CHARACTERISATION OF MICROBIAL LIPASES FROM VEGETABLE OIL POLLUTED SOIL IN IBADAN

BY

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ABSTRACT

Vegetable oil spills are becoming frequent and are potentially more challenging than petroleum hydrocarbon spills. Microbial lipases occupy a place of prominence among biocatalysts and are often used for remediation of vegetable oil spills. There is a need for extensive characterisation of lipase for the treatment of vegetable oil-polluted sites. This work was carried out to characterise lipases from lipolytic isolates and as well monitor the degradation pattern of vegetable oil.

Microorganisms were isolated from eight experimental soil samples contaminated with different types of vegetable oil, soil from an oil mill in Ibadan, and normal uncontaminated soil as a control. The isolates were characterised, identified, and screened for lipase production using standard methods. Temperature, pH and ion concentration ($NaNO_3$ and $MgSO_4$) were optimised for the lipase activity. A bacterium and a yeast with highest lipase activity, determined using a titrator were selected for further work. These enzymes were partially purified by dialysis, column and ion-exchange chromatography. Their purity and molecular weight were determined by Sodium dodecyl sulfate polyacrylamide gel electrophoresis, and their kinetics were studied. The ability of the isolates to degrade olive oil in the laboratory was monitored at five-day intervals for 25 days using two mineral salt media. Concentration of the olive oil and microbial load were monitored in the media. The fatty acid profiles were determined using gas chromatography. Data obtained were analysed using ANOVA.

Seventy-three microorganisms were isolated from the contaminated soils and identified as species of *Bacillus* (16), *Pseudomonas* (12), *Flavobacterium* (6), *Alcaligenes* (2), *Proteus* (3), *Micrococcus* (1), *Aspergillus* (9), *Penicillium* (6), *Mucor* (4), *Rhizopus* (2) *Streptomyces* (2), *Candida* (4), *Saccharomyces* (4), *Geotrichum* (1), *Kluveromyces* (1). Crude enzymes of *Pseudomonas fluorescens* and *Candida parapsilosis* had the highest lipase activity of 0.8 U/mL and 0.4 U/mL respectively. Temperature, pH, and ion concentration ($NaNO_3$ and $MgSO_4$) which had optimum enzyme activities for partially purified *Pseudomonas fluorescens* (0.85 U/mL), were 27 ^{o}C, 7.5 and 0.05 mM, and *Candida parapsilosis* (0.35 U/mL), were 35 ^{o}C, 6.5 – 7.5, and 0.05 mM respectively. The molecular mass of the purified *P. fluorescens* and *C. parapsilosis* lipases were 45 and 38 KDa respectively. The purified *P. fluorescens* and *C. parapsilosis* lipases had K_m values

of 0.8 and 1.4 mg/mL, and V_{max} of 0.7 and 1.1 µg/sec respectively. There was a ($P < 0.05$) significant reduction in the concentration of the residual oil by *P. fluorescens* on day 15 (0.0031 mol/L) compared to day 0 (0.0064 mol/L). The plate counts, 8.8 ± 0.03 and 10.3 ± 0.03, were ($P < 0.05$) significantly low on day 5 for both organisms compared to day 25. The percentage reduction of fatty acid by *P. fluorescens* and *C. parapsilosis* after 20 days was 8.2 % and 6.7 % respectively in the media used.

Lipases from *Pseudomonas fluoresecens* and *Candida parapsilosis* had potential for the degradation of fatty waste. They could therefore be employed in environmental clean up of vegetable oil spill site.

Keywords: Lipase production, Fatty acid degradation, Vegetable oil spill

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Bukola Popoola,

March, 2014

CERTIFICATION

I certify that the research work reported in this Thesis for the Degree of Doctor of Philosophy (Microbiology) was carried out in the Department of Microbiology, University of Ibadan under my supervision.

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DEDICATION

This project work is dedicated with reverence, love and thanksgiving to God my father in heaven, the reason why I live.

LIST OF ABBREVIATIONS

1. µg	Microgram
2. µm	Micrometer
3. µM	Micromolar
4. 2KG	Calcium 2-ceto-gluconate
5. ADO	Adonitol
6. ARA	L-Arabinose
7. BOD	Biological Oxygen Demand
8. C/mol	Concentration per mole
9. cDNA	Chromosomal Deoxyribonucleic acid
10. CEL	D-Celibiose
11. cfu/g	Colony forming unit per gram
12. CM	Carboxymethyl
13. COD	Chemical Oxygen Demand
14. cRNA	Chromosomal ribonucleic acid
15. DEAE	Diethylaminoethyl
16. g	Gramme
17. GAL	D-Galactose
18. Glu	D-Glucose
19. H_20	Water
20. H_20_2	Hydrogen peroxide
21. INO	Inositol
22. kDa	Kilodalton
23. K_m	Michaelis-Menten constant
24. L	litre
25. LAC	D-lactose (origine bovine)
26. LB	Luria-Bertani
27. MDG	Methyl- α-glucopyranoside
28. mg	Milligram
29. min	Minute
30. ml	Milliliter

31. mM	Millimolar
32. Mol/L	Mole per liter
33. NAG	N-Acetyl-Glucosamine
34. ng	Nanogram
35. nm	Nanometer
36. O	Aucun
37. OD	Optical density
38. PCR	Polymerase Chain Reaction
39. POME	Palm oil mill effluent
40. rpm	Revolutions per minute
41. sec	Second
42. SOR	D-sorbitol
43. sp.	Species
44. U/mL	Unit per milliliter
45. UV	Ultraviolet
46. v/v	Volume per volume
47. V_{max}	Maximum velocity (rate of reaction)
48. w/v	Weight per volume
49. XLT	Xylitol
50. XYL	D-xylose

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Vegetable Oils

Vegetable oils are oil extractions from plants and fruits such as palm nut, sunflower, soybean, coconut, rapeseed, canola, olive, castor and corn (ITOPF, 2013). There are a variety of derivatives and degrees of processing. For example, in general terms, when generated by extractions with no further processing, oil is considered crude. "De-gummed" oil is a more refined product in which the resin-like compounds are removed (ITOPF, 2013). Appropriately, the term refined oil applies to products that have been clarified and any undesirable colour and sediment removed (AAA, 2013).

1.2 Behaviour and Fate of Vegetable Oils

Like mineral oils, vegetable oils can vary significantly and when released to the marine environment will behave differently according to their individual characteristics (ITOPF, 2013).These characteristics depend on factors at the time of cultivation of the feed stock, for example, climate; the degree of processing; type and specific nature of the oil, the sea state and weather conditions at the time of the spill (ITOPF, 2013). In many instances, the influence of vegetable oil characteristics on its behaviour in the environment is not well-studied or understood. Consequently, the behaviour and fate of specific vegetable oils is somewhat more difficult to predict than that of mineral oils (AAA, 2013).

In general, at the initial stage of a spill, vegetable oils will behave similarly to mineral oils. In this regard, they tend to float and spread on the surface of water (GRT Maldives, 2012). Notably, vegetable oils tend to be less soluble in water than mineral oils; they do not undergo dispersion in the water column nor evaporate significantly (GRT Maldives, 2012).

Depending on their pour point (the temperature at which solidification commences) and the sea surface temperature, vegetable oils may form solid lumps when

spilled which will float on the water surface (AAA, 2013). These discrete lumps have little tendency to coalesce as a surface slick. Over time and depending on the prevailing conditions, the product may accumulate sediments and may sink to the seafloor (GRT Maldives, 2012).

Vegetable oils comprised primarily of triacylglycerols or fatty acids, which, in their fresh state, may be brokendown by marine bacteria (AAA, 2013). This decomposition contributes to the rancid odours, typical of vegetable oil spills (AAA, 2013). This breakdown is as a result of lipases, a class of hydrolases (enzyme) primarily responsible for hydrolysis of acylglycerides (Vakhlu and Kour). They are ubiquitous and indispensable for the bioconversion of lipids (triacylglycerol) in nature. In addition to their biological significance, lipases hold tremendous potential for exploitation in biotechnology (Vakhlu and Kour). They possess the unique feature of acting at the aqueous and non-aqueous interface which distinguishes them from esterases (Verger, 1997; Schmidt and Verger, 1998). The concept of lipases interfacial activity evolved from restriction of their catalytic activity to interface between lipid and water (Vakhlu and Kour). The catalytic activity of lipases depends largely on the aggregated state of substrates (Vakhlu and Kour). Experimental evidences suggest that the activation involves unmasking and structuring of enzyme-active-site, through conformational changes, that require presence of oil in water droplets (Vakhlu and Kour). Studies on the structure of several lipases have provided some clues for understanding their hydrolytic activity, interfacial activation and stereo selectivity of lipases (Kazlauskas and Bornscheuer, 1998). Enzymes such as proteases and carbohydrases have been used industrially for a number of years and corner the largest share of the worldwide enzyme market (Vakhlu and Kour). Whilst lipases at present account for less than 5% of the market, this share has the potential to increase dramatically via a wide range of different applications (Vakhlu and Kour).

Lipases catalyse wide range of reactions, including hydrolysis, inter-esterification, alcholysis, acidolysis, esterification and aminolysis (Vakhlu and Kour). They catalyse the hydrolysis of fatty acid ester bond in the triacylglycerol (TAG) and release free fatty acids (FFA) (Sheldon, 1993). The reaction is reversible, the direction of which depends upon the water content available in it (Vakhlu and Kour). In low water media, lipases catalyse

esterification, transesterification and interesterification (Vakhlu and Kour). Biochemical and molecular characterisation of a number of lipases of different sources has brought to light great deal of heterogeneity in them with regard to specificity, amino acid sequence and catalytic properties. Based on the inhibition of their enzyme activity by chemical modification, lipases were initially classified as serine hydrolases. The presence of serine at their active site has been shown to be enclosed in the highly conserved domain and represents the only common feature shared by all determined lipases. They can be produced easily on a large scale by growing microorganisms in a fermentor, nonetheless, their use till recently was confined largely to oleo-chemistry and dairy-based industry.

The last quarter of the 20th century witnessed unprecedented use of lipases in biotechnology, manufacture of pharmaceuticals and pesticides, single cell protein production, bionsensor preparation and in waste management (Torossian and Bell, 1991; Gandhi, 1997; Yadav *et al*., 1998, Pandey *et al*., 1999; Jaeger *et al*., 1999; Saxena *et al*., 2003). Lipases have become an integral part of the modern food industry, used in the preparation of a variety of products such as fruit juices, baked food, vegetable fermentation and dairy enrichment. They are also used in leather industry for processing hides and skins (bating) and for treatment of activated sludge and other aerobic waste products where they remove the thin layer of the fats and by so doing provide for oxygen transport. The lipid digesting preparation is employed in sewage disposal plants in USA under the trade name lipase M-Y (Meito Sangyo Co., Nagoya Japan). Lipases may also assist in the regular performance of anaerobic digesters. Nearly 1000 tonnes of lipase are used annually in detergent industry, primarily as lipid stain digesters. They are also used as flavour development agents in the preparation of cheese, butter and margarine. These hydrolases are endowed with substrate specificity that surpasses any known enzyme. This property confers on them a literally boundless potential. The growing interest in lipases is reflected by the publication of an average of 1000 research papers per year (Pandey *et al*., 1999), on different aspects of these enzymes.

Given the variety of applications, there has been a renewed interest in the development of sources of lipases. Numerous species of bacteria, yeasts and moulds produce veritable development sources of lipases with different enzymological properties and specificities but moulds are known to be more potent lipase producer (Choo *et al*.,

1997). These microorganisms produce lipases both by solid substrate and submerged fermentations (He *et al*., 2004). Because of huge variation in applications, the availability of lipases with specific characteristics is still a limiting factor. The production of microbial lipases is apparently important from economic and industrial standpoints. Moreover extracellular lipases can be produced at large scale under normal laboratory conditions.

1.3 Statement of Problem

A wide variety of industries (such as the diary and food processing) produce the effluents rich in fats, oils and greases (FOGs). High concentrations of FOGs in waste water often initiate problems in their treatment processes (Stoll and Gupta, 1997; Becker *et al*., 1999). FOGs often cause foul odours, blockage of pipes and sewer lines. These problems are solved by the preliminary refining equipment, called grease traps. Grease traps may sometimes fail to retain dissolved and emulsified FOGs allowing them to enter the water treatment system. When vegetable oils are spilled on lands which in most cases are not easily detectable, they cause deleterious effects on birds, aquatic animals and plants. Such effects include coating bird feathers, gills of aquatic animals, plants stomata, sediments and other surfaces with oil and suffocating them by oxygen depletion (the oils cause depletion of oxygen in the water column to a level below what aquatic life needs).

Vegetables oils when left to linger in the ecosystem change their chemical composition and toxicity, possibly becoming more dangerous. In view of other numerous potential applications of lipases, their availability with specific characteristics is still a limiting factor as well as high cost of the enzyme.

1.4 Justification for the Study

Vegetable oil spills are becoming more common and are potentially more challenging than hydrocarbon spills (U.S EPA, 2013). One spill can spread fast between bodies of water and the land, coming in contact with humans, plants and animals. When spilled on land, their devastating effects cannot be over emphasized. Oil pollution is a serious obstacle to photosynthesis, a fundamental life process in plant kingdom, which

affects the food chain and productivity of the sea. Oil is also absorbed by fish which ultimately reach and endanger human life.

Microorganisms are therefore useful in protecting the environment. The lipolytic activity of physiologically diverse microorganisms can be used to degrade oil spills in the environment. The ability of any fungus for instance to exist in any environment would depend largely on its ability to produce enzymes that are capable of breaking down the various complex organic materials (Oso, 1978). It is significant to isolate microbes of high potential for the biodegradation of vegetable oil. Therefore, in this study, an attempt was made to assess the bio-potentials of a few microbial species with reference to their ability to degrade lipid from vegetable oil under laboratory conditions, and as an extension to our environment in order to rid it of the possible hazards these spillages can cause. Further, the characteristics of these lipases were checked in their purified form in order to understand enzyme functions better and enhance enzyme production by applying suitable substrate as well as process parameters optimisation.

1.5 Aims and Objectives

(i) Isolation, Identification and characterisation of microorganisms from vegetable oil-contaminated soil samples.

(ii) Screening the isolates for production of lipolytic enzymes.

(iii) Studying the effect of the environment on the growth and lipolytic enzyme production by the isolates.

(iv) Characterising the lipolytic enzyme in the most prominent isolate.

(v) Purification and Molecular weight determination of the lipase enzyme.

(vi) Application and monitoring of the degradation patterns of the vegetable oil samples by the prominent isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Fats and Oils

Fats and oils are naturally-occurring substances which consist predominantly of mixtures of fatty acid esters of the trihydroxy alcohol or glycerol (Nwobi *et al*., 2006). Different fats and oils come about due to the fact that there are numerous fatty acids of various kinds and these can be combined in an infinite number of ways on hydroxyl centres of glycerol. Moreover, the physical properties of fats and oils are dependent on the nature of fatty acids involved in the ester (Aluyor *et al*., 2009). Hence, the traditional distinction of fats as solids and oils as liquids arises from the fact that due to the different chemical structures of the different fatty acids combined in the esters, the bonding forces in existence vary in strength resulting in different melting points. These differences are manifested in different chain lengths, the presence or otherwise of unsaturation as well as geometric conformation (Aluyor *et al*., 2009).

2.1.1 Triglycerides

Triglycerides are the main constituents of vegetable oils and animal fats. Triglycerides have lower densities than water (they float on water), and at normal room temperatures may be solid or liquid (Antonio, 2004). When solid, they are called ‘”fats” or “butters” and when liquid they are called “oils”. A triglyceride, also called triacylglycerol (TAG), is a chemical compound formed from one molecule of glycerol and three fatty acids. Glycerol is a trihydric alcohol (containing three – OH hydroxyl groups) that can combine with up to three fatty acids to form monoglycerides, diglycerides, and triglycerides (Antonio, 2004). Fatty acids may combine with any of the three hydroxyl groups to create a wide diversity of compounds. Monoglycerides, diglycerides, and

triglycerides are classified as esters which are compounds created by the reaction between acids and alcohols that release water (H_2O) as a by-product.

Natural fatty acids found in plants and animals are typically composed only of even numbers of C atoms due to the way they are biosynthesised from acetyl CoA Bacteria, however, possess the ability to synthesis odd and branched chain fatty acids. Consequently, ruminant animal fat contains odd numbered fatty acids, such as 15, due to the action of bacteria in the rumen.

2.2 Lipases

2.2.1 General Description

Lipases are acyl hydrolases and water-soluble enzymes that play a key role in fat digestion by cleaving long-chain triglycerides into polar lipids. Because of an opposite polarity between the enzyme (hydrophilic) and their substrates (lipophilic), lipolytic reaction occurs at the interface between the aqueous and the oil phases (Reis *et al*., 2008).

2.2.2 Lipase as Biocatalysts

Lipases (triacylglycerol acylhydrolases) are a group of enzymes, which have the ability to hydrolyse triacylglycerols at an oil-water interface to release free fatty acids and glycerol. Lipases are present in microorganisms, plants and animals (Jisheng *et al*., 2005). Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis (Joseph *et al*., 2008). This hydrolytic reaction is reversible. In the presence of organic solvents, the enzymes are effective catalysts for various inter-esterification and trans-esterification reactions. Furthermore, microbial lipases show regiospecificity and chiral selectivity (Gupta *et al*., 2003). Especially microbial lipases have different enzymological properties and substrate specificities. Many species of bacteria, yeast and moulds are found to produce lipases (Liu *et al*., 2008).

Their biotechnological potential is relying on their ability to catalyse not only the hydrolysis of a given triglyceride, but also its synthesis from glycerol and fatty acids. Therefore, microbial lipases have many industrial applications (Jaeger *et al*., 1999). The temperature stability of lipases is the most important characteristic for industrial use

(Choo *et al*., 1997). The extracellular bacterial lipases are commercially valuable, because their bulk production is much easier (Gupta *et al*., 2004). Lipase catalysed reactions are widely used in the manufacturing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics. Lipases are also used to accelerate the degradation of fatty wastes and polyurethane (Jisheng *et al*., 2005).

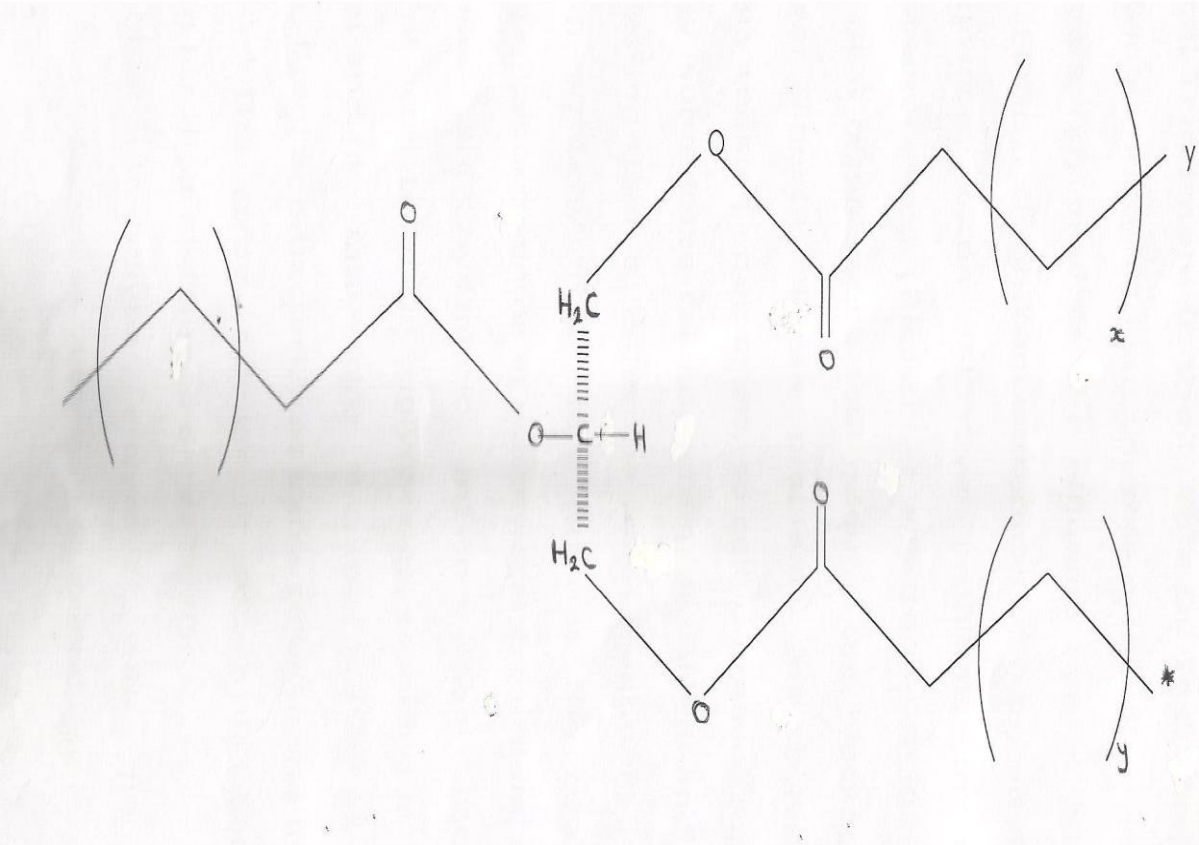

Fig. 2.1: General structure of a triglyceride

(Source: "http:/en.wikipedia.org/wiki/imageTriglyceride-General structure.png").

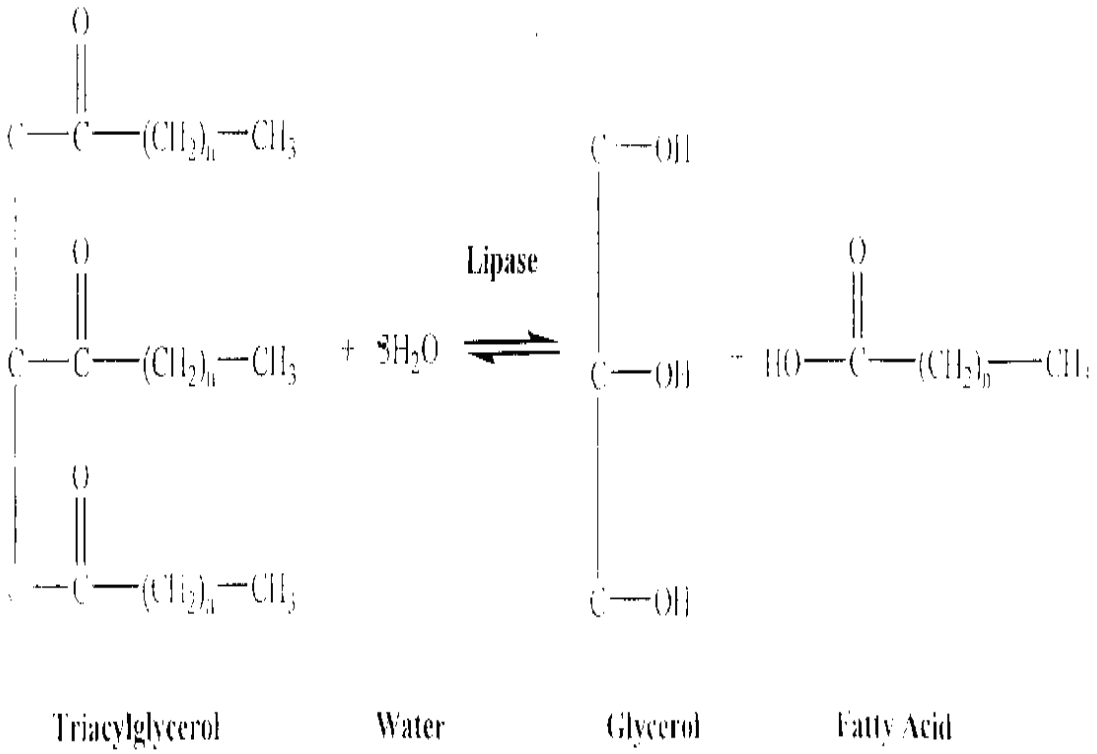

Figure 2.2. Hydrolysis or synthesis of a triacylglycerol substrate catalysed by a lipase enzyme (Thomson *et al.*, 1999).

2.2.3 Bacterial Lipases

Bacteria produce different classes of lipolytic enzymes including carboxylesterases which hydrolyze water-soluble esters and lipases which hydrolyze long-chain triacylglycerol substrates (Rosenau and Jaeger, 2000). Many bacterial species produce lipases which hydrolyse esters of glycerol with long-chain fatty acids. They act at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium. Lipases show interfacial activation, a sharp increase in lipase activity observed when the substrate starts to form an emulsion (Ece Yapasan, 2008). As a consequence, the kinetics of a lipase reaction does not follow the classical Michaelis-Menten model. With only a few exceptions, bacterial lipases are able to completely hydrolyze a triacylglycerol substrate although ester bonds are more favourable (Jaeger *et al*., 1994). Some important lipase-producing bacterial species are *Bacillus, Pseudomonas* and *Burkholderia* (Svendsen, 2000). Numerous lipase assay methods are available using coloured or fluorescent substrates which allow spectroscopic and fluorimetric detection of lipase activity. Another important assay is based on titration of fatty acids released from the substrate.

Usually enzymes are not stable in organic solvents where they tend to denature and lose their activities (Ece Yapasan, 2008). But lipases remain stable and active in organic solvents without any stabiliser. Substrates of lipase are often insoluble or partially soluble in water and thus the use of organic solvents or organic–aqueous solutions is in favour of some reactions (Zhao *et al*., 2008). Because of its catalytic ability in organic solvents, many new biotechnological applications of lipases have been identified. One of the applications is the synthesis of chirally important drugs and drug intermediates (Singh and Banerges, 2007).

Reactions catalysed by lipases are carried out in organic-aqueous interface. That process is desirable because the separation of enzyme from substrates or products is easy (Rahma *et al*., 2005). The synthesis and secretion of lipases by bacteria is influenced by a variety of environmental factors like ions, carbon sources, or presence of non-metabolizable polysaccharides (Ece Yapasan, 2008). The secretion pathway is known for *Pseudomonas* lipases with *P. aeruginosa* lipase using a two-step mechanism and *P. fluorescens* lipase using a one -step mechanism. Additionally, some *Pseudomonas* lipases

need specific chaperone-like proteins to assist their correct folding in the periplasm. These lipase-specific foldases which show a high degree of amino acid sequence homology among different *Pseudomonas* species are coded the lipase structural genes (Ece Yapasan, 2008). A comparison of different bacterial lipases on the basis of primary structure revealed only very limited sequence homology. However, some of the bacterial lipases reveal a conserved folding pattern called the alpha/beta-hydrolase fold. The catalytic site of lipases is buried inside the protein and contains a serine-protease-like catalytic triad consisting of the amino acids serine, histidine and aspartate (or glutamate) (Ece Yapasan, 2008). The active site is covered by a lid-like alpha-helical structure which moves away upon contact of the lipase with its substrate, thereby exposing hydrophobic residues at the protein's surface mediating the contact between protein and substrate (Svendsen, 2000).

2.2.3.1 Fermentation Conditions

Bacterial lipases are mostly released outside of the cell that is called extracellular enzyme. They are influenced by nutritional and physico-chemical factors; such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, stirring conditions, dissolved oxygen concentration (Rosenau and Jaeger, 2000).

The major factor for the expression of lipase enzyme is carbon source. Lipases generally are produced in the presence of lipid source such as oil, triacylglycerols, fatty acids, hydrolyzable esters, the tweens and glycerols addition to carbon source, the type of nitrogen source also influence the production of lipases. Generally, organic nitrogen source is prefered by bacteria, such as peptone and yeast extract (Gupta *et al*., 2004).

The initial pH of the growth medium is important for lipase production. Most bacteria prefer pH around 7.0 for their best growth and lipase production. The optimum temperature for lipase production is parallel with the growth temperature of the respective microorganism. It has been reported that lipases are produced in the temperature range from 20^{o}Cto 45^{o}C (Jaeger *et al*., 1999). Incubation periods change from few hours to many days until the maximum lipase production from bacteria is recorded.

2.2.3.2 Purification of Bacterial Lipases

Purification methods need to be applied in order to understand enzyme functions, its relations between environment and its 3-D structures (Reis *et al*., 2008). In addition, purification of enzymes allows amino acid sequence and the three-dimensional structure. The X-ray studies of pure lipases enable the establishment of the structure–function relationships. That knowledge provides a better understanding of the kinetic mechanisms of lipase action on hydrolysis, synthesis and group exchange of esters (Saxena *et al*., 2002). Knowledge of the three-dimensional structure of lipases plays an important role in designing and engineering lipases for specific purposes. Pre-purification steps involve concentration of culture by ultrafiltration, by ammonium sulfate or cold acetone precipitation and by extraction with organic solvents.

In further processing, affinity chromatography (especially hydrophobic interaction chromatography) is the best method since lipases are hydrophobic and have large hydrophobic surfaces in their active sites (Gupta *et al*., 2004). Alternatively, ion-exchange and size-exclusion chromatography are preferred after precipitation step because of high cost of hydrophobic resins.

2.2.4 Properties of Lipases

Lipases from several microorganisms have been studied. Generally, bacterial lipases have neutral or alkaline pH value and show activity in a broad pH range (pH 4 to pH 11). The thermal stability of lipases ranges from 20^{o}C to 60^{o}C. Stability of organic solvents is desirable in synthesis reaction. Most of bacterial lipases are stable in organic solvents (Gupta *et al*., 2004).

According to substrate specificity microbial lipases are divided into three categories; non-specific, region-specific and fatty acid-specific (Gupta *et al*., 2004). Non-specific lipases behave randomly on the triacyglyceride molecule and produce complete breakdown of triacyglyceride to fatty acid and glycerol. Conversely, region-specific lipases are 1,3-specific lipases which hydrolyse only primary ester bonds (ester bonds at atoms C1 and C3 of glycerol) and thus hydrolyse triacylglyceride to give fatty acids. Fatty acid-specific lipases display activity in the presence of fatty acid (Thomson *et al*., 1999; Gupta *et al*., 2004).

Non-specific lipases act at random on the triaclyglyceride molecule and result in the complete breakdown of triacylglyceride to fatty acid and glycerol. Examples of this group of lipases include those from *S. aureus, S. hyicus* (Davranov, 1994; Jaeger *et al*., 1994), *Corynebacterium acne* (Hassing, 1971) and *Chromobacterium viscosum* (Jaeger *et al*., 1994).

In contrast, region-specific lipases are 1,3-specific lipases which hydrolyse only primary ester bonds (i.e ester bonds at atom C1 and C3 of glycerol) and thus hydrolyse triacylglyceride to give free fatty acids, 1,2 (2,3) –diacylglyceride and 2-monoacylglyceride. Extracellular – bacterial lipases are region-specific, for example those from *Bacillus sp.* (Sugihara *et al*., 1991; Lanser *et al*., 2002), *Bacillus subtilis* 168 (Lesuisse *et al*., 1993), *Bacillus sp*. THLO27 (Dharmsthiti and Luchai, 1999), *Pseudomonas sp*. F-B-24 (Yamamoto and Fujiwara, 1988, 1995), *Pseudomonas aeruginosa* EP2 (Gilbert *et al*., 1991) and *Pseudomonas alcaligenes* 24 (Misset *et al*., 1994).

The third group comprises fatty acid-specific lipases, which exhibit a pronounced fatty acid preference. *Achromobacterium lipolyticum* is the only known bacterial source of a lipase showing fatty acid specificity (Davranov, 1994). However, lipases from *Bacillus sp*. (Wang *et al*., 2003), *Pseudomonas alcallgenes* EF2 (Gilbert *et al*., 1991) and *Pseudomonas alcaligenes* 24 (Misset *et al*., 1994) show specificity for triacylglycerides with long-chain fatty acids, while lipases from *Bacillus subtilis* 168 (Lesuisse *et al*., 1993), *Bacillus sp*. THL027 (Dharmsthiti and Luchai, 1999), *Pseudomonas sp*. ATCC21808 (Kordel *et al*., 1991), *Chromobacterium viscosus* (Horiuti and Imamura, 1977) and *Aeromonas hydrophila* (Anguita *et al*., 1993), Prefer small or medium-chain fatty acids. Lipase from *Staphylococcus aureus* 226 shows a preference for unsaturated fatty acids (Muraoka *et al*., 1982).

Another important property of lipases is their enantio-/stereoselective nature, wherein they possess the ability to discriminate between the enantiomers of racemic pair, such enantiomerically pure or enriched organic compounds are steadily gaining importance in the chemistry of pharmaceutical, agricultural, synthetic organic and natural products (Reetz, 2001). Mostly, lipases from *Pseudomonas* family fall in this category

(Reetz and Jaeger, 1998). The stereo-specificity of a lipase depends largely on the structure of the substrate, interactions at the active site and the reaction conditions (Muralidhar *et al*., 2002). The lipase from *Pseudomonas cepacia* is a popular catalyst in organic synthesis (Kazlauskes and Bornscheuer, 1998) for the kinetic resolution of racemic mixtures of secondary alcohols in hydrolysis, esterification and transesterification (Takagi *et al*., 1996; Schulz *et al*., 2000). Lipases from *Pseudomonas spp*. are used for the synthesis of chiral intermediates in the total synthesis of the anti-microbial compound chaungxiximyxin and the potent antitumor agent epothilone (Gupta *et al*., 2004). Lipases are also used in the efficient production of enantiopure (S) – Indanofan, a novel herbicide used against grass weeds in paddy fields. The synthesis of flavour and fragrance compounds such as menthol has been reported, using lipase from *B. cepacia* (Jaeger and Eggert, 2002).

Thus, bacterial lipases are highly robust enzymes, since they are active over a wide range of pH and temperature. They belong to the group of serine hydrolases and are not sulphaydryl proteins. They may be region-specific or non-specific towards triacylglycerols. Some lipases also possess fatty acid – specificity with reference to the carbon-chain length. Besides these features, the enantioselective nature of lipases provides them with an edge over other hydrolases, particularly in the field of organic chemistry and pharmaceuticals.

2.2.5 Yeast Lipases

The lipase produced by *Candida rugosa* is fast becoming one of the most industrially used enzymes. This is because of its use in a variety of processes due to its high activity, both in hydrolysis as well as synthesis. A Japanese company in 1985 used the *Candida rugosa* lipase for production of fatty acids from castor bean (Macrae and Hammond, 1985). Pandey *et al*. (1999) investigated the production of flavour in concentrated milk and creams by using microbial lipases. Organoleptically, each lipase develops a characteristic flavour. The *Candida rugosa* lipase was rated the most suitable lipase in this case. *Candida antarctica* AY30 immoblised lipase has been used for esterification of functional phenols for synthesis of lipophillic antioxidants subsequently used in sunflower oil (Pandey *et al*., 1999). Uppenberg *et al*., (1994) developed *Candida*

antarctica lipase into recombinant enzyme used for detergent formulation. The extracellular lipase produced by the asporogenic *Candida cylindracea* ATCC 14830 (CCL/CRL) hydrolyses triglycerides without specificity, both in attacked position of the glycerol molecule and in the nature of fatty acid released. This relaxed specificity vis-à-vis other lipases makes CCL/CRL particularly useful for industrial application (Lotti *et al*., 1993).

In detergent industry, yeast lipases find use as lipid stain digesters. Lipases from *Candida cylindracea* and *Candida lypolytica* (now *Yarrowia lipolytica*) are choice enzymes for the purpose (Pierce *et al*., 1990; Batenburg *et al*., 1991). Polyglycerol and carbohydrate fatty acid esters are widely used as industrial detergents and as emulsifiers in variety of food formulations (low fat spreads, ice creams, mayonnaise). Enzymatic synthesis of functionally similar surfactants has been carried out at moderate temperature (60ºC – 80ºC) with excellent regioselectivity (Vakhlu and Kour, 2006). Recently, Unichem International has launched production of isopropyl myristate, isopropyl palmitate and 2-ethylpalmitate for use of emollient in personal care products. Presently these compounds are being manufactured enzymatically using *C. cylindracea* lipase in batch bioreactor.

A promising new field is the use of microbial lipase as biosensors. Biosensors can be chemical or electronic in nature. An important analytical use of lipases is determination of lipids for clinical purpose (Pandey *et al.*, 1999). The basic concept is to utilise a lipase to generate glycerol from triacylglycerol and quantify the released glycerol or alternatively the non-esterified fatty acid by chemical and enzymatic method. This principle enables physicians precisely to diagnose patients with cardiovascular complaints. Non-specific lipases, especially of *Candida rugosa* with high specific activity has been selected to allow rapid liberation of glycerol *Candida rugosa* lipase biosensor, which optically conjugates to biorecognition group in DNA, has been developed as probe by Pandey *et al*. (1999).

2.2.6 Application and Uses of Lipases

The application of lipases in organic synthesis is tremendous. Stereoselectivity of lipases for resolution of racemic acid mixture in immiscible biphasic system has been

demonstrated (Vakhlu and Kour, 2006). Efficient kinetic resolution processes are in vogue for the synthesis of Niknomycin-B, non-steroid anti-inflammatory drugs Naproxen, ibuprofen, suprofen and ketoprofen, the potential antiviral agent lamividine (that can also be used against HIV) and enantiospecific synthesis of antitumour agents alkaloids, antibiotics and vitamins (Pandey *et al*., 1999). Hernaiz *et al*. (1997) have isolated two iso-forms, labelled A and B from *Candida rugosa* that are stereoselective.

