 ----------
BFL8 ----------
FFE ----------
GnzI ----------
GnzII TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA-AGGGGTTTCCGCCCCT 810
FFB TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA-G---------- 794
B.amyloliquefaciens TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA-GGGGGTTTCCGCCCCT 808
BFL5 TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAAGGGGGTTTCCGCCCCT 810
B.subtilis TACGCTGCTA---------- 762
FFD ----------
GnzIII TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTTAGGGGGTTTCCGCCCCT 810
BFL3 TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT-AGGGGGTTTCCGCCCCT 806
BFL7 TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT-AGGGGGTTTCCGCCCCT 810
FFC TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA---------- 786
FFA TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAG-TGTT-AGGGG-TTTCCGCCCCT 808
BFL4 TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT-AGGGGGTTTCCGCCCCT 810
BFL2 TACCCTG-TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT-AGAGGGTTTCCGCCCTT 809
BFL9 TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT-AGAGGGTTTCCGCCCTT 812
BFL1 ATCGCTAGTAATC------GCGGATC---------- 682
860 880 900
FFG TAGTG-CGCAG---------- 806
CP1 ----------
FFF TAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGA--GTACGGCCGCAAGGTTAAAA 853
GnzV ----------
GnzIV ----------
B.licheniformis TAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGGAGTACGGTCGCAAGACTGAAA 870
BFL6 ----------
BFL8 ----------
FFE ----------
GnzI ----------
GnzII TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGGAGTACGGTCGCAAGACTGAAA 870
FFB ----------
B.amyloliquefaciens TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGGAGTACGGTCGCAAGACTGAA- 867
BFL5 TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGGAGTACGGTCGCAAGACTGAAA 870
B.subtilis ----------
FFD ----------
GnzIII TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG-AGTACGGTCGCAAGACTGAAA 869
BFL3 TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG-AGTACGGTCG---------- 854
BFL7 TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGGAGTACGGTCGCAAGACTGAAA 870
FFC -AGTGTT-----TAA---------- 795
FFA TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG-AGTACGGTCGCA-GACTGAAA 866
BFL4 TAGTGCTGCAGCTAACGCATTAAGCACTCCG---------- 841
BFL2 TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG-AGTACGGTCGCAAG------- 861
BFL9 TAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGGAGTACGGCCGCAAGGCTGAAA 872

```
BFL1                 ------------------------------------------------------------

                                       920                 940
FFG                  ------------------------------------------------
CP1                  ------------------------------------------------
FFF                  CTCAAATGAATTGACGGGGCCCGCACAAGCGTG----------------- 886
GnzV                 ------------------------------------------------
GnzIV                ------------------------------------------------
B.licheniformis      CTCAAAGGAATT------------------------------------- 882
BFL6                 ------------------------------------------------
BFL8                 ------------------------------------------------
FFE                  ------------------------------------------------
GnzI                 ------------------------------------------------
GnzII                CTCAA-GGAATTGACGGGGCCCGCACAAGCGGTGGAGCATGT------- 912
FFB                  ------------------------------------------------
B.amyloliquefaciens  CTCAA-GGGAT------------------------------------- 877
BFL5                 CTCAAAGGAATTGACGGGGCCCGCACAAGC------------------- 901
B.subtilis           ------------------------------------------------
FFD                  ------------------------------------------------
GnzIII               CTCAAGGAA---------------------------------------- 878
BFL3                 ------------------------------------------------
BFL7                 CTCAAAGGAATTGAC---------------------------------- 885
FFC                  ------------------------------------------------
FFA                  CTCAAGGAATTGACGGGGGCCCGCACAACGG------------------- 897
BFL4                 ------------------------------------------------
BFL2                 ------------------------------------------------
BFL9                 CTCAAGGAATTGACGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTATT 922
BFL1                 ------------------------------------------------
n=nucleotides (total)
```

The aligned sequences were used to construct a phylogenetic tree by the neighbor-joining method emphasising the close relatedness between some of the species (Figure 3.3).

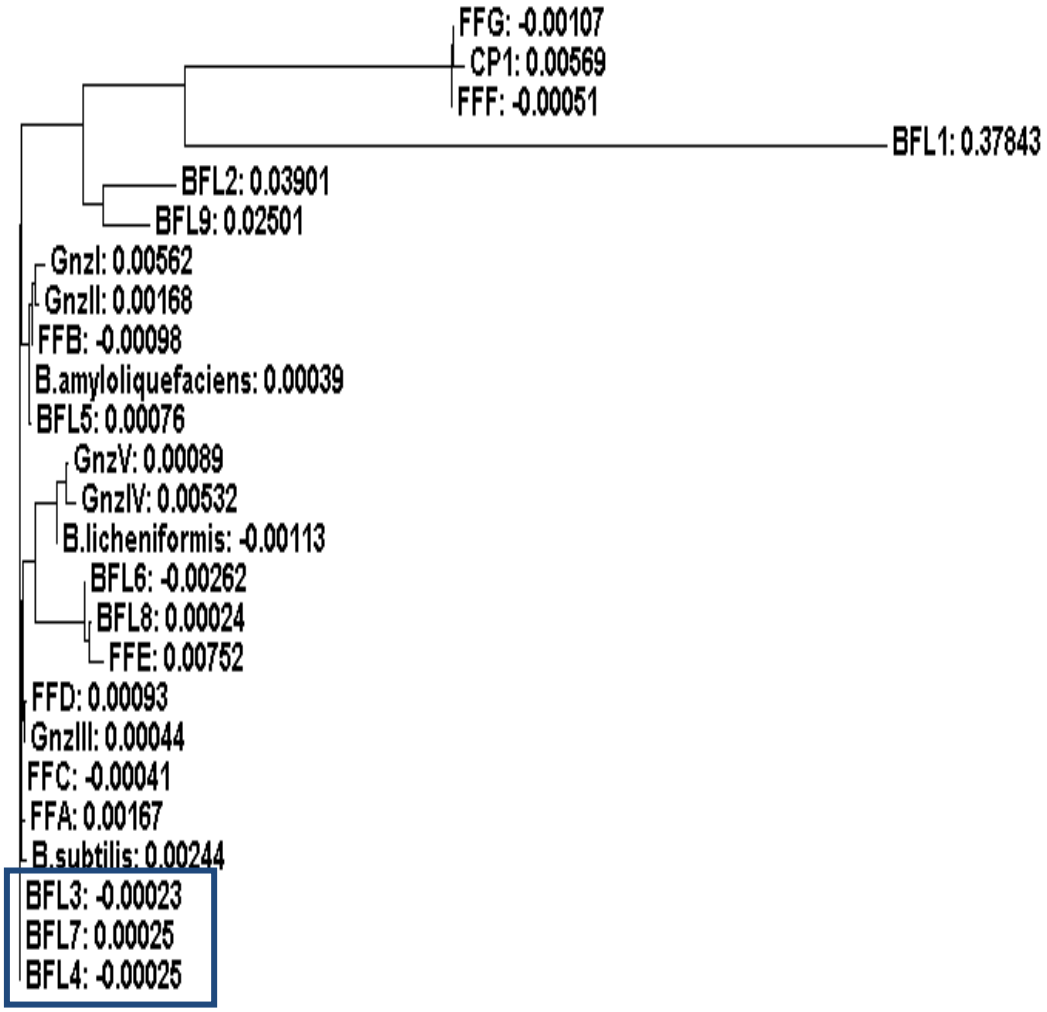


Figure 3.3.Phylogenetic tree showing the taxonomic classification of all the isolates, comparison with the controls (*B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis* and *P. putida* CP1) and closely related strains based on their 16S rRNA sequences.

3.2.4 Characterization of BFL isolates using SDS-PAGE

The nine bacterial isolates from BFL were further characterised using SDS-PAGE. The total cell protein profiles of the *Bacillus* spp. analysed by sodium sulphate polyacrylamide gel electrophoresis (SDS-PAGE) are shown in Figure 3.4. The molecular weights of the proteins of the *Bacillus* spp. were found to range from 15-170 kD. While distict patterns were obtained for BFL1 (*Bacillus circulans*), BFL2 (*Bacillus megaterium*) BFL5 (*Bacillus amyloliquefaciens*) and BFL9 (*Bacillus cereus*) similar patterns were observed for the strains belonging to the same species. Similar banding was obtained for BFL6 and BFL8 previously identified as *Bacillus licheniformis*, as well as for the BFL3, BFL4 and BFL7 previously identified as *Bacillus subtilis*.

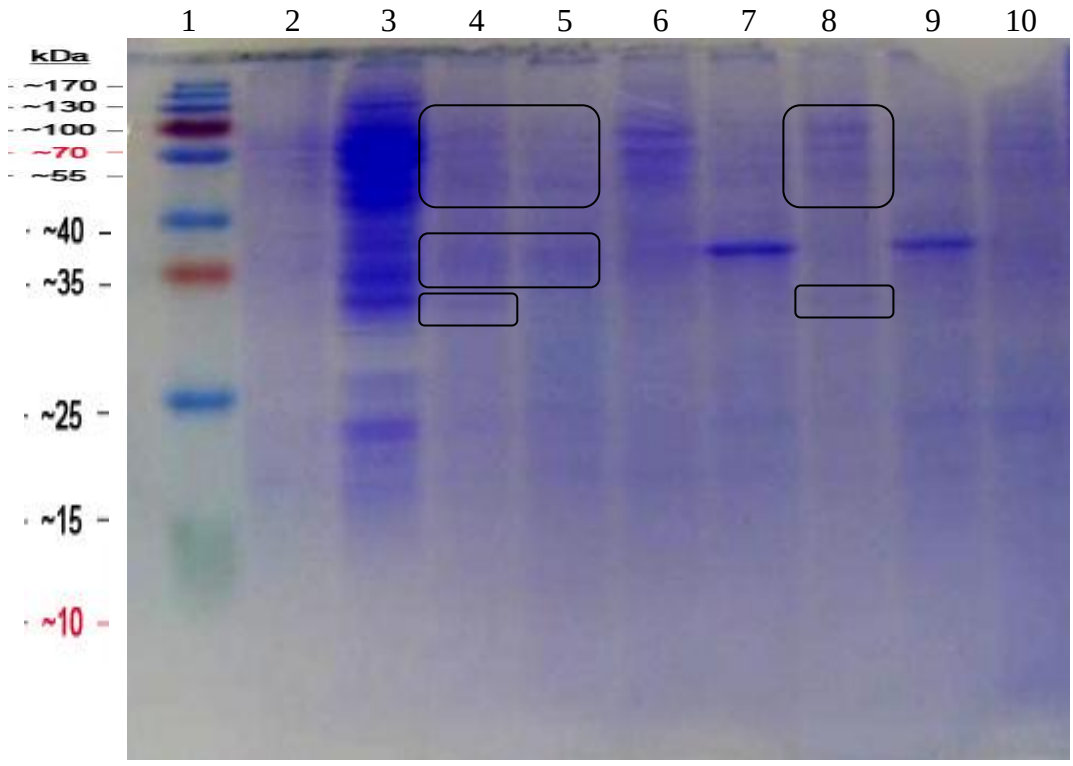


Figure 3.4 Cell protein profiles of BFL isolates by SDS-PAGE.
Lane 1: Ladder,
Lane 2: BFL1 – *Bacillus circulans,*
Lane 3: BFL2 – *Bacillus megaterium,*
Lane 4: BFL3 – *Bacillus subtilis,*
Lane 5: BFL4 – *Bacillus subtilis,*
Lane 6: BFL5 – *Bacillus amyloliquefaciens,*
Lane 7: BFL6 – *Bacillus licheniformis*,
Lane 8: BFL7 – *Bacillus subtilis*,
Lane 9: BFL8 – *Bacillus licheniformis*,
Lane 10: BFL9 – *Bacillus cereus*

3.2.5 FAME analysis for closely related BFL strains

The BFL3, BFL4 and BFL7 isolates, identified as *Bacillus subtilis* by 16S rRNA sequencing, were closely related and no differences in the obtained sequences were observed. Therefore, those strains were further analyzed according to their fatty acid profiles for confirmation of the previous physiological and biochemical methods (API, SDS-PAGE and morphological characterization) which had showed slight differentiations.

Fatty acid methyl ester (FAME) analysis of the cell-wall fatty acids from the 3 isolates, revealed the presence of 5 fatty acids identified as palmitic acid (16:0), stearic acid (18:0), elaidic acid (18:1n9t), oleic acid (18:1n9c) and linoleic acid (C18:2n6c). FAME analysis showed a clear distinction between the three organisms (Figure 3.5). The cell wall of BFL3 contained higher amounts of oleic acid and lower amounts of stearic acid and linoleic acid. Moreover, the cell-wall of the BFL3 also contained elaidic acid, which was absent in the cell wall of BFL4 and BFL7. The fatty acid profiles of BFL4 and BFL7 were very similar. However, the cell wall of the BFL4 contained higher amounts of oleic acid and linoleic acid and lower amount of palmitic acid in comparison to BFL7.

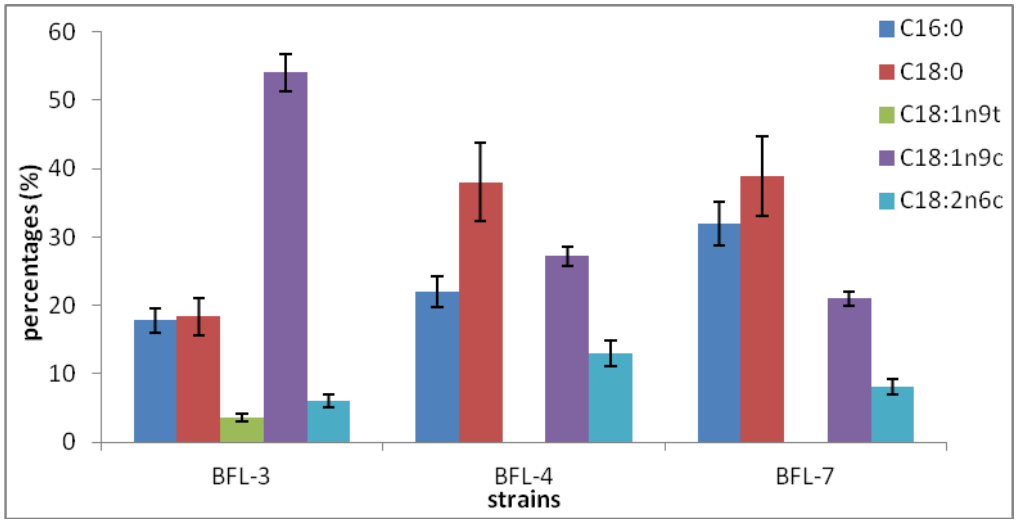


Figure 3.5 Fatty acid profiles of BFL3, BFL4 and BFL7

3.2.6 Identification of fungus present in FF

Analysis of the fungus, isolated from the FF bioaugmentation product, was conducted macroscopically, on solid and liquid medium. The organism grew readily on malt extract agar forming a colourless to grayish mycelium (Figure 3.6). The fungus grew well and sporulated at 35° C, while there was growth but no sporulation at 37° C. No growth was observed at 40° C.

Figure 3.6 Growth of fungus on malt extract agar.

When the organism was grown in liquid culture, pelleted growth of the fungus was observed. The pellets were clear, thick and resistant under agitation (Figure 3.7-A), while the structure of the pellets was more diffuse in the absence of agitation (Figure 3.7-B).

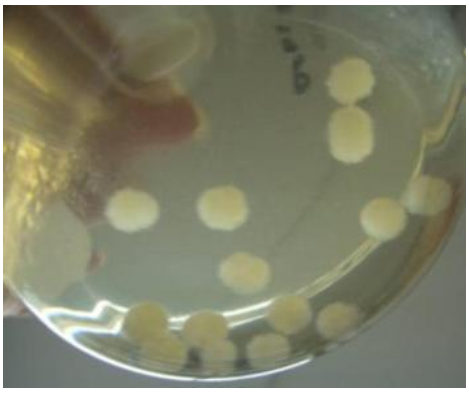
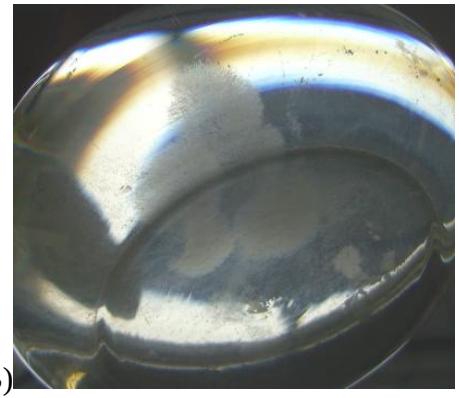
A)  B) 

Figure 3.7. Pelleted formations in the flask by FF incubated in ENM (A) agitation, (B) no agitation

Microscopic examination of the funugs (Figure 3.8) revealed the presence of septae and sporangiospores which were ellipsoidal and measured 7x5 µm. Sporangiophores were either long (> 10 mm) and seldom branched or short (1-2 mm) and frequently branched. Sporangia on long sporangiophores had at first a yellowish color which became light brown and measured up to 80 µm in diameter.

According to the colony habits and the morphological characteristics described above, the organism was identified as *Mucor circinelloides* van Tieghem and verified by DSMZ Identification Service.

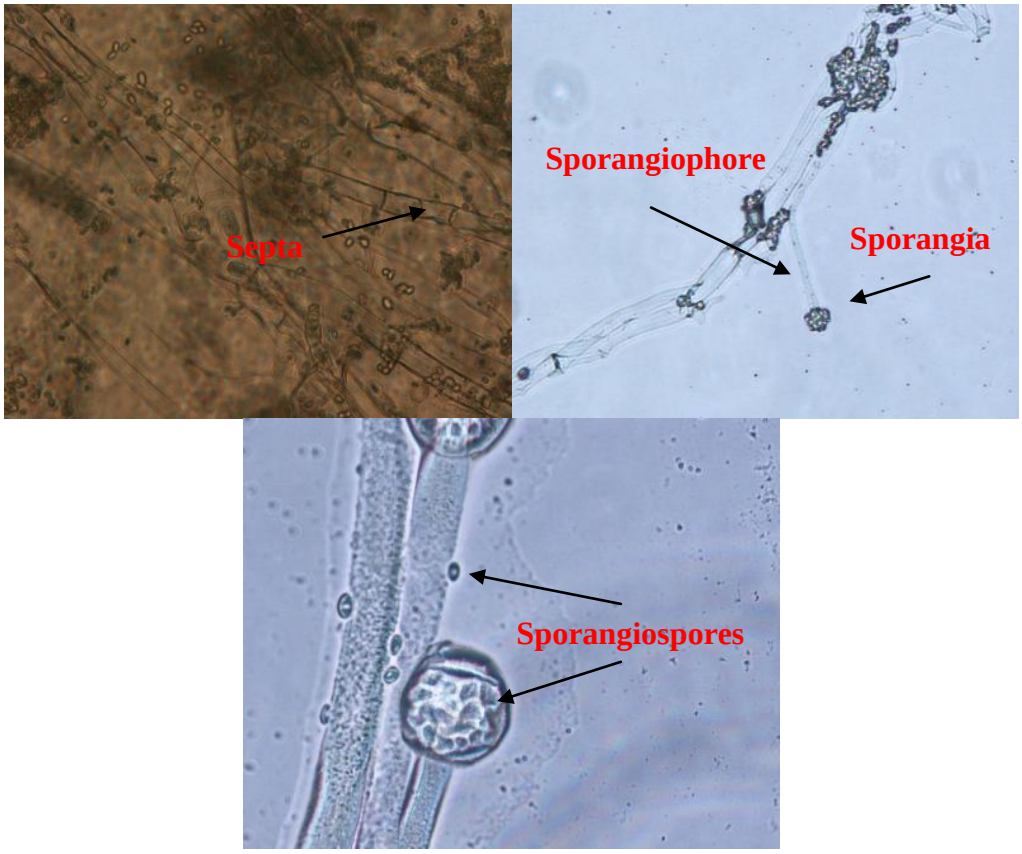


Figure 3.8. Microscopic observations of the fungus

The hydrolytic activity of the fungus was tested and presented in Table 3.15. Lipase, amylase, protease and xylanase were all produced. No substrate specificity of lipase activity was observed, as lipase was produced on all three substrates, tributyrin agar, tween 20 agar and tween 80 agar. Biosurfactant production was also observed.

Table 3.15 Enzymatic activity and biosurfactant production by the fungus

Lipase production using Tributyrin agar	+
Lipase production using Tween 20 agar	+
Lipase production using Tween 80 agar	+
Amylase production	+
Protease production	+
Xylanase production	+
Biosurfactant Production	+

+ positive

3.3 The role of the isolates in FF and BFL

The findings in Section 3.1 showed that the products FF and BFL could both degrade fats under certain environmental conditions and so it was of interest to investigate the role of constituent members of the bioaugmentation products in the biodegradation of butter and olive oil. The isolates from the two products, which were identified and characterized in Section 3.2 were tested in pure culture and in defined mixed cultures. Aerobic batch studies were carried out in minimal medium and in enriched nutrient medium at 30^{o}C for up to 13 days. The percentage fat removal was measured together with the ability of the organisms to produce biosurfactant.

FF isolates

FF was found to consist of 5 *Bacillus* spp. and 2 *Pseudomonas* spp. The seven bacterial isolates were grown individually on butter and olive oil for up to 13 days and tested for biosurfactant production and fat removal. None of the pure cultures produced any biosurfactant or degraded any fat. This was reflected in an absence of any emulsification of the fat in the flasks. A similar result was obtained with the combined *Bacillus* isolates, the combined *Pseudomonas* isolates and when all seven isolates were combined and grown on the fats.

The product had also been shown to contain a fungus. The fungus was grown alone and in combination with all the FF isolates in the two media supplemented with either fat. While the fungus could not grow well on the fats in minimal medium, very good growth coupled with good fat removal was obtained when the organism was grown in enriched medium. When the fungus was combined with all seven bacterial isolates, degradation of the fat was similar to that obtained with the fungus alone. Furthermore, the levels of fat removal corresponded with that obtained for the product as a whole (Section 3.1) suggesting that the degradative ability of the product could only be attributed to the fungus and not the bacteria.