Preparations of optically active amines that are intermediate in preparation of pharmaceuticals and pesticides have been described by Smidt *et al.* (1996). This involved reacting stereospecific N-acylamines with lipase preferably from *C. antarctica*. In an attempt to determine substrate specificity of lipases, alkyl esters of 2 aryl- propionic acid, a class of non-steroid anti- inflammatory drugs were hydrolysed with *Candida rugosa* lipase. All transformations were found to be highly selective. Lipases are also used for enantiospecific catalysis. The stereo selective enatio-discrimination of *Candida rugosa* lipase yielded optically pure propionic acid derivative in S-form. The S-form was then converted to corresponding R form, which was effective against the insect pest Tetramuchus (Pandey *et al.*, 1999)

Triglycerides, steryl esters, resin acids, free fatty acids and sterols which are lipophilic extractives (extracts) of wood (commonly referred to as pitch or wood resin) have negative impact on paper machine run ability and quality of paper. Kontkanen *et al.* (2004) in their study tested 19 commercial lipase preparations able to show degradation of steryl esters. They found lipase preparations of *Pseudomonas sp. Chromobacterium viscosum* and *Candida rugosa* were shown to have highest steryl esterase activity. All the three enzymes were able to hydrolyse steryl esters totally to completion in presence of a surfactant (thesit). Preliminary characterisation of enzymatic activity revealed that the lipase preparation of *Pseudomonas* sp. could be the most potential industrial enzyme but among yeast *Candida rugosa* lipase (CRL) ruled the roost (Kontkanen *et al*., 2004).

To introduce polymer to cellulosic material a new approach was developed by Gustavsson *et al*. (2004) using ability of a cellulose binding module of *Candida antarctica* lipase B conjugate to catalyze ring opening polymerization of epsilon-caprolactone in close proximity to cellulose fiber surface. Wang *et al*. (2003) demonstrated effective biocatalysis also by *Candida antarctica* Lipase (CAL B) in resolution of several 1-or 2-

hydroxyalkanephosphonates. The enaniomers of phosphogabob and fosfomycin were prepared using CALB-mediated resolution as key step.

2.2.7 Protein Purification and Biochemical Properties

Microbial lipases, are purified by two major purification techniques

i) Precipitation techniques (with salts, alcohols, etc.) and

ii) Chromatographic techniques (ionic, hydrophobic interaction, affinity and molecular sieving).

2.2.7.1 *Candida spp.*

There are several reports on the multiple forms of lipases produced by the microorganism. This multiplicity has been ascribed to post–transcriptional processing, existence of different genes, deglycosylation. Among yeasts *Candida albicans, Candida antarctica, Candida rugosa, Geotrichum asteroids, Geotrichum candidium, Trichosporon fermentans, Saccharomycopsis lipolytica, Yarrowia lipolytica* (formally *Candida paralipolytica*) are reported to produce multiple lipase forms.

Various strains of *Candida rugosa / cylindracea* (L.1754, ATCC 14830, DSMZ 2031) are known to produce lipase. Purification and characterization has been reported for a number of them. Two distinct lipases from *Candida rugosa* (*C. cylindrcea* L.1754) were identified and separated by high-resolution anion exchange column mono Q after ethanol extraction of crude lipase. Lipase I eluted at 0.05 MNaCl whereas lipase II eluted at 0.15 MNaCl from this column. The less anionic nature of lipase I was confirmed by native polyacryamide gel electrophoresis and isoelectricfocusing. Both the proteins have apparent molecular weight of 58 kD on SDS-PAGE. The isoelectric focusing point of lipase I and II are ~5.8 and ~5.6 respectively (Veeraragavan and Gibbs, 1989).

Chang *et al*. (1994) reported that PAGE pattern of lipolytic enzymes obtained from three commercial samples (different manufacturers) of *Candida rugosa* lipase differed. They studied the effect of culture conditions on the production of lipase from *Candida rugosa* (ATCC 14830) and proposed that culture conditions not only influence the production of lipase but also changed the pattern of formation of multiple forms of lipase. They showed presence of tween 20 and tween -80 in culture media for *Candida rugosa*

resulted in production of various forms of lipase showing different substrate specificities and thermal stabilities. The results suggest that the specificity and stability of lipase preparation can be modulated by culture condition. Chang *et a*l. (1994) proposed that multiple patterns of lipases could result from change in gene expression, variable percentage of covalently linked carbohydrates, partial proteolysis and posttranscriptional modifications. Hitherto, five genes belonging to lipase gene family have been isolated from *Candida rugosa,* which suggest that some of the multiple lipase forms that Chang *et al.* (1994) have identified, were the result of change in gene expression.

Lotti and her group in the University of Milan, Italy have been studying various aspects of lipase production by *Candida rugosa/cylindracea* for about a decade. They Lotti *et al*. (2001) undertook a flow cytometric study to evaluate growth-production process of *Candida rugosa* cells of different culture media. The yeast follows a complex pattern of lipase production depending on the presence of multiple lipase encoding genes whose expression is modulated by carbon source. The C-source employed during fermentation can act as a repressor for example glucose and sorbitol. Neutral substrates may be successfully employed in two-step fermentation, where the first step of biomass growth is followed by the induction of lipase gene expression. Most of the lipases including those from *Candida rugosa* are glycosylated in conformity with rest of the lipases (Vakhlu and Kour, 2006). *Candida rugosa* lipases are widely used in biotransformation on account of their thermal stability and substrate specificity. The percentage and nature of carbohydrate attached seem to be important in structure interaction of glycoprotein with oil water interface, thermal stability, hydrophobic hydrophilic interaction, and over all catalytic activity of lipases.

Elimination of non-covalently bound sugar produces diminution in enzymatic activity in hydrolysis of tributyrin, in synthesis of heptyl oleate and in thermal stability. The equilibrium of this enzyme with lactose or with dextran produces partial reactivation of the biocatalyst.

Benjamin and Pandey (2001) isolated and characterised three distinct forms of lipases from *Candida rugosa* (DM - 2031), produced in solid-state fermentation. Three distinct forms of extracellular lipase (lipA, lipB, and LipC) were isolated by ammonium sulphate precipitation, dialysis, ultra filtration and gel filtration using Sephadex-200. The

purification was 43-fold with specific activity 64.35 mg/ml. SDS- PAGE of purified lipase revealed three distinct bands indicating the existence of three iso-forms with apparent molecular weight of 64, 62 and 60 kD (Vakhlu and Kour, 2006). All the three forms have optimal activity at 35-40ºC and pH 7-8. Ag^{++} and Hg^{++} strongly inhibit the activity of all the iso –forms whereas Ca^{++} and Mg^{++} enhance the lipase activity. The activity of all the three forms was completely inhibited by serine protease inhibitors namely, -dichloroisocoumarin and pefabloc. Phenylmethanesulphonyl fluoride inhibited their activity partially (Vakhlu and Kour, 2006).

2.2.7.2 *Geotrichum*

Couple of species of genus *Geotrichum* are known to produce lipases most of them extracellular. Tsujisaka *et al*. (1973) purified lipase of *Geotrichum candidum* Link by means of ammonium sulphate fractionation, DEAE - Sephadex chromatography and gel filtration of sephadex G-100 and G-200. The purified enzyme was subsequently crystallized. The crystallized preparation was found to be homogenous electrophoretically as well as centrifugally. The molecular weight and pI value of the enzyme were estimated to be ~55 kD and ~4.33. The crystalline preparation contained about 7% carbohydrate and a very small amount of lipids. This lipase was active on olive oil at pH 5.6 and 7.0 at 40ºC. This enzyme maintained its stability in the pH range of 4.2 - 9.8 for 24 hrs. The enzyme is stable at temperature below 55ºC for 15 min.

Two forms of lipases have been isolated from *Geotrichum candidum* ATCC 34614 by combining of ethanol precipitation and chromatography on Sephacryl HR anion exchange and polybuffer exchange 94 (Vakhlu and Kour, 2006). The molecular weight of enzymes has been estimated to be ~56 KD. The optimum pH value and isoelectric focusing point of the iso-forms is 6.8 and 6.0 and 4.46, 4.56 for lipase I and II respectively. The enzymes are found to remain stable in the pH range 6.0 - 8.0. Monovalent ions had little effect on activity of both the enzyme whereas divalent ion at concentration above 50mM inhibited the activity in concentration dependent manner. At concentration less than 10 mM ionic detergent sodium dodecyl sulphate completely inhibited lipase activity (Veeraragavan *et al*., 1990).

Like species of genus *Candida, Geotrichum candidum* is also known to produce various forms of lipase. Purification and characterisation of different lipase iso-forms produced by various strains of *Geotrichum candidum* is difficult on account of overlapping in their physical and biochemical properties. At times the heterogeneity is result of the difference in glycosylation. The reports that appeared recently on substrate selectivity of apparently purified lipase iso-forms are contradictory (Vakhlu and Kour, 2006).

2.2.7.3. *Pseudomonas* Lipases

A number of investigations have concentrated on purification and characterisation of lipase from *Pseudomonas spp*. (Gilbert *et al*., 1991; Sharon *et al*., 1998; Dong *et al.*, 1999; Ogino *et al*., 2000). Schuepp *et al*. (1997) observed that *Pseudomonas fragi* CRDA 037 produced exo- and endolipases when grown in whey media. They obtained partially purified lipase extracts by precipitation with ammonium sulphate at 20–40% saturation for the exolipase and 20–60% saturation for the endolipase. Native PAGE of the exolipase showed the presence of a major band with a molecular weight of 25.5 kDa, whereas the endolipase showed the presence of three fractions with molecular weights of 35.5, 49 and 70 kDa. (Saxena *et al*., 2003) reported that extracellular *Pseudomonas* lipase was able to interact with alginate. Based on this observation, procedures were developed which allow rapid, simple and inexpensive concentration, stabilization and partial purification of an exolipase from a strain of *Pseudomonas aeruginosa* (Wingender *et al*., 1987). Their investigation showed that the lipase could be rapidly and almost completely harvested from the cell-free culture medium by ethanolic precipitation with alginate (about 95% of the original exolipase activity was co-precipitated). After dissolving the co-precipitate in detergent-containing buffer, exolipase and polysaccharide could be easily separated by ion exchange chromatography on DEAE-Sephadex A-25. The co-precipitation method was also successfully applied to exolipases produced by *Pseudomonas spp*. and *C. viscosum*, thus suggesting the potential use of this method in the downstream processing of lipases.

An extracellular alkaline lipase of alkalophilic *Pseudomonas pseudoalcaligenes* F-111 was purified to homogeneity using acetone precipitation and chromatography on Sephadex G-100, Fractogel phenyl 650 M and Sephadex G-100. SDS-PAGE revealed the

molecular weight of the lipase as 32 kDa and its pI was 7.3 (Lin *et al.*, 1996). A t-butyl octanoate (TBO)-hydrolyzing intracellular lipase was purified from cell-free extract of *Burkholderia spp*. by Yeo *et al*. (1998). Ammonium sulfate precipitation, three consecutive open column chromatographies and two HPLCs obtained a 74-fold purification of the lipase. Chromatography included DEAE anion exchange, Sepharose CL-6B gel filtration and a second DEAE anion exchange chromatography.

2.2.7.4 *Staphylococcus* Lipases

Several researchers have reported purification and properties of different *Staphylococcus* lipases (Muraoka *et al*., 1982; Tyski *et al*., 1983; van Oort *et al*., 1989; Oh *et al*., 1999).

Lipase (L-1) from a strain of *Staphylococcus aureus* has been purified by application of a multi-step procedure involving ammonium sulfate precipitation and hydrophobic chromatography on phenyl-Sepharose CL-4B followed by gel filtration through sepharose. Rechromatography on Sepharose CL-4B of the lipase preparation was the last step of purification.

A purified enzyme was obtained which appeared to be homogeneous by molecular sieving, polyacrylamide gel electrophoresis and sucrose gradient centrifugation. The molecular weights obtained by molecular sieving and electrophoresis in the presence of SDS were 300 and 45, respectively. Staphylococcal lipase seems to exhibit a complex structure which may explain these discrepancies (Tyski *et al*., 1983).

The *Staphylococcus hyicus* lipase gene has been cloned and expressed in *staphylococcus carnosus*. The enzyme was secreted from *S. carnosus* into the medium as a protein with an apparent molecular weight of 86 kDa. This lipase was purified by ammonium sulphate precipitation followed by a combined Sephadex G-100/G-25 column. In order to facilitate the isolation of larger amounts of homogeneous lipase, the preparation obtained after the sephadex step was incubated with various proteolytic enzymes. Following tryptic digestion, a single active band of 46kDa was obtained. This lipase was purified to homogeneity by using two DEAE-cellulose columns developed at pH 8.0 and 6.5, respectively (van Oort *et al*., 1989).

2.2.7.5 *Chromobacterium* Lipases

The crude lipase preparation of *C. viscosum* contained more than two species of lipase which differed from each other in molecular weight and isoelectric point (Sugiura *et al*., 1974). The crude enzyme preparation containing the two lipases was obtained with fractional precipitation with ethanol (50–80%) from the culture broth of *C. viscosm*

Another method for purification of the crude enzyme from the culture broth of *C. viscosum* was based on the affinity of the lipase for palmitoylcellulose (Horiuti and Imamura, 1997). This procedure was found to be simple and effective, especially for large-scale purification of the lipase. The crude lipase obtained by fractional precipitation (50–80%) with ethanol was purified by chromatography on palmitoylated gauze column with an overall recovery of 71% and an increase of 11-fold in the specific activity over the supernatant fluid of bacterial cultures .Two isoenzymes were obtained, each in a homogenous state on SDS-PAGE: one with a molecular weight of 120 kDa and a pI of 3.7 and the other with a molecular weight of 30 kDa with pI of 7.3 (Horiuti and Imamura, 1997).

The lipase of *C. viscosum* in the crude enzyme preparation was specially adsorbed on glass beads coated with various hydrophobic materials (Isobe and Sugiura, 1977). The enzyme adsorbed on a column with siliconized glass beads was not denatured and was eluted with 0.1% Triton X-100. By this method, *C. viscosum* lipases were purified 1000-fold in one step and showed two protein bands in disc electrophoresis, both with lipolytic activity. This purification method was more effective and simple than the affinity chromatography method reported earlier.

Selective separation and purification of a lipolytic preparation from *C. viscosum* were carried out by liquid–liquid extraction using a reversed micelllar system (Aires-Barros and Cabral, 1991). When techniques with organic solvents are limited by protein denaturation and solubilization, the use of liquid–liquid extraction of bio molecules employing reversed micelles was a promising solution (Naoe *et al*., 1999). This method involves a very simple procedure and requires only two steps. The first step is based on the ability of reversed micelles to solubilise proteins from an aqueous phase into the water pool of the surfactant aggregates. In the second step, the solubilised proteins are extracted

back into a new aqueous phase by changing the interactions between the protein and the reversed micellar system.

Also, extraction of *C. viscosum* lipase from aqueous solution was carried out in AOT/span 60 and in AOT/Tween-85-mixed reversed micelle systems (Yamada *et al*., 1994). Span 60 solubilised in the water pool of AOT micelles without any change in micelle shape, while Tween-85 incurred an elongation of the micelle shape. The activity of lipase improved by addition of Tween-85 and lipase activity was maximized when the molar ratio of AOT to Tween- 85 was 3:2–4:1.

2.2.7.6 *Bacillus* lipases

The extracellular lipase of *Bacillus subtilis* 168 was purified from the growth medium of an overproducing strain by ammonium sulfate precipitation followed by phenyl-sepharose and hydroxyapatite column chromatography. The purified lipase had a strong tendency to aggregate. It exhibited a molecular mass of 19 kDa by SDS-PAGE and a pI of 9.9 by chromatofocussing (Lesuisse *et al*., 1993). Schmidt-Dannert *et al*. (1997) purified two novel lipases from the thermophile *Bacillus thermocatenulatus* and found them to be of molecular weights 16 and 43 kDa. The molecular weight of 16 kDa is one of the smallest known for bacterial lipases.

In a laboratory, lipase from *B.stearothermophilus* SB1 capable of growth even at 70^{o} C has been partially purified using the aqueous two-phase system of 70% (w/v) PEG-6000 and 40% (w/v) phosphate and pH 7.0. With increase in hydrophobicity of the system by addition of NaCl, 3% (w/v), more than six fold purification of the enzyme was achieved (Bradoo *et al*., 1999).

2.2.7.7 *Acinetobacter* Lipases

Hong and Chang, (1998) purified a novel alkaline lipase of *Acinetobacter radioresistens* CMC-1 showing a broad range of specificity towards long chain triacylglycerols by ammonium sulfate precipitation (30 – 80% saturation). The excess salts were removed by applying to a PD-10 column. This was followed by anion exchange chromatography with a Mono Q and finally phenyl-Sepharose CL- 4B column chromatography. The lipase yield of 13% and purification fold of 64 were obtained.

Actinobacter nov. spp. strain KM109 produced a lipase which efficiently hydrolyses oleyl benzoate (OB). This lipase was purified to homogeneity with 213-fold purification and 0.8% yield by Sepharose CL-6B, DEAE-Sephaceln 62000SWXL and Mono Q PC chromatography (Mitsuhashi *et al*., 1999). The molecular weight was 62 kDa by SDS-PAGE under denatured–reduced conditions. However, lipase from *A. calcoaceticus* LP009 was purified by ultrafiltration and gel filtration chromatography on Sephadex G-100 by Pratuangdejkul and Dharmsthiti, (2000).

2.2.7.8 *Corynebacterium* Lipases

Lipase produced by *Corynebacterium acnes* was isolated and purified approximately 200-fold from the culture supernatant by ammonium sulphate fractionation and chromatography using Sephadex G-100. The pI of this enzyme was 3.8 (Hassing, 1971).

2.2.7.9 *Propionibacterium* Lipases

A lipase produced by *Propionibacterium* acnes hydrolyses in vivo native sebum triacylglycerols to free fatty acids (Ingham *et al*., 1981). This lipase was purified 4800-fold with a recovery of 9.5% from the crude culture supernatant. The culture supernatant was concentrated by ultrafiltration followed by gel filtration in Sephadex G-100 and ion exchange in CM Sephadex C-50 columns. The molecular weight of the lipase was 46.7 kDa by gel filtration. SDS-PAGE revealed a major protein component (Mr 41.2 kDa) together with two minor protein components (Mr 67 and 125.9 kDa).

2.3 Gene Cloning

In view of wide application of lipases referred to above and, difficulties in purifying their various isozymes due to their overlapping biochemical properties and obtaining these enzymes in bulk, attempts have been made to directly clone genes encoding them. It started with the primary objective to unravel the peptide structure of the enzyme, which is required to understand the molecular mechanism underlying the catalytic reaction and the relationship between the structure and function of lipase. Lipases

gene of number of yeasts have been cloned and expressed, hyper-expressed and bioengineered.

Candida

Lotti *et al*. (1993) reported that lipases of *Candida cylindracea* are encoded by multiple genome sequences, now on the basis of DNA-DNA homology at least seven are presumed to be present. Brocca *et al*. (1995) isolated and cloned 5 different forms of lipase from the species. The five-lipase genes comprising lipase gene family of *Candida rugosa* exhibit 80-88% pair wise identity, in nucleotide sequence. All genes encoding these isozymes were located on the same chromosome, which suggests their origin through gene duplication. Expression of cloned genes of genus *Candida* is demanding due to strong codon bias in *Candida* spp. as universal leucine sequence codon CUG is used for serine (Ohama *et al.*, 1993). CUG is used with high frequency (3% of the codon) in LIPI of *Candida cylindracea* including those corresponding to catalytic site. All the lipase sequences showed distinct pattern for several restriction sites and not minor variation as would be expected in case of alleles.

Sequence analysis performed on 5 fully sequenced genes demonstrated that the highest identity between any two-lipase genes was 88%, a value less than usually observed in allelic variants of the same gene. The available sequences of CRL correspond to isozymes derived from the strain ATCC 14830. The inconsistencies in catalytic properties reported from different laboratories were due to presence of different lipase proteins in *Candida cylindracea* whose composition and ratio may be different in different commercial lipase preparations, possibly depending on the strain and media components used (Vakhlu and Kour, 2006). Presence of multiple isoforms of lipases in *Candida* spp. is well established (Chang *et al*., 1994). Lotti and Alberghina, (2003) indicated that CRL-encoding genes are subjected to regulation at the level of transcription depending on the composition of culture medium and the physiology of culture growth. This complex family of enzymes provides *Candida rugosa* cells with large and versatile pool of catalysts to suit the composition and environment of the culture media. However significance of lipases for the growth of *Candida rugosa* growth is far from clear at present.

2.4 Environmental Pollution and the Edible Oil Industry

The vegetable oil processing industry involves the extraction and processing of oils and fats from vegetable sources. Vegetable oils and fats are principally used for human consumption, but are also used in animal feed, for medicinal purposes, and for certain technical applications. They are extracted from a range of different fruits, seeds, and nuts (MIGA, 1995). The preparations of raw materials include husking, cleaning, crushing, and conditioning. Boiling of fruits, pressing and/or solvent extraction of seeds and nuts is performed to release oil. The extraction processes are generally mechanical or involve the use of solvent such as hexane. The next phase in the processing is (a) skimming of the liquid oil phase if boiling is carried out; (b) filtration of the pressed fat if pressing is applied; and (c) separation of the crude oil. While at the same time evaporating and recovering the solvent where solvent extraction is carried out. Residues are conditioned (such as dried) and reprocessed to produce by products such as animal feed. Crude oil refining includes degumming, neutralization, bleaching, deodorization, and further refining (MIGA, 1995).

2.4.1 Waste Characteristics

With palm fruit, about 2 to 3 cubic meters waste water is generated per metric ton (m^3/t) of crude oil (MIGA, 1995). The waste water is high in organic content resulting in a Biochemical Oxygen Demand (BOD_5) of 20-35,000milligrans per liter (mg/L) and a chemical oxygen demand (COD) of 30 – 60,000mg/L (MIGA, 1995). In addition, the waste waters have high dissolved solids (10,000mg/L), oil and fat residues (5 to 10,000mg/L), organic nitrogen (500 to 800mg/L), and ash residues (4 to 5,000mg/L). Approximately, 10 to 25m^3 of wastewater is generated per metric ton of Product from seed dressing and edible fat and oil processing. Most of the solid wastes (0.7 to 0.8t/t of raw material), which is mostly of vegetable origin, can be processed into by-products or used as fuel (MIGA, 1995).

2.4.2 Pollution Prevention and Control

Good Pollution Prevention Practices in the industry focuses on these areas:

Use of citric acid instead of phosphoric acid where feasible, in degumming operations where appropriate, preference should be given to physical refining rather than chemical refining of crude oil as active clay has a lower environmental impact.

Reduce Product: losses could be reduced by better production control. Furthermore, volatile organic compounds (VOCs) in the air should be maintained below explosive limits. Hexane should be below 150mg/m^3 of air and its explosive limit is 42,000mg/m^3 (MIGA, 1995). While solvent vapors should be recovered for minimizing losses, optimize the use of water and cleaning chemicals should be optimized. There is a need for recirculation of cooling waters and waste product for use in by products such as animal feed should be collected where feasible without exceeding cattle feed quality limits (MIGA, 1995)

Continuous sampling and measuring of key production parameters allow production losses to be identified and reduced, thus reducing the waste load. Odour problems can usually be prevented with good hygiene and storage practices. Chlorinated fluorocarbons should not be used in the refrigeration system (MIGA, 1995).

2.4.3 Pollution Reduction Targets

Since the pollutants generated by the industry are very largely losses in production, improvements in production efficiency are recommended to reduce pollutant loads (MIGA, 1995).

Wastewater loads are typically 3 to 5m^3/t of feedstock, and the plant operators should aim to achieve rates lower than these at intake of effluent treatment system. Hexane, if used, should be below 50mg/L in wastewaters. The BOD_5 level should be less than 2.5kg/t of product with an aim to achieve 1 to 1.5kg/t (MIGA, 1995).

2.4.4 Treatment Technologies

Pretreatment of effluents comprises screening and air flotation to remove fats and solids; it is normally followed by biological treatment. If space is available, land treatment or pond systems are potential treatment methods. Other possible biological treatment systems include trickling filters, rotating biological contactors, and activated sludge treatment (MIGA, 1995).

Pretreated effluents can be discharged to a municipal sewerage system, if capacity exists, with the approval of the relevant authority. Odour control is by ventilation but scrubbing may also be required (MIGA, 1995).

2.4.5 Emissions Requirements

Emission levels for the design and operation of each project must be established through the Environmental Assessment (EA) process, based on country legislation and the pollution prevention and Abatement Handbook as applied to local conditions. The emission levels selected must be justified in the Environmental Assessment and acceptable to Multilateral Investment Guarantee Agency (MIGA).

The following guidelines present emission levels normally acceptable to the World Bank Group in making decisions regarding provision of World Bank Group assistance, including MIGAS guarantees; any deviations from these level must be described in the project documentation. The guidelines are expressed as concentrations to facilitate monitoring. Dilution of air emissions or effluents to achieve these guidelines is unacceptable. All of the maximum levels should be achieved for at least 95% of the time that the plant or unit is operating, to be calculated as a proportion of annual operating hours (MIGA, 1995).

2.4.5.1 Air Emissions

Odour controls should be implemented where necessary to achieve acceptable odour quality for nearby residents. Fabric filters should be used to control dust to below 50mg/Nm^3 from production units (MIGA, 1995).

2.4.5.2 Liquid Effluents

The following effluent levels should be achieved:

Table 2.1 Effluents from Vegetable Oil Processing

Parameter	Maximum Value (mg/L)
pH	6-9
BOD_5	50
COD	250
Total suspended solids	50
Oil and grease	10
Total nitrogen	10
Temperature increase	Less than or equal to 3^0C^1

[1]The effluent should result in a temperature increase of no more than 3 degrees celcius at the edge of the zone where initial mixing and dilution takes place. Where the zone is not defined, use 100 meters from the point of discharge.

(Source: "http:www.miga.org/documents/VegetableOils.pdf").

2.4.6 Monitoring and Reporting

Monitoring of the final effluent for the parameters listed above should be carried out at least weekly or more frequently if the flows vary significantly (MIGA, 1995). Monitoring data should be analyzed and reviewed at regular intervals and compared with the operating standards so that any necessary corrective actions can be taken. Records of monitoring results should be kept in an acceptable format. These should be reported to the responsible authorities and relevant parties, as required, and provided to MIGA if requested.

2.5 Environmental Impact of Edible Oil Pollution

Historically, vegetable oils have been considered relatively benign, non-toxic and therefore of limited concern to the environment. However, this generalization has been demonstrated to be incorrect as previous experience has shown that both chronic and acute pollution incidents can lead to deleterious effects (ITOPF, 2013). This awareness has led to the reclassification of many vegetable oils as category Y (hazardous) products under Annex II of the MARPOL Convention with associated limitations on their carriage.

2.5.1 Potential Environmental Impacts

Vegetable oils are harmful to the environment; like petroleum oils they produce similar environmental effects. If spilled in land, vegetable oils are almost undetectable. They cause devastating physical effects such as coating of bird feathers, gills of aquatic animals, plants sediments, plant stomata and other surfaces with oil and suffocating them by oxygen depletion (the oils cause depletion of oxygen in the water column to a level below what aquatic life needs) (ITOPF, 2013). Experimentation has shown some effects such as reduced growth rates, poor food conversion and liver impairment in fish and bivalves resulting from prolonged ingestion (ITOPF, 2013). They destroy future and existing food supplies, breeding animals, and habitats, and produce rancid odors. They create foul shorelines, clog water treatment plants, catch fire when ignition sources are present, and form products that linger in the waters and environment for many years.

2.5.1.1 Impact on Ecosystem

However, vegetable oils will also readily form solids which tend to have less smothering impact on surface organisms. A polymerised vegetable oil may form an impermeable barrier on the shoreline with potentially serious environmental and economic consequences (ITOPF, 2013). Depending on their pour point (the temperature at which solidification commences) and the sea surface temperature, vegetable oils may form solid lumps when spilled that will float on the water surface. These discrete lumps have little tendency to coalesce as a surface slick. Over time and dependent on the prevailing conditions, the product may accumulate sediment and may sink to the sea floor (ITOPF, 2013).

Vegetable oils are comprised primarily of triacylglycerols, or fatty acids, which, in their fresh state, may be broken down by marine bacteria. This decomposition contributes to the rancid odours typical of vegetable oil spills. Vegetable oils will not readily form water-in-oil emulsions but may undergo a process of polymerization to form rubbery strings and clumps (ITOPF, 2013). These deposits are highly impermeable curtailing oxygen diffusion and replenishment, dramatically slowing the degradation process and forming an anoxic layer. By this process, vegetable oils, particularly with the incorporation of sediment, may give rise to the formation of very tough and highly persistent deposits.

2.5.1.2 Impact on Food Chain, Animal and Man

Environments are made up of complex interrelations between plant and animal species and their physical environment. Harm to the physical environment will often lead to harm for one or more species in a food chain, which may lead to damage for other species further up the food chain. Where an organism spends most of its time – for example in open water, near coastal areas, or on the shoreline – will determine the effects an oil spill is likely to have on that organism (U.S EPA, 2013).

Oil settles on beaches and kills organisms that live there. It also settles on ocean floor and kills benthic (bottom-dwelling) organisms such as crabs. Oil poisons algae, disrupts major food chains and decreases the yield of edible crustaceans. In open water, fish and whales have the ability to swim away from a spill by going deeper in the water or

further out to sea, reducing the likelihood that they will be harmed by even a major spill. Aquatic animals that generally live closer to shore, such as turtles, and dolphins, risk contamination by oil that washes onto beaches or by consuming oil-contaminated prey (U.S EPA, 2013). In shallow waters, oil may harm sea grasses and kelp beds, which are used for food, shelter, and nesting sites by many different species.

Spilled oil and cleanup operations can threaten different types of aquatic habitats, with different results. Coral reefs are important nurseries for shrimp, fish and other animals as well as recreational attractions for divers. Coral reefs and the aquatic organisms that live within and around them are at risk from exposure to the toxic substances within oil as well as smothering. Exposed sandy, gravel, or cobble beaches are usually cleaned by manual techniques. Although oil can soak into sand and gravel, few organisms live full-time in this habitat, so the risk to animal life or the food chain is less than in other habitats, such as tidal flats (U.S EPA, 2013). Sheltered beaches have very little wave action to encourage natural dispersion. If timely cleanup efforts are not begun, oil may remain stranded on these beaches for years.

Tidal flats are broad, low-tide zones, usually containing rich plant, animal, and bird communities. Deposited oil may seep into the muddy bottoms of these flats, creating potentially harmful effects on the ecology of the area (U.S EPA, 2013). Salt marshes are found in sheltered waters in cold and temperate areas. They host a variety of plant, bird and mammal life. Marsh vegetation, especially root systems, is easily damaged by fresh light oils. Mangrove forests are located in tropical regions as in the Niger Delta and are home to a diversity of plant and animal life. Mangrove trees have long roots, called prop roots that stick out well above the water level and help to hold the mangrove tree in place (U.S EPA, 2013). A coating of oil on these prop roots can be fatal to the mangrove tree, and because they grow so slowly, replacing a mangrove tree can take decades.

Marshes and swamps with little water movement are likely to incur more severe impacts than flowing water. In calm water conditions, the affected habitat may take years to restore. Other standing water bodies, such as inland lakes and ponds, are home to a variety of birds, mammals, and fish. The human food chain can be affected by spills in these environments (U.S EPA, 2013). River habitats may be less severely affected by spills than standing water bodies. Spills can affect plants, grasses, and mosses that grow in

the environment. When rivers are used as drinking water sources, oil spills on rivers can pose direct threats to human health. An oil spill can harm birds and mammals in several ways: direct physical contact, destruction of food sources and habitats, and reproductive problems.

Physical contact – When fur or feathers come into contact with oil, they get matted down. This matting causes fur and feathers to lose their insulating properties, placing animals at risk of freezing to death. For birds, the risk of drowning increases, as the complex structure of their feathers that allows them to float or to fly becomes damaged.

Destruction of food resources and habitats – Even species which are not directly in contact with oil can be harmed by a spill. Predators that consume contaminated prey can be exposed to oil through ingestion (U.S EPA, 2013). Because Oil contamination gives fish and other animal unpleasant tastes and smells hence, predators will sometimes refuse to eat their prey and will begin to starve. Sometimes a local population of prey organisms is destroyed, leaving no food resources for predators. Depending on the environmental conditions, the spilled oil may linger in the environment for long periods of time, adding to the detrimental effects. In calm water conditions, oil that interacts with rocks or sediments can remain in the environment indefinitely.

Reproductive problems – oil can be transferred from birds' plumage to the eggs they are hatching. Oil can smother eggs by sealing pores in the eggs and preventing gas exchange (U.S EPA, 2013). Scientists have also observed developmental effects in bird embryos that were exposed to oil (U.S EPA, 2013). Also, the number of breeding animals and that of nesting habitats can be reduced by the spill. Long-term reproductive problems have been shown in some studies in animals that have also been exposed to oil.

2.6 Remediation of Polluted Environment in the Edible Oil Industry

Remediation is considered as the management of the contaminant at a site so as to prevent, minimize or mitigate damage to human health, property, or the environment (Lodolo, 2013). It is a broader term than Clean up in that remediation options can include physical actions such as removal, destruction and containment.

2.6.1 Types of Remediation

Biological Treatment in the Edible Oil Industry

A wide variety of industries (e.g the diary industry and food processing) produce the effluents rich in fats, oils and greases (FOGs). High concentrations of FOGs in wastewater often initiate problems in wastewater treatment processes (Stoll and Gupta, 1997; Becker *et al*., 1999). FOGs often cause foul odours, as well as blockage of pipes and sewer lines. These problems are solved by the preliminary refining equipment, so called grease traps. Grease traps may sometimes fail to retain dissolved and emulsified FOGs, allowing them to enter the water treatment system. An interesting strategy in present time is the use of lipase-producing microorganisms in wastewater treatment system (Bhumibhamon *et al*., 2002).

A further explanation of the solution to oil slicks which concentrates on the separation of water and oil was developed by researchers at the Hebrew University's Faculty of Medicine. The technique relies on the use of liposomes, a by-product of the edible oil industry, to combat oil spills. Because phospholipids have molecules that are hydrophilic (attracted to water) at one end and lipopholic (attracted to oil) at the other, the oil is prevented from automatically spreading into a thin layer on the surface of water but instead breaks up into small droplets which readily stick to solid surfaces (MFA, 2002). The surface of the water can then be cleared using any conventional absorbent material. Another advantage of the phospholipids technique is that phospholipids contain the two ingredients needed to make the oil more susceptible to attack by bacteria: phosphorus and nitrogen; hence, like the Tel Aviv University technique, phospholipids can enhance the efficacy of the use of bacteria to digest oil (MFA, 2002). Soil contamination is notoriously difficult to treat because the contaminants are often tightly bound to the soil particles.

Chemical Remediation

This involves the treatment of any contaminated soil, water or air with a chemical substance which locks up changes or breaks down the toxic compound into a safe form. Remediation by oxidation involves the injection of oxidants such as hydrogen peroxide, ozone, potassium permanganate, persulfates, oxygen gas or plain air (Wikipedia, 2014). Another example is the use of absorbent clays which are mixed, into contaminated soil to absorb pollutants, or incorporated into filters to cleanse water or air passing through. Physical removal, separation, and destruction are processes that reduce or remove the contaminant technologies and which on the other hand, control the migration of a contaminant to sensitive receptors without reducing or removing the contaminant.

Physical Remediation

A number of the technologies have been adapted from general commercial uses in other industrial sectors. These are considered "conventional" technologies. The term "innovative" refers to technologies that have been developed specially for the site remediation industry.

Factors impacting perfect remediation

Many factors affect the selection of potential remediation technologies (Wood, 2003). These include:

- contaminant type and characteristics (Properties, volume, location, exposure risk);
- Site characteristics (soil types, permeability, surface and ground water properties, climate, site infrastructures, topography, location);
- Costs (capital, operating, maintenance);
- Regulatory and public acceptance, and,
- Remediation schedule.-Approaches, consideration and prioritization.

2.7 Biodegradation and its Measurement

Biodegradation is the process by which organic substances are broken down by the enzymes produced by living organisms. Alternatively, it could be taken as the chemical

transformation of a substance caused by organisms or their enzymes. The term is often used in relation to ecology, waste management and environmental remediation (bioremediation). Organic material can be degraded aerobically with oxygen or anaerobically, without oxygen. A term related to biodegradation is biomineralisation, in which organic matter is converted into minerals (Diaz, 2008).

There are two major types of biodegradation: primary biodegradation which refers to the modification of a substance by microorganisms such that a change is caused in some specific measurable property of the substance (US Army Corps of Engineers, 1999). When the term primary biodegradation is used, it refers to minimal transformation that alters the physical characteristics of a compound while leaving the molecule largely intact. Intermediary metabolites produced may, however, be more toxic than the original substrate (wiserenewables.com, 2006). Thus mineralization is the true aim. When this happens it is referred to as ultimate or complete Biodegradation; which is the degradation achieved when a substance is totally utilized by microorganisms resulting in the production of carbon dioxide, methane, water, mineral salts, and new microbial cellular constituents (US Army Corps of Engineers, 1999).

2.8 Biodegradation Characteristics of Vegetable Oils

Lipids (fats, oils and greases) form a major part of domestic and industrial waste; hence they contribute their fair share towards environmental pollution. Sources include waste water from the edible oil refinery, slaughter houses and dairy industry products. These waste products are responsible for clogging sewer networks and unsettling the balance of waste water treatment plants (Saifudin and Chua, 2006).

The first step in the degradation of vegetable-based oils is the enzyme-catalysed cleavage of the ester bond to fatty acid. The enzymes which catalyse this biodegradation reaction include esterases and lipases which are synthesized by a wide range of microorganisms (Broekhuizen *et al*., 2003). Lipases are hydrophobic proteins that catalyze cleavage of carboxyl esterbonds in tri-,di-,and monoacylglycerols (the major constituents of animal, plant and microbial fats and oils) (Saifudin and Chua, 2006). Due to their degradative effects, lipases are applied in remediation efforts to degrade lipid-rich water. Drawbacks, however, include thermal instability of the enzyme and the high cost

of the single use of the enzyme (Saifudin and Chua, 2006). Following the first step of degradation both saturated and unsaturated fatty acids biodegrade via a process of β-oxidation.

Indeed, the biodegradation of materials also is dependent on the nature of the environment, as documented by Kaakinen *et al*. (2007) where pH adjustments of soils for instance was found to have a not incurable effect on the biodegradation of certain compounds (Kaakinen *et al*., 2007). Microbial communities are also prone to adapt to a substrate when it is a regular contaminant which has led to identification and documentation of increases in rates of transformation of hydrocarbons associated with oil-contaminated environments (Khalida *et al*., 2006). Modified strains of bacteria emerge which are characterized by the ability to degrade the substances which induce the modification (Abrashev *et al*., 2002; Mansee *et al*., 2004). However, biodegradability primarily is a function of the chemical nature of the substrate in question.

That natural and vegetable oils are biodegradable is not in doubt as documents show that "vegetable oils and synthetic esters have a much better biodegradation capacity than mineral oil under aerobic as well as anaerobic conditions" (Broekhuizen *et al*., 2003). Tests carried out severally indicate that vegetable oils undergo about 70 – 100% biodegradation in a period of 28 days (Aluyor *et al*., 2009).

In a specific comparative study carried out by Mecurio *et al.* (2003), vegetable – derived lubricants were established to be more biodegradable than comparable mineral – derived lubricants in the presence of tropical mangrove or coral reef microbial communities (Mecurio *et al*., 2003); while some others have examined the biodegradation of vegetable oils under spill conditions (Pereira *et al*., 1998; AI-Darbi *et al*., 2005; Campo *et al*., 2007). Several authors have examined the biodegradability and oxidative stability of industrial fluids obtained from vegetable oils. These include; methyl esters (Demirba, 2009); hydraulic fluids (Petlyuk *et al*., 2004; Abdalla and Patel, 2006) and lubricant (Fernando and Hanna, 2002).

However, though they are biodegradable, on-going research suggests that vegetable oil spills are becoming more common and are potentially more dangerous than hydrocarbon spills because of its toxicity. Records show that the toxicity of products such as canola oil and soybean oil actually increases significantly during aerobic

biodegradation (ITOPF, 2013). The effects of such a process in a confined, shallow environment could be significant (ITOPF, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

3.1.1 Soil Samples

Soil samples were collected from the Nursery Section of the Department of Botany, University of Ibadan. Samples were also collected from a vegetable oil mills factory located: Challenge Area, of 7^{o}21"N,3^{o}53"E Ibadan, during the raining season. Samples were taken using a soil Auger at various locations on site at a depth of about 10cm to ensure a broad spectrum of naturally -occurring microorganisms.

3.1.2 Oil Samples

Eight different types of edible oil palm kernel, vegetable (king's), soya, olive, palm, sunflower, cottonseed and groundnut, were purchased at different retail markets in Ibadan metropolis.

Hundred millilitres and 200mls of each of the oil samples were mixed thoroughly with 2kg of the soil sample collected from the Plant Nursery of the Department of Botany. Two kilogram of the uncontaminated soil was collected also at the same site. These various samples were placed in bowls for 2months.

3.2 Isolation and Culture Methods

3.2.1 Isolation of Organisms

Ten grams of each soil sample collected from the different bowls and control bowls (uncontaminated soil) and that from an oil mill were weighed into 90ml of steriledistilled water in a 250ml conical flask each. These were shaken intermittently for a period of 30minutes to dislodge organisms adhering to the soil particles. One milliliter of the solution was aseptically taken from the stock solution using sterile pipette into a tube

containing 9ml of sterile distilled water to make a 10^{-1} diluent factor. This was also mixed thoroughly and the process was repeated until 10^{-8} diluent factor was reached (Harrigan and McCance, 1976). One millilitre inoculum was aseptically transferred into a sterile Petri dish and pour-plated with the appropriate agar medium: Nutrient agar for bacterial isolation and potato dextrose agar for fungi isolation. The plates were incubated at room temperature ($27^{O}C \pm 2^{O}C$) for 24hours for bacterial isolates. Fungal isolates were also incubated at the same temperature for 3 days.

3.2.2 Maintenance of Pure Cultures

The pure cultures of the organisms were maintained on nutrient agar slants and potatoes dextrose agar slants for bacterial and fungal isolates respectively and preserved at $4^{o}C$.

3.3 Identification of Bacterial and Fungal Isolates

Bacterial isolates were identified using morphological procedures and biochemical tests. The isolated fungi were identified according to their micro-morphology, as well as colour and morphology of the sporulating structures. Glass slides preparations were done using lactophenol blue (Harrigan and McCance, 1976). Microscopic examination of the prepared slide was done using low power objective, followed by the 40X magnification objective lens. Yeast was also identified by API 20C Aux kit (Biomerieux SA).

3.3.1 Morphological Identification

The colonial morphology characteristics of size, shape, colour, elevation, surface, edge of the bacterial isolates on the plates were observed. Smears were prepared and stained for Gram's reaction and spore morphology which were then examined under the microscope for cellular and spore morphological characteristics.

3.3.1.1 Gram's Staining Technique

Gram's staining procedure was carried out. A thin smear of the bacterial isolate was made on a clear glass slide from 18 to 24 hours culture and heat-fixed by flaming. Two drops of crystal violet were added to the smear for 60 seconds after which it was washed

with tap water and Gram's iodine was added for another 60 seconds. The stain was then decolourised by flooding the slide with 95% alcohol until no more violet colouration was observed. (This lasted for 5 to 10 seconds). Two drops of safranin reagent was then added for 30 seconds to counter stain. It was rinsed again with tap water and allowed to air dry. The stained isolates were examined microscopically under the oil immersion objective to determine their Gram's reaction and cellular characteristics and also the different shapes of the bacteria cells. Gram positive cells appeared purple while Gram negative cells were red or pink.

3.3.1.2 Spore Staining

A heat-fixed smear of each bacterial isolate was prepared from 18 to 24 hours old culture. Few drops of malachite green solution were added and steam over a spirit lamp until the stain boils. The slide was allowed to cool down for some minutes and then rinsed off the stain with tap water. The slide was blot dry and examined under the microscope using the oil-immersion objectives. Spores were stained green while bacteria cells were colourless.

3.3.2 Biochemical Characterisation

Pure cultures of bacterial isolates were subjected to various biochemical tests to determine their biochemical characteristics for identity. The result of each test was recorded and the probable identity of the isolate determined using Bergey's Manual of Systematic Bacteriology (Sneath, 1986).

3.3.2.1 Gelatin Hydrolysis

Gelatin is a protein, which can be metabolised only by microorganisms capable of producing proteolytic enzymes that break it down. When broken down, gelatin loses its gelling qualities. Precisely, 15% of gelatin was prepared and mixed with 100ml nutrient broth. The mixture was dissolved in hot water bath with constant swirling. The homogenous solution was then dispensed into test tubes and sterilised; loopful of the isolate was inoculated into the cooled medium and incubated at 30°C for 7days.

Gelatinase production was detected by transferring the tubes into the freezer for 15 minutes. Tubes in which the broth remained liquefied and turned fluidy were recorded as positive. This showed gelatinase production by the isolate (Harrigan and McCance, 1976).

3.3.2.2 Indole Test

A loopful of a broth culture of bacteria isolates was inoculated into a test tube of tryptone water medium and incubated at 37°C for 5 to 7 days after which 0.5ml of Kovac's reagent (Appendix 1) was added to the tryptone water culture. A deep red colour developed in the presence of indole, which separates out in the alcohol layer within 10 minutes while no colour change indicated a negative reaction (Olutiola, 1991).

3.3.2.3 Nitrate Reduction

Nitrate peptone water consisting of peptone water and 0.1% potassium nitrate was used. 5mls portions of the medium were distributed into each test tube, containing inverted Durham tubes. The tubes were sterilised in an autoclave at 121°C for 15 minutes and allowed to cool before inoculation with isolates. Uninoculated tube served as control. All the tubes were incubated at 30°C for 5 days. The ability of the isolates to reduce nitrate to nitrites, ammonia or free nitrogen was determined by adding to each tube 0.5ml of 1% sulphanillic acid in 5N acetic acid, followed by 0.5ml of 0.6% dimethyl-naphthylanine in 5N acetic acid. The development of a red colouration indicated a positive result and the presence of gas in the Durham tube indicated the production of nitrogen gas. Absence of a colour change indicated negative reaction (Olutiola, 1991).

3.3.2.4 Urease Activity

This test was carried out to test the ability of the bacteria isolates to produce urease, an enzyme, which breaks down urea to release ammonia. After preparation of urea medium (Harrigan and McCance, 1976), it was distributed into McCartney bottles and sterilised at 121°C for 15 minutes. The bottles were allowed to cool to 45 - 50°C. Urea was then added to the basal medium to give a final concentration of 2% urea. The bottles were slanted and after cooking a little, the culture of isolates was streaked over the surface of the urea agar medium and incubated at 37°C for 7 days. If urease is produced it

hydrolyses urea to release ammonia resulting in an increase in the pH of the medium, which caused the colour of the medium to change from yellow to pink or red.

3.3.2.5 Coagulase Test

This was carried out to test the ability of the isolate to produce coagulase enzyme. A slide was marked with two sections, a loopful of normal saline (0.85% NaCl in aqueous solution) was placed in each section and a small amount of 18 to 24 hours agar culture was suspended in each drop until a homogeneous suspension was obtained. A drop of human plasma was added to one of the suspension and stirred for 5 seconds. The other was left as control. A positive result shows clumping, which will not re-suspend (Olutiola, 1991).

3.3.2.6 Catalase Test

This was carried out to test the ability of the bacteria isolate to decompose hydrogen peroxide by the production of catalase enzyme. A thick emulsion of isolates from a-24 hour old culture was made on a clean slide. A drop or two of freshly prepared 3% (v/v) hydrogen peroxide was added. The positive reaction was indicated by the production of gas bubbles while the absence of bubble indicated a negative result (Cruikshank *et al*., 1980).

3.3.2.7 Oxidase Test

Two to three drops of 1% tetramethyl paraphenylene di-amine hydrochloride were applied on a filter paper to moisten it. A smear of each bacterial isolate was made on the moistened filter paper by means of sterile inoculating loop. A positive result was recorded when the moistened filter paper turns purple after smearing within 10 seconds, while delayed reaction without purple colouration was regarded as negative (Steel, 1961).

3.3.2.8 Starch Hydrolysis

Starch agar was prepared by adding Ig of soluble starch to 100ml of nutrient agar. The mixture was homogenised and sterilised at 121°C for 15 minutes. This was then dispensed into sterile plates and allowed to set. A single streak of culture was made on the plate and incubated at 37°C for 48 hours. After incubation, the plates were flooded with Gram's iodine. A positive result was indicated by retention of the iodine colour as a clear zone

around the growth region, indicating starch hydrolysis while unhydrolised starch formed a blue-black colouration with iodine (Harrigan and McCance, 1976).

3.3.2.9 Motility Test

The agar stab method was employed. Twenty millilitres of nutrient agar was dispensed into McCartney bottles, and sterilised. After sterilisation the agar was allowed to solidify. Using a sterile inoculating needle, a colony from each of the isolates was used to make a single stab in the bottle. These were incubated at 37°C for 48 hours and the growth pattern was observed. Positive organisms were found growing away from and around the initial stab (Harrigan and McCance).

3.3.2.10 Methyl Red Test

The composition of the medium used was

Dextrose	0.5g
KH_2PO_4	0.5g
Peptone	0.5g
Distilled water	100ml

All the above components were dissolved and dispensed accordingly into McCartney bottles and autoclaved at 121°C for 15 minutes.

After 48 hours of incubation, a few drops of 10% solution of methyl red was added to the isolates. A red colour indicated a positive result while yellow colouration meant a negative result (Olutiola, 1991).

3.3.2.11 Casein Hydrolysis

This was carried out to test the ability of the isolates to hydrolyze milk protein into more soluble and transparent derivatives. Skimmed milk (1%) was added to nutrient agar and sterilised. The pour plate method was then used to inoculate the organisms on the skimmed milk nutrient agar plate. The plates were incubated at 30°C for 72 hours. Uninoculated tubes served as control. A positive result was indicated by growth of the

organisms on the Nutrient agar plates, which showed that the organisms were able to utilise or hydrolyse casein. Negative results indicated no growth (Harrigan and McCance, 1976).

3.3.2.12 Sugar Fermentation

Sugar fermentation medium (Cruickshank *et al*., 1980) was dispensed into test tubes containing an inverted Durham tube each. These were autoclaved at 115°C for 10 minutes. The tubes were allowed to cool after sterilisation and bacterial isolates were inoculated into the tubes. These were incubated at 37°C for three days. Uninoculated tube served as the control. The tubes were examined daily for change in reaction after which acid and gas (when produced) caused the phenol red to turn to yellow while gas evolution was indicated by displacement of solution in Durham tube.

3.3.2.13 Hydrogen Sulphide Production

Nutrient agar plates containing 0.2g lead acetate and 0.08 sodium thiosulphate were prepared and sterilised. A- 24 hour old culture was then inoculated into it and incubated at 30°C for 48 hours. Blackening along the line of streak of the organism on the agar plate showed a positive result. A negative reaction showed no black colouration. This suggests that the organism produce hydrogen sulphide gas from the nutrient agar (Harrigan and McCance, 1976).

3.3.2.14 Citrate Utilization

Simmon's citrate medium (see Appendix 1) was prepared and dispersed into screw capped bottles and sterilised. The content of each bottle were allowed to cool and the test organism inoculated into it. It was then incubated at 30°C for 48 hours after which the colour change was observed. A positive result was shown by a change in colour of the medium from green to blue while a negative result was indicated by a change in colour (Harrigan and McCance, 1976).

3.3.2.15 Growth at Different Temperatures

Nutrient broth was prepared into screw capped bottles and sterilised at 121°C for 15 minutes. The test organisms were inoculated into the bottles after cooling and incubated at different temperatures.

3.4 Preliminary Screening for Lipolytic Activity

Lipolytic activities of the test isolates were initially screened for on olive oil agar medium. The screening was carried out by a modified method of Gogoi *et al*. (2003). The medium consisted (g/L) of KH_2PO_4, 7.584; K_2HPO_4, 0.80; $MgSO_4.7H_2O$, 0.80; $CaCl_2$, 0.16; $(NH_4)_2NO_3$, 0.80; $FeSO_4$, 0.16; Agar, 20; Olive oil 2%, pH was maintained at 7.0, 1ml of the inoculum of each isolate was introduced into10ml of the sterilised medium in an Erlenmeyer flask, incubation was at room temperature ($27^oC \pm 2^oC$) for 7days. After incubation the olive oil agar plates were observed for visible growth, signifying hydrolysis.

In another instance, the isolates were further confirmed for lipolytic activity using a modified method of Sierra (1957). The medium containing, 0.1g $CaCl_2$, 5g NaCl all weighed and dissolved in sequence by addition of 800ml of water and the pH adjusted to 7.5 with NaOH. Thereafter, 15g of agar was added. The volume was made up to 1 liter. The mixture was sterilized by autoclaving for 15minutes at 121^oC. 1% (v/v) high refined olive oil purified by membrane filtration and 5g aniline blue dissolve in distilled water and filter-sterilized. Whitish halos around lipase -producing colonies were observed on the agar plates.

3.5 Growth studies and Production of the Enzymes

3.5.1 Growth Media

Isolates were grown in a complex basal medium whose composition was a modification of the medium of Tsujisaka *et al*. (1973) with glucose omitted. This medium contained 5% peptone, 0.1% $NaNo_3$ and 0.1% $MgSO_4$, adjusted to the desired pH, before sterilisation. Sterile olive oil (Goya) was added as carbon source.

3.5.2 Lipase Production

3.5.2.1 Preparation of Inoculum

A loopfull of the pure culture of bacteria were grown overnight in nutrient broth, yeast was also grown overnight in potato dextrose broth.

3.5.2.2 Production Procedure

One milliliter from the above preparation was inoculated into 65.0ml of sterile medium in 250ml Erlenmeyer flasks and incubated at room temperature ($27^oC \pm 2^oC$) from 24hrs to many days until the maximum lipase production was recorded. The medium was centrifuged at 30,000 g for 15min using Himac High-speed Refrigerated Centrifuge (Hitachi model CR21GII).The supernatant of the centrifuged culture broth was then decanted leaving the cells at the bottom. The cell-free extract acted as the crude lipase enzyme.

3.5.3 Growth of the Isolates

Growth of the Isolates in the growth medium was examined spectrophotometrically using a Jenway 640 UV/VIS spectrophotometer at 540nm, absorbances were measured against blank (Gojkovic, 2009).

3.5.4 Lipase Assay

Lipase activity was measured by a modification of the assay of Parry *et al*. (1966) using as substrate a 10% Olive oil-gum arabic solution emulsified by sonication for 2mins at 25watts output according to Linfield *et al*. (1985). One milliliter of cell-free fermentation broth prepared by centrifugation as describe above was added to 5ml of emulsion and incubated at room temperature for 1h with rapid stirring. Ethanol was added to stop the reaction and the free fatty acids produced were quantified by titration to pH 9.5 against 0.1N NaOH using a radiometer titration system. Blanks with 1ml of fermentation broth were employed in each experiment. Blanks ran with sterile or actual uninoculated broths were the same within experimental error. Samples were run in duplicate.

A unit of lipase activity was defined as the amount of sodium hydroxide (NaOH) used in the titration to bring the reaction mixture to a pH of 9.5 per min under the defined assay conditions. Alternatively, it is considered as the release of one micromole of free fatty acid (FFA)/min at room temperature.

3.6 Optimisation of Production Conditions

3.6.1 Effect of pH on Lipase Production

This was carried out using a modified method of Tsujisaka *et al*. (1973). Growth medium was prepared in 0.2M phosphate buffer and (0.1M citric acid mixed with 0.2M Na_2HPO_4) citrate phosphate buffer of varying pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5). The lipase activity and the growth in the culture supernatant were determined using appropriate procedure.

3.6.2 Effect of Temperature on Lipase Production

The effect of temperature on lipase activity was determined using the above method. The organism was cultivated in the growth medium at different temperatures, which ranged from 20^{o}C, 27^{o}C, 40^{o}C, 50^{o}C, 60^{o}C to 70^{o}C for 24hours. The lipase activity and growth in the culture supernatant were determined.

3.6.3 Effect of Metal ion on Lipase Production

The growth medium in which the organisms were cultivated had their metal ions varied. $ZnSO_4$, $FeSO_4$, $(NH_4)_2SO_4$, KNO_3, $AgNO_3$, $CaNO_3$, Na_2CO_3 and NaCl were used in equimolar concentration (0.1mM) instead of $MgSO_4$ and $NaNO_3$. The lipase activity and growth in the culture supernatant were then determined.

3.6.4 Effect of Aeration on Lipase Production

After inoculating the organisms into the growth medium, the flasks were continuously shaken at 27^{o}C for 24hours at varied revolutions per minute (80, 100, 120 and 140) using orbital shaker Stuart SSLI. The lipase activity and growth in the culture supernatant were then determined.

3.6.5 Time Course of Lipase Production

The organisms were cultivated in the growth medium for different periods that ranged from 24hours to 72hours. Samples were removed periodically and growth and lipase activity in the culture supernatant were determined.

3.6.6 Effect of Different Substrates on Lipase Production

To determine the suitable substrate (carbon source) for the production of lipase by the organisms, substrates such as; glycerol, soy oil, olive oil and a simple sugar (glucose) were used. They were individually tested by replacing the substrate present in the growth medium at the concentration of 2%. Thereafter, the lipase activities as well as growth in the culture supernatant were determined.

3.6.7 Effect of Different Nitrogen Sources on Lipase Production

The main nitrogen source in the growth medium was replaced by other nitrogen sources such as casein, urea and yeast extract at the same concentration (5 %). The lipase activity and growth in the culture supernatant were then determined.

3.7 Protein Estimation

The protein concentration was determined according to Lowry *et al*. (1951). Five millilitre of Reagent A (Reagent I Plus II) (Appendix 1) was added to 1ml of each culture filtrate in test tubes. The mixtures were incubated at room temperature for 10minutes. Thereafter, 0.5ml of folin reagent (BDH) was added to the mixture and incubated at room temperature for 30minutes. The optical density of each sample was taken at 670nm using Jenway 640 (UV/VIS) spectrophotometer.

3.8 Purification of the Enzymes

3.8.1 Ammonium Sulphate Purification

Based on the optimisation of production conditions, the best bacterium with the highest lipase activity and the yeast were selected for further study. These enzymes were partially purified using ammonium sulphate within zero to 100% saturation (Sharma *et al.*, 2001). The filtrates were treated with 24.3 g, 28.5 g and 15.7 g of ammonium sulphate to 0-40%, 40-80% and 80-100% saturation respectively. The mixture for each batch of percentage saturation was stirred continuously for 15minutes until the ammonium sulphate dissolved. The mixture was kept at 4^{o}C overnight in a refrigerator set at 4^{o}C after which it was centrifuged at 30,000 g for 30minutes at 4^{o}C. The supernatant was treated to the next batch until the final batch. The precipitates for each batch were pooled together and the volume adjusted to the initial volume of the culture filtrate with the appropriate buffer.

3.8.2 Dialysis

The enzyme solutions were further purified by dialysis. The procedure involved introducing the previous precipitate in solution after dissolving in the appropriate buffer phosphate buffer into a Viskin dialysis tube and dialysed extensively against the same buffer at 4^{o}C for 18hours. The clarified extract obtained was then made available for the next application, column chromatographic technique.

3.8.3.1 Dissolution of Sephadex Beads

The preparation of the gel was carried out according to the method of Ammar (1975). Sephadex G-75 was used for parking of the column.10grams of Sephadex G-75 was weighed out into a clean bowl, 50ml of sterile distilled water was added and allowed to dissolve into paste form, 300ml of the appropriate buffer was added and slurry was allowed to swell for 3 days at room temperature.

3.8.3.2 Loading of the Column

This was carried out as mentioned by Ammar (1975). The slurry was mixed together after the third day and dispensed into clean chromatography column with the use of a clean funnel. The Sephadex was allowed to compact in the column with occasional removal of the supernatant (buffer) and refilling of the column with the soaked Sephadex

(the slurry) to the zero point of the column. This was done for 48hours after which enzyme was injected into the column.

3.8.3.3 Fractionation of the Sample

The fractionation was carried out according to Ammar (1975). One millilitre of the enzyme preparation sample was carefully applied to the top of the gel and allowed to pass into the gel by running down the column. The appropriate buffer was also added without disturbing the gel surface and to the reservoir to elute the enzyme. The lipases were eluted with phosphate buffer at a flow rate of 0.5ml/min, and the fractions (10ml) were collected in each case. The eluents from the chromatographic column were also analysed for total protein and lipase activity. The fractions that showed the highest lipase activity were pooled and assayed for protein content. The specific activity of the purified enzyme was compared with that of the crude enzyme, and the purification factor was calculated.

3.8.4 Ion Exchange Chromatography (CM)

The pooled and concentrated active fractions (10ml) obtained after gel filtration were re- run on ion-exchange chromatography CM (1.5 x 15cm) equilibrated with phosphate buffer. The column was washed with two bed volumes of the same buffer and the enzyme was eluted with 0-0.5M NaCl – phosphate in a linear gradient the flow rate was adjusted to 0.5ml/min, and fractions showing lipase activity were pooled and concentrated. The specific activity of the purified enzyme was compared with that of the initial crude enzyme and the purification factor was calculated. The active fractions were stored at $4^{o}C$ until used for polyacrylamide gel electrophoresis and further enzyme characterisation.

3.8.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The enzyme samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE) on vertical electrophoresis kit SE u245 series using a discontinuous gradient gel buffer system as described by (Laemmli, 1970). The following were prepared

a. 30% acrylamide, 0.8% Bis – acrylamide, 30 grams of acrylamide and 0.8g of N, N^1 methylene Bis – acrylamide (BDH) were dissolved in 60ml distilled water in 100ml standard volumetric flask and made up to the mark. The solution was stored at 4^oC.

b. **Stacking gel buffer**

All the components were dissolved in 500ml distilled water in a 1 litre standard volumetric flask. The pH was adjusted to 6.8 with sodium hydroxide solution. The solution was then made up to mark with distilled water and stored at 4^oC.

c. **Running gel buffer**

1.5M Tris, 8mM EDTA, SDS pH 8.8 in 1 litre standard volumetric flask. The pH was adjusted to 8.8 with hydrochloric acid. The solution was then made up to mark with distilled water and stored at 4^oC.

d. **1 % Ammonium persulphate**

1 gram of Ammonium persulphate $(CNH_4)_2S_2O_8$) was dissolved in 1ml distilled water amd used immediately.

e. **Electrode buffer:** Glycine, SDS, Sodium salt EDTA

All the components were dissolved in four litres distilled water with constant stirring. The solution was kept at 4^oC.

f. **Sample buffer**

Six millilitres glycerol mixed with 1.2ml of sodium phosphate buffer pH 7.0, 1.5g of sodium diodecyl sulphate (SDS), 30mg dithiothreitol (DDT) and 10mg bromophenol blue. Distilled water was added to make up to 20ml. The mixture was dispensed into test tubes in aliquots of 1ml washed and stored at 20^oC until needed.

3.8.6 Procedure

The stacking and running gels were prepared. The latter solutions were poured into the gel assembles to a level of about 4cm below the maximum filling level. Distilled water was layered on the gel surfaces using a Pasteur pipette to ensure an even surface and also to avoid evaporation of the gel while the polymerizing gel was allowed to polymerize for 1to2 hours.

The water on the gel was poured off and the stacking gel was poured on to the polymerised gel and a comb (1.5mm thick) was gently inserted to obtain wells. It was allowed to polymerise for one hour before removing the comb. The gel was then clamped to the electrophoresis chamber. The upper and lower chambers were filled with electrode buffer and the bubbles formed were removed with asterile syringe.

The protein samples were prepared by mixing sample buffer (in a dilution of 1:1 v/v) with enzyme samples. The mixtures were then placed in a dry bath fan heated for four minutes at 95^{o}C. Equal volume of samples was applied to the bottom for the sample wells with a Hamilton syringe. Molecular weight standards (Bio rad high and low range) were then applied into wells alongside the samples.

Gel electrophoresis was conducted at 120 volts in a vertical electrophoresis apparatus for about 1.5 hours or until the bromophenol blue dye migrated to the bottom of the gel.

At the end of the electrophoresis, the gel was carefully removed from the glass plates and stained by soaking in the staining solution for 20 minutes with gentle agitation. Excess stain was removed by immersing the gel for one hour in several changes of the destaining solution each lasting for 15 – 20 minutes. The gel was then removed and allowed to dry.

3.9 Characterisation of the Partially-Purified Enzymes

3.9.1 Effect of pH on the Enzymes' Activities

This was carried out using a modified method of Tsuijisaka *et al.* (1973). The reaction medium was prepared in the appropriate buffer of varying pH (5.5, 6.0, 7.0, 7.5, 8.0, 8.5). The lipase activity at the different pH levels was then determined using the assay procedure.

3.9.2 Effect of Temperature on the Enzymes' Activities

The enzyme was cultivated in the reaction medium (adjusted to the desired pH) at varied temperature range for 24hours. The lipase activity for the varied temperature was then determined using the assay procedure.

3.9.3 Effect of Substrate Concentration on the Enzymes' Activities

This was done by varying the concentration of olive oil, using 0.5%, 1.0%, 1.5%, 2.0% and 2.5% (w/v) of the olive oil in the growth medium preparation. The enzyme activity for the enzyme at the different substrate concentration levels was then determined using the assay procedure.

3.9.4 Effect of Metal Ions on the Enzymes' Activities

Effects of the different metal ions were included at various concentrations of 0.05%, 0.1%, 0.15% and 0.2% into the assay medium. The enzyme activity for each was then determined using the assay procedure.

3.9.5 Effect of different Nitrogen Concentration on the Enzymes' Activities

This was carried out by varying the concentration of peptone. 1.0%, 2.0%, 3.0%, 4.0%, 5.0% and 6.0% (w/v) of the peptone was used in the growth medium preparation. The enzyme activity for the enzyme at the different nitrogen concentration levels was then determined using the assay procedure.

3.9.6 Effect of Agitation on the Enzymes' Activities

The enzyme-substrate mixture was incubated in a water bath at different levels of agitation (80, 100, 120, 140, 160, and 180) rpm. The enzyme activity for the enzyme cultivated at the different points was then determined using the assay procedure.

3.9b Enyme Kinetics

Km and Vmax of both enzymes were obtained from line weaver-Burk plot. To obtain K_m and V_{max} of the lipase for the substrate (olive oil), 0.5, 1.0, 1.5, 2.0, 2.5% of olive oil was mixed with 1 ml of the purified lipase respectively, using the standard enzyme assay.

3.10 Degradation of the Olive Oil in the Laboratory by the Test Organisms

This was carried out using a modified method of Gogoi *et al*. (2003). Two forms of the Mineral Salt Medium was tested, one consisting (g/L) of KH_2PO_4, 7.584; K_2HPO_4, 0.80; $MgSO_4.7H_2O$, 0.80; $CaCl_2$, 0.16; $(NH_4)_2NO_3$, 0.80; $FeSO_4$, 0.16; and Olive oil 2%, pH maintained at 7.0. The second one was also a modification of the first medium consisting (g/L) of KH_2PO_4, 7.584; K_2HPO_4, 0.80; $MnSO_4.4H_2O$, 0.80; NaCl, 0.16; $(NH_4)_2NO_3$, 0.80; $Fe_2(SO_4)_3$, 0.08;and Olive oil 2%, pH maintained at 7.0.

Hundred milliliters, each of the two Mineral Salt Medium tested was dispensed into several 250ml Erlenmeyer flasks. The medium in several flasks were autoclaved at 121^oC for 15minutes and allowed to cool. To each of the flasks was added 2ml of olive oil sterilised through membrane pore filtration, as the sole carbon source. The chosen bacterium, grown in nutrient broth overnight was used in pure cultures to inoculate the flasks. The yeast chosen (*Candida parapsilosis) was* grown in potatoe dextrose broth overnight and was also used in pure culture to inoculate the flasks. Non-inoculated medium kept under the same condition served as control. The flasks were duplicated and were incubated at room temperature on a shaken at 180rpm for 25days. The pH of the culture media was determined with the use of a pH meter. The total viable counts were carried out by plating out serial dilutions of the culture on nutrient agar plates for the bacteria and potatoe dextrose agar for the yeast and incubated at (27$^oC \pm 2^oC$).

Residual oil in the culture media was extracted with n-hexane. This was carried out by pouring the content of the flasks into a separating funnel after which 100ml n-hexane was added. After vigorous shaking and re-extracting of the aqueous phase, it was discarded while the organic phase was allowed to evaporate to a constant weight in a Petri dish, measured gravimetrically. Before evaporation, the extract was read on a UV/VIS spectrophotometer at 420nm to obtain an estimate of the olive oil content. A standard graph of oil concentration, plotted against absorbance was used for extrapolating the percent oil content. The residual oil content was further determined by gas chromatography.

3.11 Gas Chromatograph (GC)

Fatty Acid Methyl Ester Analysis

Fatty acid profile- saturated, mono- and polyunsaturated analysis were carried out by following the modified AOAC 965.49 and AOAC 996.06 official methods. 50mg of the oil sample was saponified (esterified) for five minutes at 95°C with 3.4 ml of the 0.5M KOH in dry methanol. The mixture was neutralised by using 0.7M HCL 3ml of the 14% boron triflouride in methanol was added. The mixture was heated for five minutes at temperature 90°C to achieve complete methylation process. The Fatty Acid Methyl Esters were thrice extracted from the mixture with redistilled n-hexane. The content was concentrated to 1ml for gas chromatography analysis and 1 µl was injected into the injection port of GC. The fatty acid methyl esters were separated using HP 6890 Gas chromatography analyzer powered by HP Chemstation Rev. A 09.01 [1206] Software and equipped with a Flame Ionization Detector (FID) and HP INNOWax column (30m X 0.25mm X 0.2µm film thickness). The carrier gas was nitrogen and the oven initial temperature was at 60^{o}C. The first ramping was at 12^{o}C/min for 20 min and maintained for 2 minutes. The second was at 15^{o}C/min for 3 minutes and maintained for 8 minutes. The detector temperature was 320^{o}C, while hydrogen and compressed air pressures were 22 and 35 psi respectively. The carrier gas sweeps the concentrated content through the column; the various components of the content were separated as they progress along the column and reach the end of the column at different times. A detector was used to monitor the outlet stream from the column; thus, the time at which each component reaches the outlet and the amount of that component were determined.

3.12 PHYSICO-CHEMICAL ANALYSIS

The physico-chemical properties of soil samples collected were determined for the following parameters using the procedure in the method of soil analysis (AOAC, 1980).

i. pH in Water

The soil samples were air-dried and 10 g of the samples were placed in beakers. The suspension was stirred several times over a 30 min interval and the pH of the soil in each beaker was thereafter measured with the glass electrode of a pH meter.

ii. Water Activity a_w

Soil sample was air dried for three days, thereafter 50 grammes of the soil was placed in a pre-weighed aluminum tray. The aluminium tray containing the soil sample was carefully placed in an oven and dried at 110^{o}C for 48 hrs. The tray and the oven dried soil were kept thereafter to a dessicator for 1 hr to cool. The weight of tray and the oven dried soil was then taken. The following formula was used to calculate the water activity in the soil.

$$a_w = \frac{\text{Weight of soil before oven drying}}{\text{Weight of oven dried soil}} \text{ X } 100\%$$

iii. Percentage of Organic Carbon:

Soil sample was grounded to pass through 60 mm mesh sieve and 1 g of the sieved samples was measured in duplicates into two 250 ml conical flasks. Ten millilitres of 1N $K_2Cr_2O_7$ solution was pipetted into each flask and swirled gently to disperse the soil. 20 ml of concentrated H_2SO_4 was promptly added directly unto the suspension and gently swirled immediately until the soil and the reagents have mixed. It was thereafter shaken vigorously for one minute. The flask was rotated again and allowed to stand on the bench for 30 minutes. 100 ml of distilled water was subsequently added and 3 to 4 drops of ferroin indicator was added and titrated with 0.5 N $FeSO_4$ solution in the burette. Greenish cast coloration in the solution which changed to dark green, marked the end point of the titration.

The $FeSO_4$ solution was at this point added drop by drop until the colour changed rapidly from blue to red. The blank titration (without soil) was carried out in the same manner to standardize the dichromate. The percentage organic carbon in the soil sample was calculated thus.

$$\text{Percentage of Organic Carbon} = \left(\frac{\text{me. } K_2Cr_2O_7 = \text{me. } FeSO_4 \text{ X } 0.003}{\text{Weight of air-dried soils}} \text{ X } 100 \text{ (F)}\right)$$

Correction factor, F = 1.33

[Where me. = Normality of solution X ml of solution used]

iv. Total Nitrogen

Five grammes of air-dried soil sample was grounded and transferred to a 11 cm^3 Whatman No. 2 filter paper, wrapped and dropped into a 500 ml Kjedahl flask. One heap-full of teaspoon (about 11 g) of the digestion mixture was added into the flask through a long wide stern funnel; 25 ml of concentrated H_2SO_4 was added and swirled gently until sample and acid are thoroughly mixed. The flask was then placed in the Kjeldahl digestion apparatus and the fume aspirator turned on. The mixture was heated at low heat until the organic matter was destroyed, evidenced by light gray or straw colour of the mixture. The heat was turned off, the flask removed and capped immediately. The whole unit was cooled until distilled water can be added without explosive result. Two hundred and fifty millitres distilled water was carefully added and mixed thoroughly. While holding the flask at an angle of 45^o, 75 ml of 40% NaOH was carefully and slowly added down the side of the flask to the bottom without mixing. Three pieces of mossy Zn were added and the flask attached immediately to the distillation unit at the receiving end, with a 500 ml flask containing 25 ml of 4% boric acid (plus indicator) and 250 ml of water. The Kjeldahl flask was gently swirled to mix the content at low heat.

About 200 ml of the mixture was distilled into the receiving flask. The distillate was then titrated with a standard 1M sulphuric acid until the blue colour disappeared. The end point was indicated by the solution turning pink. The volume of the acid required to titrate the blank (set up as a parallel) was subtracted from the amount required to titrate the sample. This gave the amount of nitrogen in the sample.

v. Available Phosphorus:

Five grammes of the soil samples and a scoop of refined charcoal were placed in a 250 ml conical flask. One hundred millitres of the extracted solution containing 0.5 M $NaHCO_3$ solution adjusted to pH of 8.5 with 0.1 N sodium hydroxide was added and shaken for 30 minutes. The soil suspension was then filtered through a No. 40 filter paper and 5 ml of the filtrate was pipetted into a 25 ml conical flask. 5 ml of 0.5 M ammonium molybdate solution was slowly added. The flask was shaken gently to mix the contents and the neck of the flask subsequently washed down, diluting the content simultaneously

with 22 ml of distilled water. 0.1 ml of dilute $SnCl_2$ solution was added and mixed. The transmittance of the solution was measured in the calorimeter at 660 nm, 10 minutes after adding $SnCl_2$ solution giving the value of the available phosphorus in the soil.

vi. Exchangeable Cations (Na and Mg)

Ten grammes of the soil sample was weighed (10 g) into 250 ml conical flask. 1 ml of 1N ammonium acetate was added and the flask stoppered. The suspension was manually shaken intermittently for 30 minutes. The suspension was then filtered through Whatman's No. 1 filter paper. The first 10 to 20 ml of filtrate obtained was discarded and about 25 ml of it obtained subsequently was collected in a 50 ml conical flask. A flame photometer was used to determine the concentrations of Na and Mg cations in "ppm" present in the soil sample. This was calculated as follows:

$$\text{ppm cation in soil} = \frac{\text{ppm cation in solution X vol. of extractant}}{\text{Weight of soil}} \quad \text{(X any further dil)}$$

$$\text{mg/100g of soil} = \frac{\text{ppm cation in soil}}{\text{(10 X mg wt. of cation)}}$$

3.13 BIOREMEDIATION OF VEGETABLE OIL POLLUTED SOIL

The bioremediation experiment for the treatment of vegetable oil polluted soil was carried out for 12 weeks from January to April during the dry/raining season in a nursery shed under natural environmental conditions.