BFL isolates

When the nine *Bacillus* isolates present in BFL were incubated individually or when combined in a mixture, on butter and olive oil for up to 13 days no fat degradation or biosurfactant production was observed. The product containing these isolates had shown good degradative ability on oil but not on butter (Section 3.1). When however a Gram negative bacterium, *P. putida* CP1 was introduced to the product (BFL-CP1) excellent degradation of both butter and oil was observed (Table 3.16). Further studies were carried out on BFL and BFL-CP1 to investigate the role of *P. putida* CP1 in the mixed microbial community.

Table 3.16 Percent fat removal of butter and olive oil in two media by microbes present in FF and BFL.

CULTURE	**Fat removal (%)**				**Incubation Time (Days)**
	BUTTER (7.5 g fat/L)		**OIL (8 g fat/L)**		
Product FF	MM	ENM	MM	ENM	
FFA – *B. subtilis*	–	–	–	–	13 days
FFB – *B. amyloliquefaciens*	–	–	–	–	13 days
FFC – *B. subtilis*	–	–	–	–	13 days
FFD – *B. subtilis*	–	–	–	–	13 days
FFE – *B. licheniformis*	–	–	–	–	13 days
FFF – *P. putida*	–	–	–	–	13 days
FFG – *P. putida*	–	–	–	–	13 days
Bacillus spp. (A-F) combined	–	–	–	–	13 days
Pseudomonas spp. (F,G) combined	–	–	–	–	13 days
Bacillus and *Pseudomonas* combined (A-G)	–	–	–	–	13 days
Fungus – *Mucor circinelloides*	–	94%	–	92%	13 days
Fungus and FF isolates (A-G) combined	–	92%	–	91%	13 days
Product BFL	MM	ENM	MM	ENM	
BFL1 – *B. circulans*	–	–	–	–	13 days
BFL2 – *B. megaterium*	–	–	–	–	13 days
BFL3 – *B. subtilis*	–	–	–	–	13 days
BFL4 – *B. subtilis*	–	–	–	–	13 days

BFL5 – *B. amyloliquefaciens*	–	–	–	–	13 days
BFL6 – *B. licheniformis*	–	–	–	–	13 days
BFL7 – *B. subtilis*	–	–	–	–	13 days
BFL8 – *B. licheniformis*	–	–	–	–	13 days
BFL9 – *B. cereus*	–	–	–	–	13 days
Bacillus spp. combined (1-9)	–	–	–	–	13 days
Product BFL	–	–	34%	94%	13 days
Pseudomonas putida CP1	–	–	–	–	13 days
Product BFL plus *Pseudomonas putida* CP1	88%	89%	73%	85%	7 days

- no removal

3.4 An investigation of the biodegradation of butter and oil by BFL and BFL-CP1

Fermentation studies were carried out using BFL and BFL-CP1. The degradation of butter and oil by the mixed microbial populations was investigated in aerobic batch culture at 30^{o}C for up to 13 days. Two culture media, minimal medium and enriched nutrient medium, supplemented with either butter (7.5 total fat g/L) or olive oil (8 g/L) were investigated. The inoculum size was 10^{6} cfu/ml in 100 ml culture medium. The parameters monitored included total fat, cell growth, pH, yield and fatty acid metabolism.

3.4.1 Fat Biodegradation by BFL

The results obtained following the growth of BFL on butter (7.5 total fat g/L) and olive oil (8 g/L) are described in Figure 3.9 and Figure 3.10, respectively. In the case of butter, no growth and no fat removal was observed in either of the two culture media after 13 days (312 h) of incubation. However, the fat was emulsified and the appearance of the culture media in the flasks was milky throughout the incubation. The initial pH of the culture media was 7.65-7.88. It increased to pH 9 following one day of incubation and remained almost constant for the remainder of the fermentation run.

In the case of oil, the mixed microbial population was found to degrade the fat. The colour in the flasks that contained the oil with the enriched nutrient medium changed from light brown to dark brown by the end of the incubation, while the colour in the flasks with the minimal medium remained white milky. In the minimal medium there was a lag of nine days followed by the removal of 34% of the oil. This represented a drop on the level of oil from 8g/L to 5.3 ± 0.17 g/L. When enriched medium was used, the lag in fat removal was reduced to 2 days and this was followed by a removal of 94% of the fat a decrease in the oil concentration from 8 g/L to 0.48 ± 0.0085 g/L.

During removal, the rate of fat removal was also greater for the enriched medium (0.027 ± 0.001 g fat/L/h) than for the minimal medium (0.013 ± 0.002 g fat/L/h). Growth, monitored using dry weight measurements, reflected the pattern of fat removal. Growth and the growth rate were greater in the enriched medium than in the minimal medium. The specific fat removal values (g/g/h) were the same in the two media, however the lag in the minimal medium was much longer. The yield obtained for the enriched medium however was higher than that obtained for the minimal medium. The yield coefficient, $Y_{X/S}$, was calculated based on the amount of dry weight (X) produced per oil removed (S) at the time of optimum fat removal and corresponded to 0.175 ± 0.018 g/g for the enriched medium compared with a value of 0.135 ± 0.017 g/g for the minimal medium (Table 3.17). The pattern of pH change was similar to that obtained with butter.

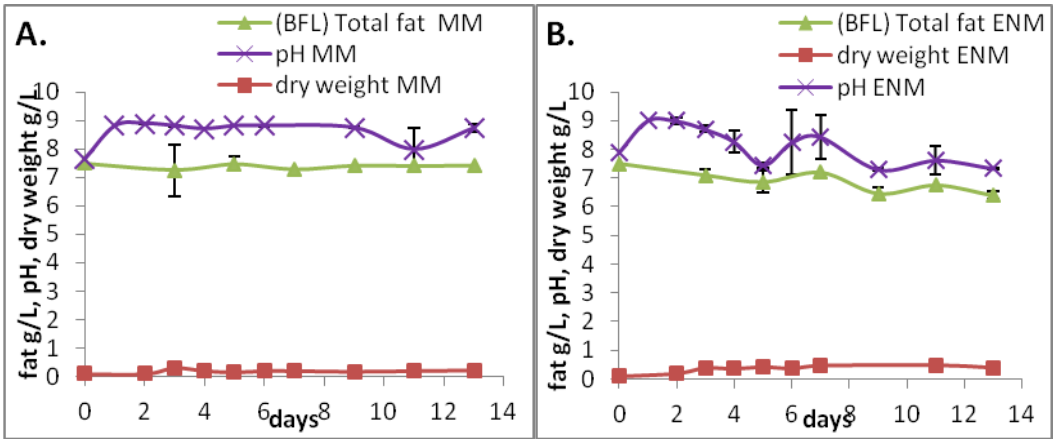


Figure 3.9 Butter removal, dry weight, pH in A) minimal medium and B) enriched nutrient medium

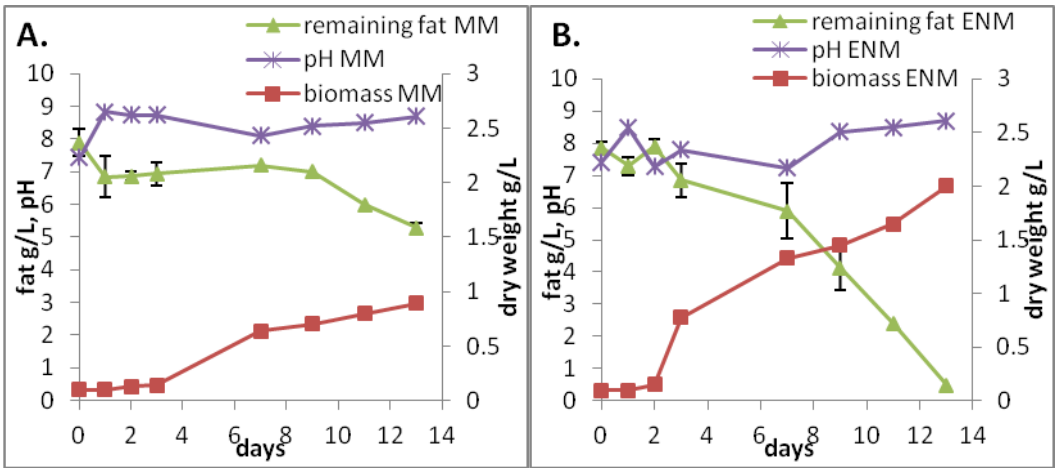


Figure 3.10 Olive oil removal, dry weight, pH in (A) minimal medium and (B) enriched nutrient medium

Table 3.17 Growth rates and fat removal rates of the olive oil

	MM (lag phase 7 days)	**ENM (lag phase 2 days)**
Growth rate (h^{-1})	0.0076	0.0099
Specific fat removal (g/g/h),	0.012 ± 0.002	0.01 ± 0.005
Fat removal rate (g/L/h)	0.013 ± 0.002	0.027 ± 0.001
Fat removal (%)	34 ± 2	94 ± 0.1
Yield ($Y_{X/S}$, g/g)	0.135 ± 0.017	0.175 ± 0.018

3.4.1.1 Fat metabolism by BFL

Following the determination of total fat, samples taken during fermentation were analysed by TLC for hydrolysis products and by GC for fatty acid composition. The extracted butter and oil, from both minimal medium and enriched medium was analysed by TLC according to the method described in 2.2.6.1 Analysis showed the presence of non-hydrolysed fat in the form of triglycerides for all four samples at the beginning of the fermentation (Figure 3.11 and Figure 3.12). Following incubation fat hydrolysis was noted in all four treatments. In the case of butter, triglycerides were present in the samples up to day 9. During that time fatty acids (B) and diglycerides (C) were also detected. No monoglycerides (D) were detected. A similar pattern was observed for the enriched medium, however, triglycerides were not detected after 5 days of incubation. When oil was the substrate, triglycerides were present until day 11 in both media. Diglycerides were detected on days 11 and 13 and free fatty acids were detected throughout the incubation period. No mono-glycerides were detected. The findings showed that fat hydrolysis did occur in each treatment regardless of fat removal. The main hydrolysis products were free fatty acids and diglycerides.

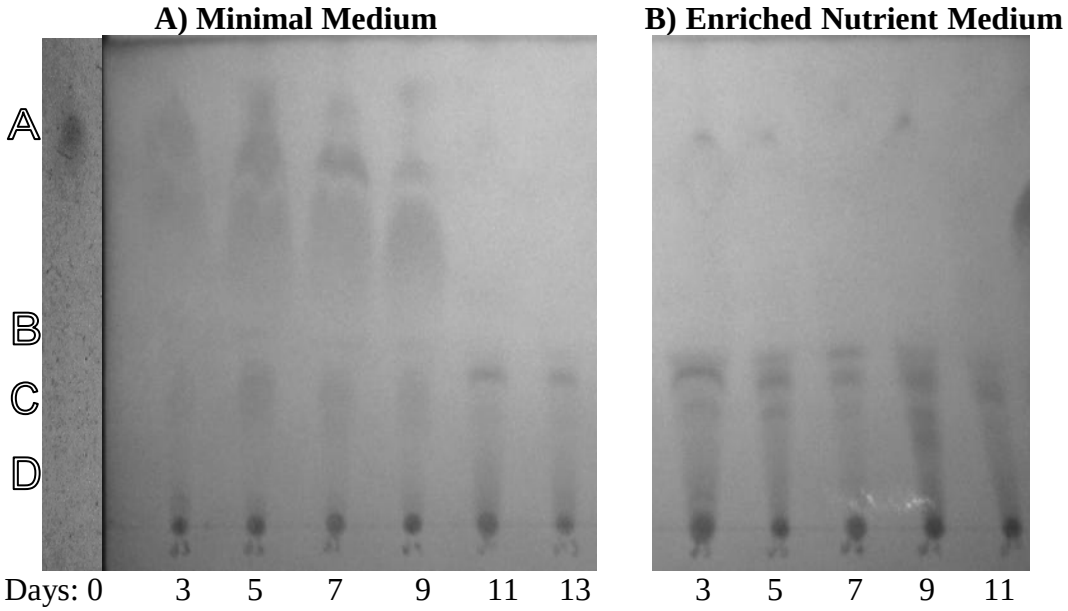


Figure 3.11 TLC analysis of the hydrolysis products of butter by BFL in A) minimal medium and B) enriched nutrient medium

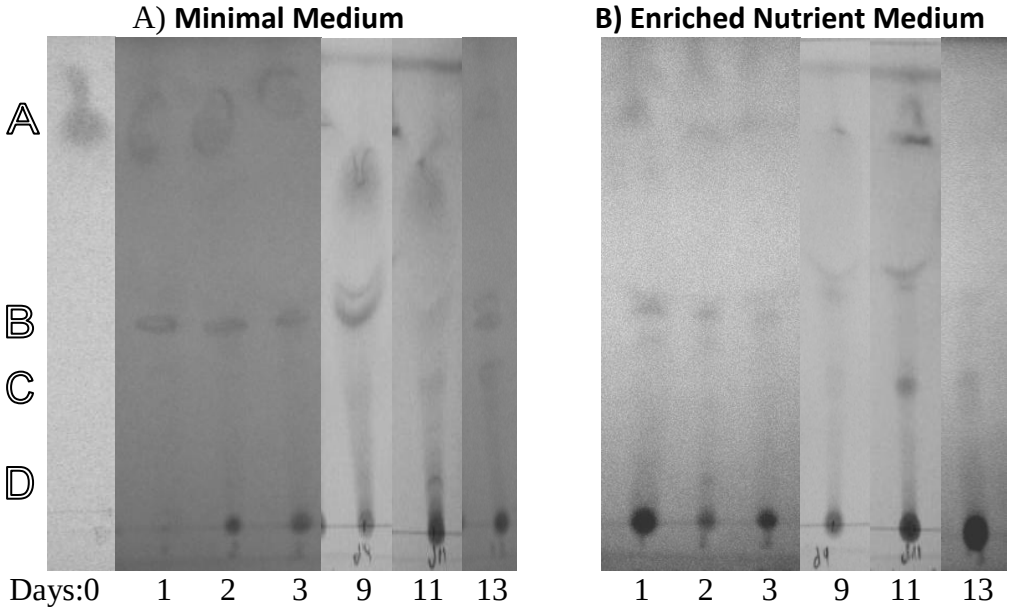


Figure 3.12 TLC analysis of hydrolysis products of oil by BFL in A) minimal medium and B) enriched nutrient medium

The fatty acid composition of the butter and oil during the fermentation was quantified by GC (Table 3.18). Traces of the fatty acid composition of the butter and oil are illustrated in Figure 3.13 and Figure 3.14, respectively. Traces of the fatty acids obtained during the fermentations are described in Appendix B.

Table 3.18 Fatty acid composition (%) in butter and olive oil

Fatty acids		butter	olive oil
Capric Acid	C10:0	1.4	–
Lauric Acid	C12:0	3.64	–
Myristic Acid	C14:0	14.2	–
Myristoleic Acid	C15:0	1.15	–
Palmitic Acid	C16:0	33.8	11.9
Palmitoleic Acid	C16:1n9c	1.64	0.77
Stearic Acid	C18:0	12	2.57
Elaidic Acid	C18:1n9t	4.25	–
Oleic Acid	C18:1n9c	25	76.74
Linoleic Acid	C18:2n6c	1.2	6.9
Arachidic Acid	C20:0	0.9	0.4
cis-11-Eicosenoic Acid	C20:1	0.97	0.7
Saturates		**66.7**	**14.87**
Monounsaturates		**31.86**	**78.21**
Polyunsaturates		**1.2**	**6.9**

The main fatty acids present in the butter were: lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), elaidic acid (C18:1n9t) and oleic acid (C18:1n9c). Other fatty acids detected by the GC at lower levels than the main seven fatty acids were: capric acid (C10:0), myristoleic acid (C14:1n9c), palmitoleic acid (C16:1n9c), linoleic acid (C18:2n6c), arachidic acid (C20:0) and cis-11-eicosenoic acid (20:1).

The main fatty acids identified in the olive oil were palmitic acid (C16:0), palmitoleic acid (C16:1n9c), stearic acid (C18:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c) and cis-11-eicosenoic acid (20:1). Arachidic acid (C20:0) was also detected in the oil by GC at lower levels.

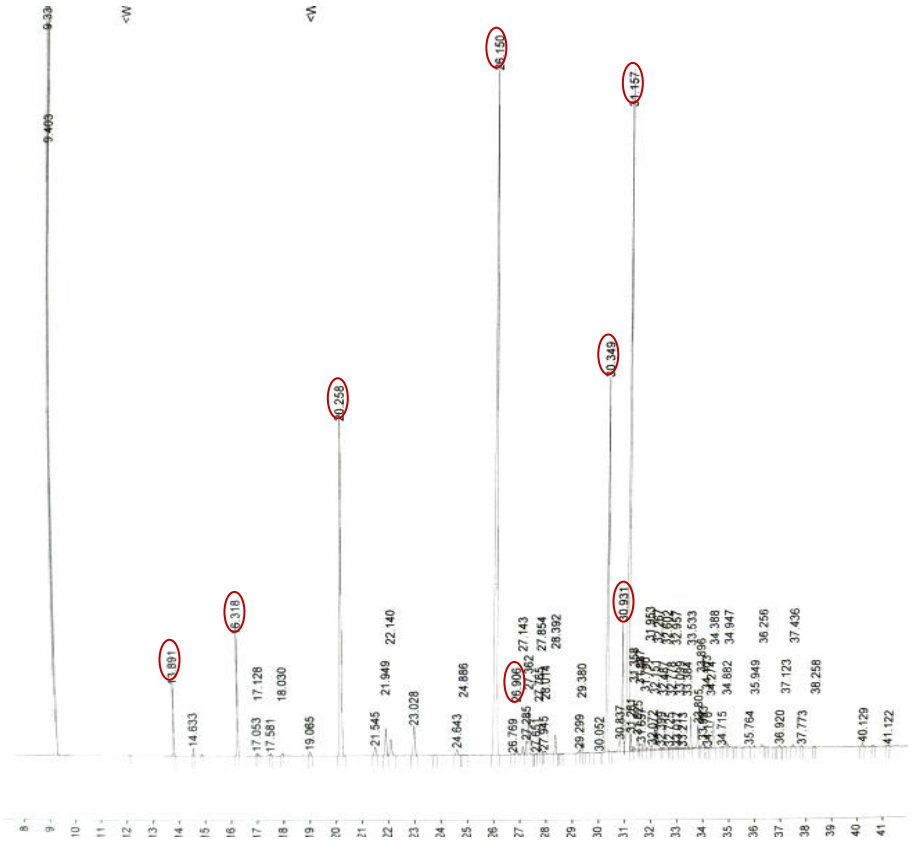


Figure 3.13. Traces of fatty acid composition of the butter, day 0.

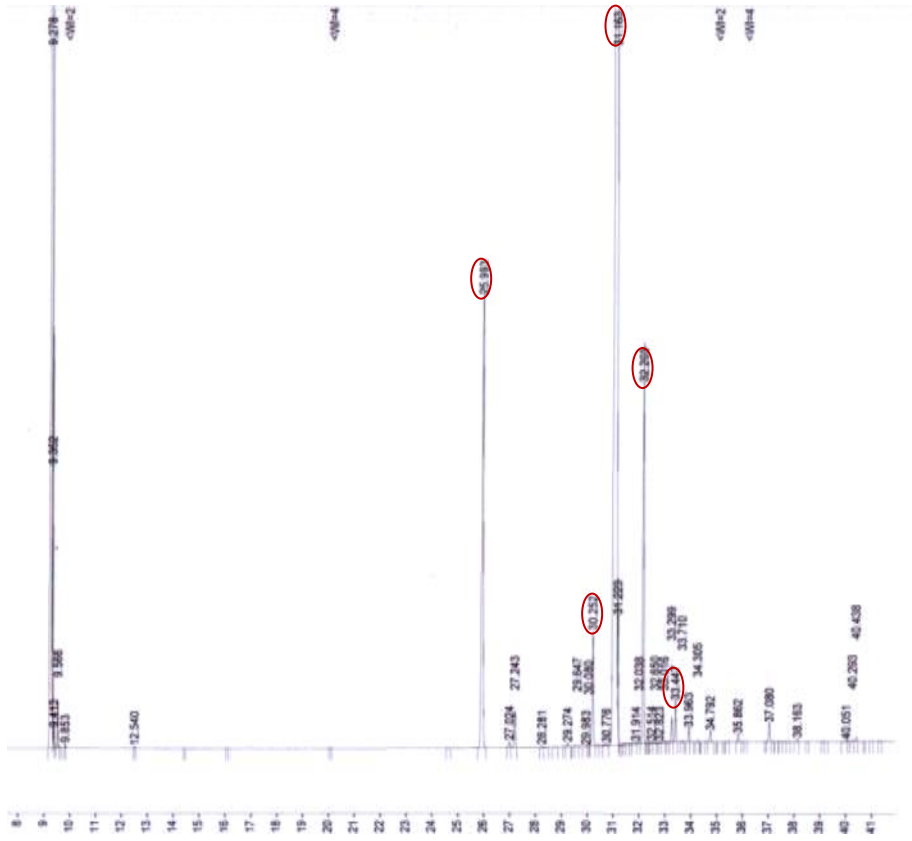


Figure 3.14 Traces of the fatty acid composition of olive oil, day 0.

The levels of each of these main fatty acids were monitored with time. The changes in the main fatty acids present in the butter are described in Figure 3.15 and Figure 3.16. There was no degradation of butter by BFL and this is reflected in the absence of change in the levels of the main fatty acids. Interestingly, linoleic acid (C18:2) and cis-11-eicosenoic acid (C20:1), were removed by the bacteria in minimal medium.

The changes in the main fatty acids present in the oil are described in Figure 3.17 and Figure 3.18. There was no degradation of the oil in the minimal medium. Of the main fatty acids present in the oil, palmitoleic acid (C16:1n9c) and cis-11-eicosenoic acid (20:1) were the only fatty acids removed. Degradation of the oil had been observed in enriched medium and this was reflected in the removal of all the main fatty acids except stearic acid (C18:0). Elaidic acid (C18:1n9t) was not present in the oil but appeared in the culture medium following 3 days of fermentation. It was removed as the fermentation progressed in both the minimal medium and the enriched medium.