3.13.1 Experimental Design for the Bioremediation Process

The bulk soil from the polluted site was measured out in 500 g each into 12 plastic containers of one litre capacity with perforated bottoms in duplicates. These cups were

then divided into two sets. The first set of six cups (in duplicates) represented the sterile soil while the other represented the non-sterile soil.

Bulk soil from unpolluted sites in the nursery area was also collected into 24 plastic containers of one litre capacity with perforated bottoms in duplicates. These cups were further divided into four sets. The first set of six cups (in duplicates) represented sterile soil purposely polluted with 20 ml each of palm kernel oil ,the second set represented its non-sterile counterpart, while the third set of 6 cups (in duplicates) represented sterile soil also purposely polluted with 20 ml each of palm oil, the fourth set represents its non-sterile counterpart.

All the sterile and non-sterile soils were further divided into four sets (each containing six cups in duplicate) representing the mixed and non-mixed in the sterile and non-sterile soils. Each of the three microbial isolates (PO1, CO1 and POC) was then added to each set of cups containing the polluted soil.

The three microbial isolates were represented as follows:

PO1-*Pseudomonas fluorescens*

CO1-*Candida parapsilosis*

POC-*Pseudomonas fluorescens* plus *Candida parapsilosis*

The experimental set-up thus follows:

2X2X3 design for all the combination of treatments used (Appendix 11).

The treatment options used on the sterile and non-sterile for all the polluted and control soils in this experiment were:

- Addition of microbial inoculum

  Po1, Co1 and Poc

- Tilling

  Mixing and non-mixing

The unpolluted control soil samples were combined in the same way for all the treatment highlighted above.

3.13.2 Inoculum Preparation and Addition

The modified method of Odokuma and Dickson (2003) was used. The two isolates (*Pseudomonas fluorescens* and *Candida parasilopsis*) from the polluted soil were used for this bioremediation experiment. They were re-introduced singly (Po1, Co1) and in combination (Poc) to inoculate the polluted and the control soils for the biodegradation process. Each of the isolates was first cultured in 100 ml Erlenmeyer flasks containing nutrient and potato dextrose broths at room temperature and 100 rpm using orbital shaker Stuart SSLI for 48 hours to increase the inoculum size.

The number of cells per ml was determined with a Marienfeld haemacytometer and standardised to obtain an inculum size of about 10^7 per ml. The culture in each flask was then dispersed in normal saline solution to make 1 litre of cell suspension, following which 20 mls of the cell suspension was inoculated into the soil in each cup. The inoculated soils were then mixed thoroughly to ensure uniform distribution of the added microbial cells.

3.13.3 Tilling

Soils with the "MIXING" treatment option were tilled twice a week throughout the 12 weeks experimental study period to improve aeration of the soil and enhance biodegradation. The soil without MIXING was left undisturbed in the cups throughout the study period.

The sterile and non-sterile polluted as well as control soils with and without tilling were represented as follows:

- Sterile with mixing (MS)
- Sterile without mixing (NMS)
- Non-sterile with mixing (MNS)
- Non-sterile without mixing (NMNS)

3.13.4 Soil Sterilisation

Polluted and unpolluted (control) soil samples were sterilised at 121^{o}C for 15 minutes for three consecutive days in order to exclude all viable microorganisms present. This was done to ensure that only the inoculated bacterial isolate will degrade the edible oil contaminant present in the soil in order to determine their biodegradative ability.

3.13.5 Addition of Water

Twenty millilitres of water was added twice a week to all the soils in the cups to prevent their drying throughout the 12 weeks experimental period.

13.14 Bioremediation

Soil samples were taken bi-weekly for 12 weeks from each bowl of each treatment option by scooping the soil at the top, middle and bottom with a sterile metal spoon so that the sample taken will be a true representation of the treated soil samples. The biodegradation process was determined by the following methods.

13.14.1 Total Viable Count Enumeration

The total viable microbial count of soil sample was determined for the period of 12 weeks in order to enumerate the ability of the microbial isolates present in the polluted soil to utilise the edible component for their growth and metabolic activity.

The pour plate technique was used, incubated at room temperature for 24 and 48 hours. Colonies that developed were counted and recorded. This was repeatedly done for 2nd, 4th, 6th, 8th, 10th and 12th weeks.

13.14.2 Spectrophotometric Analysis

The residual oil present in each soil during the bioremediation experiment was determined according to the method highlighted by Hewitt (2000). 5 g of each soil sample was extracted with 10 ml n-Hexane and allowed to stand for a few minutes. The optical density of the supernatant containing the extracted oil was then determined with a visible

spectrophotometer at 660 nm wavelength. The results obtained were then analysed graphically.

13.14.3 Gravimetric Analysis

The modified method of Nweke and Okpokwasili (2003) was used to determine the residual oil present in the soils during the bioremediation experiment. The residual oil present in the soil after 4, 8 and 12 weeks of bio-treatment was extracted as described above. The n-hexane was used as the extracting solvent at 50^{o}C. The residue obtained was then quantified in grams.

CHAPTER FOUR

RESULTS

4.1 Isolation of Organisms

Thirty-four bacterial isolates were obtained from the soil samples in the bowls contaminated with the different types of edible oil. Thirty fungal isolates and yeasts inclusive were also obtained. From the oil mill, nine bacterial and three fungal isolates were obtained. The bacterial isolates were identified as *Bacillus subtilis*, *Pseudomonas cepacia, Bacillus licheniformis, Pseudomonas fluorescens, Alcaligenes sp* and *Flavobacterium sp*. The three fungal isolates were identified as *Geotrichum candidum, Penicillium atrovenetum* and *Candida parapsilosis*. They are all presented on Tables 4.1 and 4.2

Table 4.3 shows the total heterotrophic count for bacteria and fungi isolated from the bowls contaminated with the different types of edible oil. There was notable significant difference in microbial counts in the different edible oils used to purposely contaminate the soils at both 100ml and 200ml levels. Indeed, the total heterotrophic count for bacteria from palm kernel oil was significantly higher than others. However total heterotrophic count for fungi purposely contaminated with 100ml and 200ml of vegetable and palm oils respectively were significantly higher than others.

Table 4.4 also shows total heterotrophic count for isolates from the oil mill contaminated site. All the bacterial isolates were identified with reference to Bergey's Manual of Systematic Bacteriology Sneath (1986). Table 4.5 shows the characteristics.

Tables 4.5 and 4.6 show the characteristics of the yeast isolates.

Table 4.1: Frequency of occurrence of bacterial isolates obtained from artificially-contaminated and oil mill site soil samples

Isolate Name	Type of soil /Frequency of occurrence	
	Artificially contaminated soil	Contaminated soil from oil mill site
Alcaligenes sp	2 (5.9)	1 (11.1)
Bacillus alvei	4 (11.8)	- -
Bacillus licheniformis	5 (14.7)	1 (11.1)
Bacillus megaterium	1 (2.9)	- -
Bacillus pumilus	1 (2.9)	- -
Bacillus subtilis	3 (8.8)	2 (22.2)
Flavobacterium sp	5 (14.7)	1 (11.1)
Micrococcus luteus	1 (2.9)	- -
Proteus mirabilis	1 (2.9)	- -
Proteus vulgaris	2 (5.9)	- -
Pseudomonas cepacia	2 (5.9)	2 (22.2)
Pseudomonas fluorescens	2 (5.9)	2 (22.2)
Pseudomonas putida	5 (14.7)	- -
Total	34 (100)	9 (100)

Values in parentheses represent percentage of occurrence

Table 4.2: Frequency of occurrence of fungal and yeast isolates from artificially-contaminated and oil mill site soil samples

	Type of soil/Frequency of occurrence	
Isolate	**Artificially-contaminated**	**Contaminated soil from oil mill site**
Aspergillus niger	7 (23.3)	-
Aspergillus terreus	3 (6.7)	-
Candida valida	1 (3.3)	-
Candida parapsilosis	1 (3.3)	1 (33.3)
Geotrichum candidum	-	-
Kluveromyces africanus	1 (3.3)	1 (33.3)
Mucor miehei	2 (6.7)	-
Mucor racemosus	2 (6.7)	-
Penicillium atrovenetum	-	1 (33.3)
Penicillium expansum	2 (6.7)	-
Penicillium funiculosum	1 (3.3)	-
Penicillium herquei	2 (6.7)	-
Rhizopus oryzae	2 (6.7)	-
Saccharomyces cerevisae	1(3.3)	-
Saccharomyces rouxii	3 (10)	-
Streptomyces nitrosporeus	2 (6.7)	-
Total	30(100)	3 (100)

Table 4.3: Heterotrophic bacterial and fungal count in garden soil samples contaminated with different types of edible oil

	Microbial group/volume of edible oil/count (log_{10} cfu/g)			
Sample	Heterotrophic bacterial count		Heterotrophic fungal count	
Description	100ml	200ml	100ml	200ml
Groundnut oil	4.4914^{g}	3.8451^{f}	4.6812^{c}	3.0000^{g}
Olive oil	5.2021^{d}	5.0792^{b}	3.7782^{e}	3.3010^{e}
Soya oil	5.1761^{e}	0.0000^{h}	4.6021^{d}	3.2788^{e}
Palm kernel oil	6.3222^{a}	6.1461^{a}	4.2788^{e}	4.1461^{b}
Palm oil	4.5798^{f}	4.2305^{e}	4.7324^{b}	4.2305^{a}
Cottonseed oil	5.9638^{b}	4.8692^{c}	4.8633^{a}	3.6021^{d}
Vegetable oil	5.3424^{c}	4.3802^{d}	4.8865^{a}	4.0000^{c}
Sunflower oil	5.3424^{c}	2.0000^{g}	3.7782^{e}	3.3010^{e}

Values with different superscripts are significantly different ($P < 0.05$) with respect to Duncan's Multiple Range Test.

Table 4.4: Total heterotrophic count of isolates of soil samples from oil mill contaminated site

Microbial group	Total count (log_{10} cfu/ml)
Bacteria	5.44
Fungi	5.74

Table 4.5: Morphological and biochemical characterisation of bacterial isolates obtained from vegetable oil-contaminated soil samples

Isolate Code	Gram Reaction	Cell Morphology	Catalase	Oxidase	Casein Hydrolysis	Gelatin Hydrolysis	Methyl Red	Nitrate Reduction	VP	Growth		Coagulase	Urease	Growth at pH		SH	Growth in NaCl	Citrate Utilisation	Motility	Indole Test	Glucose	Fructose	Maltose	Lactose	Sucrose	Galactose	Xylose	Arabinose	Raffinose	Rhamnose	Dulcitol	Mannitol	Probable Identity
										60°	30°			3.9	9.2																		
1. OBG	+	R	+	+	+	+	-	+	-	-	-	-	-	(+)	(+)	+	+	-	+	-	+G	-	+	+	+	+	+	+	+	+	-	+	*Bacillus licheniformis*
2. OBG	+	R	+	+	+	+	+	+	-	-	-	-	-	(+)	(+)	+	+	+	+	-	+G	-	-	+	+	-	-	+	+	-	+	*Bacillus subtilis*
3. OBG	+	R	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	+	+	+	+	+	+	(+)	-	(+)	+	*Bacillus pumilus*
4. OBG	-	R	+	-	-	+	-	+	-	-	+	-	-	-	+	-	-	-	+	-	+	+	+	+	+	-	-	(+)	-	-	(+)	-	*Flavobacterium* sp
5. OBG	-	C	+	+	-	-	+	-	+	-	-	-	+	-	+	-	+	-	-	-	+	+	+	-	+	+	+	+	-	+	-	+	*Micrococcus luteus*
6 OOB	+	R	+	+	+	+	-	+	-	-	-	-	-	(+)	(+)	+	+	-	+	-	+G	-	+	+	+	+	+	+	+	+	-	+	*Bacillus licheniformis*
7. OOB	-	R	+	-	-	+	-	+	-	-	+	-	-	-	+	-	-	-	+	-	+	+	+	+	+	-	-	(+)	-	-	(+)	-	*Flavobacterium* sp
8. OOB	-	C	+	+	-	-	+	-	+	-	-	-	+	-	+	-	+	-	-	-	+	+	+	-	+	+	+	+	-	+	-	+	*Micrococcus luteus*
9. OOB	-	R	+	+	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-	-	-	-	(+)	(+)	*Pseudomonas putida*
10. OOB	+	R	+	+	+	+	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+G	-	+	+	+	(+)	-	-	(+)	-	(+)	-	*Bacillus alvei*
11. OOB	-	R	+	+	+	+	+	-	+	-	+	+	-	-	+	-	(+)	+	+	-	+	-	+	-	+	d	(+)	-	+	+	-	+	*Pseudomonas fluorescens*
12. OOB	+	R	+	+	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+	-	+G	+	+	+	+	(+)	+	+	-	-	+	+	*Bacillus megaterium*
13. OOB	-	R	+	+	-	-	+	+	-	+	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-	-	-	-	+	(+)	*Pseudomonas cepacia*
14. OCB	+	R	+	+	+	+	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+G	-	+	+	+	(+)	-	-	(+)	-	(+)	-	*Bacillus alvei*
15. OCB	+	R	+	+	+	+	-	+	-	-	-	-	-	(+)	(+)	+	+	-	+	-	+G	-	+	+	+	+	+	+	+	+	-	+	*Bacillus licheniformis*
16. OCB	-	R	+	+	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-	-	-	-	(+)	(+)	*Pseudomonas putida*
17. OCB	-	R	+	-	-	+	-	+	-	-	+	-	-	-	+	-	-	-	+	-	+	+	+	+	+	-	-	(+)	-	-	(+)	-	*Flavobacterium* sp

Key:
- = (No Growth)
R = Rod shape C= Cocci shape d = delayed G = Gas production VP= Voges Proskeur OBG= Bacteria isolated from groundnut oil - contaminated soil + = Positive OOB = Bacteria isolated from olive oil - contaminated soil OCB = Bacteria isolated from cottonseed oil - contaminated soil (+) = Weakly positive OSB= Bacteria isolated from sunflower oil - contaminated soil OPRB = Bacteria isolated from palm kernel oil - contaminated soil OPB = Bacteria isolated from palm oil - contaminated soil OSOB = Bacteria isolated from soya oil- contaminated soil OVB = Bacteria isolated from vegetable oil- contaminated soil
SH= starch hydrolysis

Isolate Code	Gram Reaction	Cell Morphology	Catalase	Oxidase	Casein Hydrolysis	Gelatin Hydrolysis	Methyl Red	Nitrate Reduction	VP	Growth		Coagulase	Urease	Growth at pH		SH	Growth in NaCl	Citrate Utilisation	Motility	Indole Test	Glucose	Fructose	Maltose	Lactose	Sucrose	Galactose	Xylose	Arabinose	Raffinose	Rhamnose	Dulcitol	Mannitol	Probable Iden
										60°	30°			3.9	9.2																		
18. OSB	-	R	+	-	-	+	-	+	-	-	+	-	-	-	+	-	-	-	+	-	+	+	+	+	+	-	-	(+)	-	-	(+)	-	*Flavobacteri*
19. OSB	-	R	+	+	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-	-	-	-	(+)	(+)	*Pseudomona*
20 OSB	-	R	+	+	-	-	+	+	-	+	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-	-	-	-	+	(+)	*Pseudomona*
21. OSB	+	R	+	+	+	+	-	-	-	-	+	-	-	+	-	+	+	-	+	+	+G	-	+	+	+	(+)	-	-	(+)	-	(+)	-	*Bacillus alve*
22. OPB	+	R	+	+	+	+	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+G	-	+	+	+	(+)	-	-	(+)	-	(+)	-	*Bacillus alve*
23. OPB	-	R	+	-	+	+	+	+	+	-	-	+	+	+	(+)	-	+	+	+	-	+G	+	-	-	(+)	+	+	-	-	-	-	-	*Proteus mira*
24. OPB	+	R	+	+	+	+	+	+	-	-	-	-	-	(+)	(+)	+	+	+	+	-	+G	-	-	+	+	+	-	-	+	+	-	+	*Bacillus subt*
25. OSOB	-	R	+	+	+	+	+	-	+	-	+	+	-	-	+	-	(+)	+	+	-	+	-	+	-	+	d	(+)	-	+	+	-	+	*Pseudomonas*
26. OSOB	-	R	+	+	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-	-	-	-	(+)	(+)	*Pseudomona*
27. OSOB	-	R	+	-	+	+	+	+	+	-	-	+	+	+	(+)	-	+	+	+	+	+G	+	+	-	+	+	+	-	-	-	-	-	*Proteus vulg*
28. OPRB	-	R	+	-	-	+	-	+	-	-	+	-	-	-	+	-	-	-	+	-	+	+	+	+	+	-	-	(+)	-	-	(+)	-	*Flavobacteri*
29. OPRB	-	R	+	-	+	+	+	+	+	-	-	+	+	+	(+)	-	+	+	+	+	+G	+	+	-	+	+	+	-	-	-	-	-	*Proteus vulg*
30. OPRB	-	R	+	+	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-	-	-	-	(+)	(+)	*Pseudomona*
31. OPRB	+	R	+	+	+	+	-	+	-	-	-	-	-	(+)	(+)	+	+	-	+	-	+G	-	+	+	+	+	+	+	+	+	-	+	*Bacillus lich*
32. OVB	+	R	+	+	+	+	-	+	-	-	-	-	-	(+)	(+)	+	+	-	+	-	+G	-	+	+	+	+	+	+	+	+	-	+	*Bacillus lich*
33. OVB	+	R	+	+	+	+	+	+	-	-	-	-	-	(+)	(+)	+	+	+	+	-	+G	-	-	+	+	+	-	-	+	+	-	+	*Bacillus subt*
34. OVB	-	R	+	+	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-	-	-	-	(+)	(+)	*Pseudomona*

Key:

- = (No Growth) R = Rod shape C= Cocci shape d= delayed G = Gas production VP= Voges Proskeur OBG= Bacteria isolated from groundnut oil - contaminated soil + = Positive OOB = Bacteria isolated from olive oil - contaminated soil OCB = Bacteria isolated from cottonseed oil - contaminated soil (+) = Weakly positive OSB =Bacteria isolated from sunflower oil - contaminated OPRB = Bacteria isolated from palm kernel oil - contaminated soil OPB = Bacteria isolated from palm oil - contaminated soil OSOB = Bacteria isolated from soya oil- contaminated soil OVB = Bacteria isolated from vegetable oil- contaminated soil SH= starch hydrolysis

Table 4.6: Biochemical characterisation of the yeast isolates from vegetable oil-contaminated sites

Sample code	Cell morphology	Urease	Glucose	Xylose	Galactose	Lactose	Maltose	Manitol	Melibose	Sucrose	Raffinose	Arabinose	Nitrate assimilation	Growth 20°C	37°C	50°C	Acid production (glucose)	0.1% actidione	Gelatin liquifiation	0.01% actidione	Probable organisms
OCY1	Oval	-	+	+	+	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	*Saccharomyces cerevisiae*
OCY2	Oval	-	+		+	W+	+	-	-	W+	W+	-	+	+	+	-	+	-	-	-	*Saccharomyces rouxii*
OCY3	Oval	-	+	+	-	-	-	-	-	+	-	-	+	+	+	-	+	-	-	+	*Candida valida*
OCY4	Oval	-	+	+	+	-	-	-	-	-	-	W+	+	+	+	-	+	-	-	-	*Kluveromyces africanus*

Key:

- = No growth + = Positive W+ = Weakly positive OCY = Yeast isolated from contaminated site

Table 4.7: Biochemical characteristics of a yeast isolate from vegetable oil-contaminated soil samples using API 20C

AUX

Sample code	O	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	**Probable organisms**
	-	+	+	+	+	+	+	-	+	-	+	+	+	-	-	+	+	+	+	-	*Candida parapsilosis*

Key: O - Aucun, GLU - D-Glucose, GLY – Glycerol, 2KG – Calcium 2-ceto-Gluconate, ARA –L-Arabinose, XYL – D-Xylose, ADO – Adonitol, XLT – Xylitol, GAL – D-Galactose, INO-Inositol, SOR-D-Sorbitol, MDG-Methyl-α D-Glucopyranoside, NAG-N-Acetyl-Glucosamine, CEL-D-Celiobiose, LAC-D-Lactose (origine bovine) MAL-D-Maltose, SAC-D-Saccharose, TRE D-Trehalose, MLZ D-Melezitose, RAF D-Raffinose.

4.2 Fungal Identification

Aspergillus terreus:

Colonies reached 3 cm diameter in ten days, growth was brown velvety on the plates. Conidiophores were smooth walled, hyaline, with hemispherical vesicles; metulae present in conidial heads were strictly columnar; conidia were globose to slightly ellipsoidal and smooth-walled.

Aspergillus niger:

Colonies reached 3 cm diameter in ten days, typically black powdery on plates. Condiophores arising from long, broad, thick-walled was brownish. The foot cells were branched; conidia were in large, radiating heads, mostly globose irregularly and roughened.

***Mucor racemosus*:**

Colonies were smoke grey on plates. Some *sporangiophores* were short, while some others tall but they were hardly differentiated into layers, walls were slightly encrusted, with sympodial and monopodial branches, the latter short, perpendicular and some what recurved. Watts sporangia at first hyaline, later becoming brownish, wall spinulose, rupturing at maturity. Columellae were shown to be ovoid, oval and pale brownish. Sproangiospores were broad-oval and greyish. Chlamydospores were numerous in the sporangiophores (even in the columellae), barrel-shaped to sub globose and slightyly yellowish.

***Penicillium expansum*:**

Colonies fast growing, reaching 5 cm diameter in 14 days. Candiophores in fresh isolates typically loosely synnematous, giving the colony a zonate appearance, light green and reverse colourless to yellow-brown. Candiophores were smooth-walled, pencilli was two-to-three-stage brancced with numerous, usually some what appressed metulae. Phialide tip thin-walled, the first five or so conidia adhering to the phialide were weakly de-limited and hyaline. Conida subglobase, smooth-walled, odour was aromatic, fruity, suggesting apples.

***Penicillium funiculosum*:**

Growth spread broadly on colonies, reached 5.5 cm diameter in 12 days. Sporulating areas yellow-green, the funiculose or tufled habit of the colonies was not well developed and odour was earthy. Yellow vegetable mycelium were present on the colony surface, reverse was pink to deep red. Conidiophore stipes was smooth or nearly so, conidia ellipsoidal to subglobose, apiculate and delicately roughened.

***Penicillium herquei*:**

Phralides had an abruptly tapering neck. The conidiophores were strictly two-stage branched with very dense metulae and a coarsely roughened stipe. Colonies grew rather restrictedly, reaching 3cm diameter in 14 days on the plate. The abundant aerial mycelium was yellow and the conidial areas were yellowish green. Reverse was dark yellowish green. Conidia were shown in more or less tangled chains ellipsoidal, and almost smooth-walled.

***Rhizopus oryzae*:**

Colonies were very fast growing about 1cm high with tendency to collapse, pale or dark brown-grey, stolons hyaline, at their ends usually producing a rhizoid with 4-8 branches and 1-5 sporangiophores; other sporangiophores arising directly from stolons or aerial hyphae. Sporangia wall diffluent, opaque, brown-grey to black. Columella and apophysis together ± glubose, soon collapsing after spore release.

***Penicillium atrovenetum*:**

Colonies grew some what restrictedly, reaching 2 cm diameter in ten days, with bright bluish green, later deep greyish blue conidial areas; reverse intensly yellowish brown, later turned dark green to blackish with the pigment diffusing into the agar plates. Conidiophores were partly rough-walled; penicilli typically with an additional divergent branch; metulae some what divergent.Conidia globose and coarsely warty.

Mucor miehei:

Colony was 1mm in height, deep olive gray; stolons had roughened walls; rhizoids were weakly developed. Sporangiophores were sympodially branched, with slightly encrusted, brownish walls; sporangia were grey, glittering with encrusted walls which rupture at maturity; columellae obovoid to slightly pyriform, brownish to mouse grey with small protusions; collars poorly defined to none. Sporangiospore subglobose, a few ellipsoidal, zygospores (homathallic) formed in the aerial mycelium, globose to slightly compress.

Streptomyces nitrosporeus:

There was rapid spore formation, spore chains typically flexious, formed green to black vegetative growth on the media. The organism was inhibited by Streptomycin. Biochemical characterisation showed that the organism fermented glucose, xylose, arabinose, rhamnose and galactose, but did not ferment fructose, raffinose, mannitol, insitol, salicin and sucrose.

Geotrichum candidum:

Colonies very fast growing, reaching 5.0-0.6 cm diameter in 5 days on agar plates. They are white butyrous and odour often fruity. Their advancing hyphae are dichotomously branched, conidial chains are mostly aerial, erect or decumbent.

4.3 Screening for Lipolytic Activity

Table 4.8 shows the result of the screening experiment for qualitative production of lipase by the selected isolates using olive oil agar medium. Results show that these selected organisms showed lipolytic activity by production of halos around lipase-producing colonies seen on the agar plates.

Table 4.8. Results of the qualitative screening of isolates for lipolytic activity

Isolates	Lipolytic activity
Alcaligenes sp	+
Bacillus licheniformis	+
Bacillus subtilis	+
Candida parapsilosis	+
Flavobacterium sp	+
Pseudomonas cepacia	+
Pseudomonas fluorescens	+

Key: + Positive presence of halos around the colonies

4.4. Optimization of production conditions

Effect of pH on growth and lipase production by the isolates

Figure 4.1 shows the effect of pH on growth of the selected bacteria; at pH 5.5, with the exception of *Pseudomonas cepacia*, most of the bacteria did not grow, whereas majority grew best at pH 7.0-9.0. Figure 4.2 shows the effect of pH on lipase production by these organisms. Lipase production was best in most of the organisms at pH 7.0-8.0. In the case of the yeast studied for the growth and enzyme production, this is as shown in Figure 4.3, maximum growth was achieved at pH 8.5, whereas best enzyme production was recorded at pH 8.0 (0.4 U/ml).

Effect of temperature on growth and lipase production by the selected isolates

As temperature increased from 20^{o}C, growth in majority of the organisms decreased as `shown in Figure 4.4. While the growth of *Pseudomonas fluorescens*, *Pseudomonas cepacia* and *Bacillus subtilis* reached a peak at 27^{o}C, *Flavobacterium sp*. and *Bacillus licheniformis*, had maximal growth at 40^{o}C. In each instance, the peak was followed by a gradual descent to the minimum. In Figure 4.5, majority of the isolates recorded their best enzyme production at 27^{o}C, followed in each case by a sharp decline in production. Figure 4.6 shows that for *Candida parapsilosis*, as temperature increases, growth and lipase yield decrease.

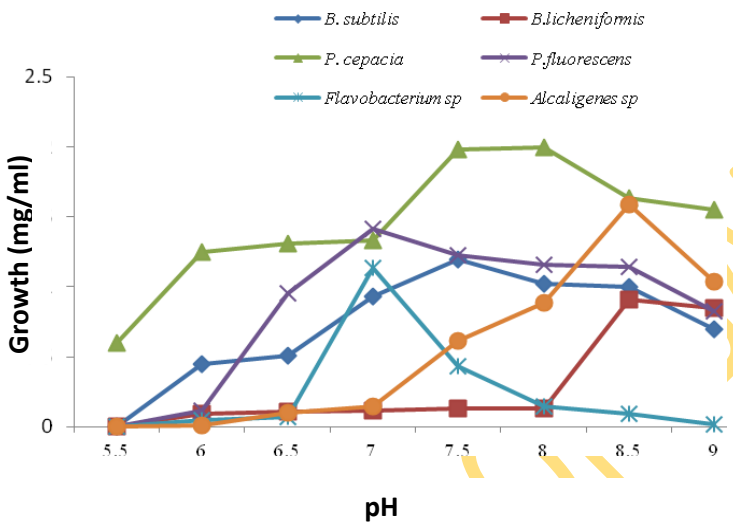

Figure 4.1. Effect of pH on growth of some selected bacterial isolates

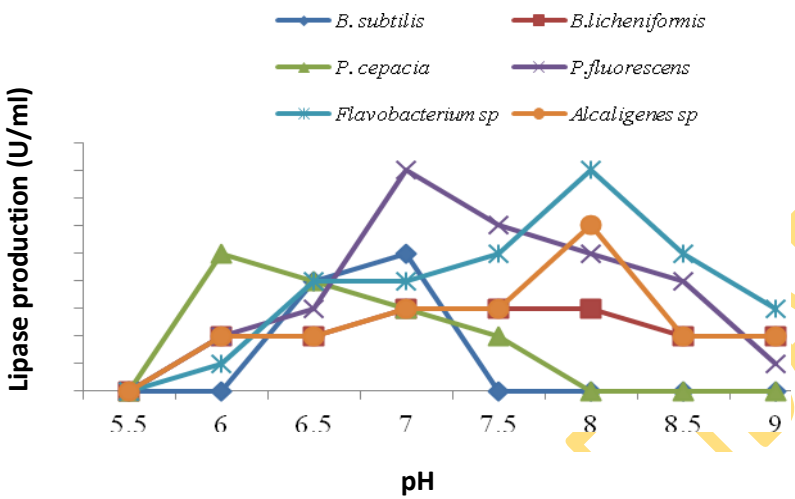

Figure 4.2. Effect of pH on lipase production by some selected bacterial isolates

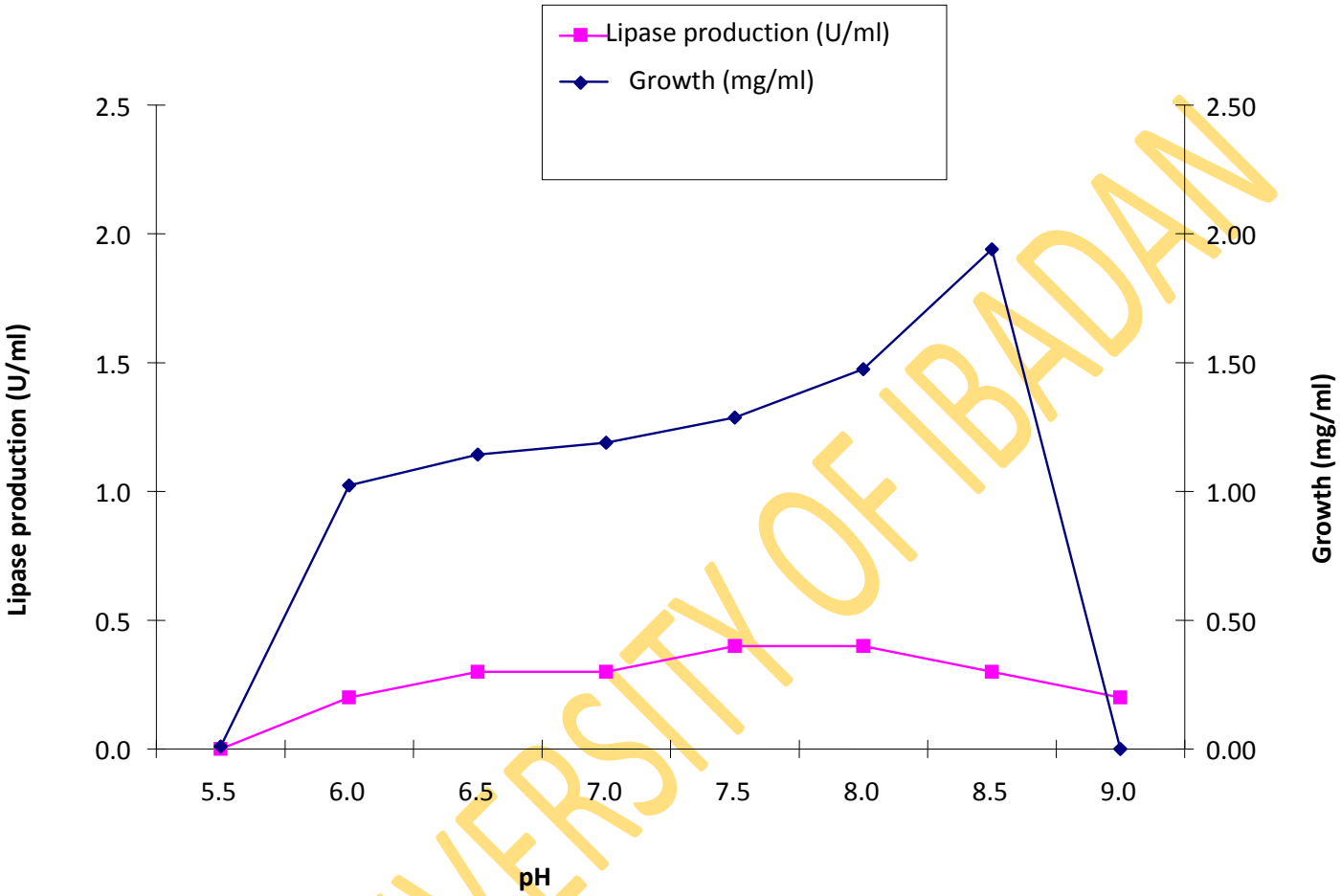

Fig 4.3: Effect of pH on growth and lipase production of *Candida parapsilosis*

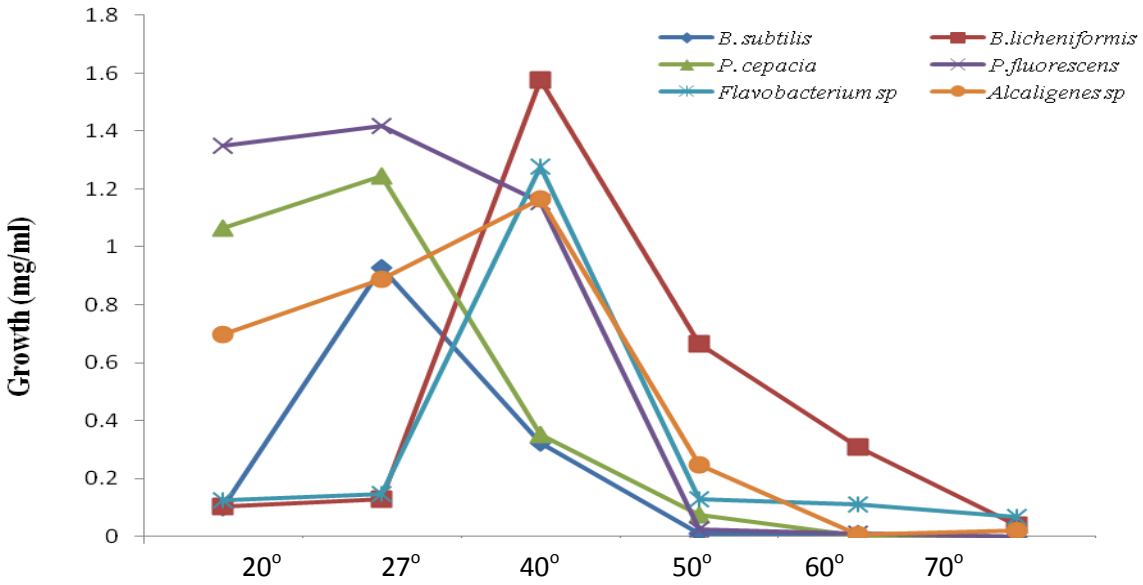

Fig. 4.4: Effect of temperature on growth of some selected bacterial isolates

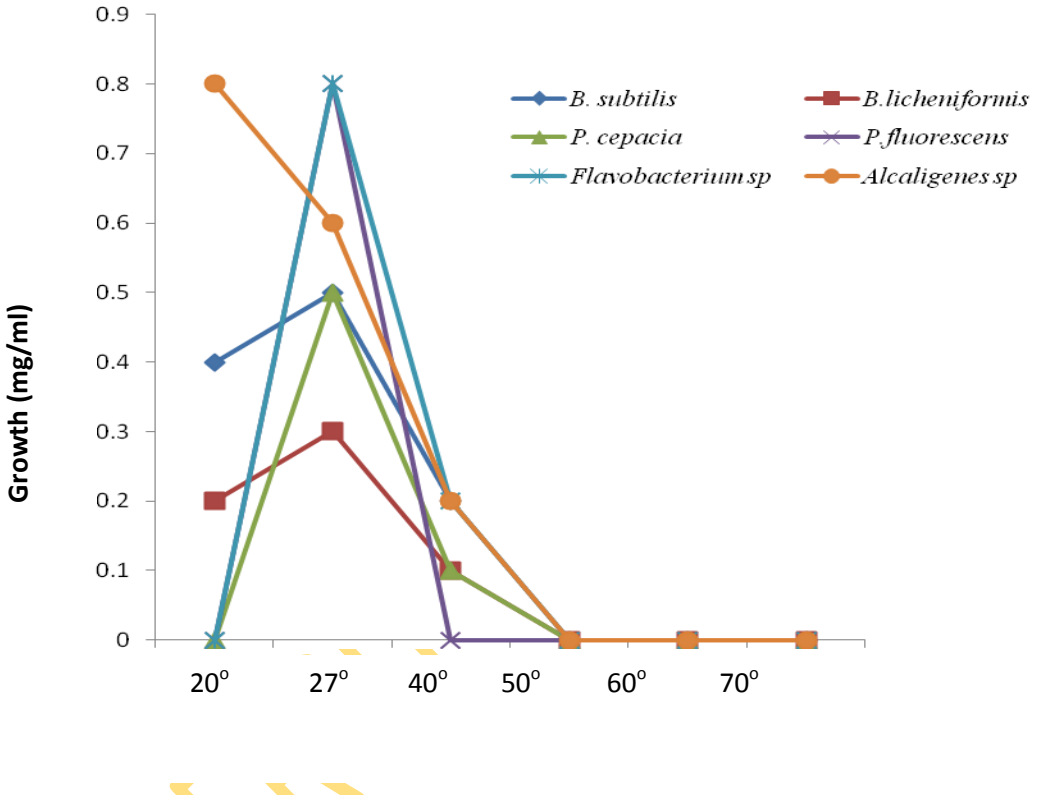

Fig. 4.5: Effect of temperature on lipase production by some selected bacterial isolates

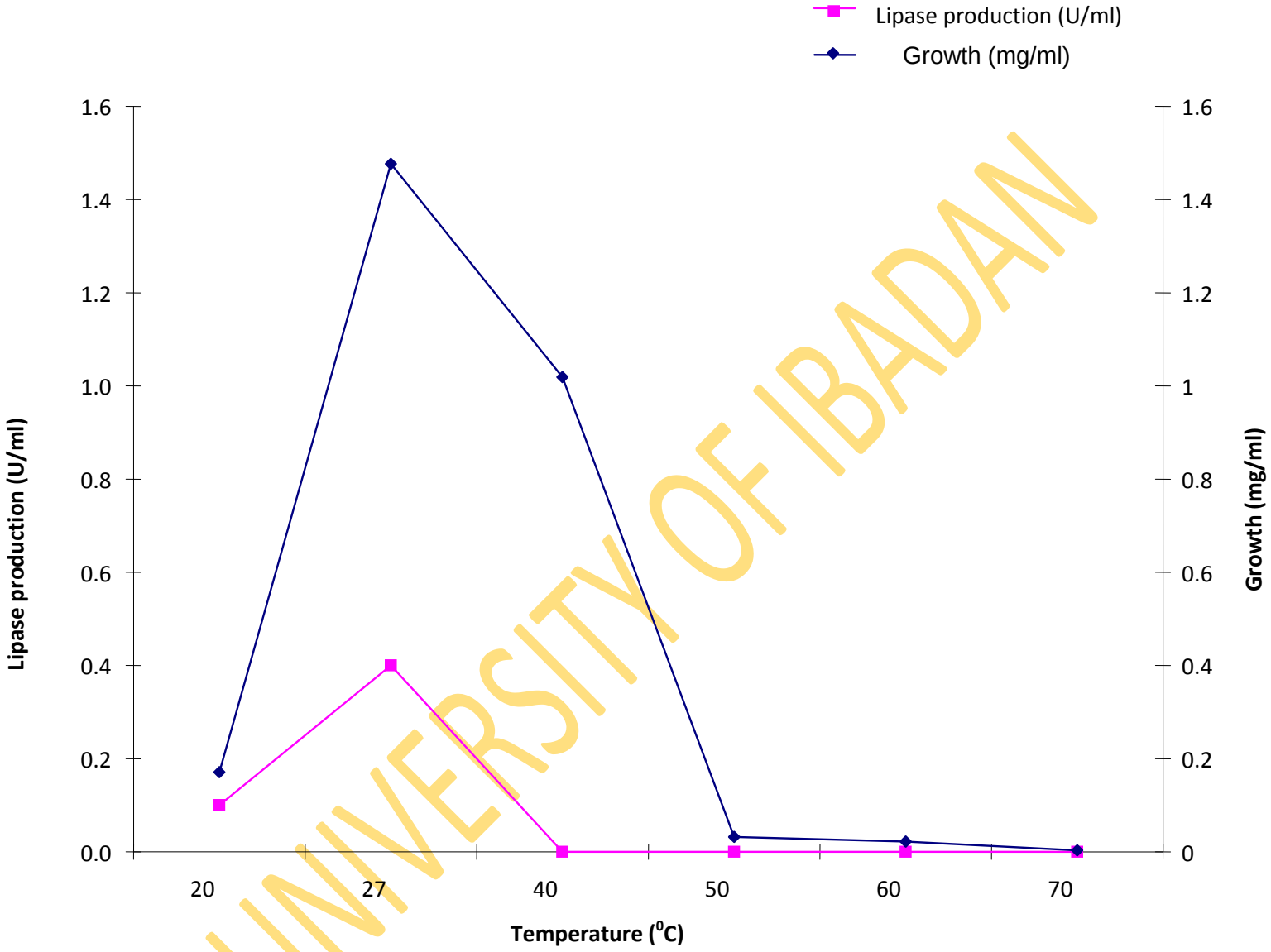

Fig 4.6: Effect of temperature on growth and lipase production by *Candida parapsilosis*

Effect of metal ions on growth and lipase production of isolates

Figure 4.7 shows the effect of cations on growth of the organisms. Growth was not generally supported by $AgN0_3$ in all the organisms selected but supported by $KN0_3$. Figure 4.8 shows that lipase production was inhibited by some cations but $MgS0_4$ generally supported lipase production.

Figure 4.9 shows effect of cations on growth and enzyme activity of *Candida parapsilopsis* both growth and enzyme activity was inhibited by $AgN0_3$, but $(NH_4)_2S0_4$ supported both 1.757 mg/ml and 0.5 U/ml respectively. Figures 4.10 and 4.11 show the effect of anions on growth and enzyme activity of the different organisms. Figure 4.12 shows that lipase production was supported by $NaNO_3$ in all the organisms selected.

Effect of aeration on growth and lipase production of isolates

Growth increased with increase in agitation speed for *Bacillus subtilis*, *Bacillus licheniformis* and *Flavobacterium* sp but in *Pseudomonas cepacia*, *Pseudomonas fluorescens* and *Alcaligens sp.* growth decreased after reaching a peak at 100 rpm. This is as shown in Figure 4.13.

Figure 4.14 shows that in most of the organisms studied, agitation did not support release of lipase production except in *Bacillus licheniformis.* In the case of *Candida parapasilopsis,* growth was supported at increased agitation speed up to 100 rpm thereafter there was decrease in growth, agitation did not support lipase production in *Candida parapsilosis* as shown in Figure 4.15.

Time course of growth and lipase production

Growth was supported best at 48 hours of incubation in majority of the organisms investigated, with the exception of *Bacillus licheniformis* and *Flavobacterium sp.* which grew best at 72 hours of incubation as shown in Figure 4.16. In all the organisms, lipase production was supported best at 24 hours of incubation as seen in Figure 4.17. Growth increased for *Candida parapsilosis* with 1.8 mg/ml but lipase production decreased to 0.0 U/ml at 72 hours of incubation as shown in Figure 4.18.

Effect of different carbon sources on growth and lipase production by the isolates

Figure 4.19 shows the effect of different substrates on growth of organisms while Figure 4.20 shows that of lipase production. While glucose supported growth slightly, lipase production in *Bacillus subtilis*, *Bacillus licheniformis* and *Pseudomonas cepacia* was nil as shown in Figure 4.20. Figure 4.21 shows the effect of the different carbon sources on growth and lipase production of *Candida parapsilopsis* glucose enhanced lipase production (0.5U/ml) whereas it was inhibited by glycerol

Effect of different nitrogen sources on growth and lipase production by the isolates

Figure 4.22 shows the effect of different nitrogen sources on growth of the organisms. Peptone generally supported growth in all the organisms studied. Figure 4.23 shows that of enzyme production, also generally supported by peptone. Figure 4.24 shows the effect of the different nitrogen sources on growth and lipase production by *Candida parapsilosis* Urea did not support growth (0.004 mg/ml) however yeast extract supported growth but not lipase production (0.0 U/ml).

Based on the optimisation of production conditions, two isolates were chosen for further study.

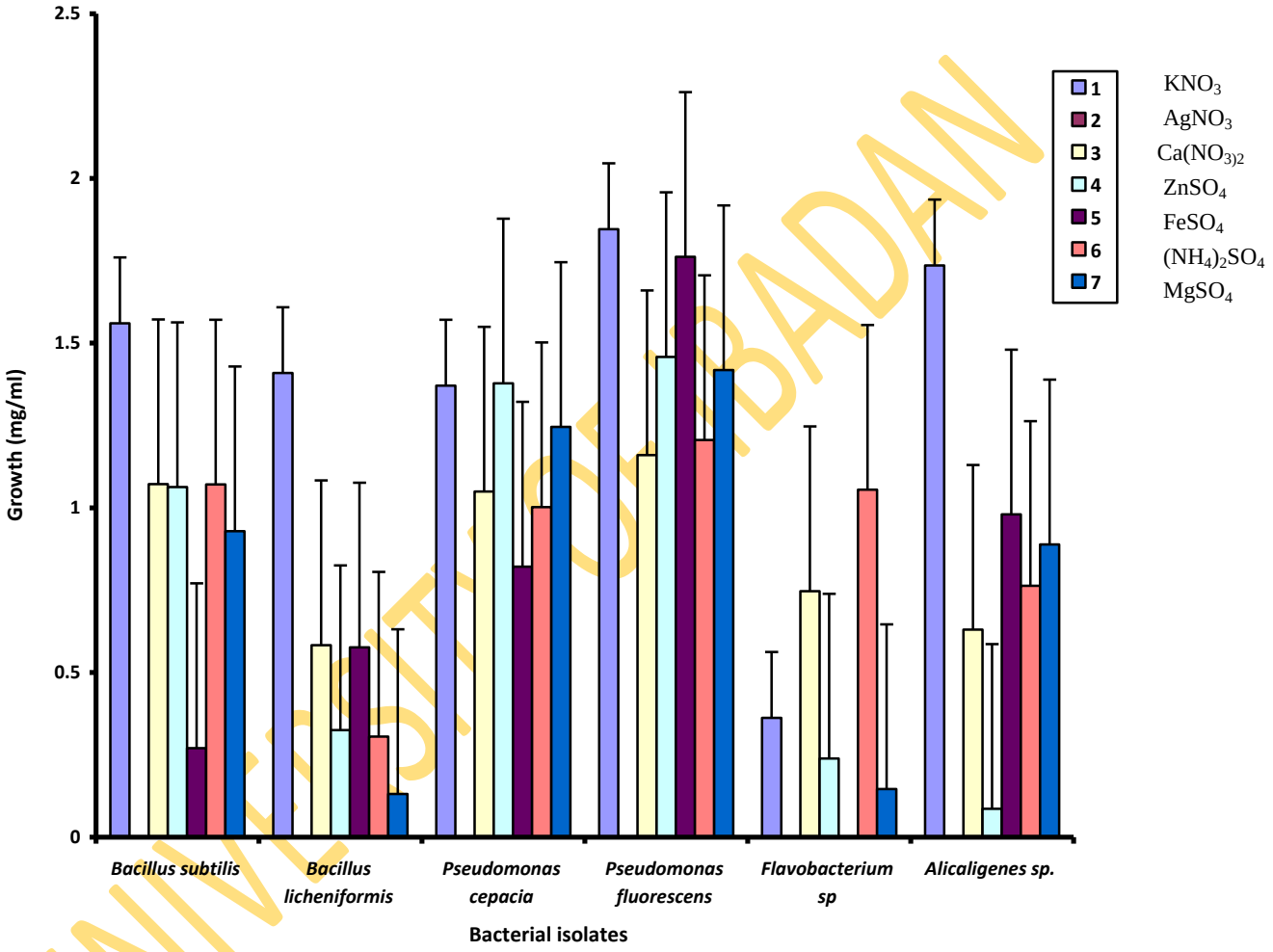

Fig. 4.7: Effect of 0.1% different cations on growth of some selected bacterial isolates

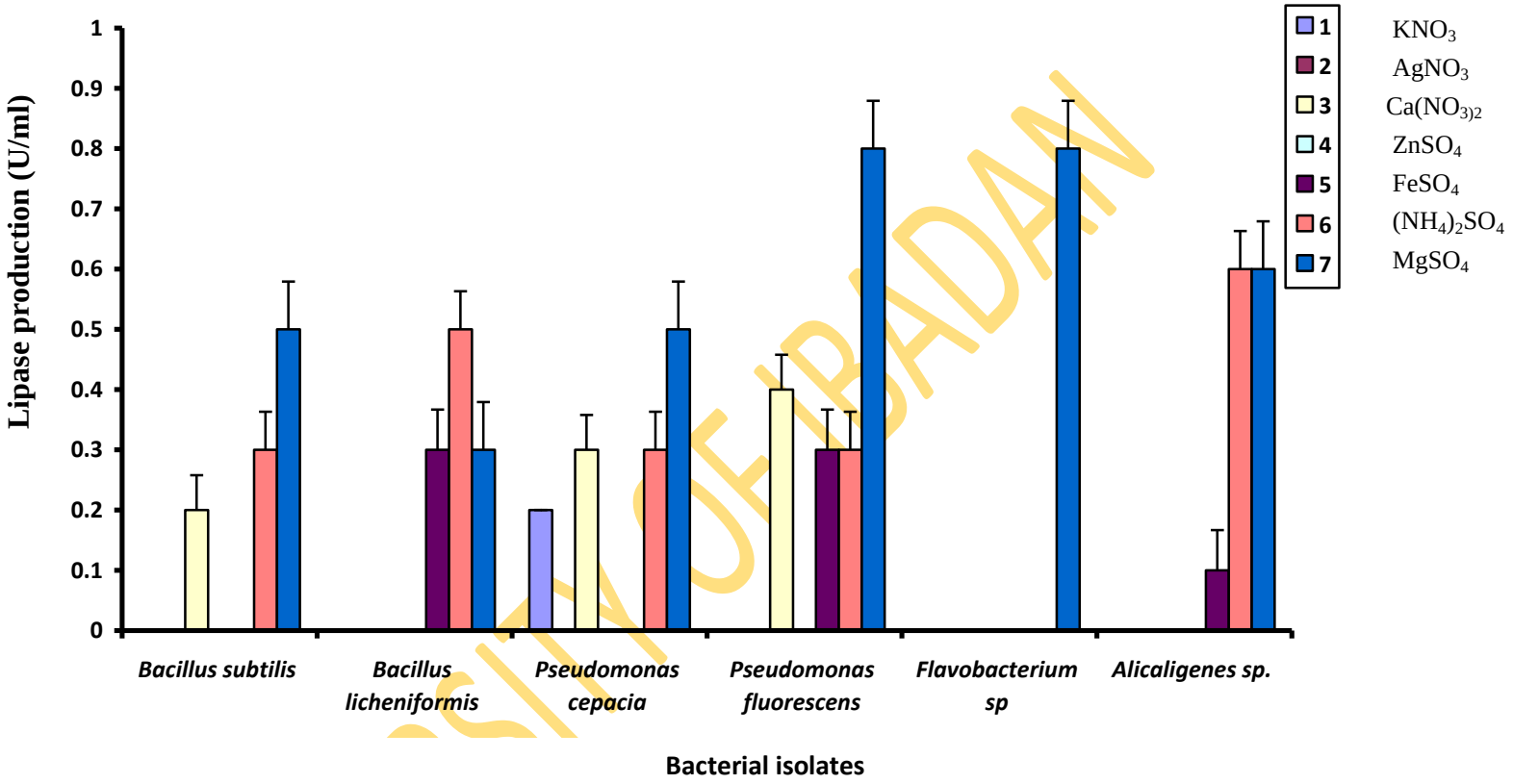

Fig. 4.8: Effect of 0.1% different cations on lipase production by some selected bacterial

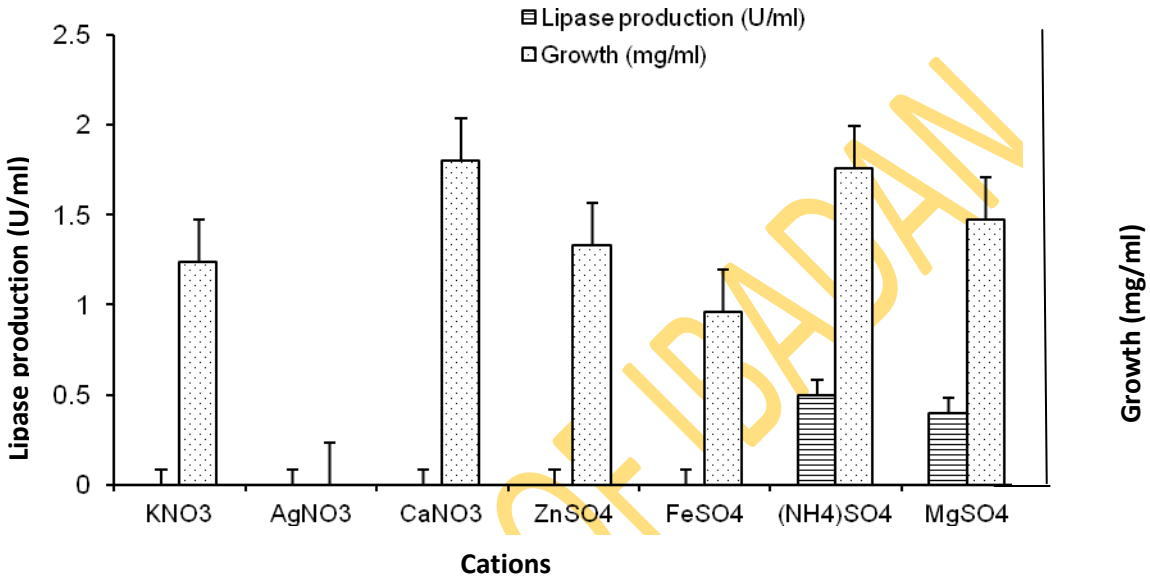

Fig. 4.9: Effect of 0.1% different cations on growth and lipase production by *Candida parapsilosis*

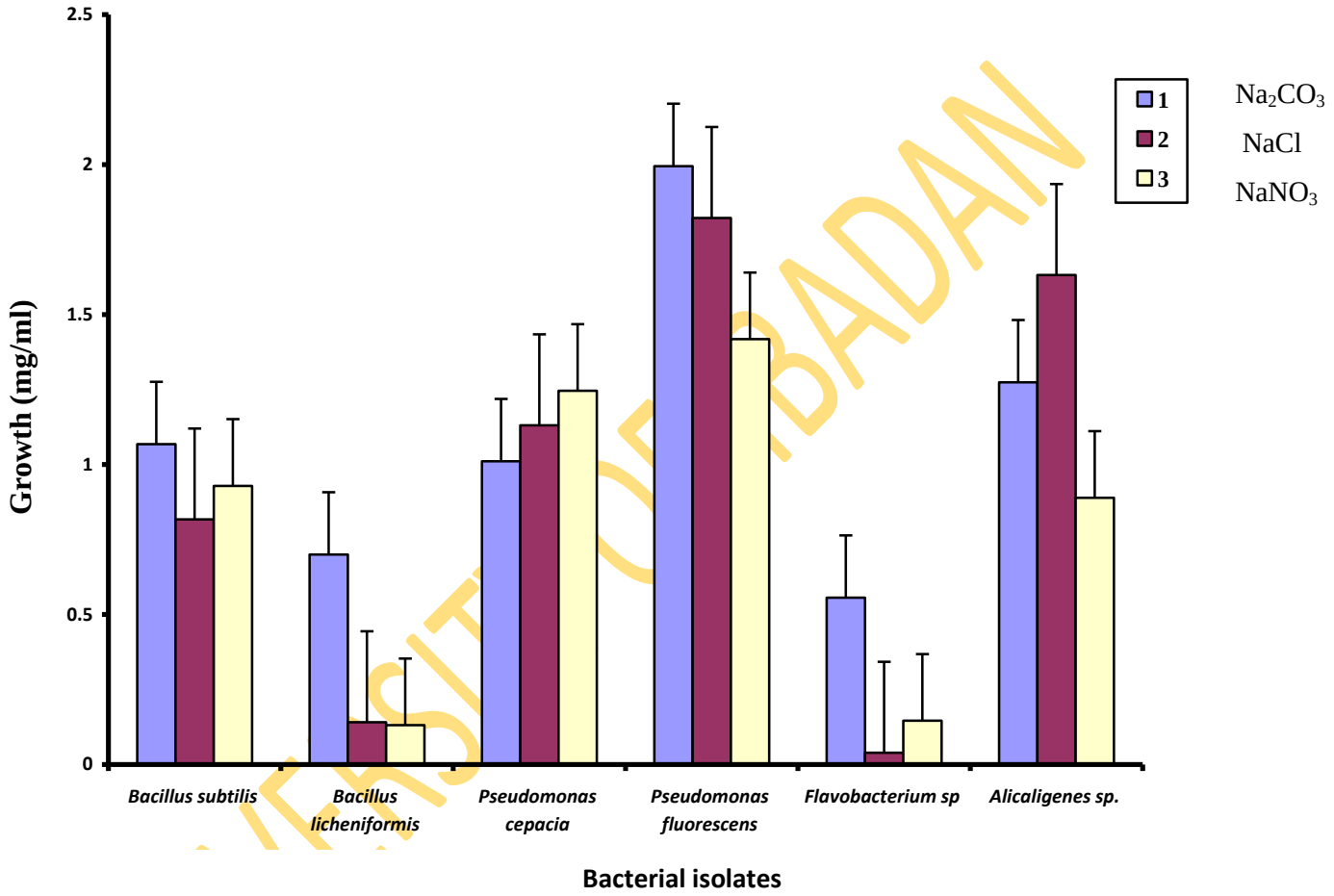

Fig. 4.10: Effect of 0.1% different anions on growth of some selected bacterial isolates

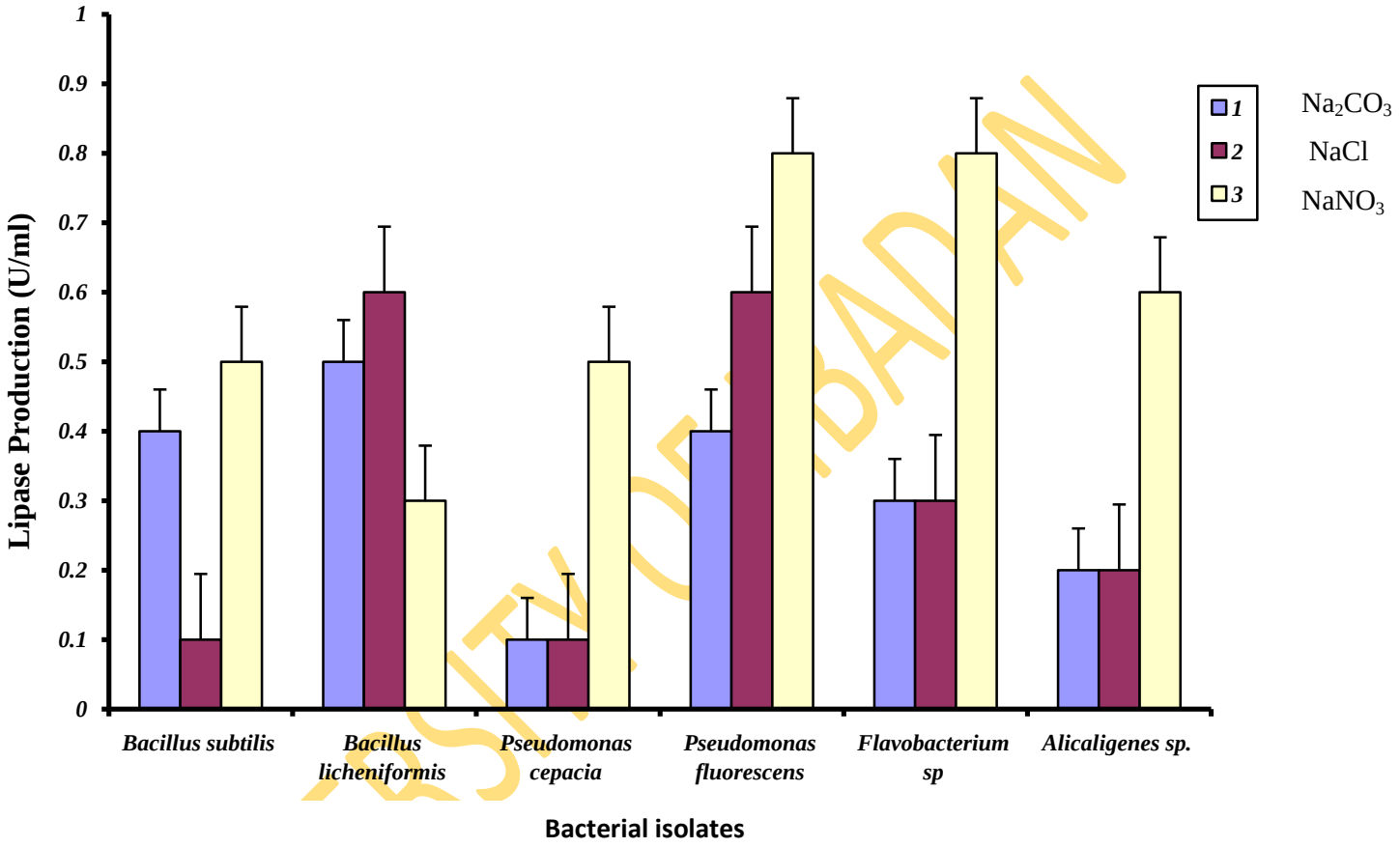

Fig. 4.11: Effect of 0.1% different anions on lipase production by some selected bacterial isolates

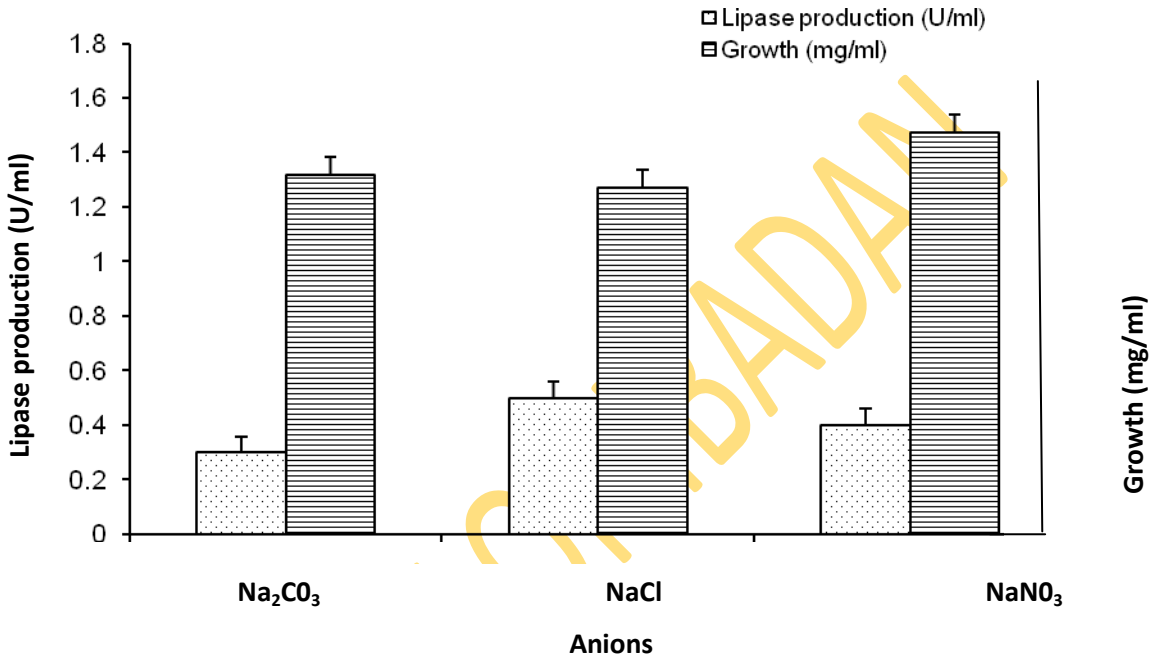

Fig. 4.12: Effect of 0.1% different anions on growth and lipase production by *Candida parapsilosis*

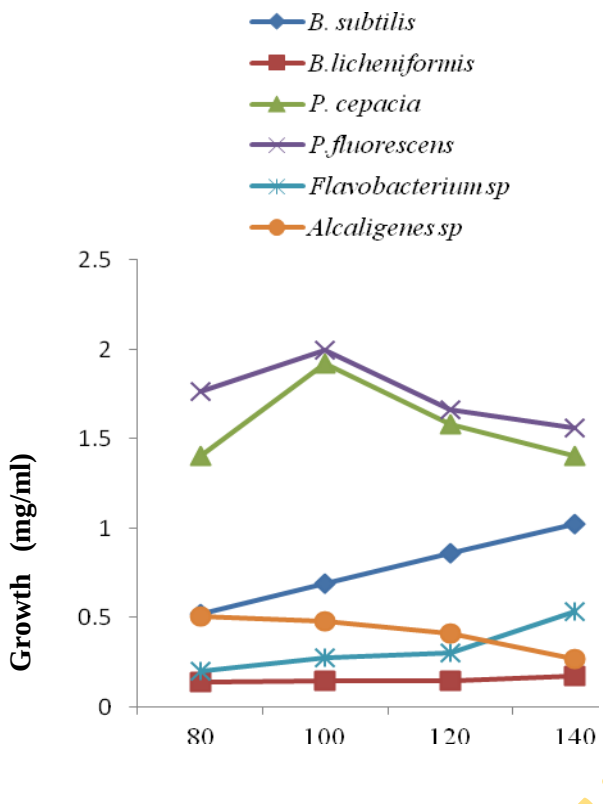

Fig. 4.13: Effect of agitation on growth of some selected bacterial isolates

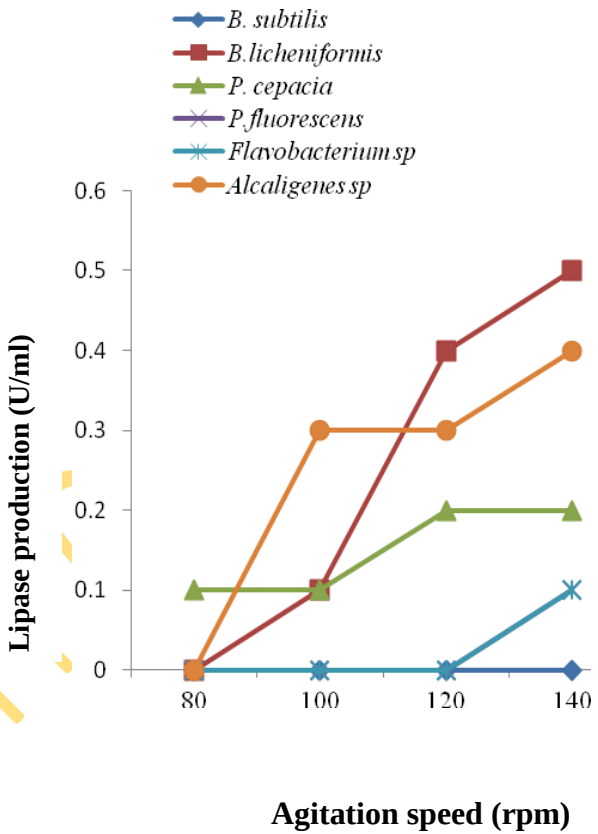

Fig. 4.14: Effect of agitation on lipase production by some selected bacterial isolates

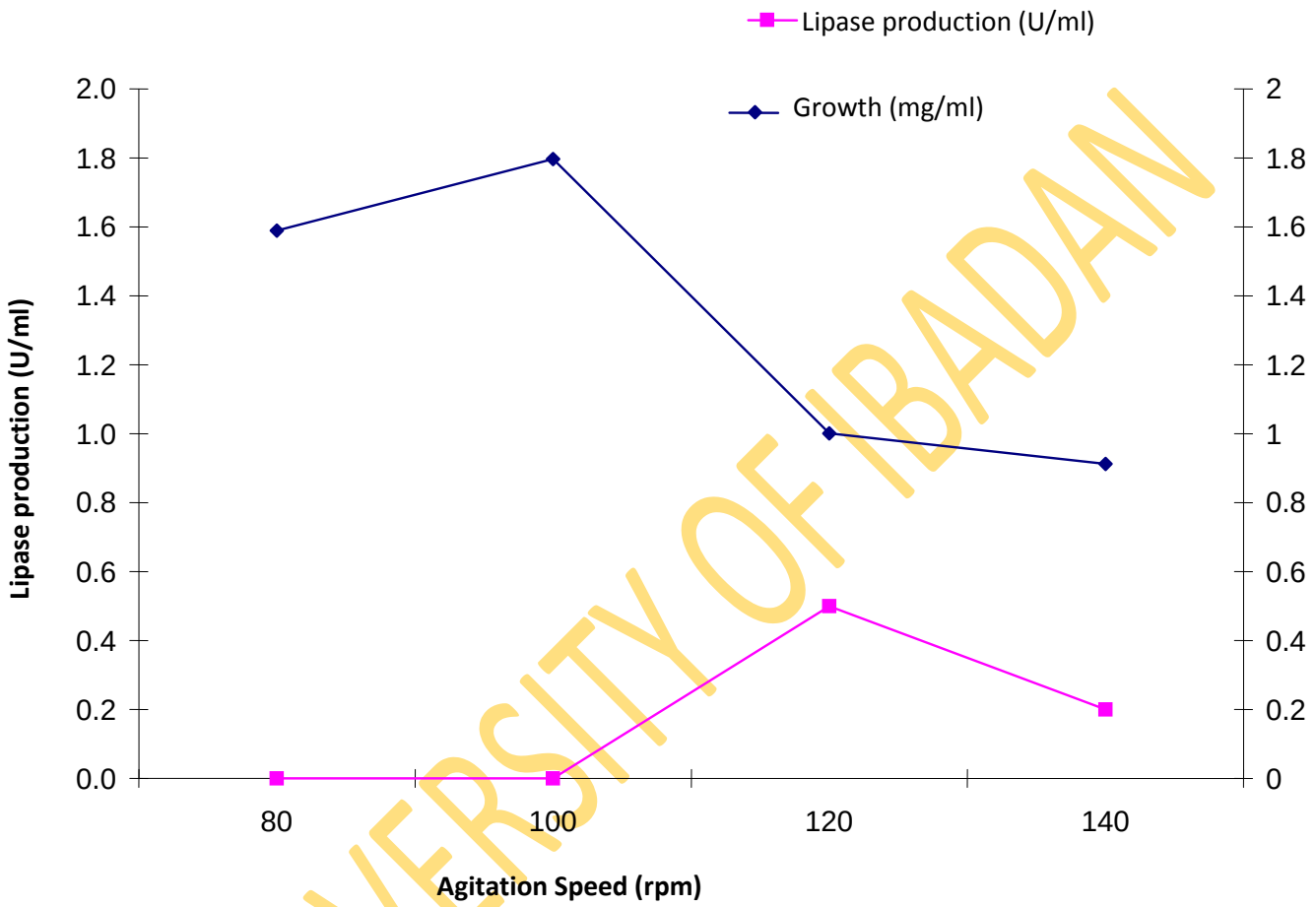

Fig. 4.15: Effect of agitation on growth and lipase production by *Candida parapsilosis*

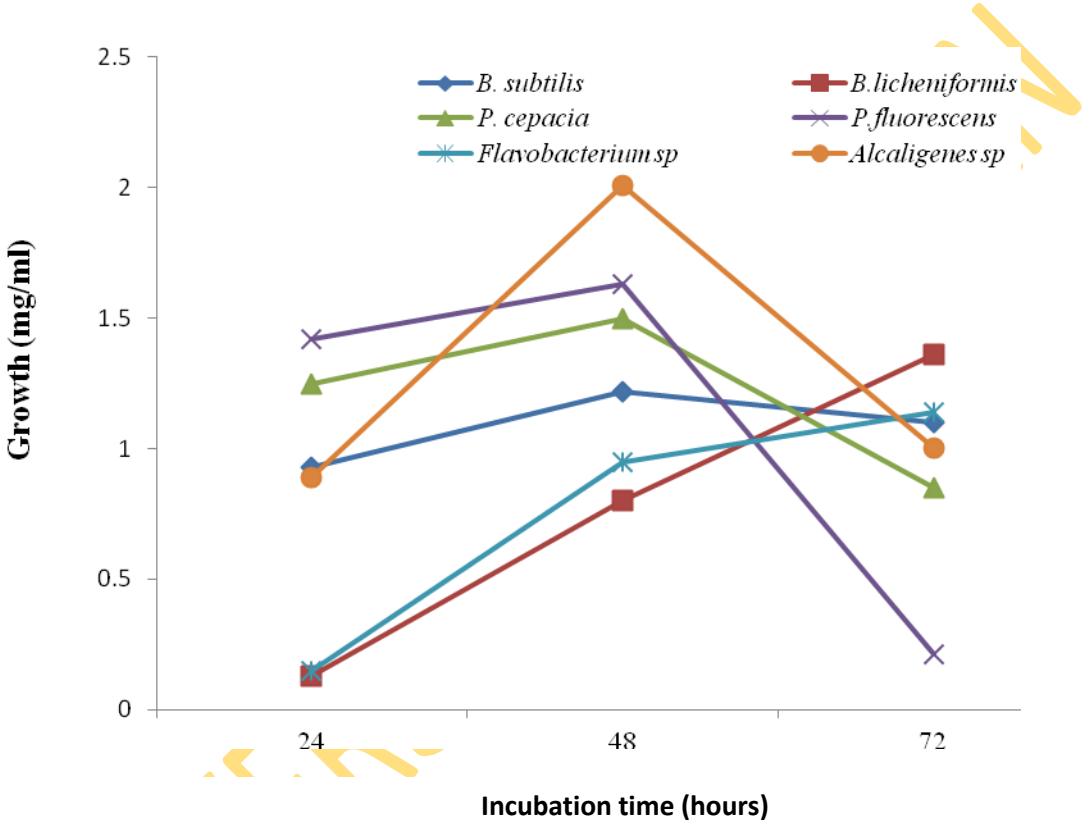

Fig. 4.16: Effect of incubation time on growth of some selected bacterial isolates