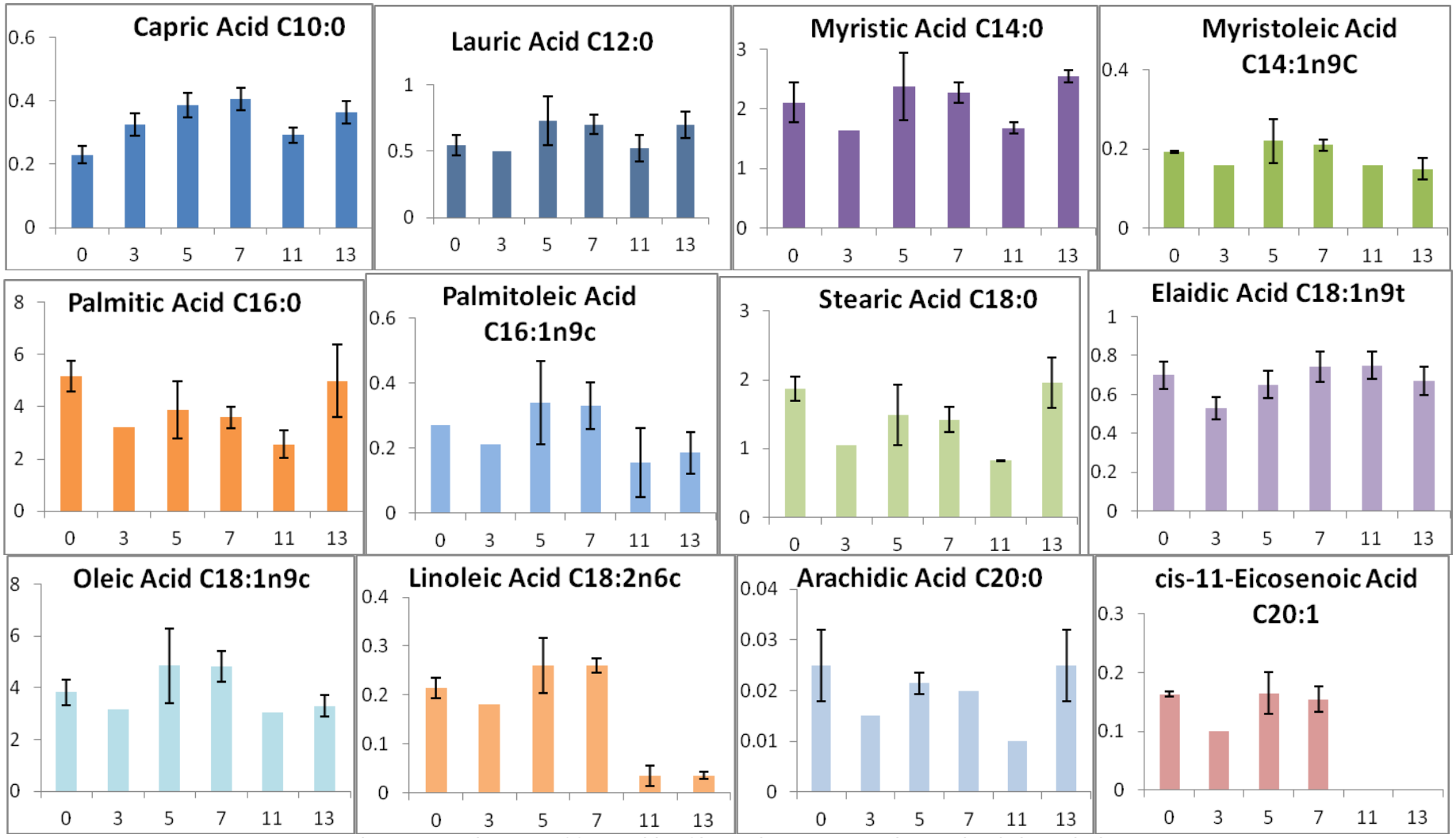


Figure 3.15 Changes of fatty acids of butter by BFL grown in MM (mg/ml per day)

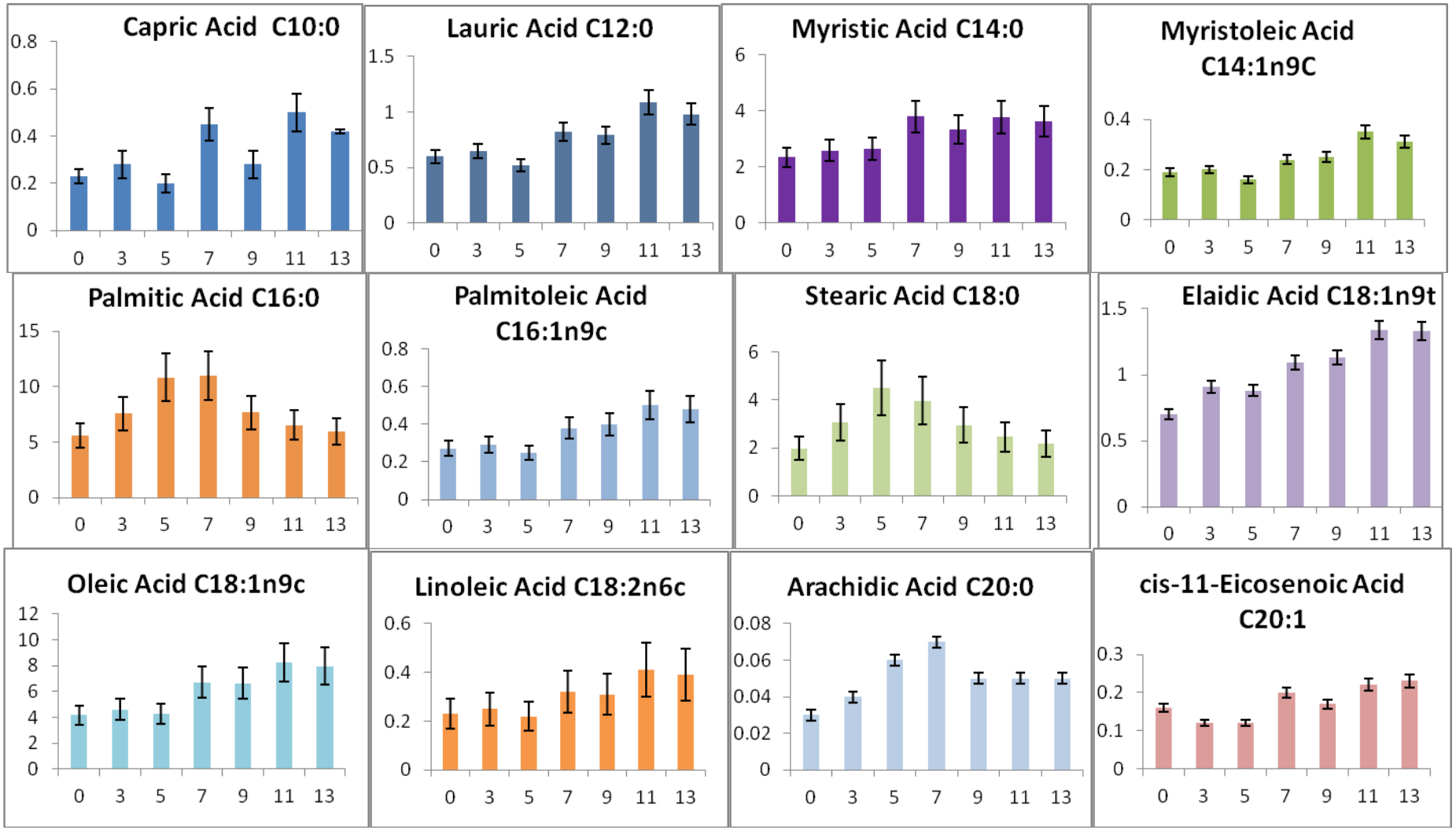


Figure 3.16 Changes of fatty acids of butter by BFL grown in ENM (mg/ml per day)

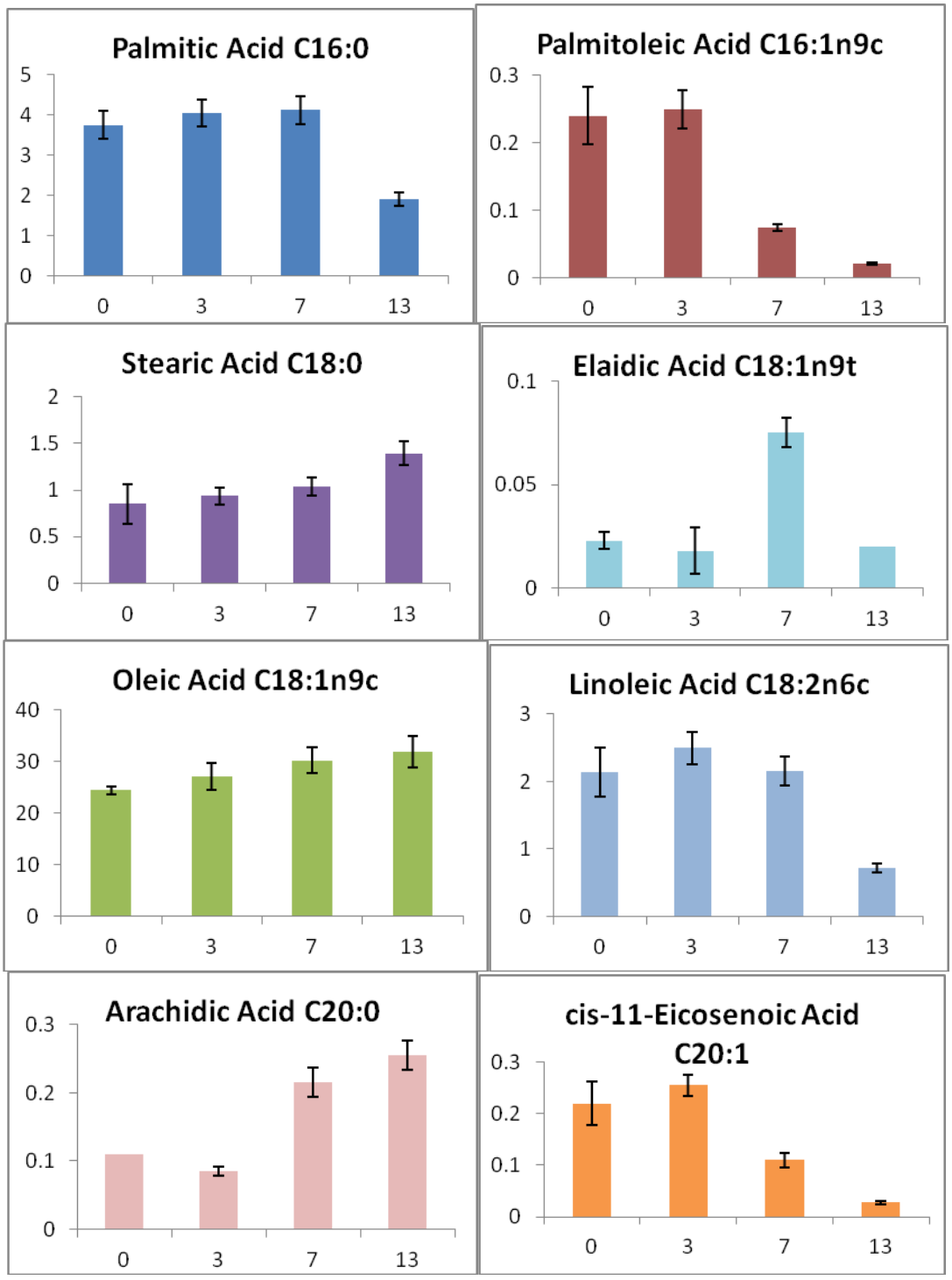


Figure 3.17 Changes in the composition of fatty acids of oil by BFL in MM (mg/ml per day)

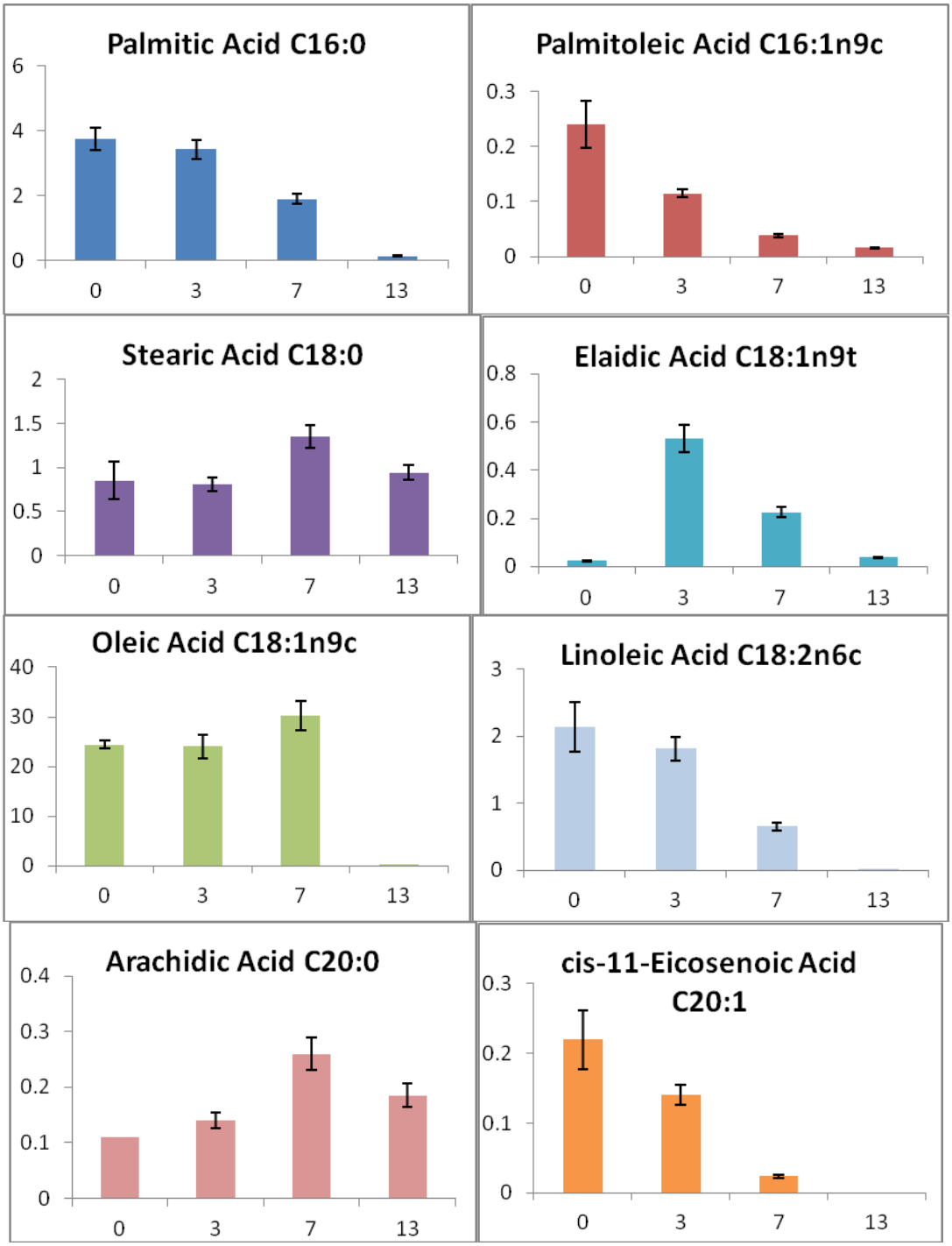


Figure 3.18 Changes in the composition of fatty acids of oil by BFL in ENM (mg/ml per day)

3.4.2 Fat Biodegradation by BFL-CP1

The BFL bioaugmentation product had limited ability to degrade fats however the addition of *P. putida* CP1 to the mixture had greatly enhanced its degradative ability. Biodegradation of butter (7.5 g/L) and olive oil (8 g/L) was monitored for a period of up to 13 days at 30° C and 150 rpm in the enriched nutrient medium. The flasks were sampled at regular intervals over a period of 13 days and fat removal, bacterial growth, intracellular lipid and pH were monitored.

The fat was well emulsified in the medium and changes in the color of the medium were observed throughout the incubation time indicating modification of the fat by the bacteria. The patterns of bacterial growth, fat removal, intracellular fat and pH are shown in Figure 3.19. The results show similar patterns for both butter and oil. There was no detectable lag in the removal of the butter or the oil. Most (88%) of the fat is removed in 6 – 7 days in both cases and at a similar rate of 0.05 g fat/L/h (Table 3.19). The remainder of the fat was removed more slowly and at 13 days 92 – 94% of the fat was removed. The organisms grew rapidly in the first 2-3 days reaching an optimal level of biomass, 2.3 - 2.6 ± 0.038 g/L, after 3 days of incubation (72 h) with a growth rate of 0.04 h^{-1}. The levels of biomass were maintained at this level until day 7, when most of the substrate was removed, and then declined.

The specific rate of fat removal was 0.0138 ± 0.0006 g/g/h (or 0.3312 ± 0.0151 g/g/day) for butter and 0.018 ± 0.003 g/g/h (or 0.43 ± 0.1 g/g/day) for the oil. The yield coefficient, $Y_{X/S}$, was calculated based on the amount of dry weight (X) produced per oil removed (S) at the time of optimum fat removal and corresponded to 0.3738 ± 0.0063 g/g for butter and 0.31 ± 0.034 g/g for oil.

Intracellular lipid comprised a maximum value of 1.38 ± 0.54 g/L after 2 days incubation on butter and 0.816 ± 0.26 g/L after 3 days incubation on oil. The bacteria consumed the accumulated fats on entering the stationary phase of growth. Lipid content decreased to approximately 0.1 g/L at day 6 (144 h) and the levels remained low at 0.03 ± 0.004 g/L until the end of the fermentation.

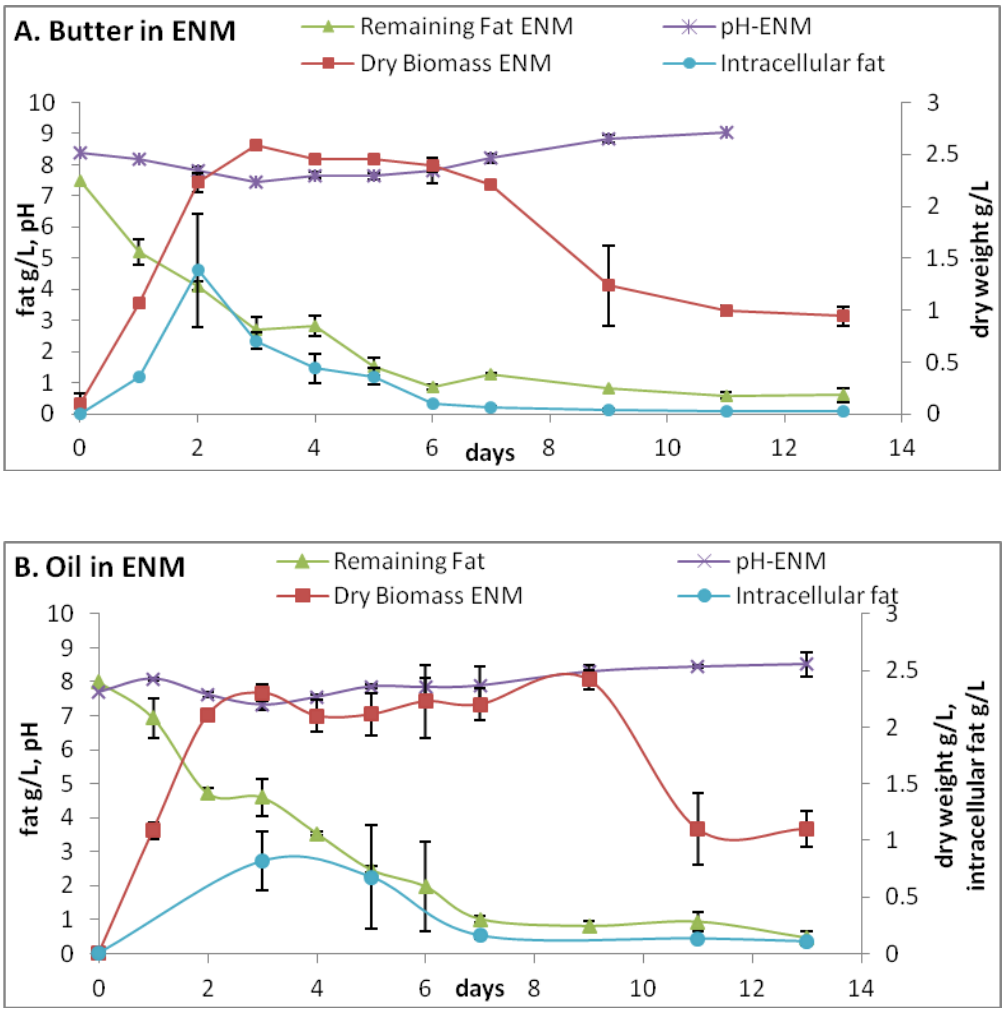


Figure 3.19 Curves of fat removal, dry weight, pH and intracellular fat during cultivation of BFL-CP1 in butter (A) and olive oil (B).

Table 3.19 Growth rate, fat removal rate, specific fat removal rate and yield during the incubation of BFL-CP1 in butter and oil (144h).