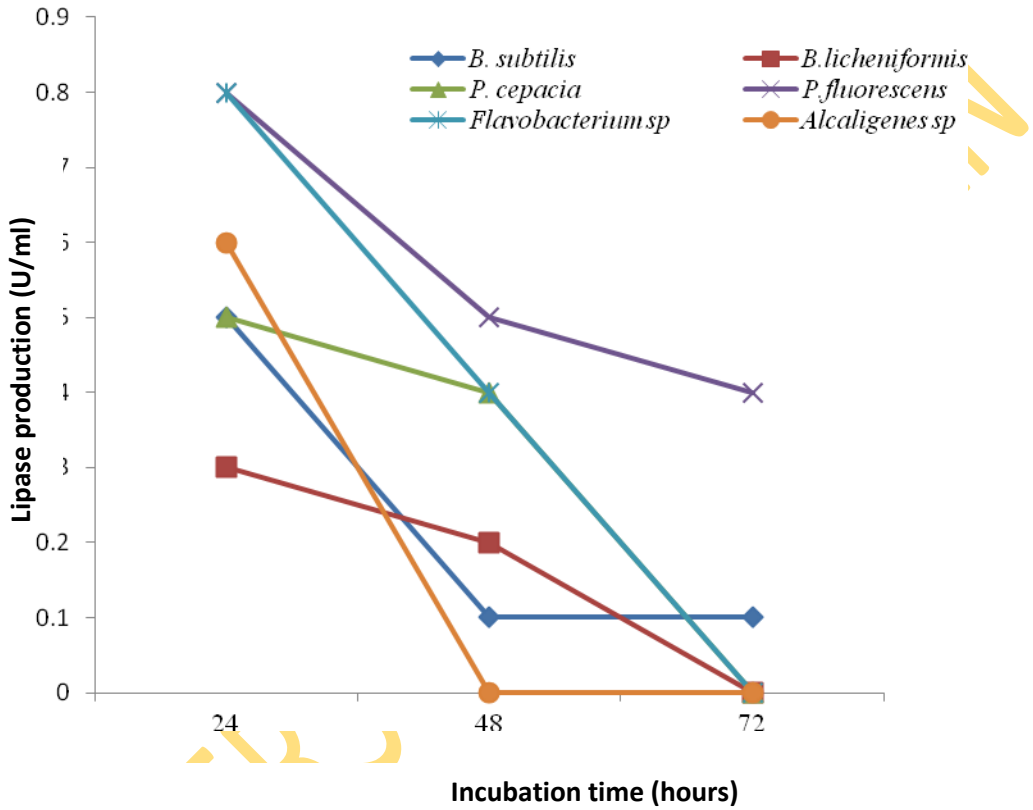

Fig. 4.17: Effect of incubation time on lipase production by some selected bacterial isolates

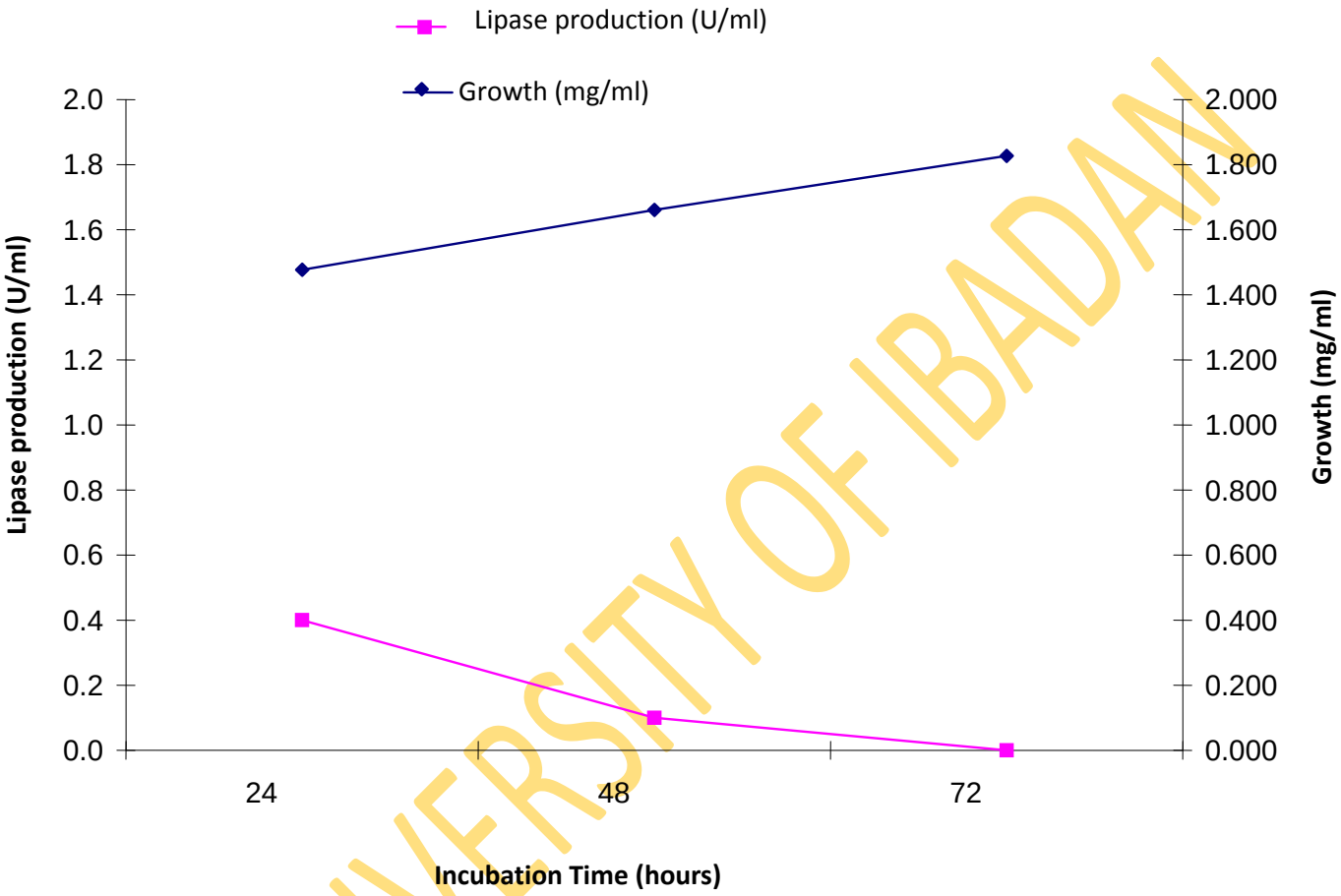

Fig.4.18: Effect of Incubation time on growth and lipase production by *Candida parapsilosis*

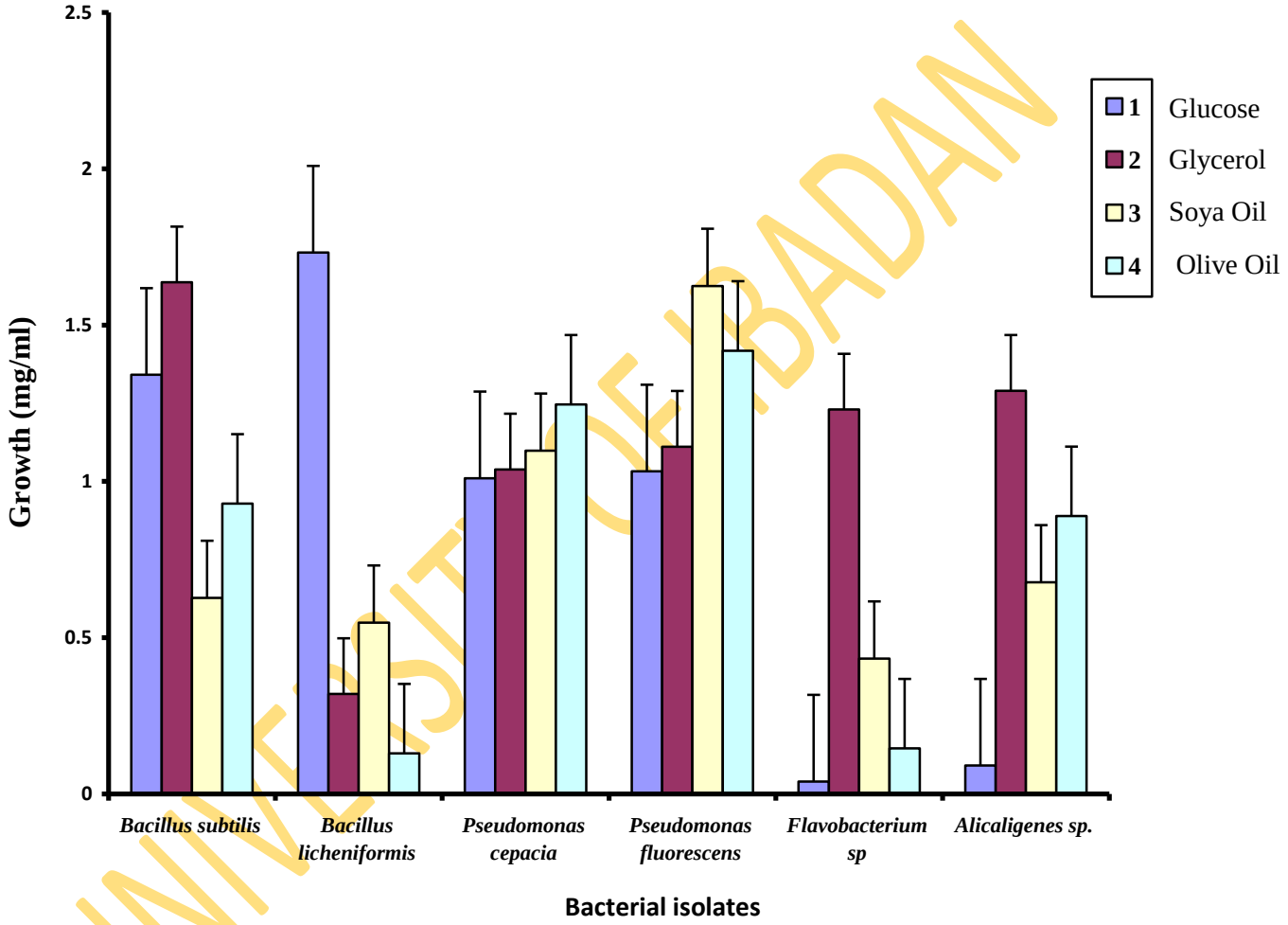

Fig. 4.19: Effect of 2% different carbon sources on the growth of some selected bacterial isolates

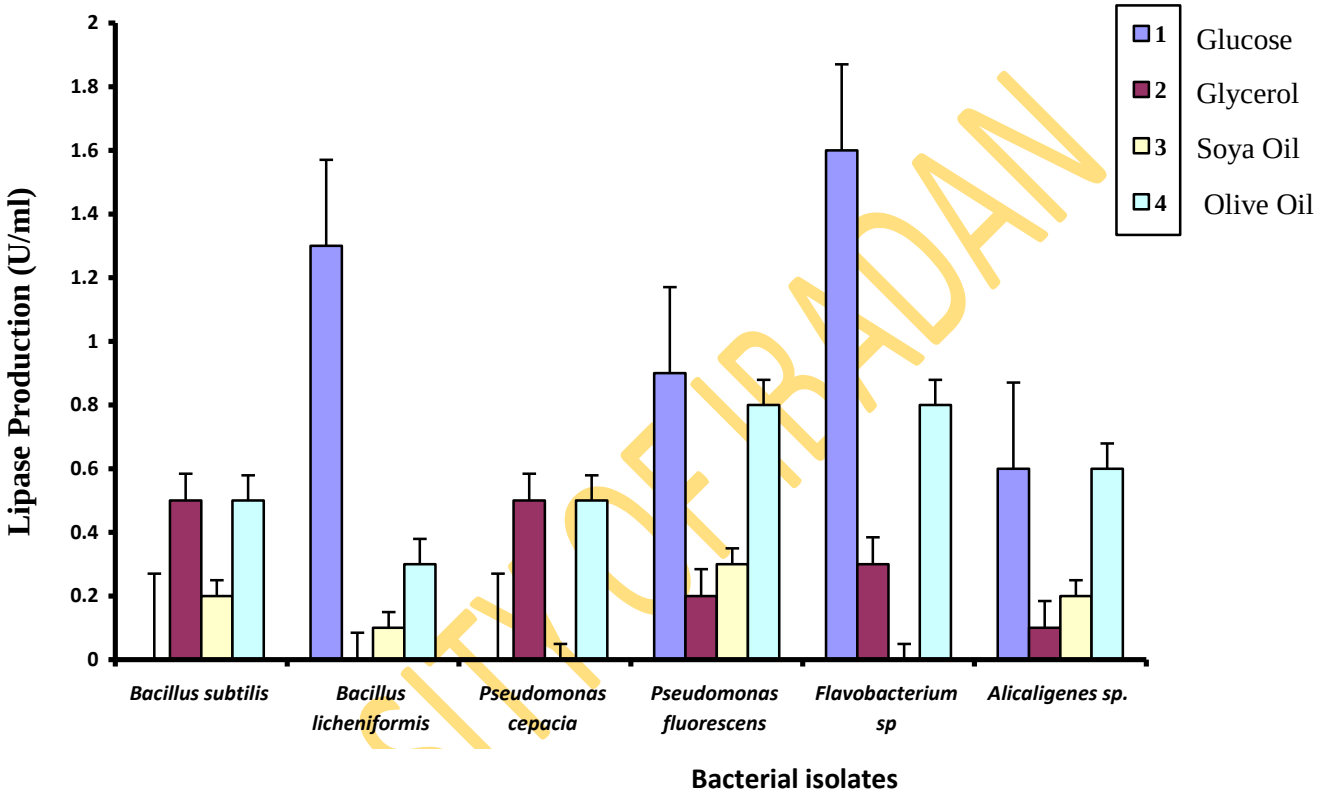

Fig.4.20: Effect of 2% different carbon sources on lipase production by some selected bacterial isolates

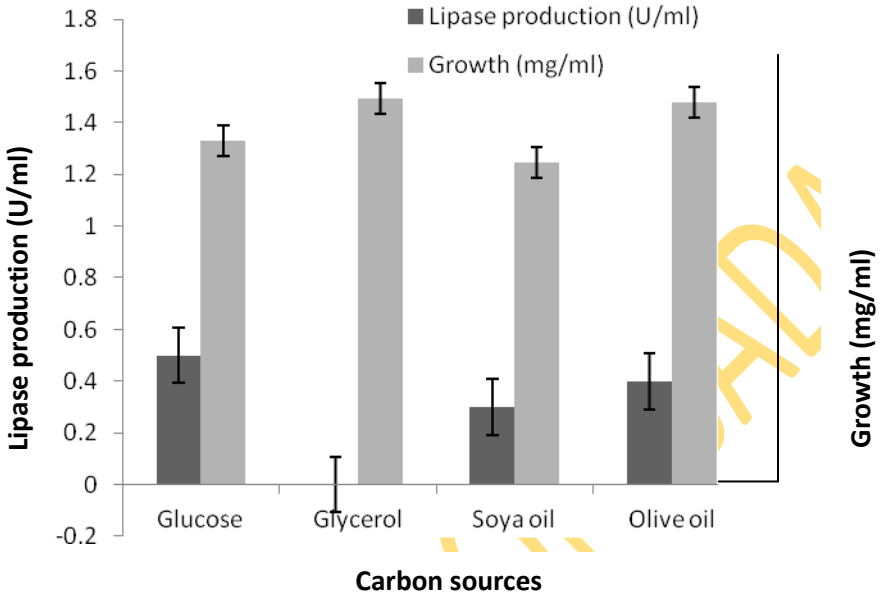

Fig. 4.21: Effect of 2% different carbon sources on growth and lipase production by *Candida parapsilosis*

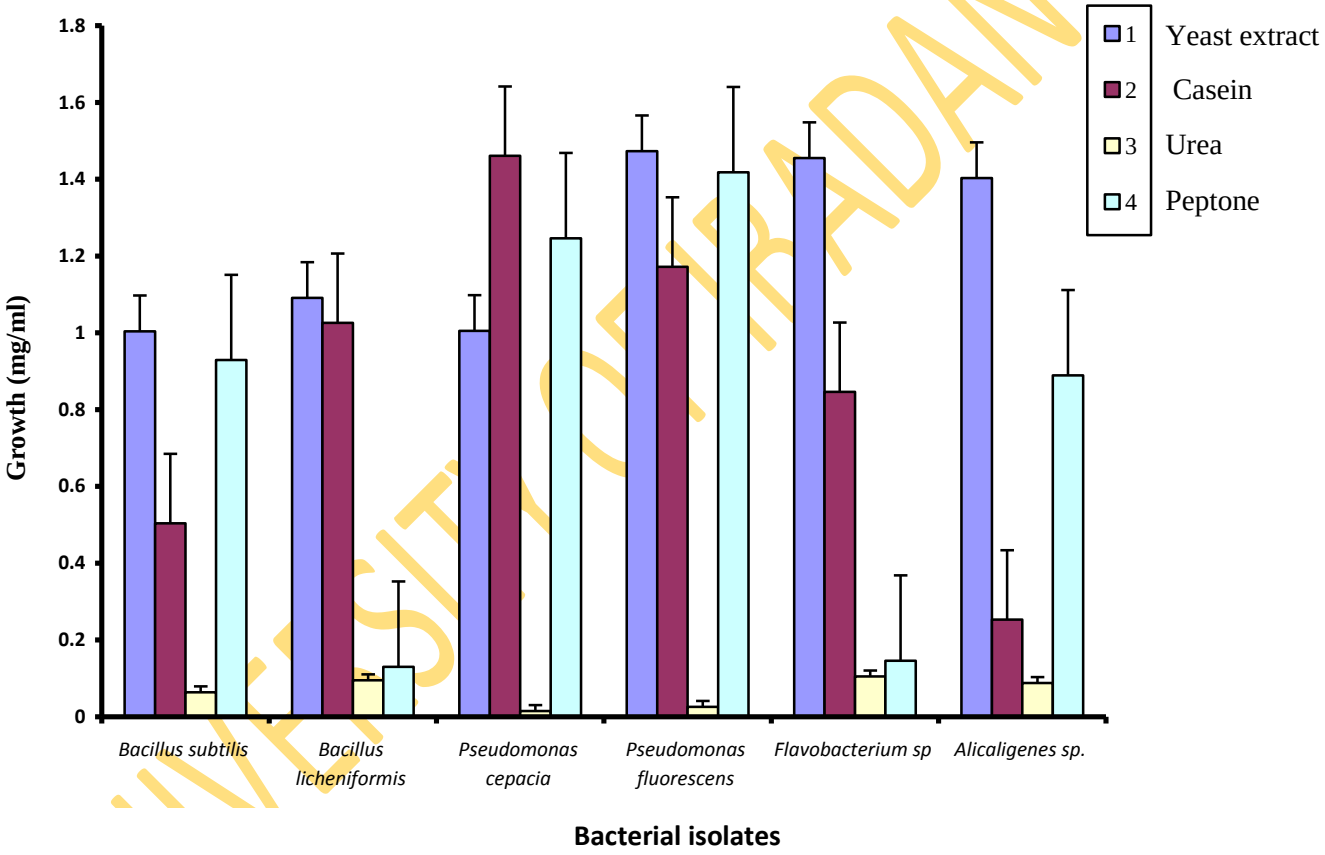

Fig.4.22: Effect of 5% different nitrogen sources on growth of some selected bacterial isolates

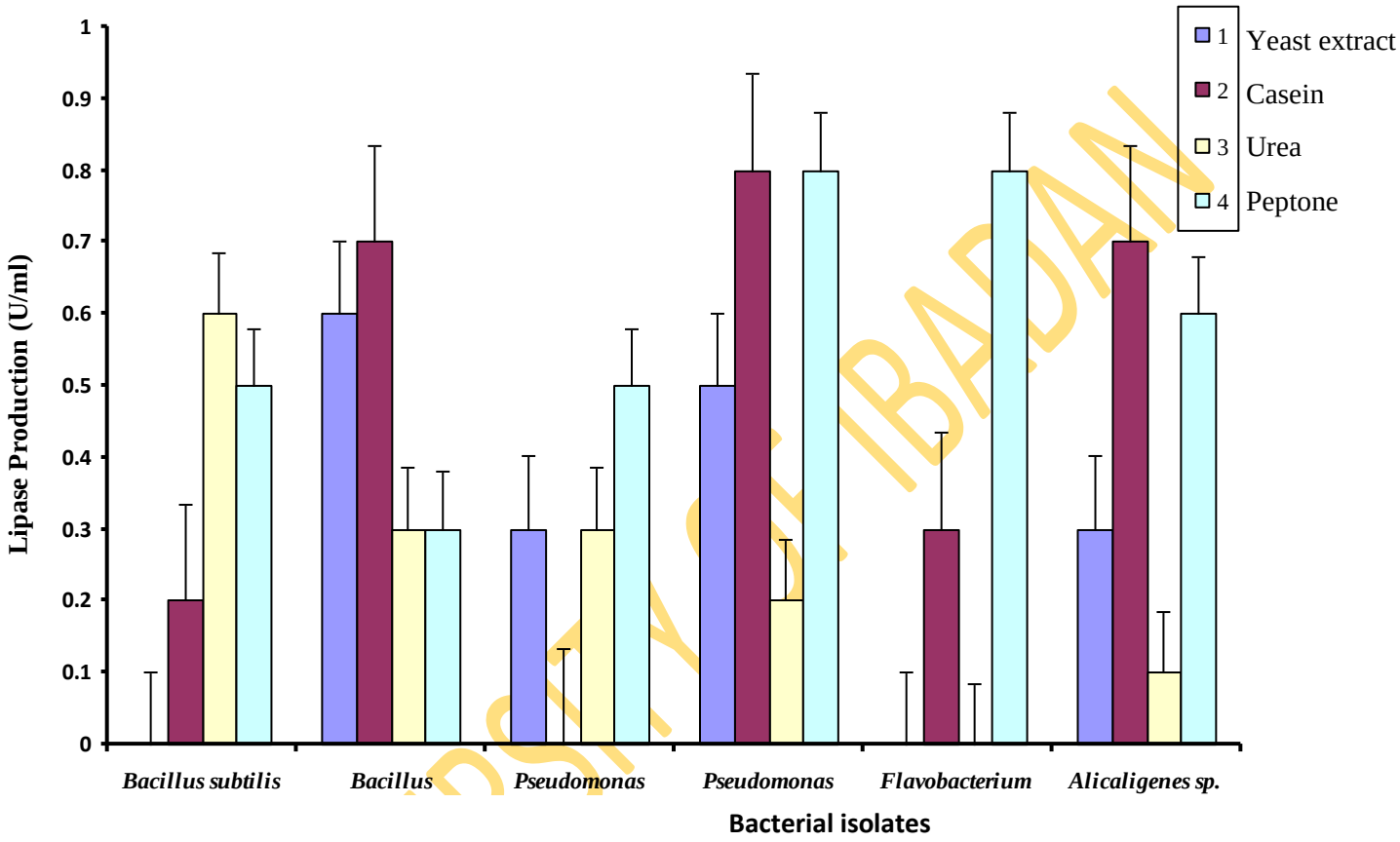

Fig. 4.23: Effect of 5% different nitrogen sources on lipase production by some selected bacterial isolates

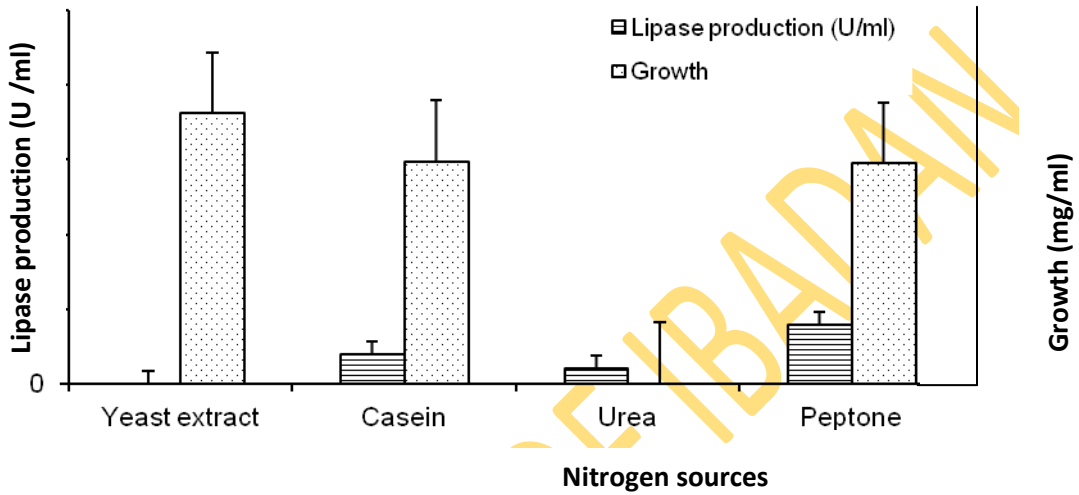

Fig. 4.24: Effect of 5% different nitrogen sources on growth and lipase production by *Candida parapsilosis*

4.5 Purification of the extracellular lipases

Lipase was precipitated to 100% saturation using ammonium sulphate. The result shows *Pseudomonas fluorescens* had maximum specific activity of 0.410 U/mg with purification fold of 1.03 as shown in Table 4.9a. The ammonium sulphate precipitated enzymes were dialysed. Dialysis raised the specific activity of 0.73 U/mg with 1.85 purification fold as also shown in Table 4.9a.

The purification fold of the lipases increased to 3.60 and 5.14 respectively for *Pseudomonas fluorescens* and *Candida parapsilosis* after applying Sephadex G-75 column chromatography technique. A total of 30 fractions of 10 ml each were collected, the two major peaks for *Pseudomonas fluorescens* (tubes no 13 and 22) were pooled together, whereas tubes no 8 and 21 which had specific activities of 1.43 U/mg and 1.56 U/mg respectively, were pooled together in the case of *Candida parapsilosis.* These fractions were loaded onto ion exchange chromatography (CM) equilibrated with 7.5 phosphate buffer (0.5 M, pH 7.5), a total of 30 fractions of 10 ml each were also collected. A major peak was observed in tube 7 in the case of *Pseudomonas fluorescens*, the peaks were observed in tubes 8 and 13 in the case of *Candida parapsilosis*. These are shown in Figures 4.25 to 4.28.

Table 4.9a. Purification levels of the lipolytic extract of *Pseudomonas fluorescens*

	Total Protein (mg)	Enzyme Activity	% Yield	Specific Activity $(Umg^{-1})^1$	Purification Fold
Crude Enzyme	2.013	0.80	100	0.397	1
Ammonium Sulphate Precipitated	1.2183	0.50	62.5	0.410	1.033
Dialysed Extract	0.6127	0.45	56.25	0.734	1.849
Gel Filtration (Sephadex 75)	0.2802	0.40	50	1.428	3.597
Ion-Exchange	0.0944	0.30	37.5	3.178	8.005

Table 4.9b. Purification levels of the lipolytic extract of *Candida parapsilosis*

	Total Protein (mg)	Enzyme Activity	% Yield	Specific Activity $(Umg^{-1})^1$	Purification Fold
Crude Enzyme	1.3153	0.40	100	0.304	1
Ammonium Sulphate Precipitated	0.3641	0.30	75	0.824	2.711
Dialysed Extract	0.2326	0.25	6.25	1.075	3.536
Gel Filtration (Sephadex 75)	0.1280	0.20	50	1.563	5.141
Ion-Exchange	0.083	0.15	37.5	1.807	5.945

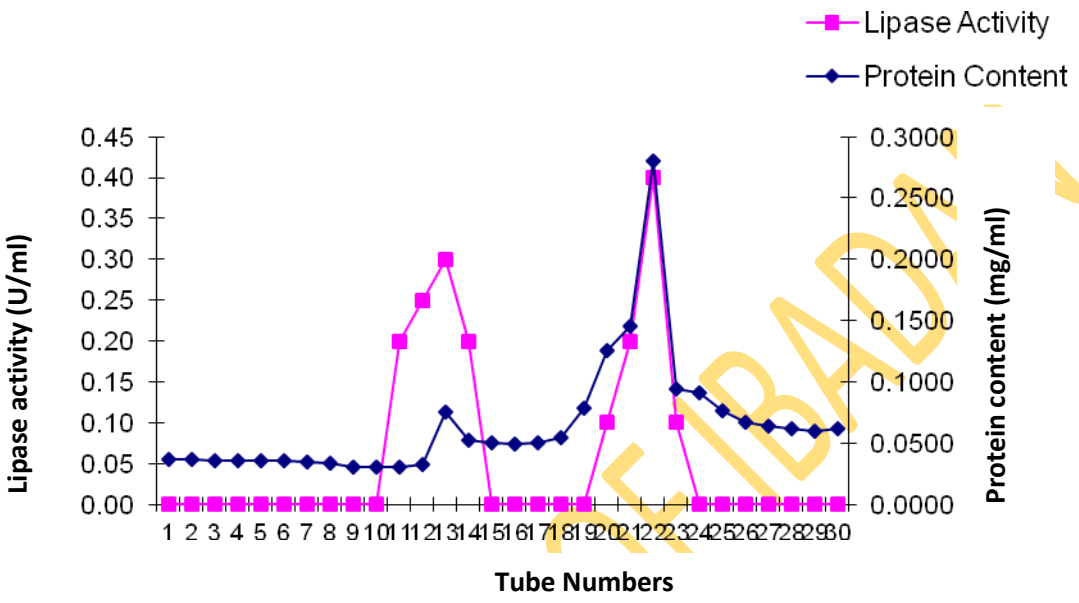

Fig. 4.25: Separation of proteins in concentrated extracts of *Pseudomonas fluorescens* by gel filtration and enzymic activity of the fractions towards oleic acid

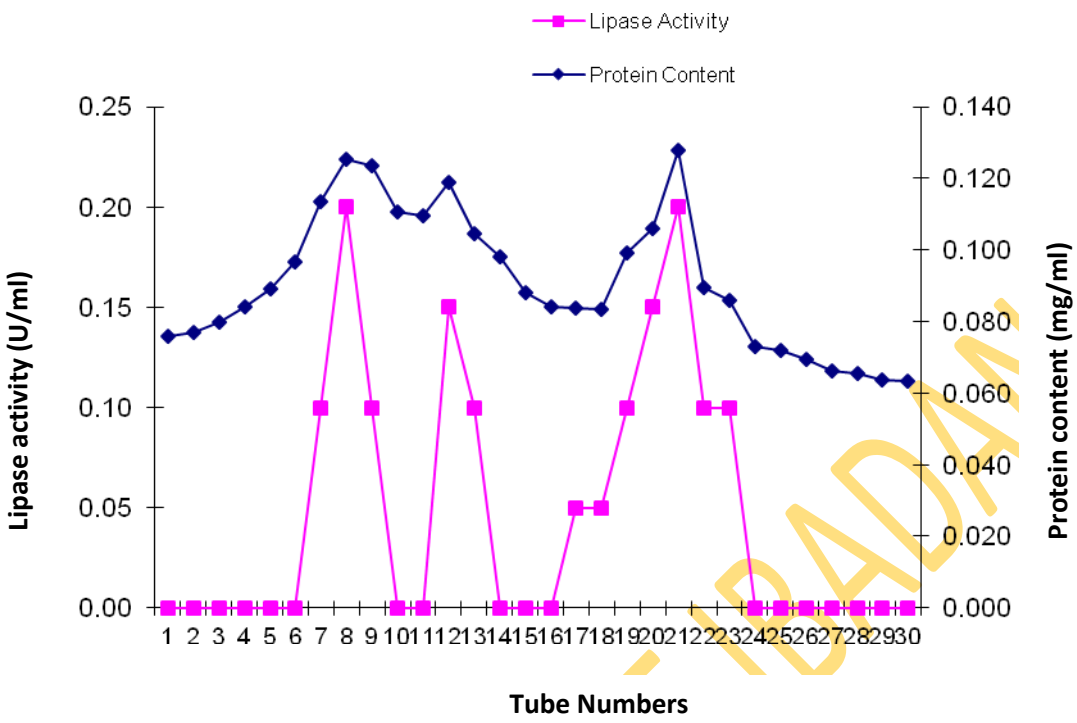

Fig 4.26: Separation of proteins in concentrated extracts of *Candida parapsilosis* by gel filtration and enzymic activity of the fractions towards oleic acid

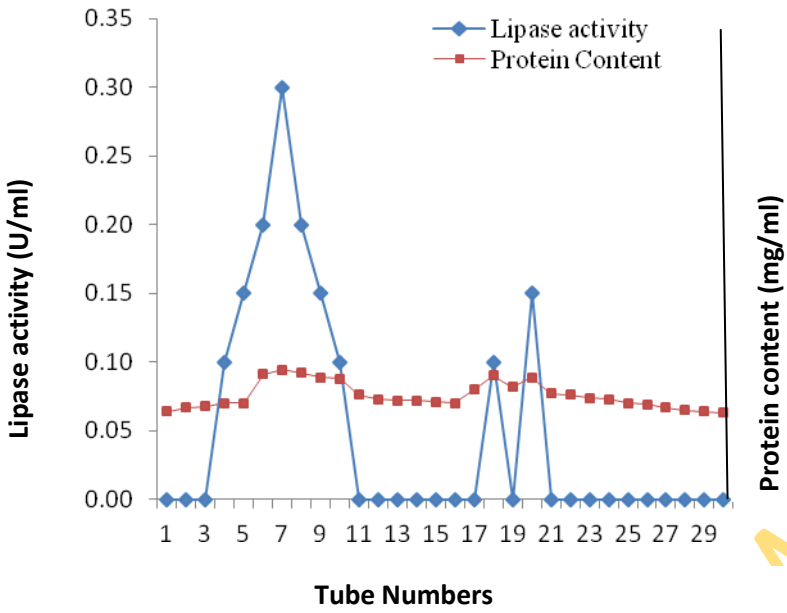

Fig 4.27: Separation by Ion-exchange chromatography of high molecular weight proteins(fractions 10-24) obtained from separated extracts of *Pseudomonas fluorescens* by gel filtration and enzymic activity of the fractions towards oleic acid

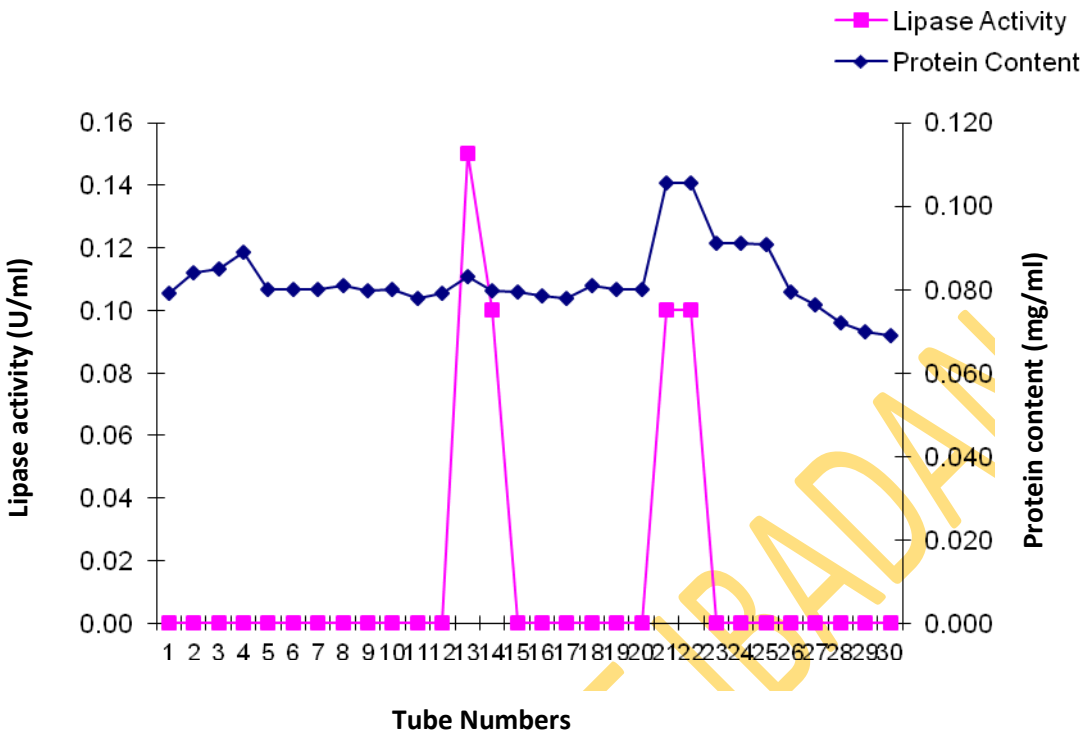

Fig 4.28: Separation by Ion-exchange chromatography of high molecular weight proteins (fractions 6-24) obtained from separated extracts of *Candida parapsilosis* by gel filtration and lipolytic activity of the fractions towards oleic acid

SDS-PAGE analysis shows bands of purified lipase after partial purification. When compared with low molecular weight protein markers, the molecular weight of the purified lipases were found to be approximately 38 KDa for *Candida parapsilosis* and within the range of 35 to 50 KDa for *Pseudomonas fluorescens* based on the position of the protein marker as shown in Figures 4.29 and 4.30 respectively.

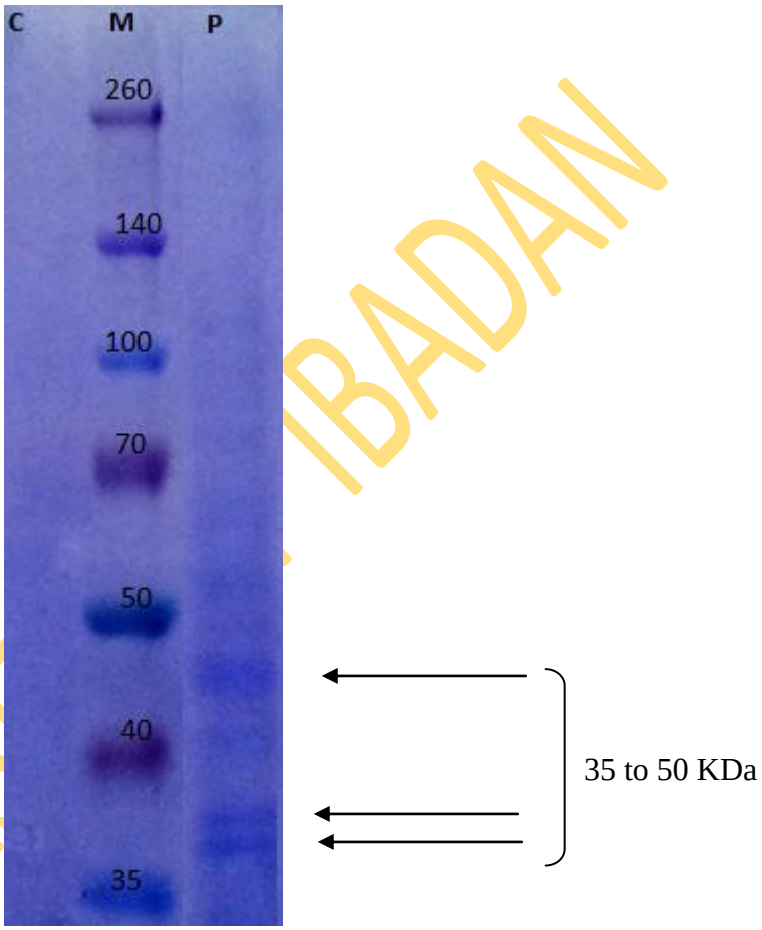

Fig. 4.29: SDS-PAGE of partially purified lipase.The lanes are as follows: M, marker proteins with relative molecular masses indicated on the right; P, lipase of *Pseudomonas fluorescens*

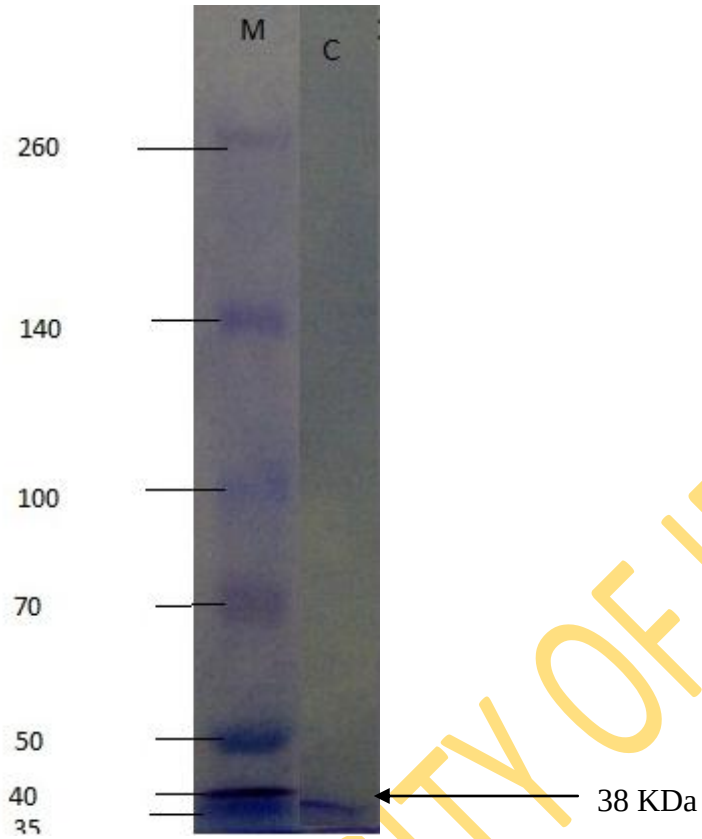

Fig. 4.30: SDS-PAGE of partially purified lipase.The lanes are as follows: M, marker proteins with relative molecular masses indicated on the right; C, lipase of *Candida parapsilosiss*

4.6 Characterisation of the partially purified enzymes

Effect of varied pH on lipolytic activity of partially purified enzyme is as shown in Figure 4.31. The mean value at 7.5 pH 7.5 was the optimum for *Candida parapsilosis* and *Pseudomonas fluorescens* and at pH 5.5, the enzymes did not have any lipolytic activity. In both cases, activity increased gradually from pH 5.5 to reach the peak at 7.5. Beyond this point, a sharp decline in activity was observed.

Figure 4.32 shows the effect of temperature on lipolytic activity of partially purified enzymes, *Pseudomonas fluorescens* enzyme had its optimum temperature at 27^{o}C, while *Candida parapsilosis* was at 35^{o}C; at 25^{o}C, both did not have any lipolytic activity.

The result of monitoring of the effect of different concentrations of partially purified enzyme on lipolytic activity is shown in Figure 4.33. At 0.5ml, *Candida parapsilosis* had optimum activity (0.35U/ml) and no activity at 2ml (0.00 U/ml) whereas *Pseudomonas fluorescens* had optimum activity at 1.0ml (0.50 U/ml).

Figure 4.34 shows the effect of different concentrations of metal ions on lipolytic activity of the partially purified enzyme. In the presence of different concentration of $MgSO_4$, *Candida parapsilosis* recorded the highest lipolytic activity at 0.05% concentration (0.35 U/ml), *Pseudomonas fluorescens* also had highest lipolytic activity recorded at 0.05% concentration (0.85 U/ml). They both had no activity at 0.2 % concentration.

Figure 4.35 shows the effect of different concentrations of the nitrogen source used (peptone) on lipolytic activity of the partially purified enzyme. They both had their highest activity recorded at 5% concentration (0.30 U/ml and 0.50U/ml).

The ffect of agitation on lipolytic activity of partially purified enzyme is shown in Figure 4.36, *Candida parapsilosis* had optimum recorded activity at 140rpm (0.50 U/ml) and no activity at 180 rpm, *Pseudomonas fluorescens* had highest recorded activity at 100rpm (0.70 U/ml) and at 120rpm (0.40 U/ml) but from 140rpm, no activity was recorded.

Figures 4.37 to 4.40 show comparison of crude and partially purified enzyme activity. Figure 4.37 shows that *Candida parapsilosis* had significantly higher lipolytic activity for both forms of enzyme at 0.5 ml enzyme concentration, whereas at 2.0 ml

enzyme concentration they had the least. Comparing the two forms of enzyme in *Pseudomonas fluorescens* at 0.5 ml enzyme concentration, crude enzyme had no activity whereas the partially purified form had activity (0.30 U/ml).

Figure 4.38 shows the comparison of the effect of temperature on the enzyme form. Partially purified enzyme of *Candida parapsilosis* had no activity up to 25^{o}C, whereas its crude counterpart had. However, from 35^{o}C to 40^{o}C, the crude form of enzyme had no activity but the partially purified enzyme had. In the case of *Pseudomonas fluorescens*, at 20^{o}C, both forms of enzyme had no activity.

Figure 4.39 shows the comparison of the effect of nitrogen source. Both enzyme forms of these organisms had their highest activity at 5.0 g concentration of peptone. Figure 4.40 shows the comparison of the effect of metal ions ($NaN0_3$) on the enzyme forms. At 0.15 %, *Candida parapsilosis* had higher activity for the partially purified form than its crude counterpart, whereas the reverse was the case in *Pseudomonas fluorescens.* At 0.2 %, both enzyme forms for the two organisms had no activity.

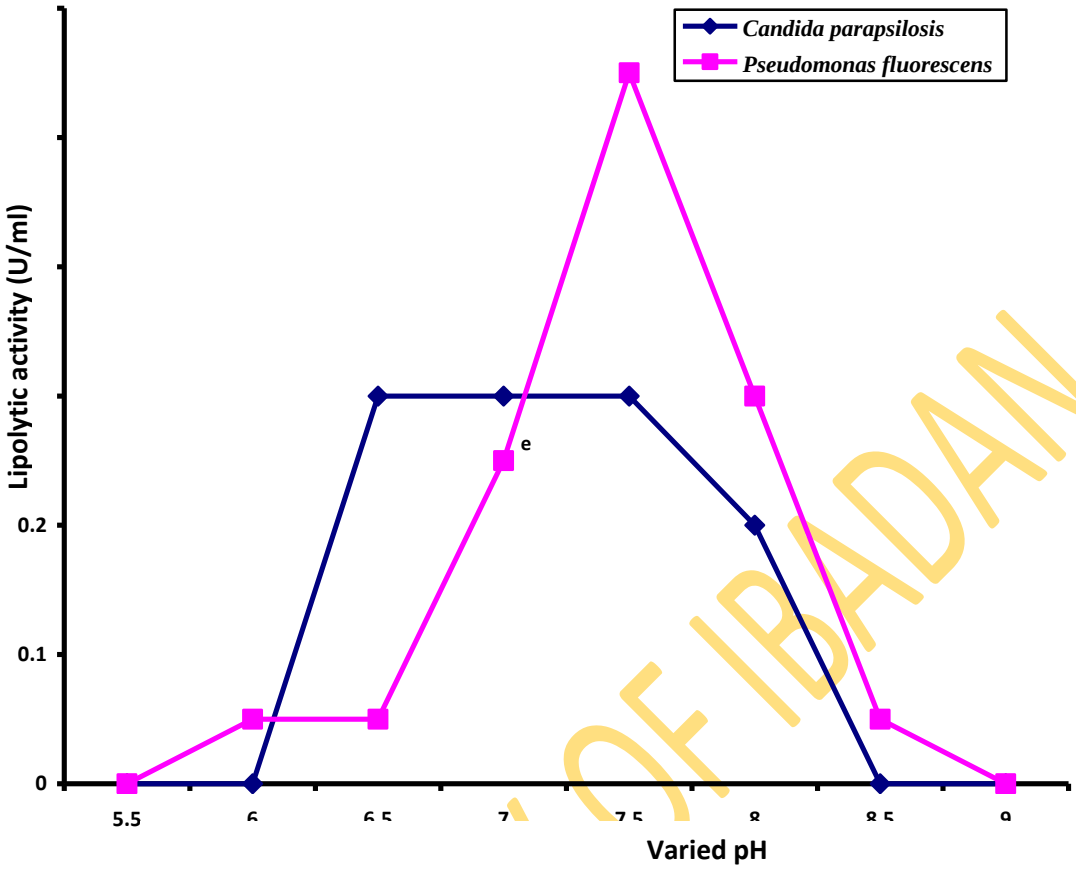

Fig. 4.31: Effect of varied pH on lipolytic activity of partially purified enzymes of *Candida parapsilosis and Pseudomonas fluorescens*

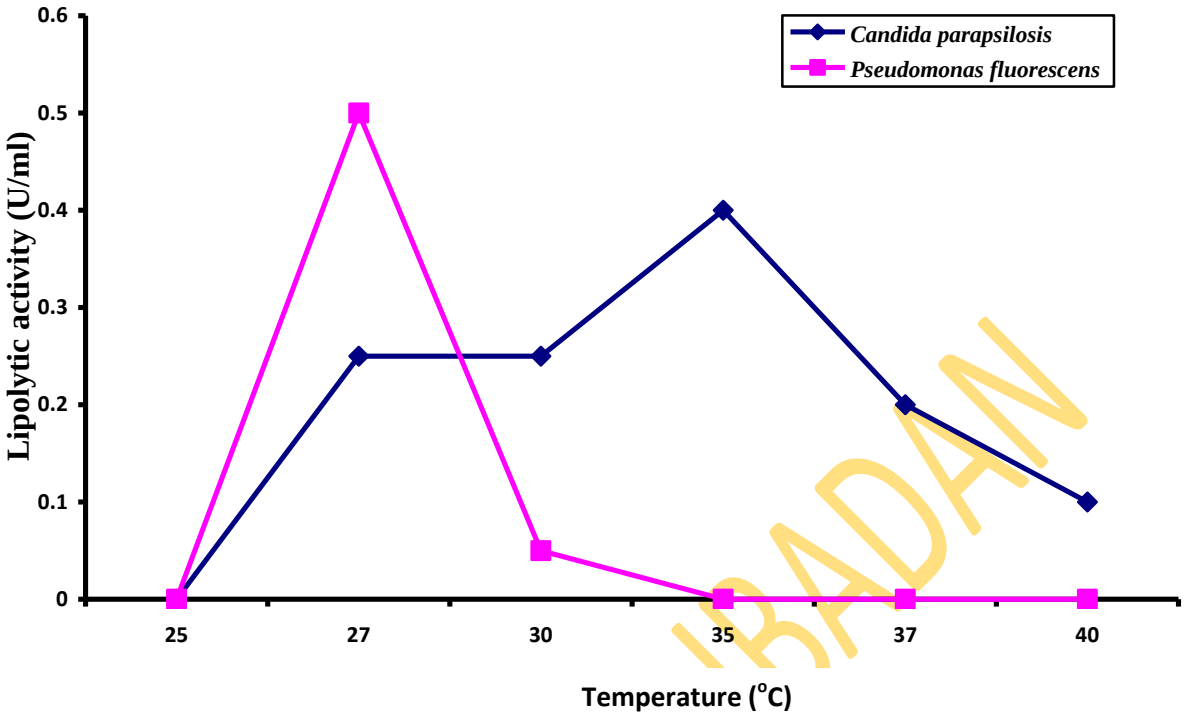

Fig. 4.32: Effect of temperature on lipolytic activity of partially purified enzymes of *Candida parapsilosis and Pseudomonas fluorescens*

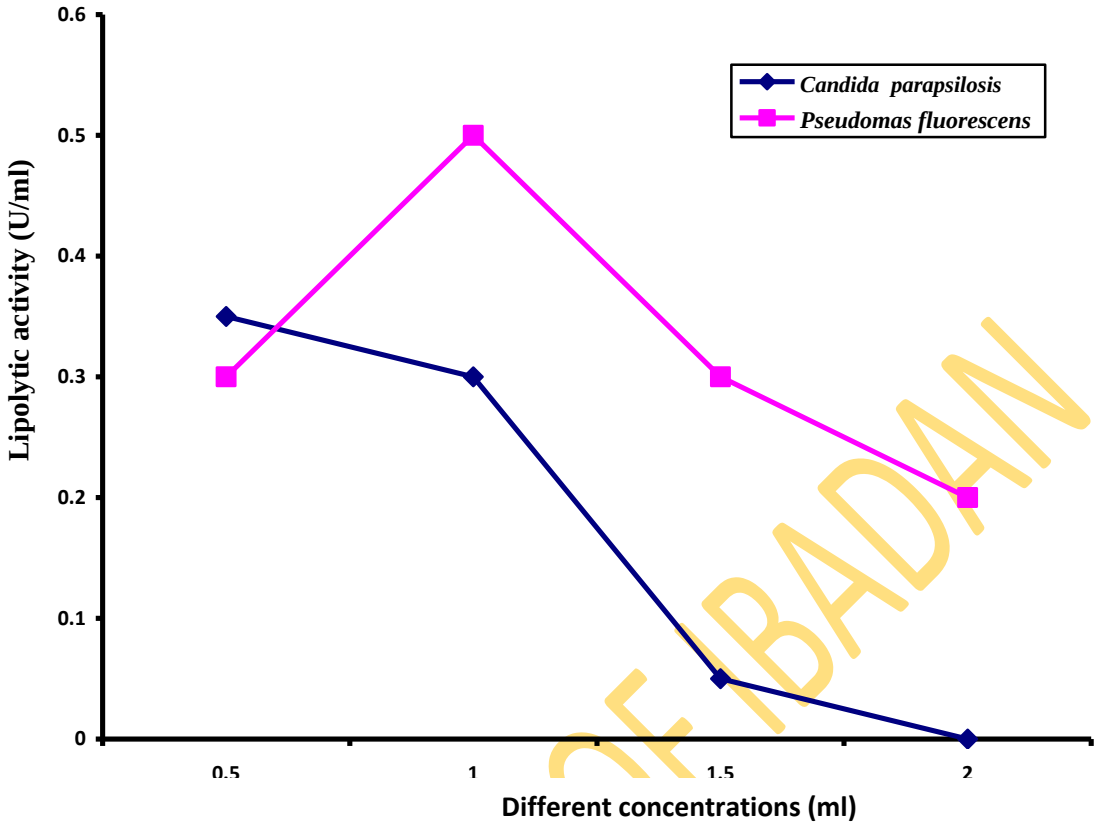

Fig. 4.33: Effect of different concentrations of partially purified enzymes on lipolytic activity of *Candida parapsilosis and Pseudomonas fluorescens*.

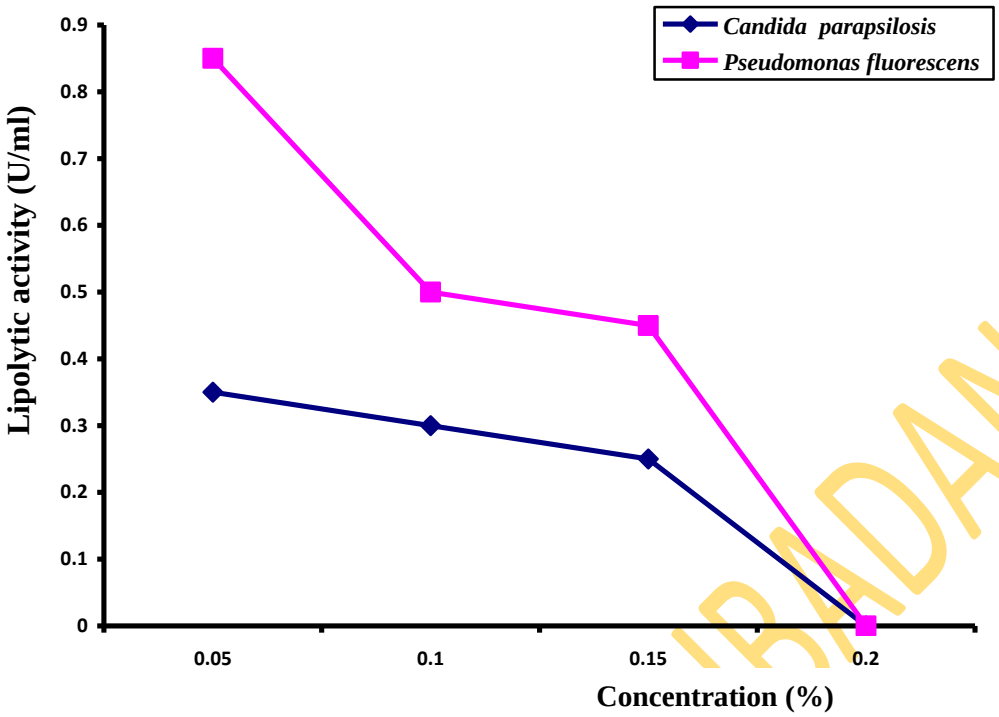

Fig. 4.34: Effect of different concentrations of ion ($MgSO_4$) on lipolytic activity of partially purified enzymes of *Candida parapsilosis and Pseudomonas fluorescens*

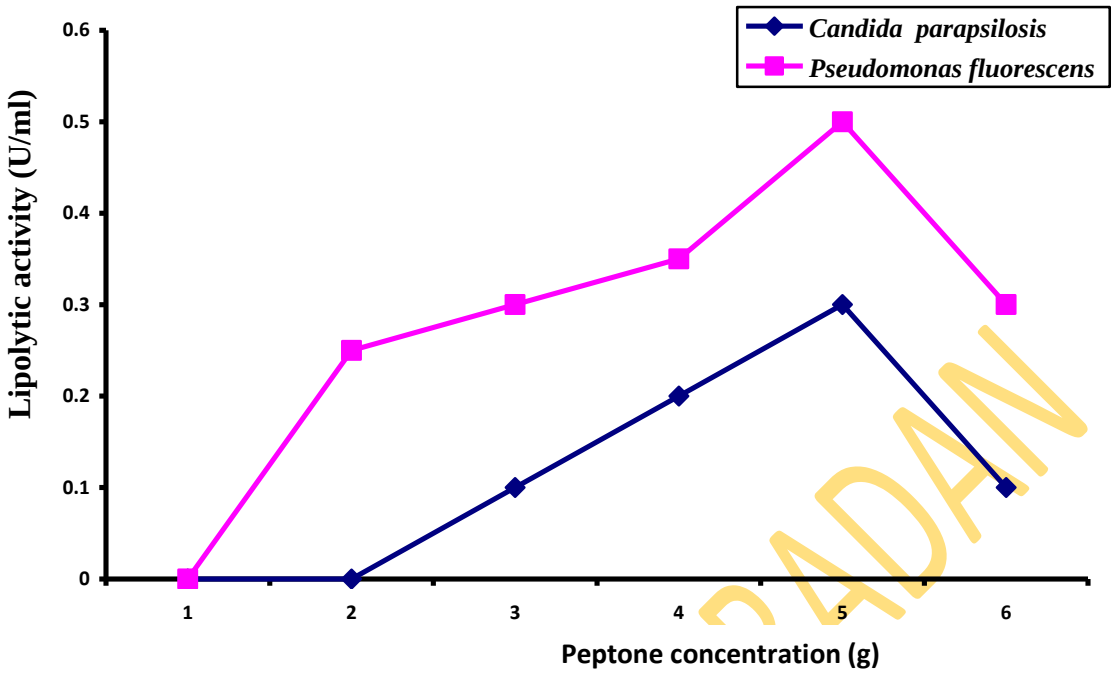

Fig. 4.35: Effect of different concentrations of nitrogen source (peptone) on lipolytic activity of partially purified enzymes of *Candida parapsilosis* and *Pseudomonas fluorescens*.

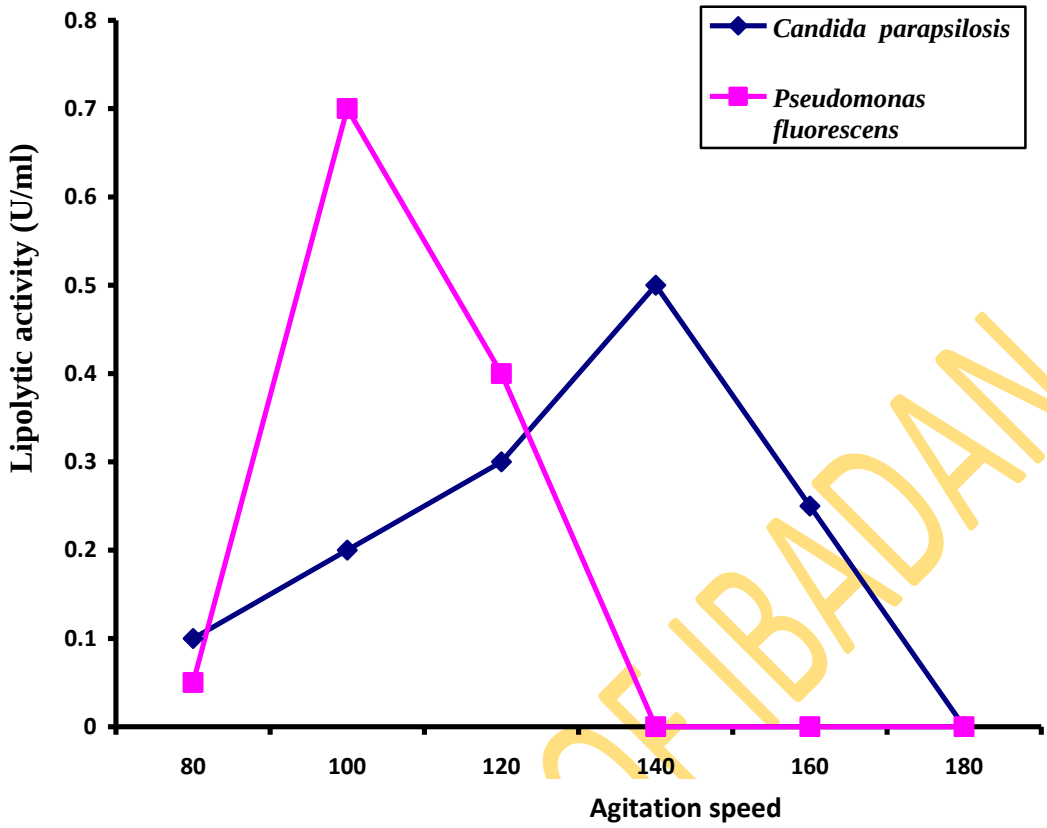

Fig. 4.36: Effect of agitation on lipolytic activity of partially purified enzymes of *Candida parapsilosis* and *Pseudomonas fluorescens*.

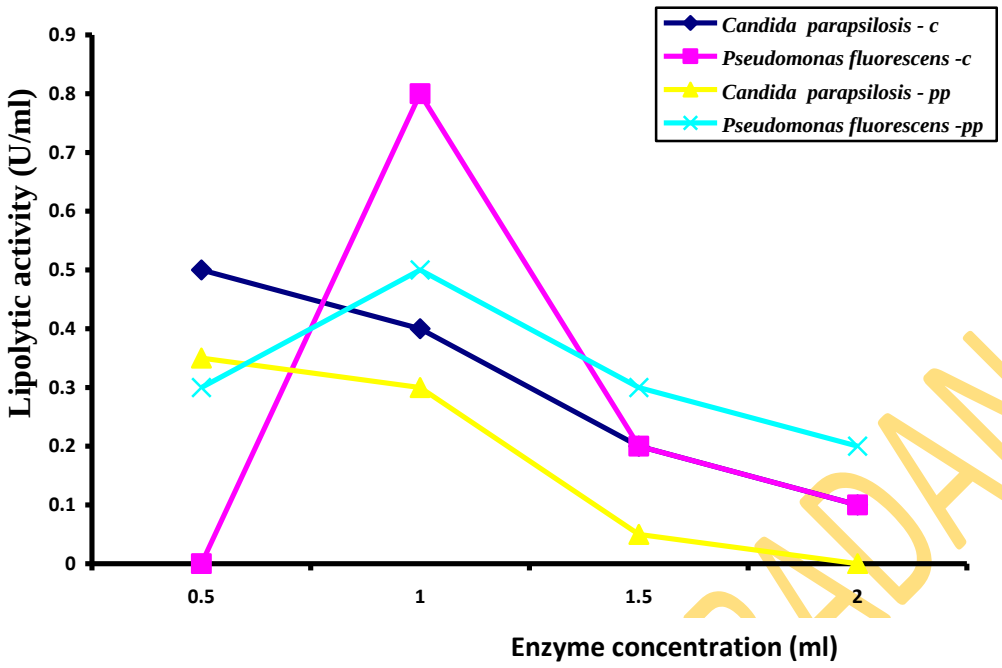

Fig. 4.37: Comparison of the effect of enzyme concentration on the activity of crude(c) and partially purified(pp) lipase of *Candida parapsilosis* and *Pseudomonas fluorescens*

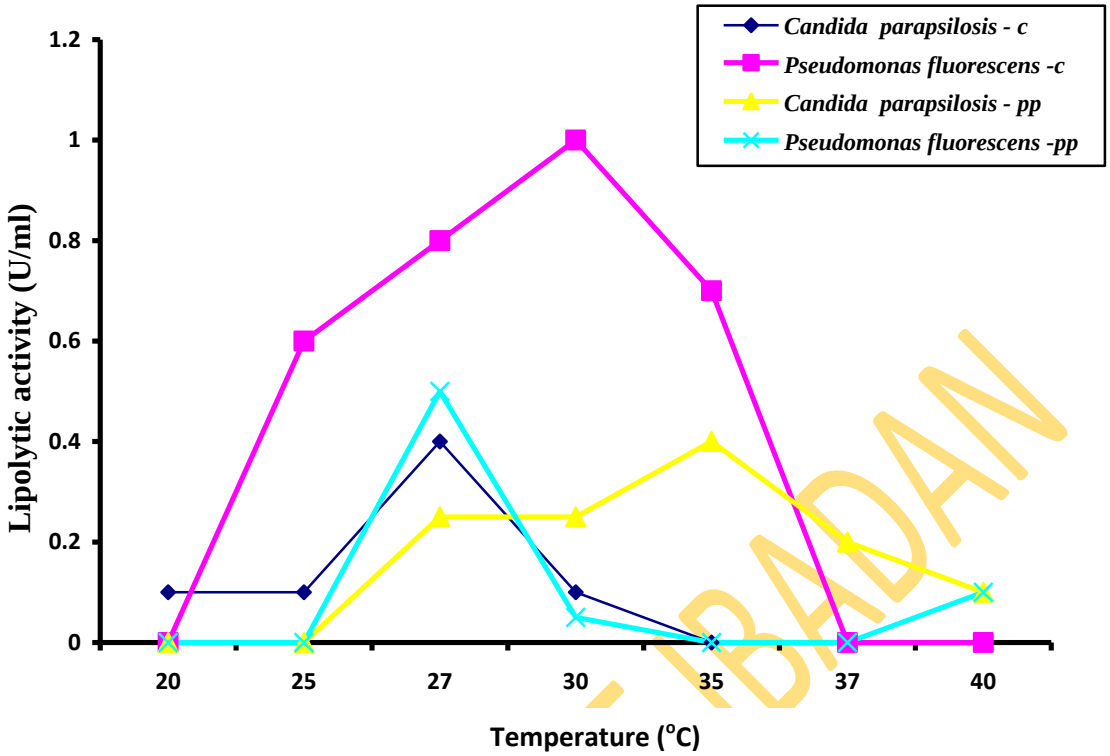

Fig. 4.38: Comparison of the effect of temperature on activity of crude(c) and partially purified(pp) lipase of *Candida parapsilosis* and *Pseudomonas fluorescens*

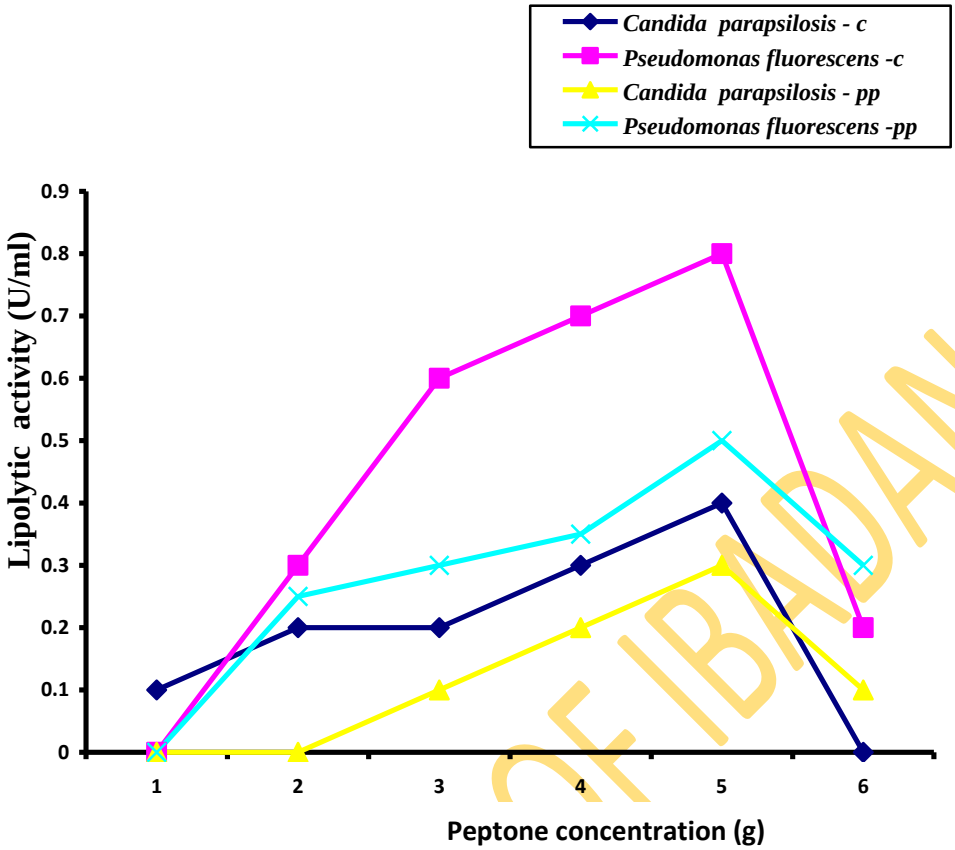

Fig. 4.39: Comparison of the effect of nitrogen source on activity of crude(c) and partially purified(pp) lipase of *Candida parapsilosis* and *Pseudomonas fluorescens*

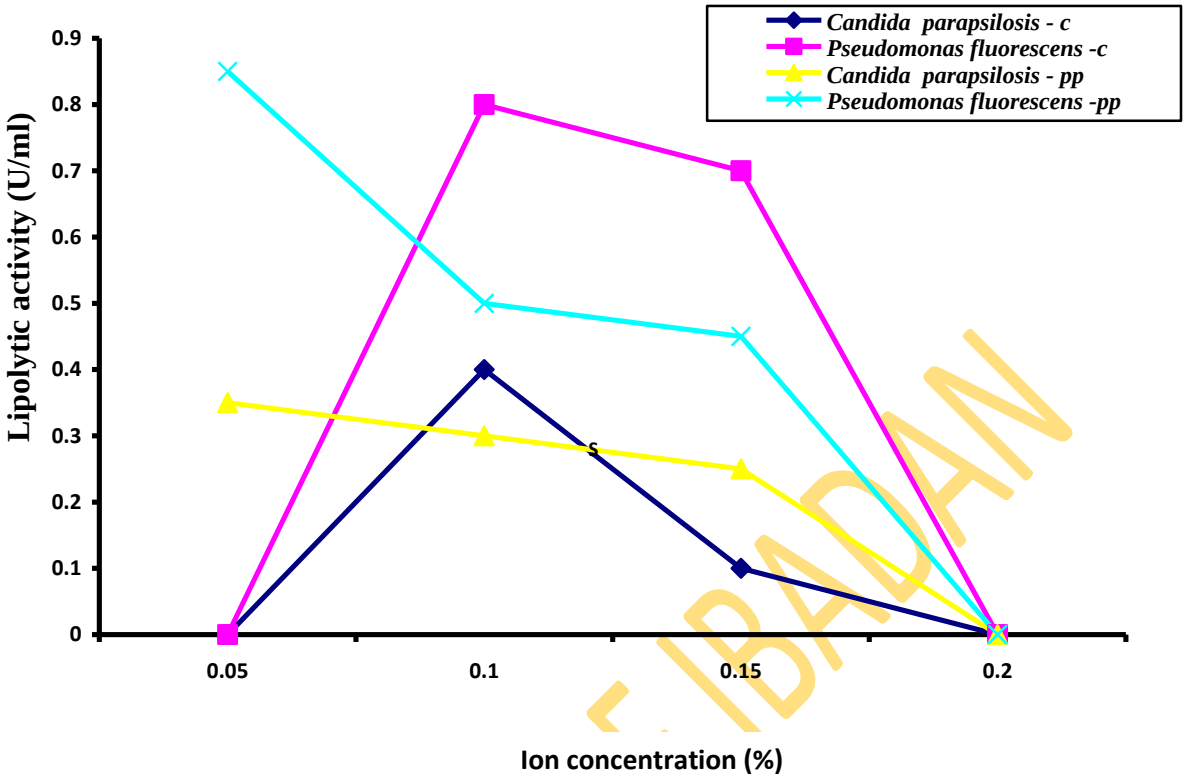

Fig. 4.40: Comparison of the effect of ion ($NaNO_3$) on the activity of crude(c) and partially purified(pp) lipase of *Candida parapsilosis* and *Pseudomonas fluorescens*

4.7 Enzyme Kinetics

K_m and V_{max} of *Pseudomonas* and *Candida parapsilosis* were obtained from Lineweaver-Burke plot of the reciprocal of substrate concentration against that of measured rate of hydrolysis. Results showed that apparent K_m and V_{max} values of the two lipases to be 0.7670 mg/ml and 0.702 µg/s respectively for purified *Pseudomonas fluorescens* while purified *Candida parapsilosis* lipase had 1.428 mg/ml and 1.095 µg/s respectively. These are shown in Figures 4.41 and 4.42.