	Butter ENM	**Olive Oil ENM**
Growth rate (h^{-1})	0.0437 ± 0.0004	0.0422 ± 0.0003
Specific fat removal (g/g/h)	0.0138 ± 0.0006	0.018 ± 0.003
Fat removal rate (g/L/h)	0.0512 ± 0.0005	0.05 ± 0.006
Fat removal (%)	88.5 ± 1.28 (d6)	87.5 ± 1 (d7)
	92 ± 3 (d13)	94.3 ± 3.56 (d13)
Yield ($Y_{X/S}$, g/g)	0.3738 ± 0.0063	0.31 ± 0.034

3.4.2.1 Fat metabolism by BFL-CP1

During the fermentation, the extracted lipids were analyzed by TLC for their hydrolysed products (Figure 3.20) and by GC for their fatty acid composition. The fats were initially represented as triglycerides on day 0 (A). Triglycerides continued to be detected for up to 6 days when most of the fat was removed. During this period free fatty acids (B) and diglycerides (C) were also detected and continued to be detected until day 13 showing good fat hydrolysis in both systems. Monoglycerides (D) were not detected suggesting either the absence of these breakdown products or the rapid uptake of the hydrolysed products.

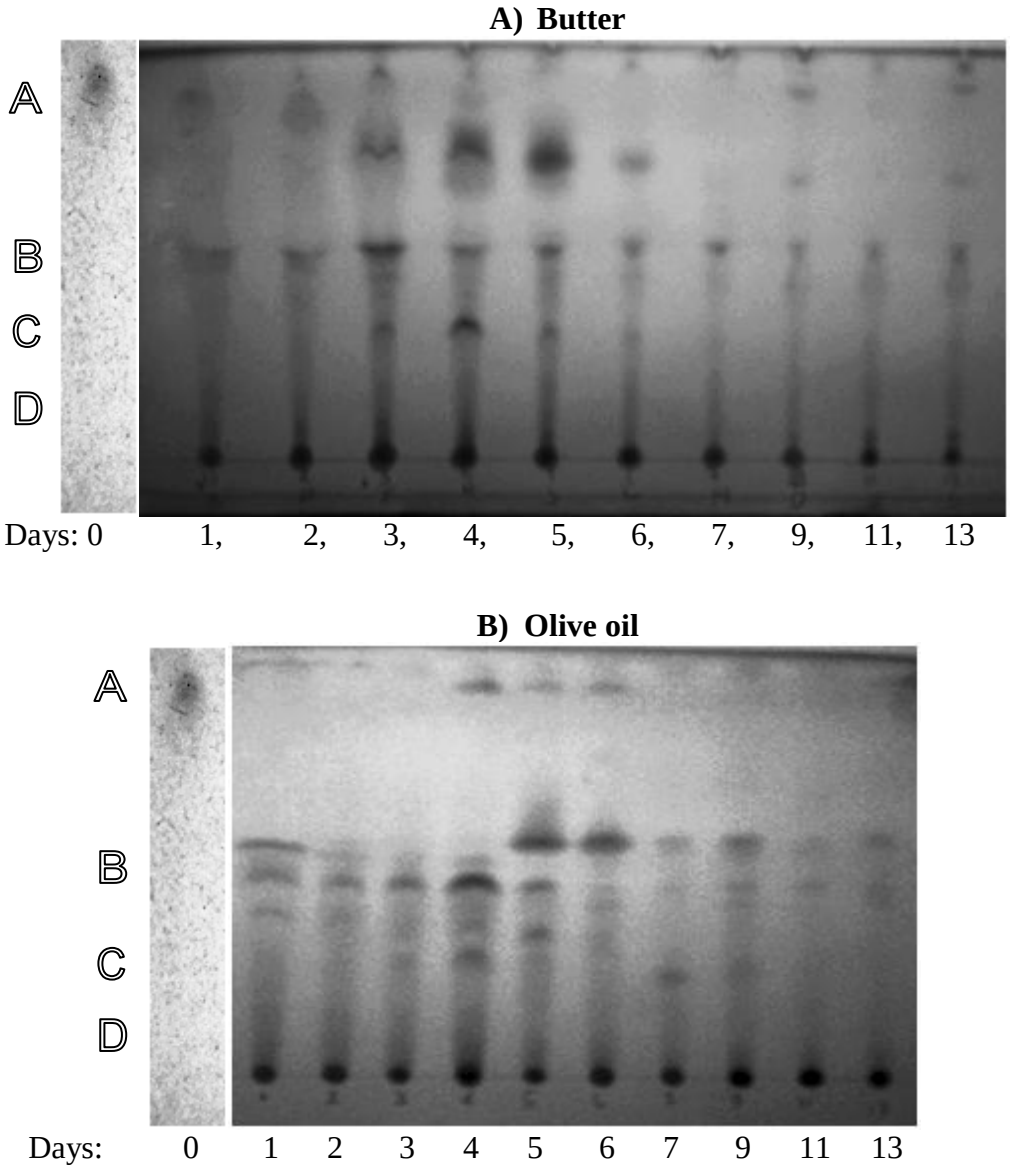


Figure 3.20 Hydrolysis of A) butter and B) olive oil by BFL-CP1

The fatty acid composition of the extracted butter and oil at the beginning of the fermentation quantified by GC were as detected in Section 3.4.1.1. The main fatty acids in the butter were lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9c) and elaidic acid (C18:1n9t). Other fatty acids detected by the GC at lower levels than the main fatty acids were: capric acid (C10:0), myristoleic acid (C14:1n9c), palmitoleic acid (C16:1n9c), linoleic acid (C18:2n6c), arachidic acid (C20:0) and cis-11-eicosenoic acid (20:1).

The main fatty acids present in the olive oil were palmitic acid (C16:0), palmitoleic acid (C16:1n9c), stearic acid (C18:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c) and cis-11-eicosenoic acid (20:1). Arachidic acid (C20:0) was also detected in the oil by GC at lower levels. As in the fermentation studies with BFL in section 3.4.1.1, elaidic acid (C18:1n9t) was detected on day 3 of incubation and it was consumed by the end of the incubation period.

All the main fatty acids were rapidly consumed in both the butter and the oil in the first 5 – 7 days which corresponded with the period of maximum fat removal. The pattern of removal of the stearic acid was different. While it had not been consumed by BFL, the levels of this fatty acid increased initially on day 3 and then decreased. While elaidic acid (C18:1n9t) was not detected in the oil at time 0, its levels rose on day 3 and then decreased.

All the other fatty acids present in lower levels were also consumed during the fermentation except arachidic acid (C20:0) which continued to be detected at the end of the fermentation runs at similar levels to that detected at the beginning.

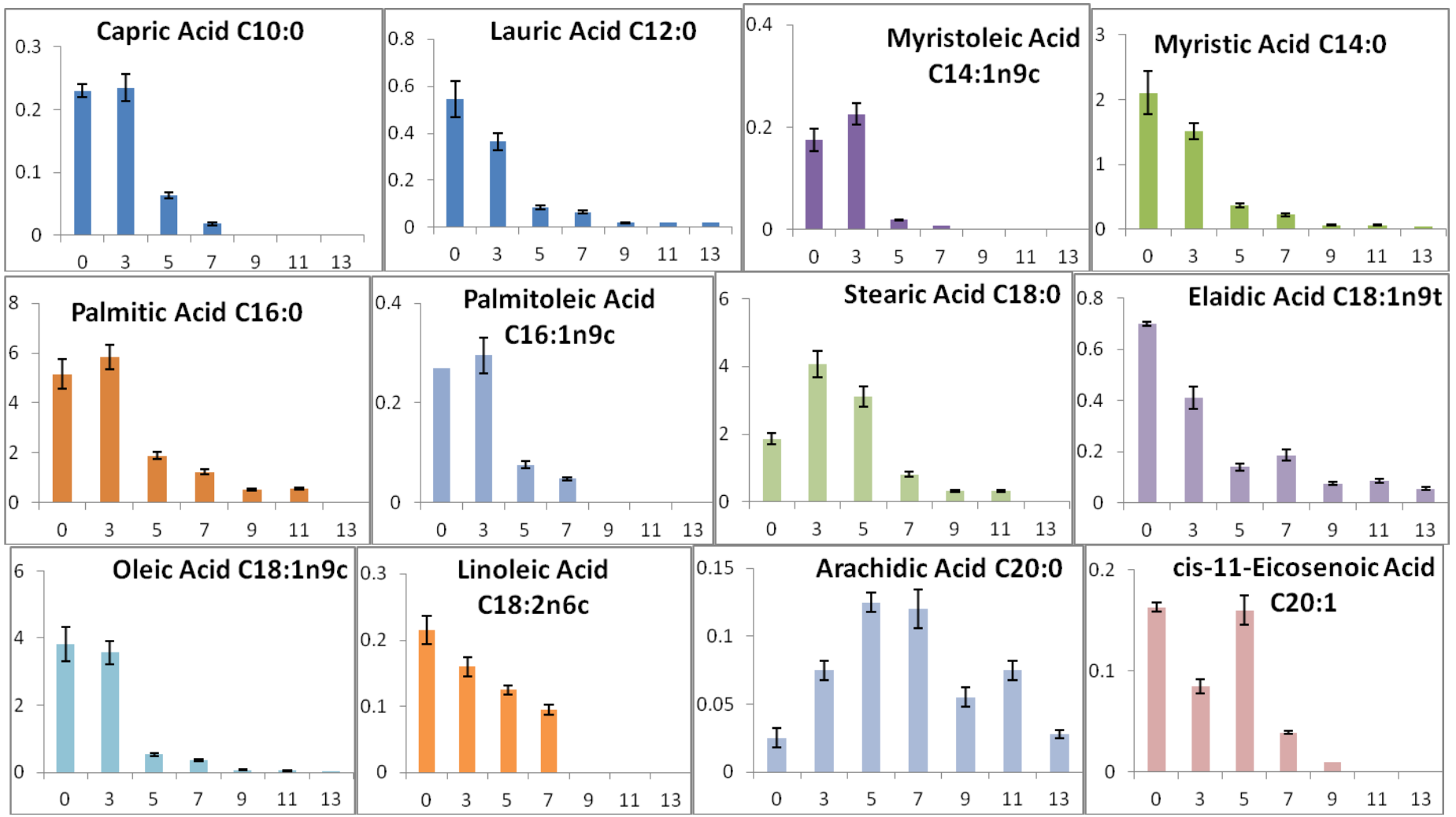


Figure 3.21 Metabolism of fatty acids of butter by BFL-CP1 (mg/ml per day)

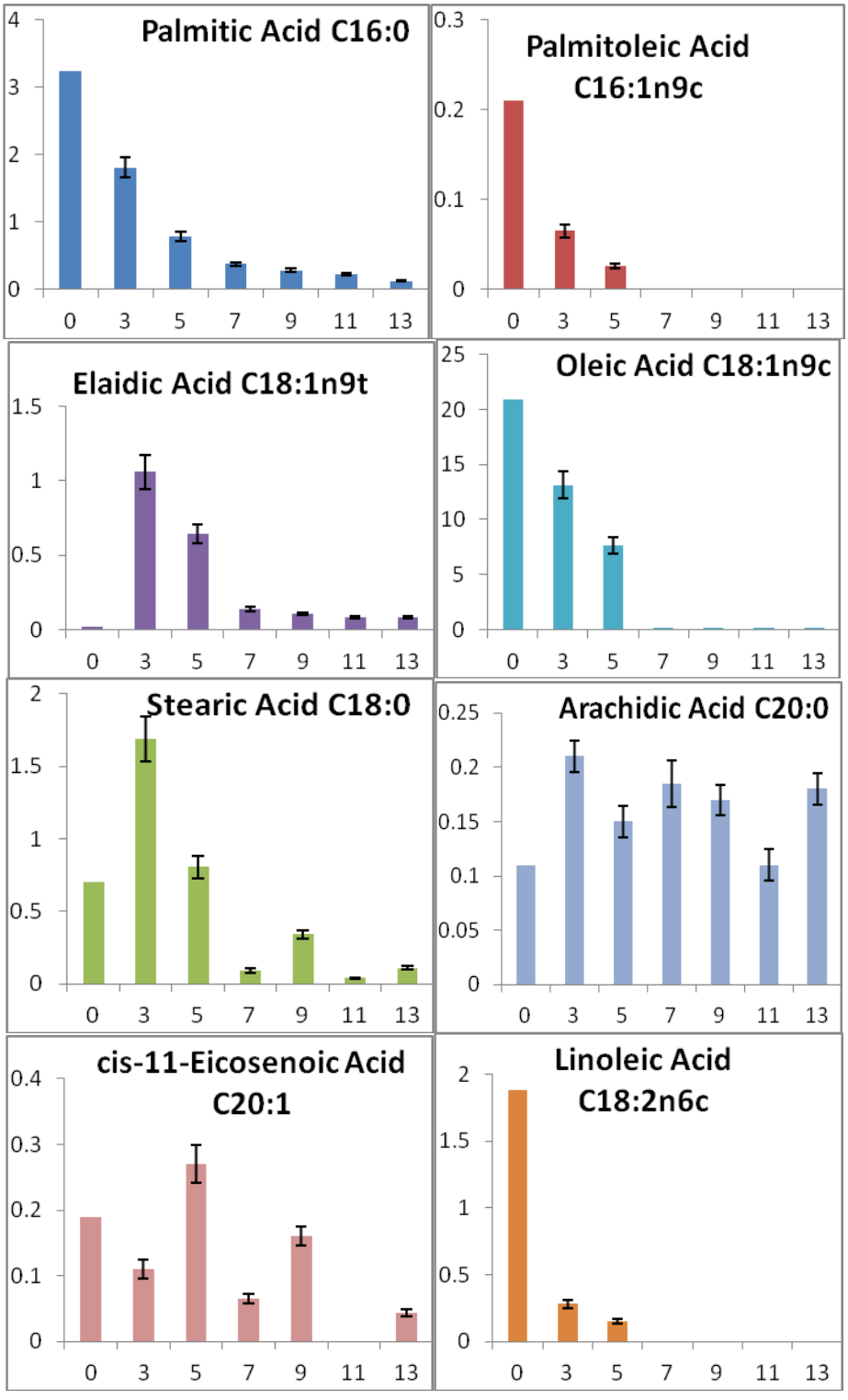


Figure 3.22 Metabolism of fatty acids of oil by BFL-CP1 (mg/ml per day)

Investigating the role of P. putida CP1 in the mixed culture

It had been observed that good fat degradation was achieved when *P.putida CP1* was added to the commercial bioaugmentation product BFL which comprised 9 strains of *Bacillus.* In order to further investigate this result the relative roles of the bacteria was investigated.

When BFL and BFL-CP1 were grown on olive oil (8 g/L) at 30° C and 150 rpm in the enriched nutrient medium, observations of colony morphology showed that *Bacillus* spp. could be detected throughout the run in the case of BFL (Figure 3.23), however in the case of BFL-CP1, *P.putida* CP1 dominated the population after day 1 and continued to be the dominant population up to day 13 (Figure 3.24).

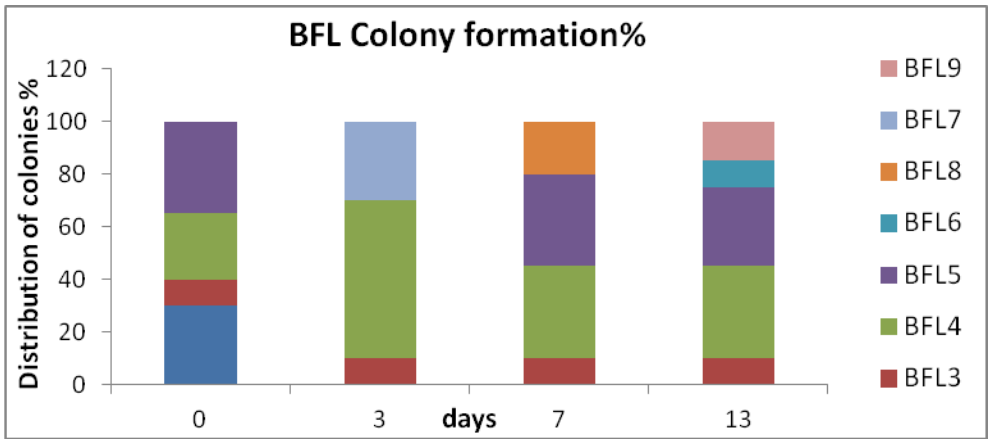


Figure 3.23 Bacterial changes of BFL in olive oil in ENM

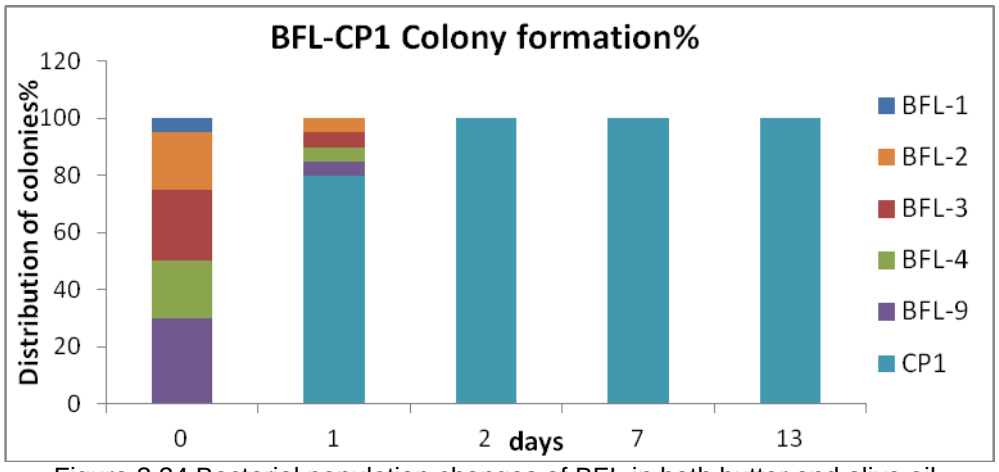


Figure 3.24 Bacterial population changes of BFL in both butter and olive oil.

When the *Bacillus* spp. and the *P.putida* were monitored using selective plating techniques, a similar result was obtained. The *Bacillus* species were found to grow in the absence *of P.putida* – when BFL was used. However when BFL-CP1 was used the *Bacillus* population was detected but did not show significant growth. The gfp labeled form of *P. putida*, *P.putida* CP1::Tn7-*gfp* showed a steady increase in the numbers of this organism (Figure 3.25).

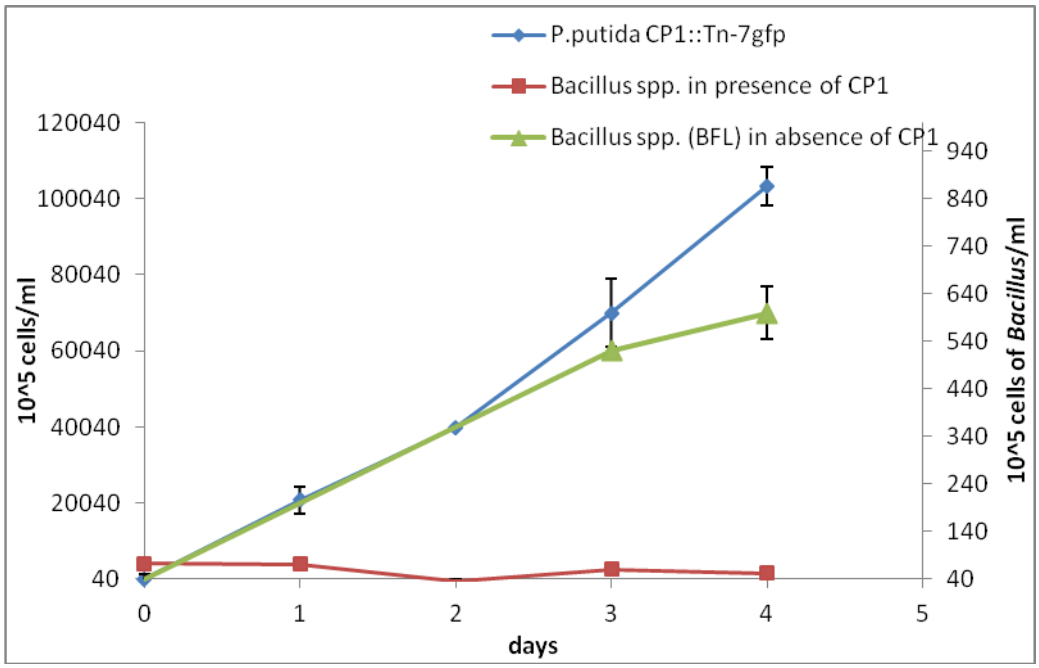


Figure 3.25 Determination of bacterial cells numbers and populations of *P. putida* CP1::Tn7-*gfp* and *Bacillus* spp. in presence and absence of *P. putida* CP1

The results obtained for emulsification, fat degradation and fatty acid metabolism suggest that while the *Bacillus* spp. could hydrolyse the fat, fat metabolism was largely due to the presence of the Gram negative organism.

It was noted that BFL-CP1 formed aggregates in the medium unlike BFL which was more dispersed.

A. B.

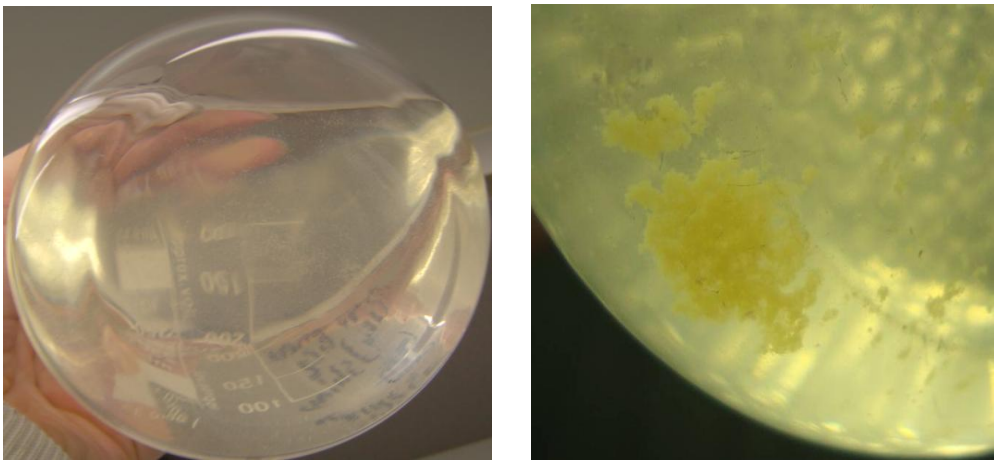


Figure 3.26 Appearance of BFL (A) and BFL-CP1 (B) in the culture medium

The clumps formed by the combination of BFL with the *Pseudomonas putida* strain were observed microscopically (Figure 3.27).

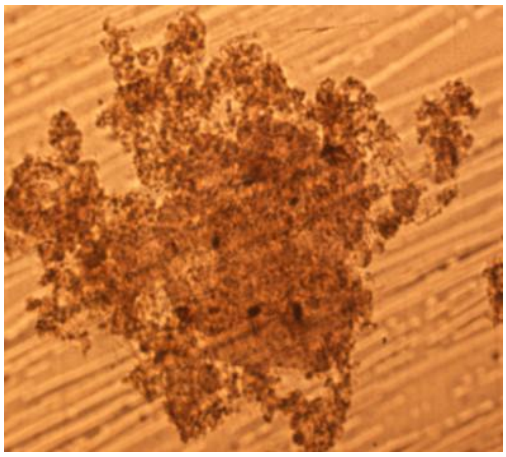


Figure 3.27 Microscopically observation of BFL-CP1 clumps (40x and 10x)

A biochemical examination of the aggregates showed the presence of EPS comprising carbohydrate, protein and some DNA (Figure 3.28). This aggregative response is thought to enable the introduced bacterium to compete satisfactorily in the mixed microbial community.

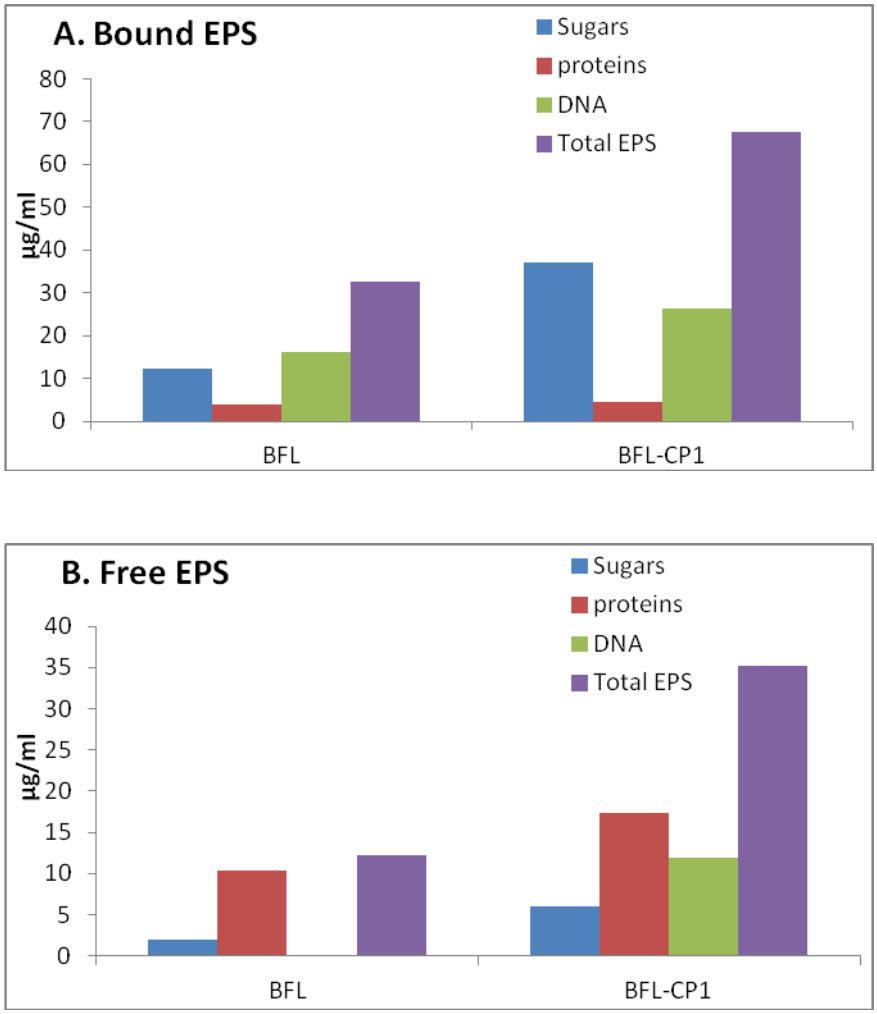


Figure 3.28 Concentration of cell-bound EPS (A) and free EPS (B) of BFL and BFL-CP1

4 DISCUSSION

Three commercial bioaugmentation products were investigated for their ability to degrade FOG. The products were produced for use in grease traps. Operators of grease traps have a reluctance to use biological products and so it was of interest to investigate their degradative ability. The three commercial bioaugmentation products were supplied in different forms - BFL in powder form, Gnz in liquid and FF in tablet form. Preparation of the products was carried out according to the manufacturer's instructions.

The three bioaugmentation products were tested under laboratory conditions. In deciding on a suitable substrate to represent FOG, butter was selected as a hard fat and olive oil as a soft fat. The fat was supplied as the sole carbon source in a minimal medium which had been used by Loperena *et al.* (2006 and 2009). Given the complexity of waste in a grease trap, it was also decided to investigate degradation using an enriched medium (Brooksbank *et al.*, 2007). The bioaugmentation products also contained some nutrients.

Few studies in the literature have reported the degradation of fats and oils by commercial bioaugmentation products. Salome and Bonvallot (1994) tested five different products under laboratory conditions using olive oil as the substrate. No significant degradation of the oil was observed. In this study, two products, FF and BFL, promoted degradation of oil under certain environmental conditions in the laboratory, while the Gnz did not perform any degradation.

Saravia *et al*. (2002) investigated the degradation of butter oil by a bio-augmentation product (6 g/100ml, 10^6 cells/ml). Unlike this study, the fat was first emulsified with a homogenizer and while incubation was carried out in minimal medium at 30° C a high agitation speed, 300 rpm, for 20-40 h. was used. Interestingly, the product which comprised species of the genus *Bacillus* performed well and similar results to those obtained with BFL and FF were reported. Up to 82-95% of the butter oil was degraded however the concentration of butter oil at 0.2-1 g/L was much lower than this study. The fat removal rate was calculated as approximately 0.0225 g/L/h which was similar to the fat removal rate obtained using BFL and olive oil in minimal medium (0.017 g/L/h) and

enriched nutrient medium (0.027 g/L/h) and when FF was grown on butter and olive oil in enriched medium (approx. 0.022 g/L/h).

Brooksbank *et al*. (2007) examined the ability of a commercial microbial supplement F69 to degrade various fats and oils (10 ml/L) in 100 ml of enriched nutrient medium at 30° C and 130 rev/min. While they tested a number of multi-species supplements, they reported that only one product was capable of enhancing fat degradation. With this product they obtained lower removal percentages with longer incubation periods than the oil removal obtained with BFL and FF in the enriched nutrient medium after 13 days of incubation. They reported 40% and 62% degradation of lard and soya oil, respectively, after 21 days, and 40% degradation of sunflower and rapeseed oil was obtained after 28 days incubation.

Loperena *et al.* (2007) investigated the degradation of dairy effluent using both a commercial inoculum and an activated sludge inoculum from a dairy wastewater treatment plant. Both inocula showed similar removal efficiency as described by COD values. However, a higher population diversity and greater metabolic rate was noted for the activated sludge, indicating the superior adaptation of this inoculum to the effluent.

The source of the inoculum in a bioaugmentation product is important. Many researchers have found promising results in fat biodegradation using bioaugmentation products where the constituent microbes were isolated from a related polluted area.

Wakelin and Forster (1997) examined the degradation of vegetable oils, lard and grease (8 g/L) from a fast-food restaurant grease trap by pure and mixed cultures (5% v/v) in 200 ml culture medium after 8 days. The pure cultures were *Acinetobacter* sp., *Rhodococcus rubra*, *Nocardia amarae* and *Microthrix parvicella* and these were compared with a mixed culture isolated from a grease-trap, MC1, and with activated sludge. The cultures, obtained at the end of the activated sludge studies, described as 'acclimatised activated sludge', were subsequently re-inoculated into fresh culture media, in order to investigate FOG

removal performance. The performance of the *Acinetobacter* sp. was good, with FOG removal values ranging from 51 to 67%. The removal efficiency of the mixed culture, MC1, ranged from 29% for rapeseed oil to 73% for the restaurant grease. However, the acclimatised activated-sludge achieved >90% removal efficiency.

Chipasa and Medrzycka (2008) carried out degradation studies of 2 g/L refined rapeseed oil by activated sludge in a mineral medium using Tween 80 as an emulsifying agent. 85% fat removal was observed with a removal rate of 0.06-0.07 g/L/h.

An important consideration in reported biodegradation studies is the use of emulsification prior to fat degradation. Emulsification increases the interaction between microbial enzymes and lipids and thus enhances lipid degradation ability. While good fat degradation was obtained by Mongkolthanaruk and Dharmsthiti (2002) and Prasad and Manjunath (2011) emulsification of the fat prior to degradation had taken place in both cases.

Many authors have reported degradation of various fats and oils and differences in the degradation capabilities of the mixed populations used. It has been suggested that the more highly saturated the fat, the greater the challenge for biodegradation (Papanikolaou *et al.*, 2007).

Tano-Debrah *et al*. (1999) investigated degradation of a variety of oils and fats, 10 - 20 g fat in 100 ml basal medium using 2 - 4% inoculum (approx. 4.8 x 10^8 cells/ml). The fats were homogenized in the medium prior to inoculation. The mixed culture used comprised 15 bacterial isolates from fatty wastewater samples. Good fat degradation, ranging from 67 to 90%, was observed after 7 days incubation. The degradability of the fats and oils was found to be linked to the degree of saturation of the fats. There was 24% removal of sheafat which comprised half saturated and half unsaturated fatty acids and 69% removal of lard which contained lower concentrations of saturates.

The biodegradability of the lard was similar to a range of oils. The highest oil removal was reported for olive oil at 73%, followed by corn oil at 67% and grapeseed, sesame oil, peanut oil and salad oil in the range 55-60%. All the oils contained low levels of saturates. Olive oil differed from the other vegetable oils in that it contained higher amounts of monounsaturated fatty acids, while the others contained higher amounts of polyunsaturated fatty acids.

Cipinyte *et al*. (2009) investigated the fat degradation ability of lipase-producing strains in an enriched medium supplemented with 0.1% of animal fats and vegetable oils. During 24 h of cultivation at 30° C at 200 rpm, the pure isolates degraded the 25-45% of the hard fat (tallow and lard) and 40-58% of the soft fat (sunflower oil and olive oil).

Butter is a dairy product made by churning fresh or fermented cream or milk. It is generally used as a spread and a condiment, as well as in cooking, such as baking, sauce making and pan frying. Butter consists of butterfat, milk proteins and water. Butter consists of 80% total fat and the rest is water.

Analysis of the butter by GC showed five main saturated fatty acids: capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0). Unsaturated fatty acids present in butter were identified as: elaidic acid (C18:1n9t) and oleic acid (C18:1n9c). However, further studies with pure standards of vaccenic acid (C18:1n11t) and elaidic acid (C18:1n9t) are required to confirm the identity of the peak of elaidic acid in butter. Previous studies with this column were unable to distinguish the two fatty acids. Myristoleic acid (C14:1n9c), palmitoleic acid (C16:1n9c), linoleic acid (C18:2n6c) and cis-11-eicosenoic acid (20:1) were also detected in butter at lower concentrations. In total the saturated fatty acids comprised 66% of the butter composition, while the unsaturated fatty acids comprised only the 34% of the fatty acid composition of the butter, which finding was in agreement with Graf (1976). Butter comprises higher levels of saturated fatty acids than lard and tallow (Table 4.1) (Graf, 1976). Therefore, butter was chosen to represent a hard fat for the biodegradation studies.

Table 4.1 Fatty acid composition (%) in lard and tallow (Graf, 1976)

Fatty acids		Lard	Tallow
Myristoleic Acid	C15:0	1	3
Palmitic Acid	C16:0	25-28	26
Palmitoleic Acid	C16:1n9c	3	3
Stearic Acid	C18:0	12-14	14
Oleic Acid	C18:1n9c	44-47	47
Linoleic Acid	C18:2n6c	6-10	3
Saturates		**38-43**	**43**
Monounsaturates		**47-50**	**50**
Polyunsaturates		**6-10**	**3-4**

The main unsaturated fatty acids identified in the olive oil were palmitoleic acid (C16:1n9c), oleic acid (C18:1n9c), linoleic acid (C18:2n6c) and cis-11-eicosenoic acid (20:1). The main saturated fatty acids present were palmitic acid (C16:0) and stearic acid (C18:0), while arachidic acid (C20:0) was also detected in the oil by GC at lower levels. The unsaturated fatty acids comprised 85% of the composition of the oil, while the saturated fatty acids comprised only 15% of the composition.

Olive oil contains much lower amounts of polyunsaturated fatty acids than other vegetable oils found in the literature, such as sunflower oil, corn oil and soybean oil (Table 4.2). However, it contains higher levels of oleic acid than the other oils. Oleic acid has been described as the main fatty acid found in FOG (Lv *et al.*, 2011; Long *et al.,* 2012) and so was thought to be a good representative of the oils for the biodegradation studies.

Olive oil is used throughout the world, but especially in Mediterranean countries. Greece is the world's third largest producer of olive oil (behind Italy and Spain) and Greeks are by far the largest consumers of olive oil in the world. The average olive oil consumption of every single Greek man, woman and child is over 26 litters per person annually. In Greece today, olive oil production accounts for approximately 10% of the total agricultural production. The olive and its oil are

not only ubiquitous in Greece, but a vital part of the regular diet (www.oliveoiltimes.com).

Table 4.2 Fatty acid composition (%) of vegetable oils (Edem, 2002)

Fatty acids		**Palm**	**Soybean**	**Corn oil**	**Sunflower**
Caproic acid	C6:0	–	–	–	–
Caprylic acid	C8:0	–	–	–	–
Capric acid	C10:0	–	–	–	–
Lauric acid	C12:0	0.2	–	–	–
Myristic acid	C14:0	1.1	0.1	–	–
Palmitic acid	C16:0	44	11	12.2	6.5
Stearic acid	C18:0	4.5	4	2.2	4.5
Oleic acid	C18:1	39.2	23.4	27.5	21.1
Linoleic acid	C18:2	10.1	53.2	57	66.2
Linolenic acid	C18:3	0.4	7.8	0.9	–
Arachidic acid	C20:0	0.1	–	0.1	0.3
Saturates		**49.9**	**15.1**	**14.5**	**11.3**
Monousaturates		**39.2**	**23.4**	**27.5**	**21.1**
Pollyunsaturates		**10.5**	**61**	**57.9**	**66.2**

In order to monitor fat metabolism, the levels of extracellular total fat were determined. The method of choice was that described by Brooksbank *et al.* (2007). Total fat determination first involves the use of a solvent for extraction of the fat from the aqueous phase. The solvent that has been most widely used for the extraction of lipids from aqueous media is hexane (Shikoku-Chem, 1994; Tano-Debrah *et al.*, 1999; El-Bastawy *et al.*, 2005; Rashid and Imanaka, 2008). Papanikolaou *et al.* (2001) reported the use of petroleum ether and chloroform, while Brooksbank *et al*. (2007) extracted the remaining lipids using dichloromethane although they reported that this solvent did not achieve satisfactory recovery of the fat. Mongkolthanaruk and Dharmsthiti (2002), Wakelin and Forster (1996) as well as Prasad and Manjunath (2011) who studied degradation of high concentrations of fat, used 1,1,2-trichloro-trifluoroethane

(freon). Chipasa and Medrzycka (2008) used chloroform for the extraction of the remaining lipids. In this study hexane extraction combined with chloroform extraction was found to be most suitable. The extraction stage was always followed by a centrifugation step as the fat was generally highly emulsified. Even when no fat degradation was observed for BFL, the contents of the flasks were milky white denoting emulsification of the fats.

The challenge posed to bacteria by hard fats as distinct to oils was borne out by the results obtained using BFL. No degradation of butter was observed for the mixture of 9 *Bacillus* spp. while 34% degradation of olive oil in the minimal medium and 94% degradation in the presence of the enriched nutrient medium were observed. A lag period preceded degradation of the olive oil. This lag was considerably longer, 7 days, in the minimal medium than in the enriched medium (2 days). The rate of oil removal was also slower in the minimal medium,0.013 g/L, than in the enriched nutrient medium, 0.027 g/L, even though the specific rate of fat removal was the same in both media due to the increase in growth in the richer medium. The difference on the degradation rates in the two media can be explained because of the different N:P ratio, which in the case of the minimal medium the ratio was 1:4, while in the enriched medium the ratio was 10:1. Clearly, the increase of nitrogen source highly enhanced fat degradation as has been reported previously by Tano-Debrah *et al.* (1999) who demonstrated the effects of the presence of other carbon and/or nitrogen sources on the degradability of the fats and oils. They noted that the presence of glucose together with a nitrogen source increased the degradability, enhancing growth. Similarly, degradation of both fats by the fungus isolated from FF, *Mucor circinelloides*, was only observed in the presence of the enriched nutrient medium.

The most important hydrolytic enzymes for wastewater treatment are lipases, amylases and proteases. Lipases are most important when considering the biodegradation of FOG. The gel-diffusion assay has been widely used to qualitatively determine hydrolysis and to quickly screen various lipase-producing microorganisms most commonly using tributyrin as a substrate (Thomson *et al.*, 1999; Gupta *et al.*, 2003; Rashid and Imanaka, 2008; Mohan *et al.*, 2008; Hasan *et al.*, 2009) or Tween 20/80 agar plates (Gupta *et al.*, 2003;). Quantitative methods,

such as titrimetry, colorimetric and spectrophotometric assays are simple and accurate, however, they are laborious and time consuming and some of the substrates are quite expensive (Gupta *et al.*, 2003). Therefore, for quick and reproducible screening of a number of strains for lipase production tributyrin agar plates were used. Tween 20 agar and Tween 80 agar plates were also used as different substrates for lipase production screening. Smith and Haas (1992) suggested that two different lipid substrates should always be used in screening. Tributyrin is a triglyceride composed of butyric acid (C4) and glycerol. Tween 20 and Tween 80 are polyoxyethylene sorbitol esters with lauric acid (C12) and oleic acid (C18) primary fatty acids, respectively. The majority of the BFL bacteria showed high lipolytic activity in tributyrin agar and less on Tween 20 agar and little or no activity on Tween 80 agar. These results indicated the substrate specificity of the lipases and their preference for the short-chain fatty acids and are in agreement with previous reports (Eggert *et al.,* 2002; Chen *et al.,* 2004). Generally, the substrate specificities of lipases include: fatty acid specificity, positional specificity and stereospecificity (Song *et al.,* 2008). Chen *et al.* (2004) showed that extracellular *Bacillus* lipase hydrolysed different chain length fatty acids with a preference for short-chain fatty acids. They noted that in some cases diacylglycerols and monoacylglycerols were hydrolysed faster than triacylglycerols.

Fat hydrolysis was detected using TLC analysis. The TLC method for analysis of hydrolysis products has been widely used and reported (Matsumiya *et al.,* 2007; Cipinyte *et al*., 2009; Fuchs *et al.,* 2011). The method is a quick and inexpensive way to demonstrate lipid hydrolysis in contrast to HPLC which is expensive, time consuming and not environmentally friendly as many solvents are needed. The need to choose a suitable detector is also a challenge. The most recent HPLC equipment use UV detectors which are unsuitable when mixed lipids are to be analysed. UV detection gives very different responses for different fatty acids. Therefore, HPLC methods for mixed fatty acid analysis generally requires refractive index (Funchs *et al.,* 2011). TLC profiles obtained in this study were compared to the results reported by Matsumiya *et al.* (2007) and Cipinyte *et al*. (2009). In all cases, triglyceride hydrolysis to free fatty acids and diacylglycerides was observed. High levels of fat removal were accompanied by total hydrolysis of

the triglycerides and this was indicated by an absence of triglycerides in the TLC method.

Fat hydrolysis was not always accompanied by fatty acid uptake. Fatty acid metabolism was determined using GC analysis. In the case of BFL there was preferential utilization of the unsaturated fatty acids over the saturated fatty acids. The long chain unsaturated fatty acids, palmitoleic acid (C16:1n9c), linoleic acid (C18:2) and cis-11-eicosenoic acid (C20:1) were preferentially used above the long chain saturated fatty acids, palmitic acid (16:0), stearic acid (18:0) and arachidic acid (20:0). Given the relatively higher levels of saturated fatty acids in the butter, this result supported the limited ability of BFL to degrade the hard saturated fat. A further difference in the metabolism of the fats by BFL was observed with the appearance of elaidic acid (C18:1n9t) following incubation of the mixed culture on the olive oil – after three days in the case of the enriched medium and seven days in the minimal medium (Figures 3.17 and 3.18). The appearance of elaidic acid (C18:1n9t) was accompanied by the disappearance of palmitoleic acid (C16:1n9c). This suggests that bioconversion of palmitoleic acid possibly through biohydrogenation, elongation, desaturation and isomerisation to elaidic acid occurred. Desbois and Smith (2010) reported that free fatty acids with double bonds in *cis* orientation tend to have greater antibacterial activity than free fatty acids with double bonds in *trans* orientation. Therefore, alterations in the ratio of *trans/cis* fatty acids of oil by bacteria may be indicative of a protection mechanism against toxicity and account in part for the increase in biomass observed by day 7 in MM and day 3 in ENM (Figure 3.10). The relative enrichment in stearic acid that accompanied the removal of elaidic acid as the fermentation progressed to day 13 in both media may also be an indication that BFL is capable of hydrogenation activity.

These results are in agreement with those reported by Chipasa and Medrzycka (2008) and Novak and Klaus (1973), who found that the utilization rate of fatty acids is different and depends on the length and degree of unsaturation of their carbon chains. Novak and Klaus (1973) determined the substrate utilization rates of fatty acids myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acid by microorganisms. They also found that the maximum

utilization rates of 16 and 18 carbon saturated fatty acids were lower than those of unsaturated fatty acids with the same chain length. Moreover, Brooksbank *et al.* (2007) and Sun and Wakeham (1994) also demonstrated the preferential degradation of unsaturated fatty acids over saturated fatty acids of the same carbon chain length.

Studies were carried out to identify the members of the mixed microbial communities. All the isolates were bacteria except one organism, a fungus, which was identified as *Mucor circinelloides*. The bacteria were distinguished initially on the basis of colony and cell morphology, Gram stain and spore stain and a number of biochemical tests. Twenty one different strains of bacteria were isolated and 19 were members of the genus *Bacillus*.

A number of different approaches were used to identify the bacteria to species level and to distinguish between species. Although diagnostic keys and tables for Bacilli have been available for a long time, the identification of these bacteria is still considered to be complicated and in many laboratories is taken no further than "aerobic spore-forming rod" or "*Bacillus* species" (Logan and Berkeley, 1984). One of the most widely used approaches is the use of miniaturized systems which contain a battery of tests such as the BIOLOG (Biolog Microbial ID systems, www.biolog.com/microbialid) and the API (Biomerieux) systems.

These systems contain a set of dehydrated reagents which can rapidly test for the reactions of the bacteria to a range of sugars, amino acids, organic acids and other physiological and biochemical characteristics and give results in a fast and efficient manner. In this study API 50CHB combined with API 20E were used for the identification of *Bacillus* spp. and API 20NE for identification of the Gram negative isolates. The API system revealed the presence of 11 *Bacillus subtilis/amyloquefaciens*, 5 *Bacillus licheniformis*, 1 *Bacillus circulans*, 1 *Bacillus megaterium*, 1 *Brevibacillus laterosporous*, 1 *Aeromonas hydrophila* and 1 *Pseudomonas putida*.

However, the taxonomy of environmental *Bacillus* isolates using biochemical or metabolic identification tests has been reported to face some difficulties and to lack reliability (Garland and Mills, 1991). Many of the bacteria cannot be identified effectively by phenotypic characteristics due to their physiological, biochemical and ecological specificity. Depending on extracellular conditions, their metabolism can change and lead to different metabolic pathways due to their need to adapt to changing environmental conditions. Some authors have reported that this system offers only a preliminary taxonomic screening test for *Bacillus* species (Baillie *et al*., 1995; De Paolis and Lippi, 2008).

Studies have reported misidentification by BIOLOG and API systems. Five strains of the closely related *B. cereus/thuringiensis* group were misidentified as *B. anthracis* using BIOLOG (Baillie *et al*. 1995). 20% of all the strains of bacilli examined during the study gave unreadable reaction profiles due to false-positive reactions. Oka *et al*. (2000) also got false positive identification for *Arthrobacter globiform* when using the BIOLOG system.

The BFL9 isolate was identified by the API 50CH system as *Brevibacillus laterosporous* with 99% similarity. However, interpretation of some of the reactions was difficult and depending on their interpretation the organism could be identified as *Bacillus cereus* with 91% similarity. While some of the bacteria were clearly distinct in terms of some of their reactions, the API system failed to distinguish them. These included 11 *Bacillus subtilis/amyloquefaciens* and 5 *Bacillus licheniformis*. Gordon *et al*. (1973) regarded *B. amyloliquefaciens* as a synonym of *B. subtilis* and more recently Logan and Berkeley (1984) and Jeyaram *et al.* (2011) failed to distinguish *B. subtilis* from *B. amyloquefaciens.* In this study, the API identification system also lacked the ability to distinguish between control strains of *B. subtilis* and *B. amyloquefaciens.*

Boyd *et al.* (2005) evaluated the API 50CH identification system comparing the identification profiles of 97 *Lactobacillus* isolates with the identification obtained using whole-chromosomal DNA probes. They reported that the API 50CH system misidentified 33 of the 97 isolates as either *L. acidophilus* or *L. fermentum*. These two species belong to different groups and are not very closely related. *L.*

acidophilus belongs to Group I of the lactic acid bacteria being an obligatory homofermentative organism, while *L. fermentum* belongs to Group III being an obligatory heterofermentative organism. The API 50CH database also identified 7 out of 20 *L. vaginalis* isolates as *L. fermentum*. They also reported that over half of the 97 isolates yielded an uninterpretable or doubtful API profile. Jeyaram *et al.* (2011) also compared the API identification system with genomic methods and reported misleading identification of few *Bacillus* spp. by the API 50CHB system. Furthermore, taxonomic bacterial changes have been detected. Increasingly new bacterial species have been identified in addition to old species being reformed to new genus, resulting in a problematic characterization of genus with phenotypic tests indicating the importance of using further identification methods (Ercolini, 2001; Coeuret *et al*., 2003).

The difficulty of phenotypic methods to reliably identify bacteria has led to the development of molecular alternatives based on microbial DNA sequencing (Reva *et al*., 2001). Molecular methods have been widely used for the characterization of bacteria or for the confirmation of phenotypic identification (Ercolini, 2001; Coeuret *et al*., 2003).

Molecular techniques involving bacterial DNA extraction and subsequent PCR amplification and analysis of the 16S rRNA gene have been routinely used in identifying bacterial species and have been used to distinguish Bacillus species and related strains being a very accurate and rapid identification method (Ash *et al*., 1991; Wu *et al*., 2006; De Paolis and Lippi, 2008; Loperena *et al*., 2009; Zheng *et al*., 2008; Xiong *et al*., 2010; Ibrahim *et al*., 2009). Use of a combination of various metabolic and molecular methods is highly recommended to ensure a definite identification of *Bacillus* strains (De Paolis and Lippi, 2008). Therefore, genotypic-based identification was used to verify the identification obtained using the API system.

The use of 16S rRNA in the classification of bacterial species has been well established and 16S rRNA gene sequencing is now the gold standard of bacterial identification (La Duc *et al*., 2004; Mohamed *et al*., 2006). 16S rRNA genes are highly conserved among all organisms and various unique species-specific

regions allow for bacterial identification. Analysis of the full-length of the 16S rRNA gene is probably the best and most accurate tool for a detailed classification within some members of *Bacillus* groups (Mohamed *et al*., 2006) although partial analysis of the 16S rRNA gene is easier and more economical. Goto *et al*. (2000) and Mohamed *et al*. (2006) analysed the hypervariant region (HV region) and it proved highly specific for each type of strain and a very reliable and efficient way for rapid identification of *Bacillus* to species level.

The primers used must be species specific for an accurate identification and to distinguish between species. A number of studies have reported limited success because the primers used were not species specific (Oguntoyinbo, 2010). In this study, amplification of the 16S rRNA gene was performed satisfactorily using the universal primers, pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAG GAGGTGATCCAGCCGCA-3'). These primers code for almost the full length of the 16S rRNA gene although in terms of distinguishing the *Bacillus* strains, it was found that the most conserved region of the 16S rRNA gene was the 50-500 bp region. This finding was in agreement with Goto *et al.* (2000) and Clarridge III, 2004, who found that region satisfactory for differentiation to species and strain level. The universal primers pA and pH were also satisfactorily used by Vardhan *et al*. (2011), Kebria *et al.* (2009), Scheldeman *et al.* (2004), Gomma and Momtaz (2007), Das and Bissoyi (2011) and Yan *et al.*(2006) for *Bacillus* spp. Loperena *et al*. (2009) amplified almost full-length 16S rRNA gene fragments using eubacterial primers and obtained good results: 27F (5'-AGAGTTTGATC(A/C)TGGCTCAG-3') (same as the pA) and 1492R (5'-ACGG(C/T)TACCTTGTTACGACTT-3').

Extraction of the DNA was conducted according to a modified method of Mohamed *et al*. (2006) and Gevers (2001) based on the phenol/chloroform extraction protocol. The best result for the lysis of Gram positive cells was obtained by treating the cells with lysozyme followed by 10-20% SDS and incubation at 37° C. This was a variation of methodology described in the literature (Chassy and Giuffrida, 1980; Sadaie *et al.*,1997; Goto *et al.*, 2000; Gevers 2001; Wang *et al.*, 2001; Mohamed *et al.*, 2006). Gram negative bacteria

being surrounded by a lipopolysaccharide (LPS) layer which prevents the action of lysozyme were treated with EDTA prior to DNA extraction.

A number of studies have shown interest in using commercial kits for the extraction of genomic DNA for the identification of *Bacillus* spp. (Rashid and Imanaka, 2008; Kebria *et al.*, 2009; Vardhan *et al*., 2011), however, manual genomic DNA extraction is more accurate producing higher yield and is less costly. An extraction kit was used to recover or concentrate DNA fragments (50bp-10kb) from agarose gels, PCR or other enzymatic reactions. The method used a chaotropic salt, guanidine thiocyanante to dissolve agarose gel and denature enzymes. DNA fragments in chaotropic salt solution bind to the glass fibre matrix of the spin column. After washing of the contaminants, the purified DNA fragments are eluted by addition of low salt elution buffer. Salts, enzymes and unincorporated nucleotides are effectively removed from reaction mixtures without phenol extraction or alcohol precipitation. Typical recoveries are 60-80%. Modification on the purification of the PCR product using the kit according to the manufacturer's instruction was carried out by using a vacuum drier in order to ensure complete removal of the ethanol, which may inhibit the sequencing. The vacuum drier was also used in order to increase the concentration of the DNA. Some of the obtained sequences were of smaller length than the expected. This could be due to the preparation of the template.

PCR was performed following methods described by Gomma and Momtaz (2007) and Vardhan *et al*. (2011). The clear bands obtained on the resulting gel demonstrated that optimum conditions had been achieved using the modified method which involved using the higher annealing temperature of 55^{o}C and not 33^{o}C.

The isolate FFG had been identified as *Aeromonas hydrophila* using the API system but was identified as *Pseudomonas putida* using the genotypic method. The identification of the BFL9 isolate had been ambiguous, *Brevibacillus laterosporous* or *Bacillus cereus,* using the API system. However the organism was identified as *Bacillus cereus* using 16S sequencing. Similarly, the API system failed to distinguish between *B. subtilis* and *B. amyloliquefaciens* however

the genetic approach did resolve the identity of the bacteria as *B. subtilis* or *B. amyloliquefaciens.*

The isolates BFL6 and FFE, were identified as *Bacillus licheniformis* using both the API and the 16S methods. The organisms had been isolated from different sources and, while the API profiles were the same, sequence alignment showed a subtle difference at the nucleotide level. Closely related taxa are often extremely similar in their 16S rRNA sequences, such as some members of the *Bacillus cereus* group (*B. anthracis, B. cereus* and B. *thuringiensis*) (La Duc *et al*., 2004; Mohamed *et al*., 2006). Phenotypic characters such as β-hemolytic activity can be used to distinguish between *B. cereus* and *B. thuringiensis* in the first instance and *B. anthracis* in the second instance. La Duc *et al*. (2004) used the analysis of the *gyrB* gene for the identification of members of the *B. cereus* group and it was proved more highly differential than 16S. Similarly, Chun and Bae (2000) and Wang *et al.* (2007) used the *gyrA* and *gyrB* genes, respectively, for the *Bacillus subtilis* group. They reported lower percentages of sequence similarities than comparing the 16S rRNA gene. In this study, it was of great interest to sequence the full 16S rRNA gene and to demonstrate the great similarities between strains isolated from different sources.

It was found that bacteria closely related belonging to the same species performed demonstrated different metabolic activities. For instance, BFL3, BFL4, BFL7, FFA, FFC FFD and GnzIII strains identified as *Bacillus subtilis* had differences in their sequences using sequence alignment. The phylogenetic tree using the neighbor-joining method, emphasised their close relatedness. However, the BFL3, BFL4 and BFL7 demonstrated higher amylase activity than the rest, while the FFA, FFD and GnzIII showed hydrolytic activity on Tween20 at pH 5.5. The enzymatic activity of the *Bacillus subtilis* isolates was different among the different strains, while it was observed that the isolates identified as *Bacillus licheniformis* (BFL6, BFL8, FFE, GnzIV and GnzV) demonstrated in general low enzymatic activity with subtle differences. The pattern of hydrolytic activity for the *Bacillus amyloliquefaciens* isolates (BFL5, FFB, GnzI and GnzII) was also discrimatory. The BFL5 isolate showed higher amylase activity and positive cellulase activity, while the other strains did not demonstrate any cellulase

activity. Moreover, the FFB, GnzI and GnzII showed hydrolytic activity on Tween 20 at pH 5.5, while the GnzI and GnzII performed the highest. The hydrolytic activity of the isolates on different pH showed that the majority of the *Bacillus* spp. could not perform any activity on Tween20 at pH 5.5, while only few strains among the same species showed positive results. Using different substrate and pH for the lipase activity it was demonstrated the significance of the different strains as it was reported by Mohan *et al.* (2008).

Three isolates, BFL3, BFL4 and BFL7 were identified as *Bacillus subtilis* using both approaches to identification. The bacteria had been distinguished on the basis of colony and cellular reactions. Sequence alignment did not distinguish between the strains and the phylogenetic tree demonstrated the very close relationship between the three isolates. However, their metabolic activity was different. BFL3 and BFL4 showed higher lipase activity on Tween 20 (pH 7.5) than the BFL7. Moreover, those strains demonstrated positive cellulase activity and lipase activity on Tween 80 (pH 7.5) in contrast to the BFL7. It was of interest to see if other approaches such as SDS-PAGE and FAME could distinguish between the strains.

Kaynar and Beyatli (2008) examined the total cell protein profiles of 30 *Bacillus* spp. using SDS-PAGE. The results they obtained confirmed that the patterns of total cell proteins can be used to study and compare strains of *Bacillus* spp. Total cell protein profiles of the 9 BFL *Bacillus* spp. were analysed by SDS-PAGE according to a modified method Kaynar and Beyatli (2008). A higher percentage polyacrylamide gel was used in this study for the protein electrophoresis in order to get better resolution. Members of six different species were examined. The profiles were found to be different and specific for each species. These results are in agreement with the study of Kaynar and Beyatli (2008).

However, subtle differences were observed in the protein profiles of the strains BFL3, BFL4 and BFL7 identified as *B. subtilis*. The BFL4 was similar to BFL3 between 35-40 and 50-100 kDa, but it lacked proteins between 25-35 kDa, while BFL7 was similar to BFL3 between 25-35 kDa and 50-100 kDa, but not between 35-40 kDa. As the molecular weight of the cellulase has been reported between

35-55 kDa (Han *et al.*, 1995; Li *et al.*, 2008), no clear bands were observed in that range for the BFL7 confirming that this strain did not produce that enzyme.

FAME is a widely used technique for *Bacillus* identification because the bacterial fatty acids are highly conserved and the method is an easy, cheap and rapid identification tool (Kämpfer 1994; Dawyndt *et al*., 2006; Slabbinck *et al*., 2010). The first genus-wide fatty acid methyl ester (FAME) analysis of the genus *Bacillus* was done by Kämpfer (1994) who concluded that fatty acid analysis had a potential for species differentiation within the genus *Bacillus*. In this study fatty acid methyl esters of five fatty acids present in BFL3, BFL4 and BFL7 were analysed by GC. Three methods for derivatization were investigated as described by Moss and Dees (1975), Heipieper and Bont (1994) and Fakhruddin and Quilty (2006). The method described by Heipieper and Bont (1994) was found to be most satisfactory. FAME analysis showed resolution among the BFL3, BFL4 and BFL7.

Slabbinck *et al*. (2008) first applied artificial neural networks for genus-wide FAME-based identification of the genus *Bacillus*. The results showed a significant improvement in *Bacillus* species identification, indicating that machine learning techniques would be a promising tool for FAME-based classification and identification of bacterial species. Identification of FAME profiles can be carried out using a commercial system such as the MIDI system (Newark, Delaware, USA). The system is a fully automated gas chromatographic analytical system, that has been routinely used for bacterial identification (Sasser, 1990; Slabbinck, *et al*., 2008 and 2009).

A fungus was identified in the product FF. The organism, *Mucor circinelloides*, showed good degradation of butter and oil in the enriched medium. The bacterial isolates when grown in pure culture were unable to degrade either fat. Unlike the bacteria, the fungus was seen to produce surfactant. The production of surfactant by the microorganisms was determined using a modification of the drop collapse method of Youssef *et al*. (2004). The use of a drop collapse technique for the screening of surfactant-producing microorganisms has been reported previously (Bodour and Miller-Maier, 1998; Youssef *et al*., 2004; Tugrul and Cansunar,

2005) suggesting it as a sensitive and easy method to test for biosurfactant production. This method has been mainly applied as qualitative way to screen biosurfactant-producing microorganisms (Youssef *et al*., 2004; Tugrul and Cansunar, 2005).

In the case of the fungus, the biosurfactant was first extracted from the medium according to the method described by Das *et al.* (2008). The absence of biosurfactant in the bacterial cultures might have been due to a lack of sensitivity of the method. In this study two methods were evaluated and standard curves were constructed using SDS according to Bodour and Miller-Maier (1998). One method involved using a 24-microwell lid, 100 µl mineral oil and 20 µl sample, while the other method involved a 96-microwell lid, 20 µl mineral oil and 7µl sample. There was a clear linear correlation between the SDS concentration and the drop diameter in the range 250 – 2500 mg/L for the first method, while the results of the second method were not clear. Therefore, the first method was used during the experiments as it was found more sensitive and accurate. Youssef *et al.* (2004) reported that this method may not be as sensitive in detecting low concentrations of biosurfactants since they found that 16 strains that were negative for biosurfactant production by the drop collapse method, actually produced low concentrations of biosurfactant, 50 – 60 mg/L. This may explain the inability of this method to detect biosurfactant production, if any, by the bacterial isolates in 100 ml media, as the SDS in this method was detectable for values higher than 250 mg/L (Figure 2.2).

The degradation of fat by the fungus was seen to be similar to that obtained with FF suggesting that the degradative ability of the product was attributed in the main to the fungus. Dublin City Council have drafted a procedure for the approval of additives for FOG treatment (Dublin City Council, 2012). They stipulate that the product must be bacteria and so products containing fungi are not suitable. For this reason no further studies of FF were conducted and studies on the bioaugmentation products focused on BFL, the other product showing potential for FOG removal and which was found to only contain bacteria.

The addition of *Pseudomonas putida* CP1 into the BFL product optimised fat degradation. While no degradation of butter was obtained by BFL, BFL-CP1 showed good butter removal. Degradation of the olive oil was also enhanced by the addition of *Pseudomonas putida* CP1 to BFL. While both mixed cultures, BFL and BFL-CP1, removed 92 – 94% of the olive oil, the rate of fat removal was significantly greater for BFL-CP1.

The fat removal rate for BFL-CP1 on butter and oil was 0.05 g/L/h. This result compared very favourably with values reported in the literature. A rate of fat removal of 0.03 g/L/h was calculated for the results reported by Loperena *et al*. (2009) who investigated fat removal using a native microbial population. They had used a lower fat concentration than this study and the fat was emulsified prior to degradation. A fat removal value of 0.06 g/L/h was calculated for the findings of Cipinyte *et al*. (2009) who investigated oil removal by a mixed culture. The value was very similar to that obtained in this study however the concentration of fat used by Cipinyte *et al*. (2009) was much lower than this study.

The challenge of the unemulsified fat in the medium was demonstrated by the use of the pure isolates and the mixture of isolates of FF and BFL, while in the reported studies the fat was homogenised prior fat degradation. The fat entering the grease traps from the kitchen sink are most likely emulsified due to the use of soaps and detergents. However, the presence of the nutrients in the BFL enhanced biosurfactant production and fat emulsification by the bacteria, succeeding this challenge and the fat removal obtained by BFL-CP1 was highly competitive to the previously reported studies conducted in the laboratory.

The higher and faster fat removal values by BFL-CP1 were also reflected on the faster growth rate and higher yield coefficient in comparison to the low growth rate and yield coefficient obtained by BFL, 0.17 g/g. The yield obtained by BFL-CP1, 0.31 g/g and 0.37 g/g in oil and butter, respectively, were close to the range of yield, 0.41-0.67 g/g, reported for growth of *Acinetobacter* sp. (Wakelin and Forster, 1997). However, the fat removal percentages by the *Acinetobacter* sp. were not as high ranging between 51 – 67.5%. Similary, the mixed inoculum MC1 performed higher yield coefficients (0.39 – 0.75 g/g), even though the fat removal

percentages reported by Wakelin and Forster (1997) were not as high as in this study. Those results, in comparison to the results in this study, implied the differences in the cell metabolism for the utilisation of various FOG substrates and for biomass yield.

When fat hydrolysis was monitored using TLC, the pattern of hydrolysis was similar for BFL and BFL-CP1. However, fatty acid metabolism was found to differ. When BFL was used there was limited uptake of the main fatty acids. In the case of BFL-CP1 all the main fatty acids of both fats were rapidly consumed by day 5 – 7 days incubation which corresponded with the period of maximum fat removal. The metabolism of oleic acid gave rise to an increase in the levels of stearic acid in both treatments however, while stearic acid was not taken up by BFL it was metabolised by BFL-CP1. This result was also observed by Pereira *et al*. (1998) and Chipasa *et al*. (2008). Elaidic acid (C18:1n9t) was detected in the butter but not in the oil at the beginning of the fermentation. In the case of the oil, elaidic acid (C18:1n9t) was detected after a few days incubation and was removed by both the BFL and the BFL-CP1.

It was pointed out that because BFL could degrade the olive oil and not the butter, the product preferentially metabolised unsaturated fatty acids. In the case of BFL-CP1 however, the ability of the product to degrade both fats suggested that the mixed culture had no preference for saturated or unsaturated fatty acids.

The microbial composition of any consortium used to treat waste fats, oils and grease has been shown to be of particular importance. In this study it was shown that the combination of a *Pseudomonad* species with a number of *Bacillus* species produced the best result. The results suggest that while the Bacillus species could hydrolyse the fats, complete fat metabolism only took place in the presence of *Pseudomonas putida* CP1.

Chappe *et al.* (1994) examined the ability of four commercial bioaugmentation products to biodegrade fat. They found that while Gram-negative bacteria present in the bioaugmentation products effectively assimilated fatty acids, the Gram-positive bacteria were almost always inhibited or destroyed in the presence of

fatty acids and their activity was limited to hydrolysis. Nisola *et al*. (2009) demonstrated good fat degradation in a grease trap using immobilised strains of *Pseudomonas aeruginosa.* He suggested that a combination of *Pseudomonas* species with *Bacillus* species would be more effective. Loperena *et al.* (2009) also showed that a combination of *Bacillus* sp. and *Pseudomonas* sp. and an *Acinetobacter* sp, was very effective for fat degradation and suggested that this could be attributed to a cooperative activity between members of the Genus *Bacillus* and the genus *Pseudomonas*. The results in this study were in agreement with Sheu and Freese (1972, 1973) and Desbois and Smith (2010) who pointed out that Gram negative bacteria can utilize long-chain fatty acids as a carbon source. They convert them to the acyl-coenzyme A (CoA) derivative and then metabolize them by β-oxidation. However, *B. subtilis* cannot utilize long-chain fatty acids. They reported that fatty acids inhibit the growth and oxygen consumption by *Bacillus* species in nutrient medium by inhibiting the transport of amino acids, keto acids and others through the cell membrane. The degree of inhibition depends on the chain length of the fatty acid, therefore the inhibition increases with chain length of the fatty acids.

Fatty acids, when assimilated by BFL-CP1 were accumulated the first 2-3 days of incubation and simultaneously fatty acids were completely degraded via the β-oxidation pathway. The absence of lipid accumulation in the BFL-CP1 at the end of the fermentation indicated that complete degradation of the two fats occurred and therefore assimilated lipids underwent β-oxidation.

The microbial population was studied while monitoring fat degradation in order to monitor *Pseudomonas putida* CP1 in the BFL-CP1 mixture and also to investigate the population dynamics. When using the plate count technique to determine the numbers of bacteria, an interesting observation was made. The colony morphology of the bacteria suggested a distinct population shift during the fermentation. When BFL was grown on fat, *Bacillus* spp. could be detected throughout the run. However, when BFL-CP1 was grown on fat, *P.putida* CP1 dominated the population after day 1 and continued to be the dominant population up to day 13.

Loperena *et al*. (2007) also observed changes in the population of a commercial inoculum during the incubation in a bioreactor using the plate count technique and observing colony morphology. They used genomic fingerprinting to confirm their findings. In this study, FAME was investigated as a method to study the population dymanics of the mixed culture. However, the use of hexane to wash the biomass interfered with the fatty acid esterification and so the method was not further explored.

P. putida CP1 had previously been successfully labeled in the laboratory with green fluorescent protein. The labeled bacterium, *P.putida* CP1::Tn7-*gfp,* had been used in earlier bioaugmentation studies (Mc Laughlin *et al*., 2006). Using the organism with BFL and also using selective culture conditions for the *Bacillus* species, it was demonstrated that while the *Bacillus* species could grow on fat when BFL was used, the population was merely maintained in the mixture in the presence of *P. putida* CP1. The *Pseudomonas* sp. however grew vigorously on fat in the BFL-CP1 mixture. This supported the finding that the Bacillus species hydrolysed the fat while the Pseudomonad species metabolised the hydrolysed fatty acids.

Sonderkamp *et al*. (2001) and Loperena *et al*., (2009) reported the same phenomenon. They observed the dominance of one strain in a mixed population. They suggested that the mixed microbial population brought about a change in the nutrients resulting in the dominance of a particular strain. This phenomenon has also been described as referred cometabolism (Atlas and Bartha, 1998). According to this interaction, the active population that oxidised the substrate and which yielded a detectable level of lipase activity were the *Bacillus* spp.. This population did not assimilate the hydrolysed products and did not increase in biomass. The hydrolysed products, after the cometabolic transformation by the Bacillus spp., were available to the *Pseudomonas putida* CP1 resulting in a fast decrease of the substrate and an increase in population size of the *Pseudomonas putida* CP1.

An interesting observation was made when *P. putida* CP1 was added to BFL. The mixed population was found to form aggregates. A biochemical examination of the aggregates showed the presence of EPS comprising carbohydrate, protein and

some DNA. This phenomenon might have resulted from a combination of the peptidoglycan layer of the Gram positive cells and the lipopolysaccharide layer of the Gram negative bacterium. Another possibility is that the aggregates formed due to the charge between the cell-wall fatty acids of the *Bacillus* spp. and the hydroxyl fatty acids (3-OH) which have been reported to be present in the cell-wall of Gram negative bacteria (Kunitsky *et al.*, 2006).

Aggregation of *P.putida* CP1 has been described by Farrell and Quilty (2002), and Fakhruddin and Quilty (2006). McLaughlin et al., (2006) showed that *P.putida* CP1 could attach to activated sludge flocs thereby enhancing bioaugmentation. An aggregative response is thought to enable the introduced bacterium to compete satisfactorily in the mixed microbial community. Hansen *et al.* (2007) reported that when *P. putida* was cultured together with an *Acinetobacter* sp. strain C6, the *P. putida* attached to the other strain and having a higher growth yield it outcompeted the *Acinetobacter* sp. This interaction is also categorised as a commensal interaction (Atlas and Bartha, 1998; Christensen, et al. 2002; Hibbing, et al. 2009). Similar result was observed when the *Pseudomonas putida* was added to the *Bacillus* spp. (BFL). The *P. putida* CP1 had higher growth yield and outcompeted the *Bacillus* spp. whose cells numbers remained stable, while when the BFL was incubated alone the numbers slowly increased after a lag phase. However, higher values of fat removal and biomass were obtained only when *Bacillus* spp. and *Pseudomonas putida* CP1 were combined.

The *Bacillus* spp. and the *Pseudomonas putida* CP1 interacted successfully promoting higher and faster fat removal. The results in this study emphasise the importance of careful consideration when choosing the members of a mixed microbial community for use in bioaugmentation.

5 MAIN FINDINGS

- Of the three bioaugmentation products tested, only two, FF and BFL, showed potential for fat degradation. The bioaugmentation product in liquid form, Gnz, did not degrade either hard or soft fat.

- The three products comprised a mixed population of bacteria and FF also contained a fungus. The bacteria present in BFL and Gnz all belonged to the genus *Bacillus*. FF contained members of the genera *Bacillus* and *Pseudomonas*.

- All the bacteria produced a range of hydrolytic enzymes. However, none produced biosurfactant

- Identification of the bacteria to species level using 16S rRNA sequencing gave better resolution than the API system and distinguished the isolates to strain level using multiple sequence alignment.

- The distinction between closely related *Bacillus subtilis* strains was supported by SDS-PAGE and FAME analysis

- The degradative ability of FF was attributed largely to the presence of the fungus identified as *Mucor circinelloides*

- BFL did not degrade the hard fat. The product only partially (34%) degraded the olive oil in minimal medium. In the presence of an additional carbon source, 94% oil removal was obtained after 13 days incubation with a fat removal rate 0.03 g/L/h after a 2 day lag period. The growth rate was 0.0099 h^{-1}

- When *Pseudomonas putida* CP1 was added to the BFL product (BFL-CP1), both unsaturated and saturated substrates were rapidly consumed. No lag was observed in the fat removal and up to 88% fat was removed after 7 days incubation with a fat removal rate of 0.05 g/L/h. The growth rate was 0.04 h^{-1}

- Fat hydrolysis e.g. BFL growing on butter, was not always accompanied by fatty acid metabolism.

- The main fatty acids present in the butter were: lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), elaidic acid (C18:1n9t) and oleic acid (C18:1n9c). Other fatty acids detected by the GC at lower levels than the main seven fatty acids were: capric acid (C10:0), myristoleic acid (C14:1n9c), palmitoleic acid (C16:1n9c), linoleic acid (C18:2n6c), arachidic acid (C20:0) and cis-11-eicosenoic acid (20:1) – 66% saturated fatty acids.

- The main fatty acids identified in the olive oil were palmitic acid (C16:0), palmitoleic acid (C16:1n9c), stearic acid (C18:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c) and cis-11-eicosenoic acid (20:1). Arachidic acid (C20:0) was also detected in the oil by GC at lower levels – 85% unsaturated fatty acids.

- BFL could degrade the unsaturated fatty acids under certain environmental conditions, but could not degrade the saturated fatty acids. However, BFL-CP1 could degrade both saturated and unsaturated fatty acids.

- Monitoring the population dynamics in BFL and BFL-CP1 indicated that a cooperative activity took place between the *Bacillus* species and the *Pseudomonas* species thus enabling effective fat degradation

Proposals for future study

Investigations on;

- Degradation of FOG by BFL-CP1 in the laboratory and the field under a variety of environmental conditions
- Degradation of FOG by other environmental bacterial isolates
- Immobilised bacteria for use in grease traps

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APPENDIX A

16S rRNA sequences of all bacterial isolates and controls

>FFB -- 12..805 of sequence
CTATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGG
TGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAA
TACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTT
ACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC
GTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTT
CAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGC
AGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAA
CTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGA
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTAG

>BFL5 -- 14..914 of sequence
CTATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGG
TGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAA
TACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTT
ACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC
GTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTT
CAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGC
AGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAA
CTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGA
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTAAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCC
GCCTGGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC

>BFL4 -- 11..851 of sequence
GCTATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGG
GTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACT
TACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGT
TCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCG
CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAA
ACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCC
G

>GnzI -- 12..559 of sequence
GCTATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGG
GTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACT
TACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGT
TCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAC
CACCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCG
CATGTTCT

>GnzV -- 14..632 of sequence
ATACATGCTAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAAT
ACCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTT
GCAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAAACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAACAACGCCGC
GTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTT
CGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGC
AGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAA
CTGGGGAACTTGAGTGCAG

>BFL3 -- 14..867 of sequence
TACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGA
GTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATAC
CGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACA
GATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCG
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
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AGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGA
ATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGG
CGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTG
GGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAG
ATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCG
AAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCT
GGGGAGTACGGTCG

>GnzII -- 13..924 of sequence
GCTATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGG
GTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACT
TACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
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CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGT
TCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
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AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGG
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GCCTGGGGGAGTACGGTCGCAAGACTGAAACTCAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGT

>GnzIII -- 11..888 of sequence
CTATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGG
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TACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTTGGCTACCACTT
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GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCC
GCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAGGAA

>FFG -- 13..818 of sequence
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GGAATCCTTGAGATTTTAGTGCGCAG

>BFL8_pA -- 12..189 of sequence
TGCTATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGAC
GGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGC
TAATACCGGATGCTTGATTGAACCGCATGGTTCAATTATAAAAGGTGGCTTTTACCTA

>GnzIV -- 12..496 of sequence
CTATACATGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGG
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TGCAGATGGACCCGCGGCGCATTAGCTAGTTGGGGAGGTAACGGCTCACCAAGGCGACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAACAACGCCC
CGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGT
TCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCC

>FFC -- 13..807 of sequence
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TACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTT
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GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC
GTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTT
CGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGC
AGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAA
CTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGA
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTTAA

>FFA -- 14..910 of sequence
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ATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACT
TACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGT
TCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCG
CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAA
ACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAGTGTTAGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGC
CTGGGGAGTACGGTCGCAGACTGAAACTCAAGGAATTGACGGGGGCCCGCACAACGG

>BFL6 -- 14..187 of sequence
TATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAAT
ACCGGATGCTTGATTGAACCGCATGGTTCAATTATAAAAGGTGGCTTTTGCCTA

>Pseudomonas putida CP1 -- 14..662 of sequence
TACACATGCAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGA
GTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACC
GCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTA
GGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCT
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GTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAG
GTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTG
TTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC
AGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAA
GTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAG
TACGGTAGAGGGTGGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAAT

>B.subtilis -- 15..776 of sequence
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ATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACT
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TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGT
TCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCG
CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAA
ACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAG
GAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACGCTGCTA

>B.amyloliquefaciens -- 18..894 of sequence
ATACATGCTAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAAT
ACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTA
CAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATG
CGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCG
TGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTC
AAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCA
GGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAAC
TGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAG
AGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAG
CGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGC
CTGGGGGAGTACGGTCGCAAGACTGAACTCAAGGGAT

>FFE -- 54..188 of sequence
TTATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAA
CTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATTATAAAA
GGTGGCTTTTGCCTA

>B.licheniformis -- 16..897 of sequence
CTATACATGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGG
TGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAA
TACCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACT
TACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
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CGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGT
TCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
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AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGG
CGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAAGTGTTAAAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCC
GCCTGGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATT

>BFL1
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TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAG
CAAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCTCTGACACTCCTAGAGATAGGACGTTCCCCTTCGGGGGACAGAGTG
ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA
CGTGCTACAATGGATGGTACAAAGGGCAGCAAAACCGCGACGTCGAGCAAATCCCATAAAA
CCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATC
GCGGATC

>BFL2 -- 16..876 of sequence
CTATACATGCAGTCGAGCGAACTGATTAGAAGCTTGCTTCTATGACGTTAGCGGCGGACG
GGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCT
AATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCAC
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CAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCG
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CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCG
CCTGGGGAGTACGGTCGCAAG

>BFL9 -- 11..932 of sequence
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GGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGC
TAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCA
CTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAAC
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CCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCT
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TGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACT
CCGCCTGGGGGAGTACGGCCGCAAGGCTGAAACTCAAGGAATTGACGGGGGCCCGCACAA
GCGGTGGAGCATGTGGTTTATT

>FFF -- 13..898 of sequence
CTACACATGCAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTG
AGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATAC
CGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCT
AGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTC
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GGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGCTAATACCTTGCT

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CAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTA
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ACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTG
GAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGAGTACGGC
CGCAAGGTTAAAACTCAAATGAATTGACGGGGCCCGCACAAGCGTG

>FFD -- 16..747 of sequence
TATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGG
TGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAA
TACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTTGGCTACCACTT
ACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC
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CGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGC
AGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAA
CTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGA
GCGAAAGCGTGA

>BFL7 -- 12..896 of sequence
GCTATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGG
GTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACT
TACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGT
TCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCG
CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAA
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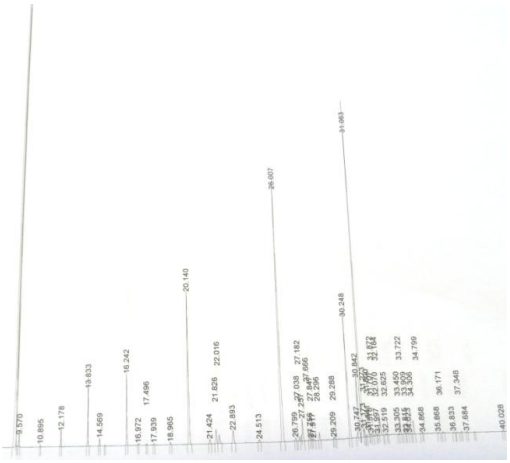
AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCC
GCCTGGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGAC

APPENDIX B

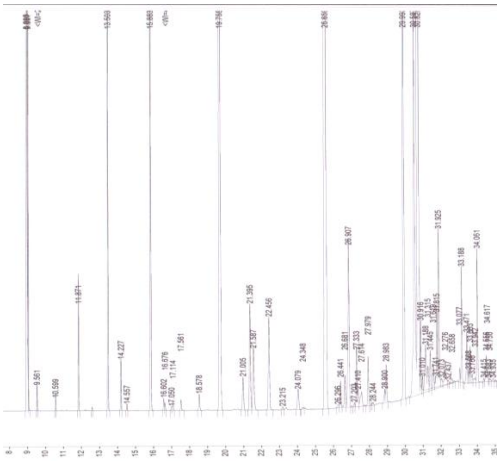
FATTY ACID TRACES

Traces of fatty acids of butter in MM throughout the incubation of BFL

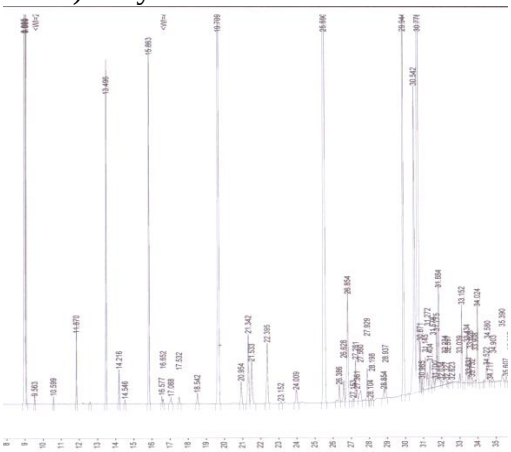
A) Day 3



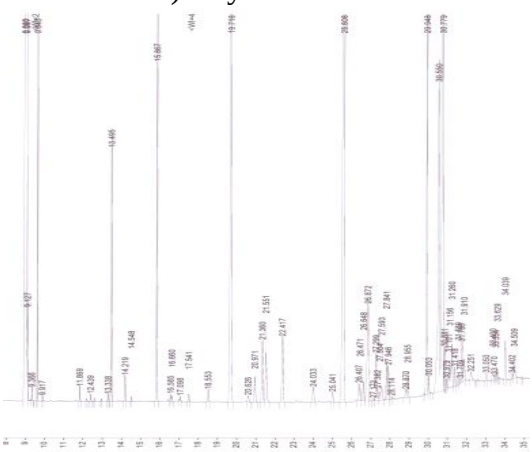
B) Day 5



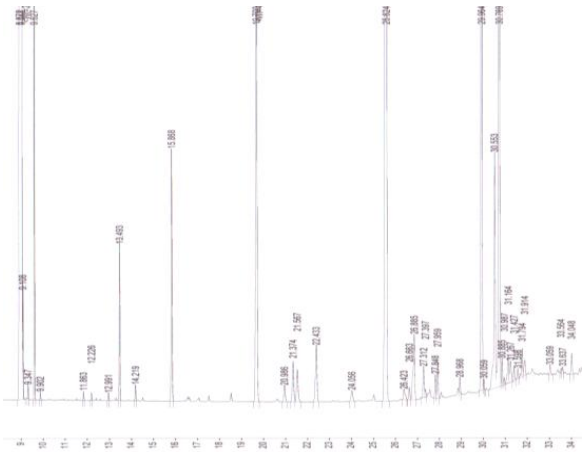
C) Day 7



D) Day 11



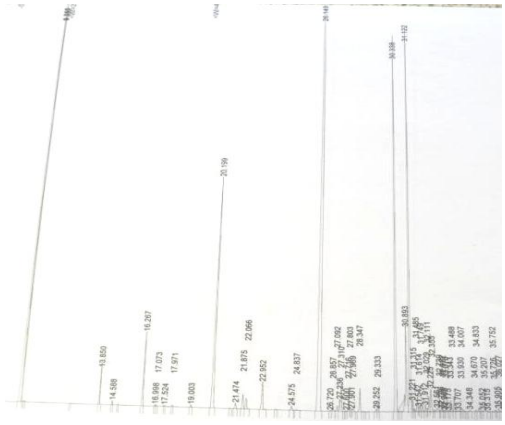
E) Day 13



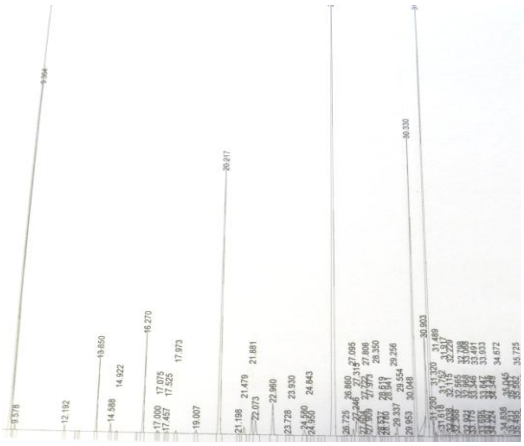
Traces of fatty acids of butter in ENM throughout the incubation of BFL

A) Day 3

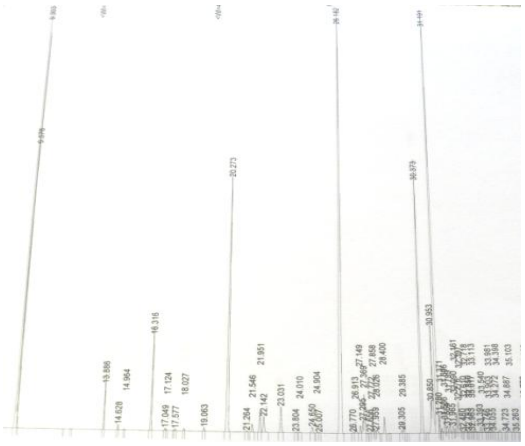
B) Day 5



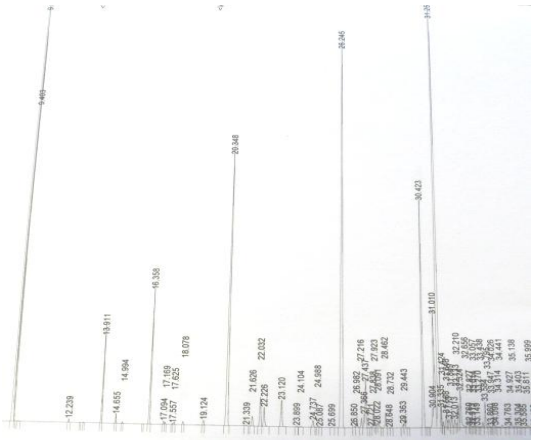
C) Day 7



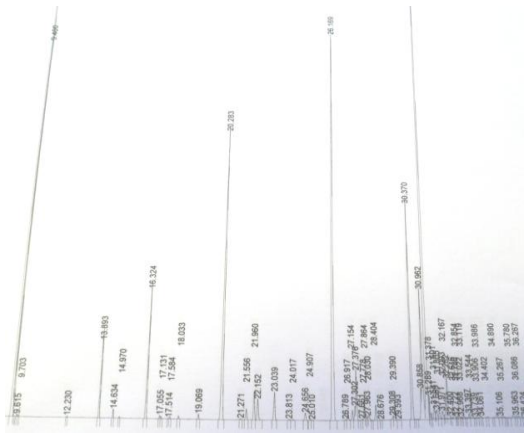
D) Day 9



E) Day 11

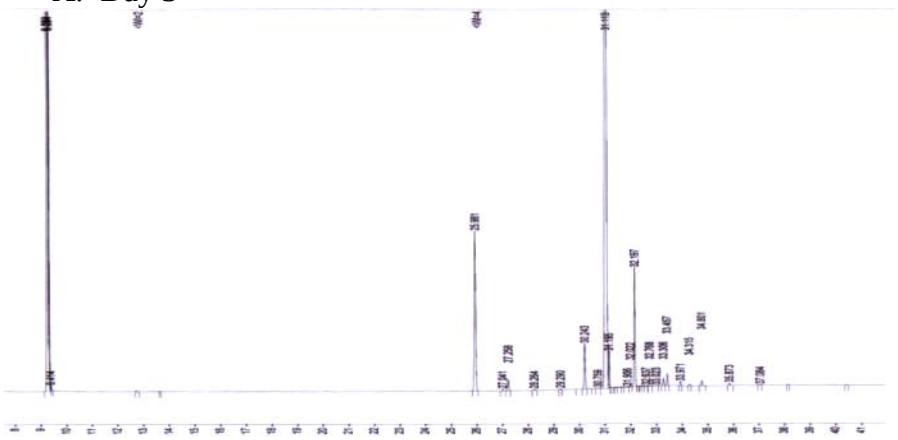


F) Day 13

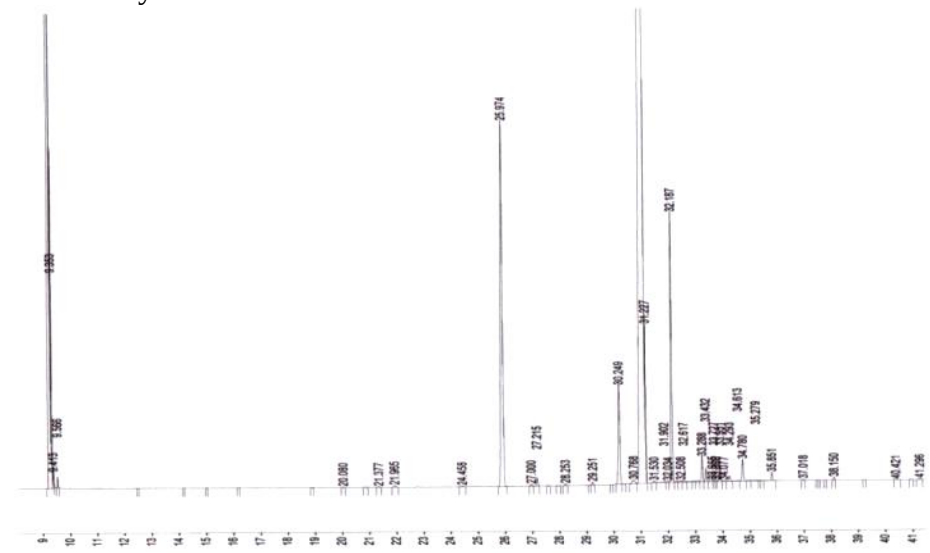


Traces of fatty acids of oil in MM throughout the incubation of BFL

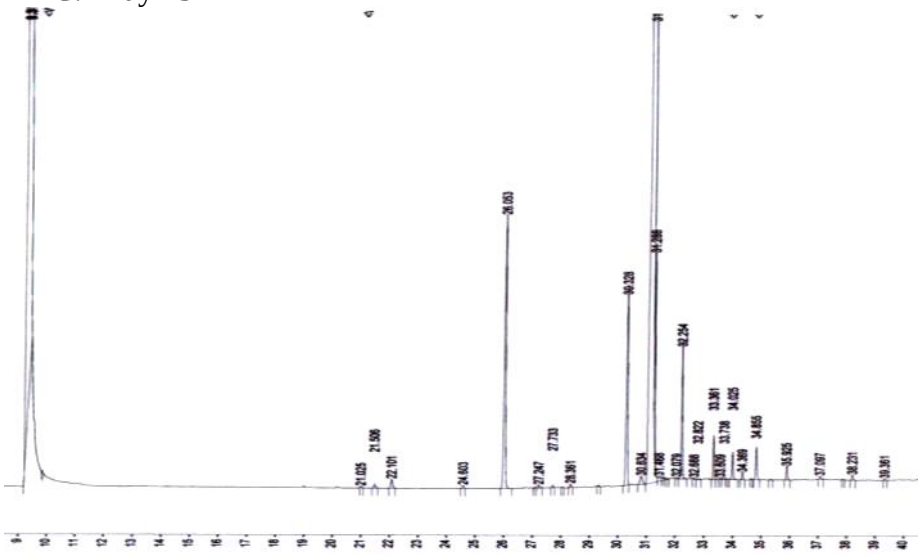
A. Day 3



B. Day 7

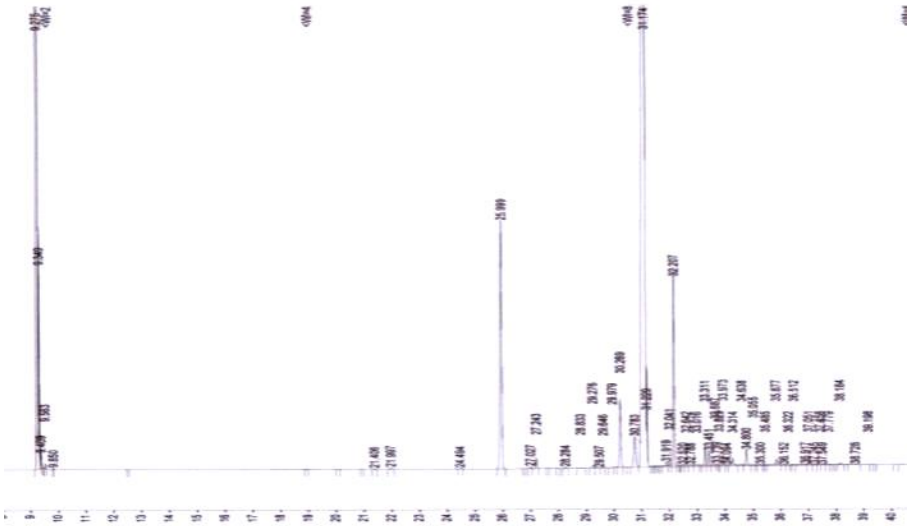


C. Day 13

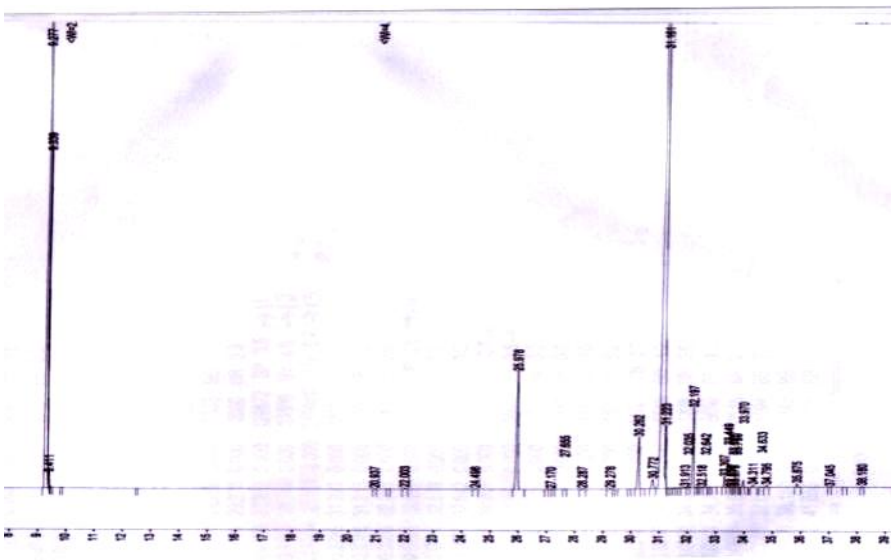


Traces of fatty acids of oil in ENM throughout the incubation of BFL

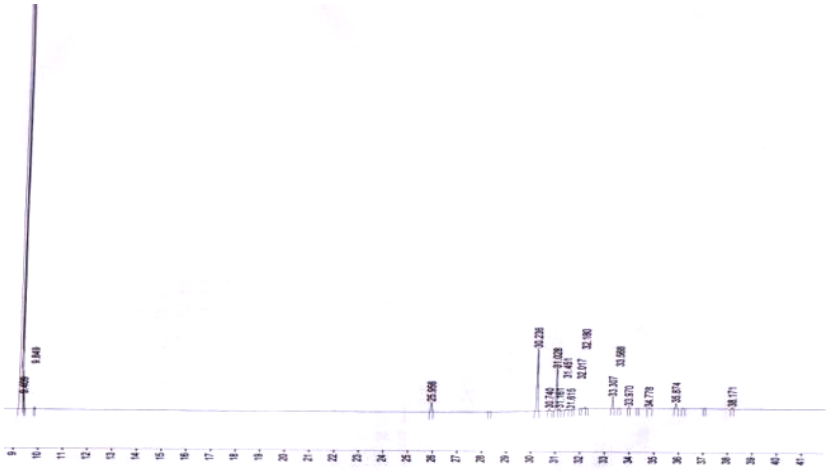
A. Day 3



B. Day 7



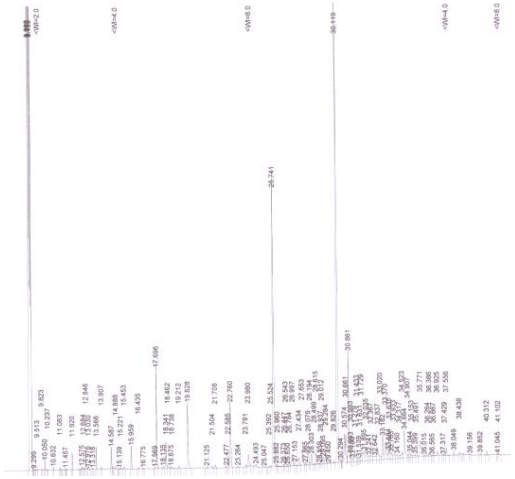
C. Day 13



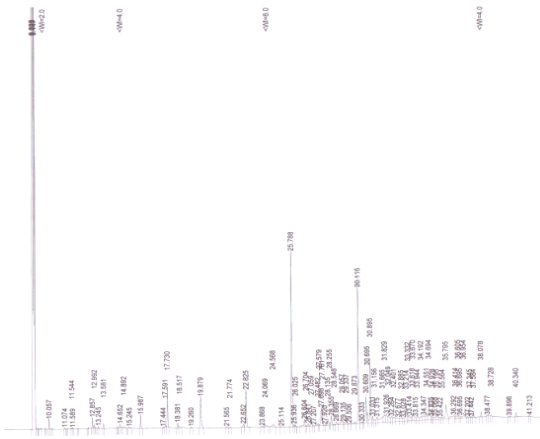
Traces of fatty acid composition of butter in enriched nutrient medium throughout the incubation with BFL-CP1

A) Day 3

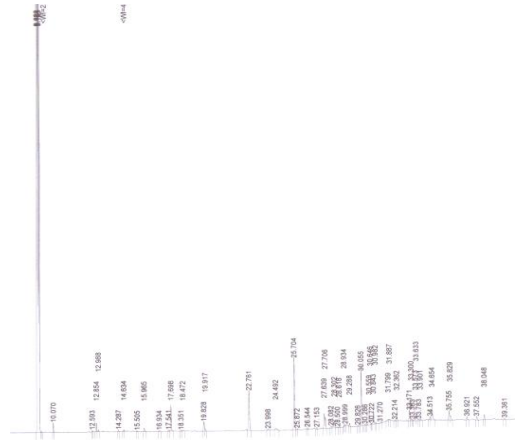
B) Day 5



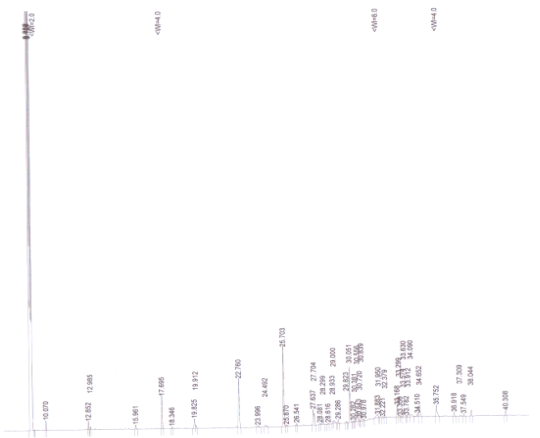
C) Day 7



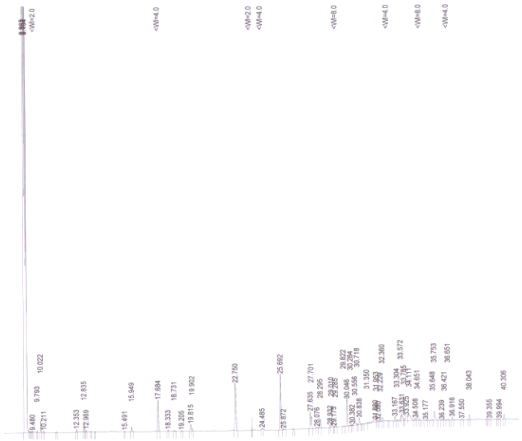
D) Day 9



E) Day 11

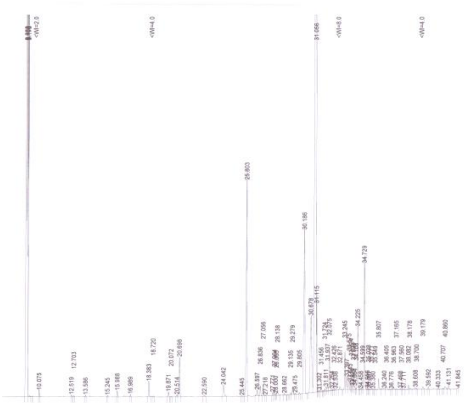


F) Day 13

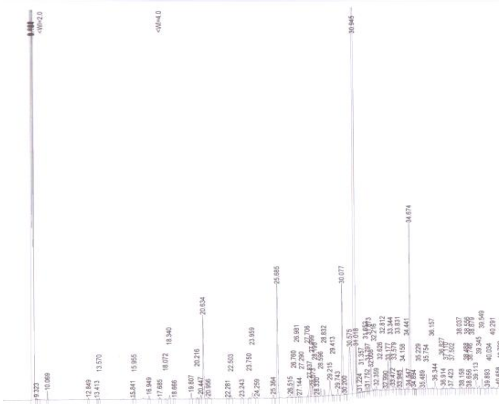


Traces of fatty acid composition of oil in ENM throughout incubation with BFL-CP1

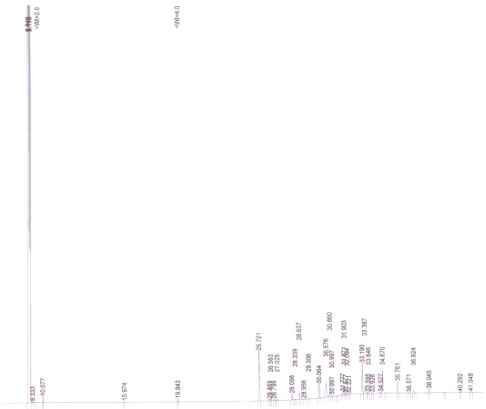
A) Day 3



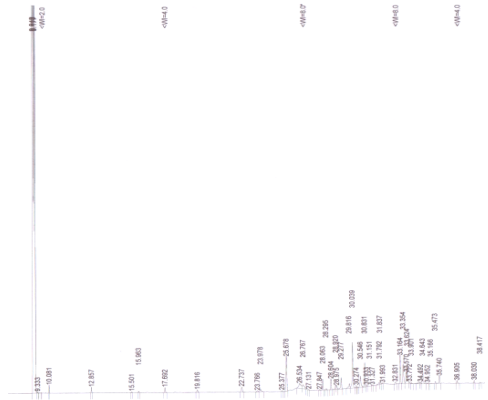
B) Day 5



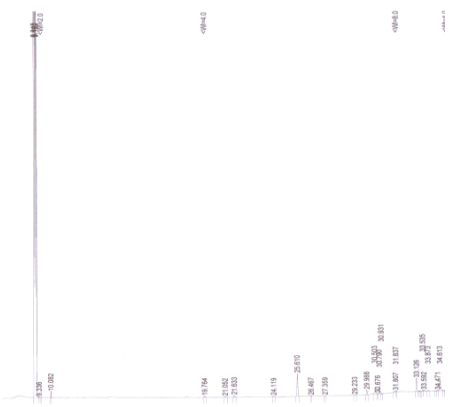
C) Day 7



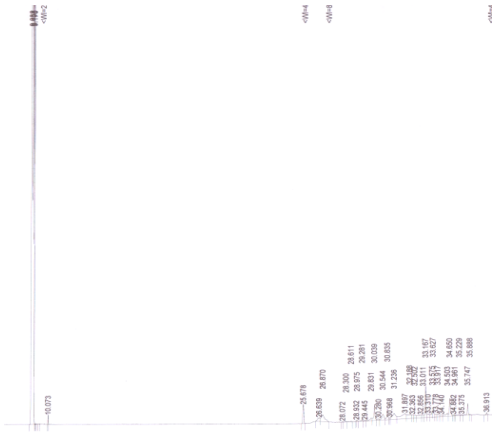
D) Day 9



E) Day 11



F) Day 13



APPENDIX C

RESEARCH PRODUCTS

ORAL PRESENTATIONS:

1. Tzirita, M., Papanikolaou, S. and Quilty, B. 2012. The growth of Yarrowia lipolytica on fats and oils. International Conference on Sustainable Energy and Environmental Protection (SEEP2012), Dublin City University, Ireland.

2. Tzirita, M. and Quilty, B. 2011. An investigation of mixed microbial populations for use in the treatment of waste fats, oils and greases (FOGs). IV International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2011), Torremolinos-Malaga, Congress Center of Torremolinos, Spain.

3. Tzirita, M., Papanikolaou, S. and Quilty, B. 2010. The degradation of fat using a bioaugmentation product. Environ Meeting: 20th Irish Environmental Researcher' Colloquium, Limerick, Limerick Institute of Technology, Ireland.

4. Tzirita, M., Papanikolaou, S. and Quilty, B. 2010. Fat Degrading Bacteria for use in Grease Traps. 8th Annual EPA Post-graduate research seminar, Convention Centre Dublin, Ireland.

5. Tzirita, M., Papanikolaou, S. and Quilty, B. 2009. A Bioaugmentation Product for Fats, Oils and Greases Degradation. Food Chemistry Days, Food and Environment, Athens, Evgenidou exhibition center, Greece.

POSTER PRESENTATIONS:

1. Tzirita, M. and Quilty, B. 2012. The Addition of a *Pseudomonas* Species Ensures Degradation of Butter by a Bioaugmentation Product. IWA World Congress on Water, Climate and Energy 2012, Convention Centre Dublin, Ireland.

2. Tzirita, M. and Quilty, B. 2011. An investigation of three commercial bioaugmentation products for the treatment of waste fats, oils and greases (FOGs). 9th Annual EPA Post-graduate research seminar, Ireland.

3. Tzirita, M., Papanikolaou, S. and Quilty, B. 2011. The biodegradation of olive oil by a mixed bacterial population. Biotechnology Research Day, Dublin, Dublin City University (DCU), Ireland

4. Tzirita, M., Papanikolaou, S. and Quilty, B. 2010. The Development of a Bioaugmentation Product for the treatment of waste Fats, Oils and Greases (FOGs). Green Chemistry conference, Dublin, DCU, Ireland.

5. Tzirita, M., Papanikolaou, S. and Quilty, B. 2010. An evaluation of three commercial bioaugmentation products for their ability to degrade Fats, Oils and Greases (FOGs). Irish Division of the Society for General Microbiology Autumn: Insect-mediated microbial diseases of humans and animals; Current problems and future threats, Maynooth, National University of Ireland (NUI), Ireland.

6. Tzirita, M., Papanikolaou, S. and Quilty, B. 2009. A Novel Bioaugmentation Product for Fats, Oils and Greases (FOGs) Degradation. Environ Meeting: 19th Irish Environmental Researcher' Colloquium, Waterford, Ireland, Waterford Institute of Technology, Ireland.

7. Tzirita, M., Papanikolaou, S. and Quilty, B. 2008. A Novel Bioaugmentation Product for Fats, Oils and Greases (FOGs) Degradation. Biotechnology Research Day, Dublin, Dublin City University, Ireland.

Conference Proceedings

1. Tzirita, M. and Quilty, B. 2012. An investigation of mixed microbial populations for use in the treatment of waste fats, oils and greases (FOGs). In Microbes in Applied Research: Current Advances and Challenges, pp18-22. Edited by Mendez-Vilas, World Scientific Publishing Co. Pte. Ltd. ISBN: 978-981-4405-03-4

2. Tzirita, M. and Quilty, B. 2012. The addition of a *Pseudomonas* species ensures degradation of butter by a bioaugmentation product", In Proceedings of IWA World Congress on Water, Climate and Energy 2012, 13th to 18th May 2012, Convention Centre Dublin, Ireland,

3. Tzirita, M., Papanikolaou, S. and Quilty, B. 2012, "The growth of Yarrowia lipolytica on fats and oils", In Proceedings of the 5th International Conference on Sustainable Energy and Environmental Protection 5th to 8th June 2012 – Part II: Environment & Clean Technologies, pp.223-228. Edited by Abdul Ghani Olabi and Khaled Benyounis, Dublin City University, Ireland.

Papers in preparation

The degradation of fats, oils and grease (FOG) by a commercial bioaugmentation product

Enhanced fat degradation following the addition of a *Pseudomonas* species to a commercial bioaugmentation product used in grease traps

The identification of bacteria present in bioaugmentation products using a range of biochemical and molecular techniques.