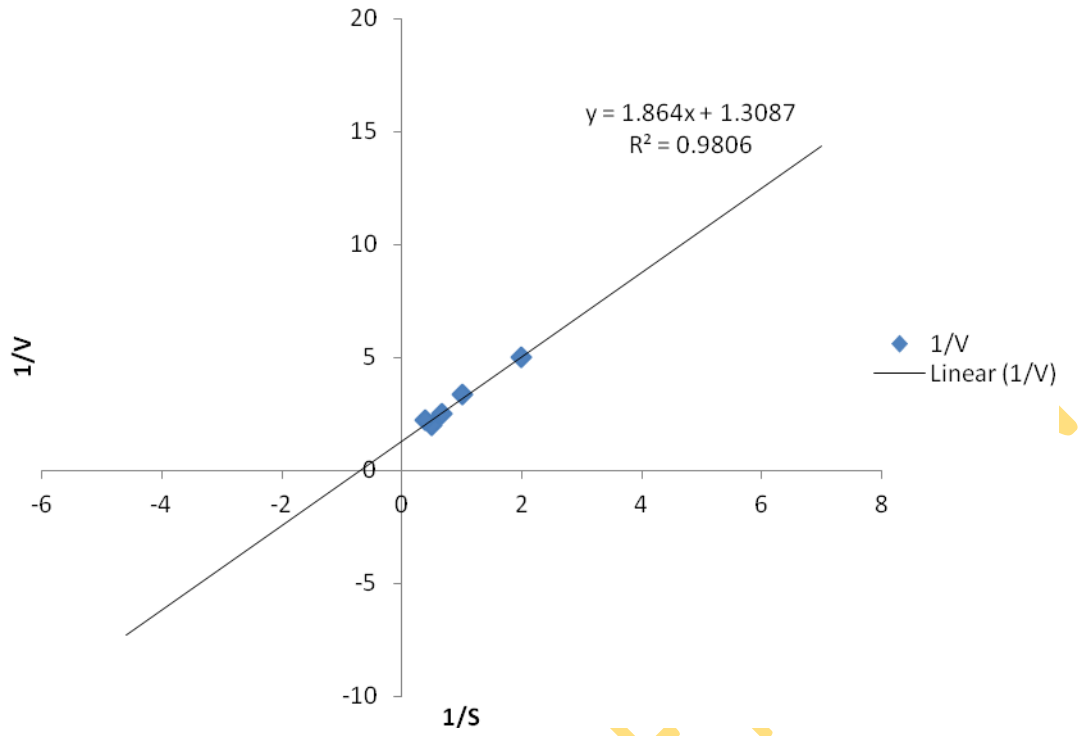

Fig. 4.41: Lineweaver-Burke plot of the reciprocal of lipase activity of *Pseudomonas fluorescens* against that of substrate concentration

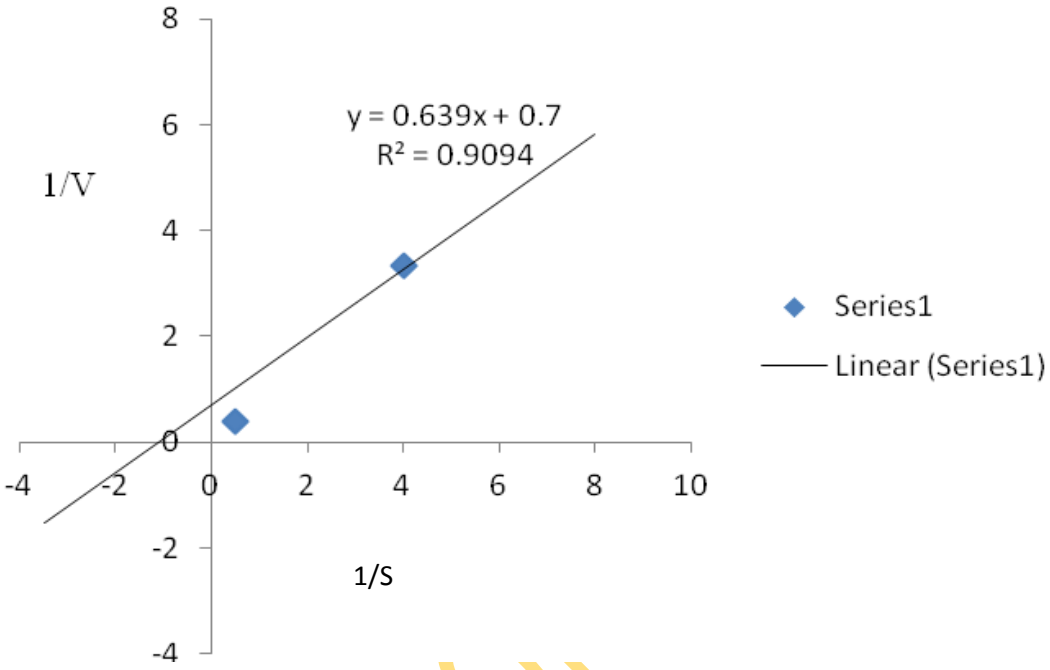

Fig. 4.42: Lineweaver-Burke plot of the reciprocal of lipase activity of *Candida parapsilosis* against that of the substrate concentration

4.8 Degradation of the Olive Oil in the Laboratory by Test Organisms

Table 4.10 shows that the concentration of residual oil of *Pseudomonas fluorescens* from medium 1 was significantly lower on day 15 during the period of 25 days but for *Candida parapsilopsis* on day 0 the concentration was significantly lower than others. For culture medium 2 concentration of oil recovered from *Pseudomonas fluorescens* culture medium was significantly higher on day 20, it has the least concentration on day 15 and day 0 as shown on Table 4.11.

Table 4.12 shows the plate count of the isolates from culture medium 1. On day 20 for *Pseudomonas fluorescens,* the value 12.97 ($\log_{10}$cfu/ml) was significantly higher than those of other days and day 5 was the least. *Candida parapsilosis* had plate count increase over the entire period of degradation. In the case of isolation from medium 2, isolation from *Pseudomonas* culture medium shows increase in plate count over the period of the degradation, this also applies to *Candida parapsilosis*, as shown on Table 4.13, with significant difference over the period of degradation.

Table 4.14 shows pH of culture medium 1. For *Pseudomonas fluorescens* on day 15, the pH was significantly lower than others and significantly higher on days 10 and 20. *Candida parapsilosis* had lower pH value on day 25. Table 4.15 shows that of culture medium 2, there was no significant difference for *Pseudomonas fluorescens* on the 5th and 25th days. pH for culture medium of *Candida parapsilosis* shows no significant difference from days 15 to 25, it also shows a decrease in pH value as shown on Table 4.15.

Table 4.10. Residual oil concentration in culture medium (1) degraded by individual soil microorganism over 25 days

	Degradation period (days) / Concentration (mol/l)					
	0 Day	5th Day	10th Day	15th Day	20th Day	25th Day
Pseudomonas fluorescens	0.00064 ± 0.0000^{b}	0.0114 ± 0.0003^{a}	0.0081 ± 0.0006^{b}	0.0031 ± 0.0011^{c}	0.0075 ± 0.0009^{b}	0.0067 ± 0.0007^{b}
Candida parapsilosis	0.0064 ± 0.0000^{d}	0.0130 ± 0.0004^{a}	0.0098 ± 0.0005^{b}	0.0079 ± 0.0002^{c}	0.0093 ± 0.0001^{b}	0.0091 ± 0.0003^{b}

Means with different superscripts across the rows are significantly different at $P \leq 0.05$ using Duncan's Multiple Range Test

Culture medium 1 consisting (g/L) of KH_2PO_4, 7.584; K_2HPO_4, 0.80; $MgSO_4.7H_2O$, 0.80; $CaCl_2$, 0.16; $(NH_4)_2NO_3$, 0.80; $FeSO_4$, 0.16; olive oil 2%, pH maintained at 7.0.

Table 4.11. Residual oil concentration in culture medium (2) degraded by individual soil microorganism over 25 days

	Degradation period (days) / Concentration (mol/l)					
	0 Day	5th Day	10th Day	15th Day	20th Day	25th Day
Pseudomonas fluorescens	0.00064 ± 0.0000^{b}	0.0105 ± 0.0003^{ab}	0.0096 ± 0.0003^{ab}	0.0066 ± 0.0004^{ab}	0.0133 ± 0.0021^{a}	0.0096 ± 0.0022^{ab}
Candida parapsilosis	0.0064 ± 0.0000^{c}	0.0140 ± 0.0001^{a}	0.0075 ± 0.0007^{bc}	0.0096 ± 0.0011^{b}	0.0091 ± 0.0004^{b}	0.0094 ± 0.0013^{b}

Means with different superscripts across the rows are significantly different at $P \leq 0.05$ using Duncan's Multiple Range Test

Culture medium 2 consisting (g/L) of KH_2PO_4, 7.584; K_2HPO_4, 0.80; $MnSO_4.4H_2O$, 0.80; NaCl, 0.16; $(NH_4)_2NO_3$, 0.80; $Fe_2(SO_4)_3$, 0.08; olive oil 2%,

Table 4.12. Plate count of the isolates in culture medium (1) during degradation over 25 days

	Degradation period (days) / Count (log_{10}cfu/ml)				
	5th Day	10th Day	15th Day	20th Day	25th Day
Pseudomonas fluorescens	8.81±0.0335^{e}	10.39±0.0266^{d}	12.57±0.0118^{c}	12.97±0.0118^{a}	12.78±0.0035^{b}
Candida parapsilosis	10.27±0.0353^{e}	10.85±0.0061^{d}	10.98±0.0067^{c}	8.77±0.0037^{b}	9.51±0.0335^{a}

Means with different superscripts across the rows are significantly different at P≤ 0.05 using Duncan's Multiple Range Test

Table 4.13. Plate count of the isolates in culture medium (2) during degradation over 25 days

	Degradation period (days) / Count (log_{10}cfu/ml)				
	5th Day	10th Day	15th Day	20th Day	25th Day
Pseudomonas fluorescens	7.91±0.0053^{e}	9.80±0.1990^{d}	10.59±0.1110^{c}	11.90±0.0055^{b}	12.70±0.0000^{a}
Candida parapsilosis	8.76±0.0150^{d}	8.92±0.0131^{c}	9.64±0.0396^{b}	9.71±0.0211^{ab}	9.77±0.0074^{a}

Means with different superscripts across the rows are significantly different at P≤ 0.05 using Duncan's Multiple Range Test

Table 4.14. pH changes in the culture medium (1) under degradation by individual soil microorganism over 25 days

	Degradation period (days) / pH				
	5th	10th	15th	20th	25th
Pseudomonas fluorescens	5.05±0.05^{ab}	5.25±0.05^{a}	4.90±0.10^{c}	5.20±0.00^{a}	5.10±0.00^{ab}
Candida parapsilosis	4.15±0.05^{b}	4.55±0.05^{a}	4.45±0.05^{a}	3.85±0.05^{c}	3.55±0.05^{d}

Means with different superscripts across the rows are significantly different at P≤ 0.05 using Duncan's Multiple Range Test

Table 4.15. pH changes in the culture medium (2) under degradation by individual soil microorganism over 25 days

	Degradation period (days) / pH				
	5th	10 th	15th	20th	25th
Pseudomonas fluorescens	3.70±0.20^{ab}	4.25±0.05^{a}	3.65±0.05^{b}	4.25±0.05^{a}	3.95±0.05^{ab}
Candida parapsilosis	4.20±0.10^{b}	4.55±0.05^{a}	3.50±0.10^{c}	3.70±0.10^{c}	3.50±0.00^{c}

Means with different superscripts across the rows are significantly different at P≤ 0.05 using Duncan's Multiple Range Test

4.9 Fatty Acid Methyl Ester Analysis

On Table 4.16, the percentage composition of the released components of fatty acid degraded by *Pseudomonas fluorescens* and *Candida parapsilosis* using medium 1 over 25days is shown. It can be seen that Oleic acid has the highest percentage composition (73.1831%) starting from day 5, the percentage composition of oleic acid reduced to 72.2548%, whereas stearic acid for instance increased from 4.5241% to 5.5839% on day 10.

After 15days of fermentation, percentage composition of oleic acid reduced to 72.039. From 72.0390% percentage oleic acid composition was drastically reduced to 67.8111% as seen on day 20. However, on the 20th day, oleic acid increased a little from 67.8111% to 69.4782% on the 25th day, stearic acid composition however increased over the period of 25days from 4.5241% on day 5 to 8.7102% on day 25 as shown on Table 4.16. In the case of *Candida parapsilosis*, percentage oleic acid on the 5th day was 74.8731%. Day 10 recorded 72.5577% of oleic acid composition. The composition was 71.0757% on the 15th day, it was reduced substantially by the 20th day to 69.2278%. However, on the 25th day, it came up to 70.0411% shown on Table 4.16.

In case of the released fatty acid degraded by *Pseudomonas fluorescens* from culture medium 2 the percentage composition of oleic acid on day 5 was 73.4490%, this is shown in Table 4.17. After 10 days, it was 72.1077%. On the 15th day it was higher 72.6759%, however on day 20 it was brought down to 69.2945% by *Pseudomonas fluorescens* in culture medium 2. Finally, on day 25, it was 69.2167%. In the case of fermentation by *Candida parapsilosis*, by day 5, the percentage of oleic acid was 74.4584%, however by the 10th day, oleic acid composition reduced to 73.3139%, which was further reduced to 71.4433% on the 15th day. Day 20 marked a reduction in oleic acid composition to 69.4455%. Increase in oleic acid percentage composition occurred on 25th day of fermentation by *Candida parapsilosis*, as shown on Table 4.17.

Table 4.18 shows the overall percentage oleic acid released from the residual oil by *Pseudomonas fluorescens* and *Candida parapsilosis* over 25days. The breakdown pattern of the oil can be seen by the pattern of reduction in the oleic acid.

4.10 PHYSICO-CHEMICAL PARAMETERS

The physico-chemical parameters of the polluted and unpolluted soil samples were shown in Table 4.19. The oil mill polluted soil sample was blackish, while the unpolluted garden soil was dark–brown. As can be seen all the parameters measured showed values higher in oil mill polluted soil than in the unpolluted soil.

Table 4.16: Percentage composition of released fatty acid degraded by *Pseudomonas fluorescens* (PF) and *Candida parapsilosis* (CP) (from culture medium 1) over 25 days

		Degradation period (days) / organisms / percentage composition									
S/N	**Fatty acid**	**5**		**10**		**15**		**20**		**25**	
		PF	**CP**	**PF**	**CP**	**PF**	**CP**	**PF**	**CP**	**PF**	**CP**
1	FGN1	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}
2	FGN2	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}
3	FGN3	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}
4	F001	0.0698^{j}	0.0820^{i}	0.0834^{h}	0.1090^{f}	0.2382^{e}	0.0900^{g}	0.3169^{d}	0.3822^{c}	0.4531^{b}	0.5658^{a}
5	F002	11.5095^{i}	10.1880^{j}	11.7429^{h}	11.7739^{g}	13.8403^{e}	13.7205^{f}	16.4771^{b}	15.7671^{d}	16.6447^{a}	16.3746^{c}
6	F003	0.0792^{b}	0.0941^{a}	0.0513^{d}	0.0674^{c}	0.0226^{f}	0.0304^{e}	0.0111^{h}	0.0211^{g}	0.0000^{i}	0.0000^{i}
7	FCS1	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}
8	FCS2	4.5241^{j}	4.5799^{i}	5.5839^{h}	5.5983^{g}	5.8617^{f}	6.2702^{e}	8.8069^{a}	7.6284^{d}	8.7102^{b}	8.2155^{c}
9	FCS3	73.1831^{b}	74.8731^{a}	72.2548^{d}	72.5577^{c}	72.0390^{e}	71.0757^{f}	67.8111^{j}	69.2278^{i}	69.4782^{h}	70.0411^{g}
10	FCS4	9.4021^{a}	9.0634^{b}	9.2607^{c}	8.8472^{d}	6.8863^{f}	8.25074^{e}	5.9675^{h}	6.040^{g}	4.091^{j}	4.0864^{j}
11	FSF1	0.9986^{a}	0.8426^{b}	0.7397^{c}	0.6762^{d}	0.5112^{e}	0.4394^{g}	0.3587^{h}	0.4528^{f}	0.2796^{j}	0.3047^{i}
12	FSF2	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0000^{a}
13	FSF3	0.0620^{d}	0.0731^{c}	0.0747^{b}	0.0978^{a}	0.0000^{a}	0.0000^{a}	0.0000^{e}	0.0000^{e}	0.0000^{e}	0.0000^{e}
14	FP01	0.0467^{d}	0.0553^{h}	0.0562^{g}	0.0735^{f}	0.1627^{a}	0.0333^{j}	0.0795^{e}	0.1517^{b}	0.1082^{d}	0.1302^{c}
15	FP02	0.0251^{e}	0.0296^{d}	0.0315^{c}	0.0405^{b}	0.0879^{a}	0.0186^{f}	0.0000^{g}	0.000^{g}	0.0000^{g}	0.0000^{g}
16	Lignoceric	0.100^{i}	0.1187^{h}	0.1209^{g}	0.1584^{f}	0.3499^{a}	0.0715^{j}	0.1714^{e}	0.3285^{b}	0.2348^{d}	0.2816^{c}

Means with different superscripts across the rows are significantly different at $P \leq 0.05$ using Duncan's Multiple Range Test

Table 4.17: Percentage composition of released fatty acid degraded by *Pseudomonas fluorescens* (PF) and *Candida parapsilosis* (CP) (from culture medium 2) over 25 days

		Degradation period (days) / organisms / percentage composition									
S/N	**Fatty acid**	**5**		**10**		**15**		**20**		**25**	
		PF	**CP**	**PF**	**CP**	**PF**	**CP**	**PF**	**CP**	**PF**	**CP**
1	FGN1	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}
2	FGN2	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}
3	FGN3	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}
4	F001	0.0625 ^{j}	0.0815 ^{g}	0.0720 ^{i}	0.0721 ^{h}	0.1170 ^{f}	0.1564 ^{e}	0.2948 ^{d}	0.4040 ^{c}	0.6352 ^{a}	0.6020 ^{b}
5	F002	10.8173 ^{i}	10.2901 ^{j}	11.1341 ^{h}	11.1909 ^{g}	14.0435 ^{e}	13.4261 ^{f}	15.6081 ^{c}	15.2642 ^{d}	16.3984 ^{a}	16.0694 ^{b}
6	F003	0.0712 ^{b}	0.0937 ^{a}	0.0440 ^{d}	0.0443 ^{c}	0.0278 ^{g}	0.0421 ^{e}	0.0098 ^{h}	0.0323 ^{f}	0.0000 ^{i}	0.0000 ^{i}
7	FCS1	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}
8	FCS2	4.4830 ^{j}	4.5545 ^{i}	5.4319 ^{g}	5.1792 ^{h}	6.0439 ^{e}	6.0156 ^{f}	8.7267 ^{b}	7.8235 ^{d}	8.9695 ^{a}	8.4980 ^{c}
9	FCS3	73.4490 ^{b}	74.4584 ^{a}	72.1077 ^{e}	73.3139 ^{c}	72.6759 ^{d}	71.4433 ^{f}	69.2945 ^{i}	69.4455 ^{h}	69.2167 ^{j}	70.0905 ^{g}
10	FCS4	9.8983 ^{b}	9.5437 ^{c}	10.2956 ^{a}	9.4090 ^{d}	6.3022 ^{f}	8.1514 ^{e}	5.5103 ^{h}	6.0909 ^{g}	4.1199 ^{j}	4.1804 ^{i}
11	FSF1	1.0083 ^{a}	0.7033 ^{b}	0.6716 ^{c}	0.5457 ^{e}	0.5944 ^{d}	0.4714 ^{f}	0.3329 ^{h}	0.4674 ^{g}	0.2956 ^{j}	0.2074 ^{i}
12	FSF2	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}	0.0000 ^{a}
13	FSF3	0.0559 ^{d}	0.0727 ^{a}	0.0642 ^{c}	0.0646 ^{b}	0.0000 ^{e}	0.0000 ^{e}	0.0000 ^{e}	0.0000 ^{e}	0.0000 ^{e}	0.0000 ^{e}
14	FP01	0.0420 ^{j}	0.0547 ^{f}	0.0482 ^{h}	0.0436 ^{i}	0.0528 ^{g}	0.0794 ^{d}	0.0706 ^{e}	0.1490 ^{b}	0.1151 ^{c}	0.1415 ^{a}
15	FP02	0.0226 ^{f}	0.0292 ^{b}	0.0270 ^{e}	0.0272 ^{d}	0.0289 ^{c}	0.0429 ^{a}	0.0000 ^{g}	0.0000 ^{g}	0.0000 ^{g}	0.0000 ^{g}
16	Lignoceric	0.0898 ^{j}	0.1182 ^{f}	0.1037 ^{i}	0.1046 ^{h}	0.1137 ^{g}	0.1711 ^{d}	0.1522 ^{e}	0.3231 ^{a}	0.2495 ^{b}	0.2108 ^{c}

Means with different superscripts across the rows are significantly different at $P \leq 0.05$ using Duncan's Multiple Range Test

Table 4.18: Percentage of oleic acid released from residual oil by microbial isolates over 25 days

S/N	Sample Nature	Percentage Oleic Acid
1	1P5	73.1831
2	1P10	72.2548
3	1P15	72.0390
4	1P20	67.8111
5	1P25	69.4782
6	2P5	73.4490
7	2P10	72.1077
8	2P15	72.6759
9	2P20	69.2945
10	2P25	69.2167
11	1C5	74.8731
12	1C10	72.5577
13	1C15	71.0757
14	1C20	69.2278
15	1C25	70.0411
16	2C5	74.4537
17	2C10	73.3139
18	2C15	71.4433
19	2C20	69.4455
20	2C25	70.0905

Key

1 – Culture medium 1
2 – Culture medium 2
P – *Pseudomonas fluorescens*
C – *Candida parapsilosis*
5,10,15,20 and 25 – Period of degradation in days

Table 4.19: Physico-chemical analysis of soil samples

Parameter	Unpolluted Garden Soil (Control)	Oil Mill Polluted Soil (Sample)
Nitrogen (g/kg)	1.34	2.21
Total organic carbon	11.4	23.4
Sodium (Cmol/kg)	0.49	1.35
Magnesium (Cmol/kg)	1.72	11.04
Available phosphorus (mg/kg)	16.20	24.15
pH in water	6.5	7.7
Water holding capacity (%)	12.2	42.4
Colour	Dark-brown	Blackish

4.11 Bioremediation Experiment

4.11.1 Total Viable Count Enumeration:

The growth of microbial species in all the soil samples increased from 2 to 12 weeks in almost all the experiments. However, by the 12th week, there was recorded decrease in growth in the non-sterile soil of the oil mill sample with and without mixing.

Figure 4.43 shows total viable microbial count in sterile soil samples from oil mill with and without mixing. Averagely, there was significant increase from the second week to the twelfth week. Figure 4.44 shows total viable microbial count in non-sterile soil samples from oil mill with and without mixing, there was also increase in microbial count from the second to the tenth, but on the twelfth week there was decrease in microbial counts.

Comparatively, the microbial count in sterile control soil samples with and without mixing presented in Figure 4.45 was almost constant between the 2nd and 4th week and then gradually decreased from the 4th to the 8th week. Thereafter, a gradual increase was observed from the 10th to the 12th week, although there were some exceptional cases as in soil treated with *Pseudomonas fluorescens*, mixed on the 6th week. Figure 4.46 which show the total viable microbial count in non-sterile soil samples (control) with and without mixing also shows a similar growth pattern with the sterile counterpart.

The result of sterile soil sample with and without mixing option, from palm oil is shown in Figure 4.47. There was a general increase in total viable counts from the 2nd week to the 12th week, in the non- sterile counterpart as shown in Figure 4.48. There was a steady increase from the 2nd week to the 8th week and subsequently, a gradual decrease from the 10th to the 12th week.

The counts recorded in the sterile soil samples from palm kernel oil contaminated samples with and without mixing relatively increased over the 12 weeks of bioremediation. In the non-sterile soil sample counterpart, there was also steady increase from the 2nd week to the 8th week. However, from the 10th week there was gradual decrease in counts.

4.10.2 Gravimetric analysis of residual oil from bio-remediated soil samples

Figure 4.49 shows the total recoverable residual oil weight in sterile oil mill sample with and without mixing. At the beginning of the experiment, the weight of

extract from the oil mill polluted soil sample was 0.025g. They all reduced by the 4th week but decreased slightly by the 8th week before increasing on the 12th week, although the weight difference is negligible. However, there were some exceptions as shown on the figure. The non-sterile counterpart shows initial increase by the 4th week followed by decrease in their residual weight on the 8th week, followed by some showing increase again by the 12th week, whereas others showed decrease in residual weight by the 12th week (e.g POC non-mixed).

Figure 4.53 shows the total recoverable residual oil in sterile unpolluted sample with and without mixing. At the beginning of the experiment, the weight of extract from the unpolluted soil sample was 0.025g. All reduced by the 4th week and then increased slightly over the 12 weeks period, although the weight difference is negligible, however, PO1 non-mixed reduced over the period of the experiment. The non-sterile counterpart shown in Figure 4.54 shows that there was gradual decrease in residual weight over the 12 weeks period of remediation with an exceptional case seen in PO1 mixed which showed increase by the 8th week before a final drastic reduction by the 12th week, also the weight differences are almost negligible.

In the case of sterile soil sample purposely polluted with palm kernel oil with and without mixing, the total residual oil weight as shown in Figure 4.55 shows progressive decreases in weight from start through eight weeks before it significantly reduced by the 12th week, however, CO1 mixed is an exception. In the non-sterile counterpart shown in Figure 4.56 the same pattern of reduction was seen.

Figure 4.57 shows total recoverable residual oil in sterile palm oil contaminated soil sample with and without mixing, a progressive reduction over the 12 weeks period of remediation was noted, although some had slight increase from 4th to 8th week before finally reducing in weight on the 12th week. The same progressive reduction over the 12 weeks period of remediation was observed in the non-sterile counterpart however, PO1 non-mixed is an exception, its weight increased from the 4th week to the 8th week, before reducing drastically on the 12th week.

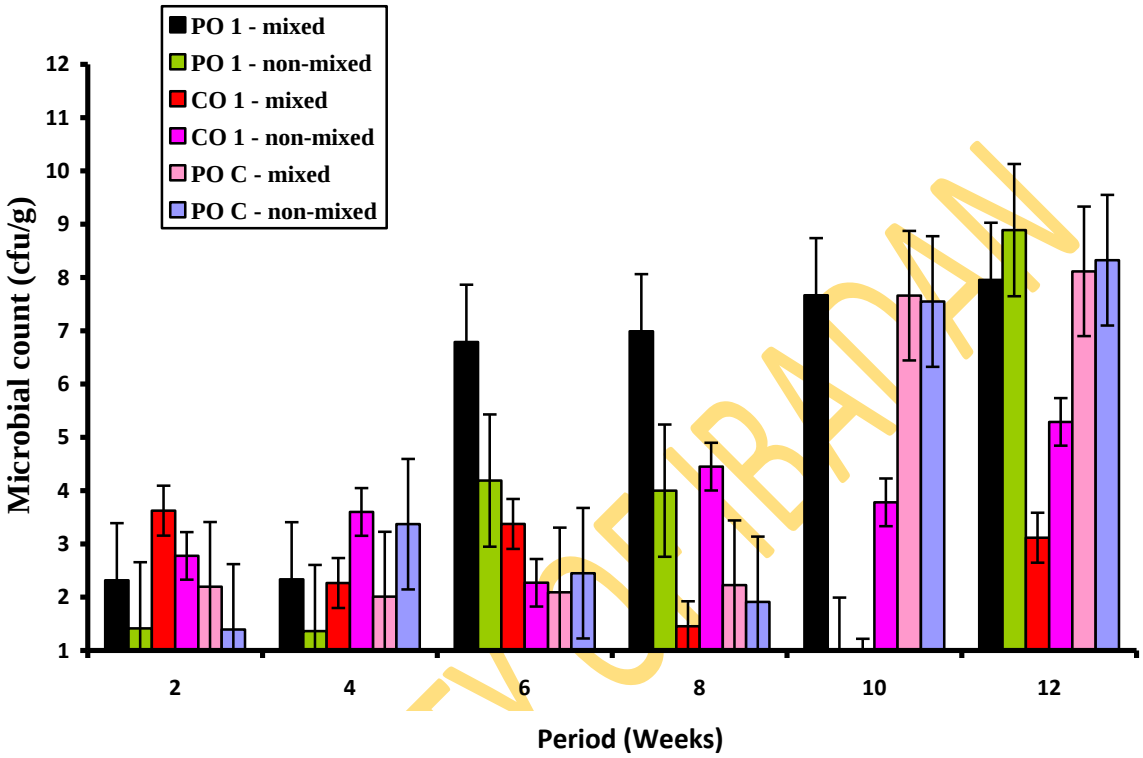

Fig.4.43: Total viable microbial count in sterile soil samples from oil mill with and without mixing

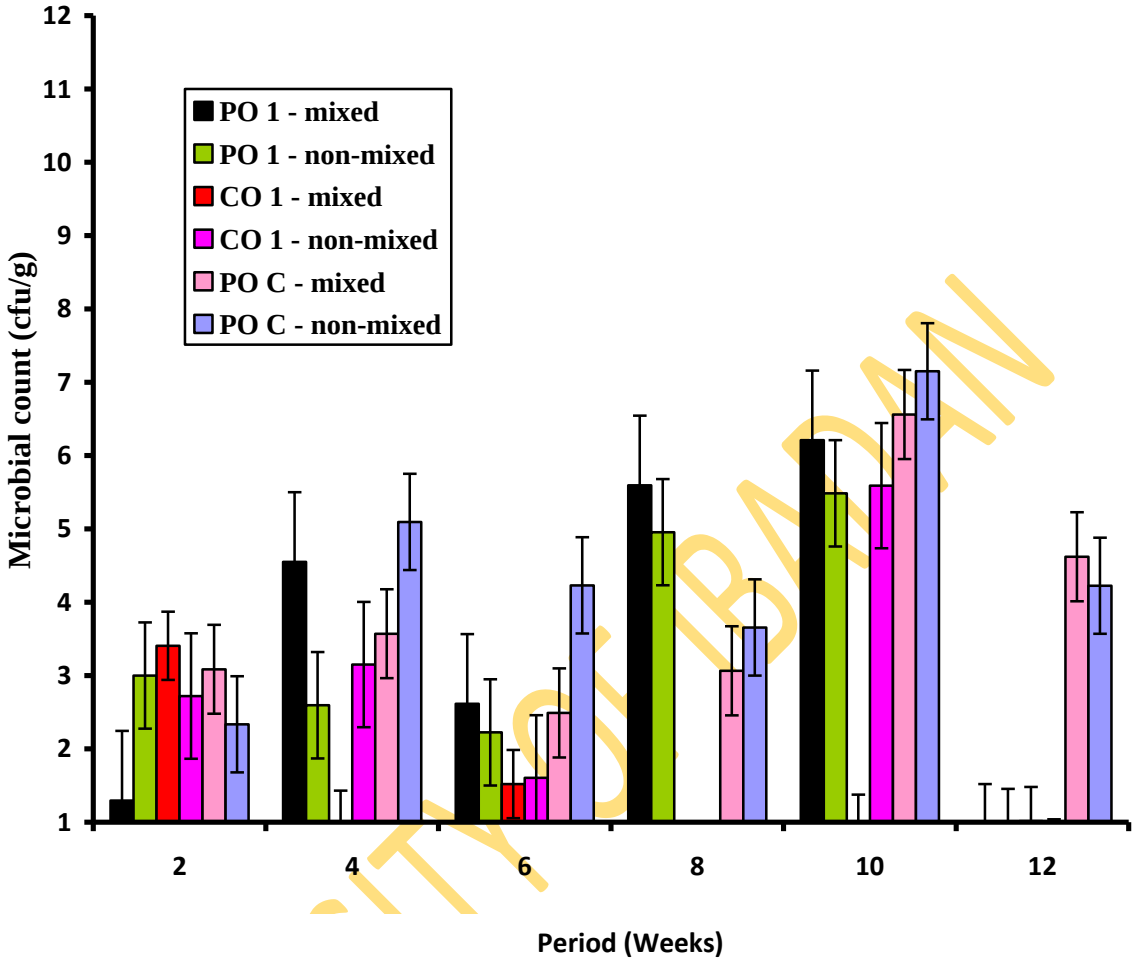

Fig.4.44: Total viable microbial count in non-sterile soil samples from oil mill with and without mixing

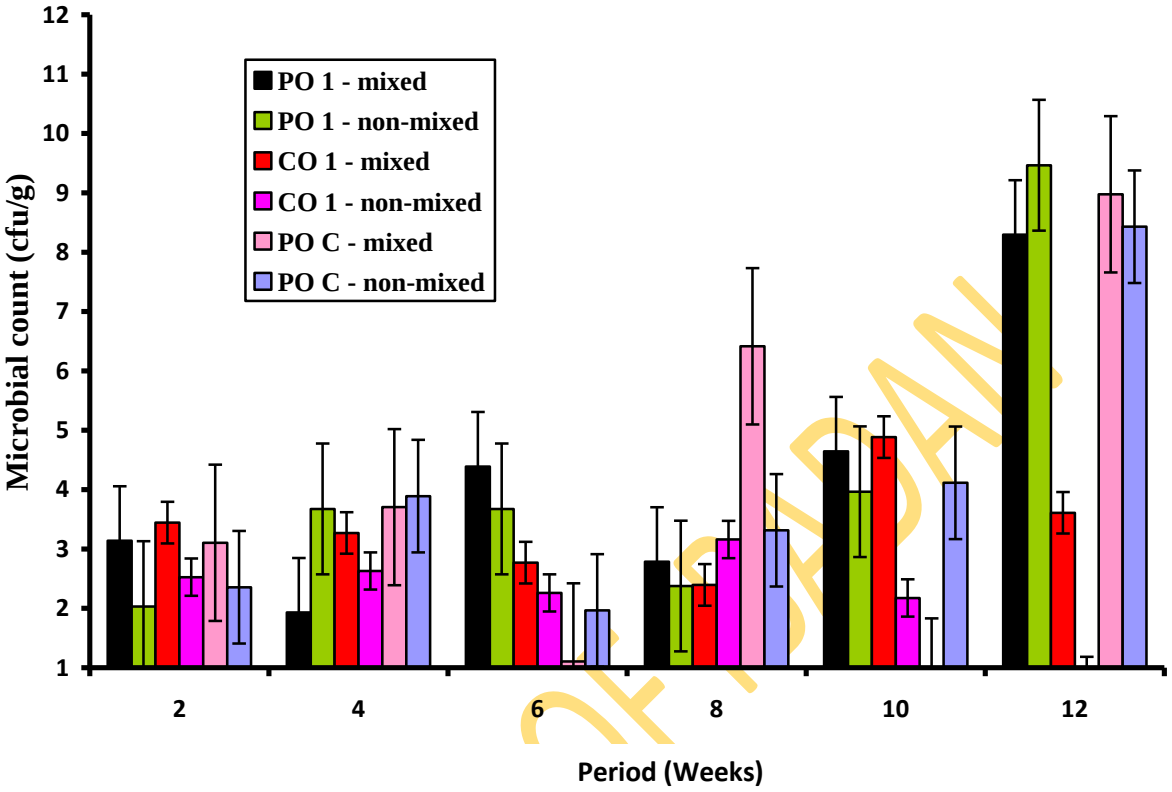

Fig.4.45: Total viable microbial count in sterile soil samples (control) with and without mixing

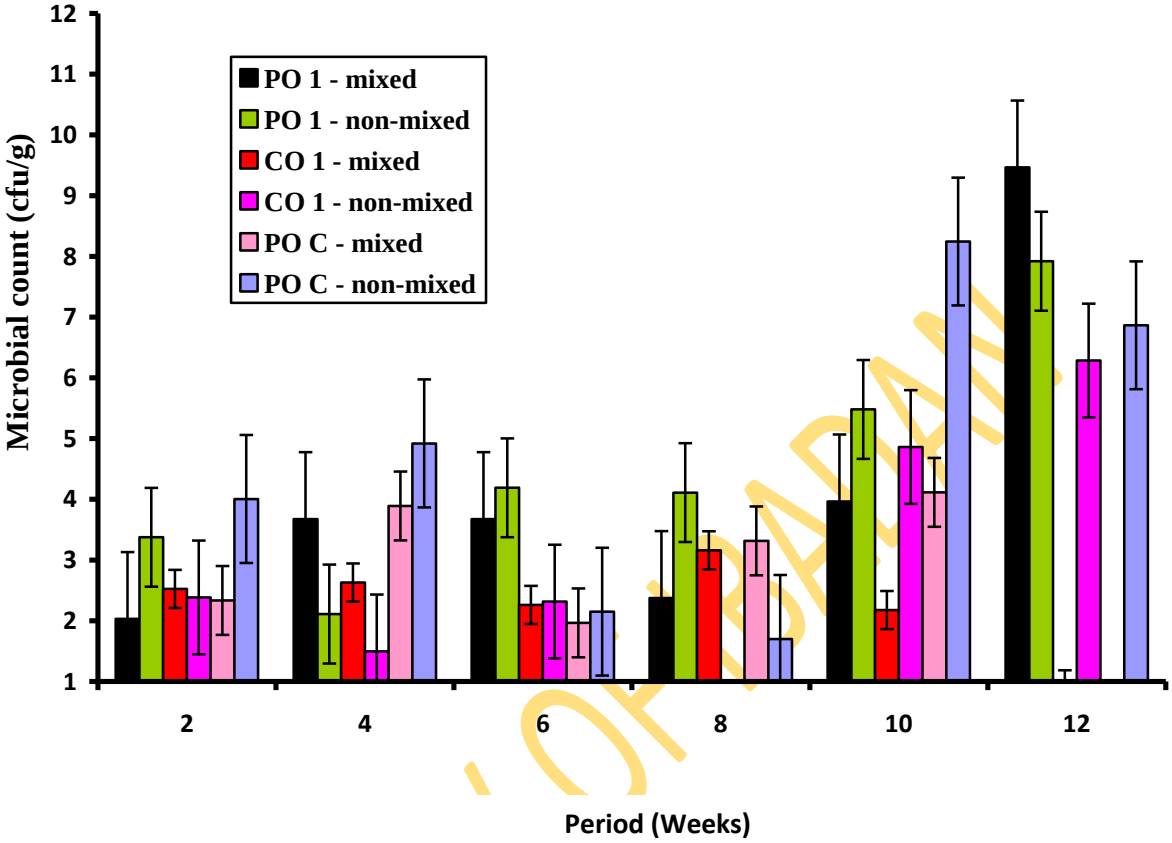

Fig.4.46: Total viable microbial count in non-sterile soil samples (control) with and without mixing

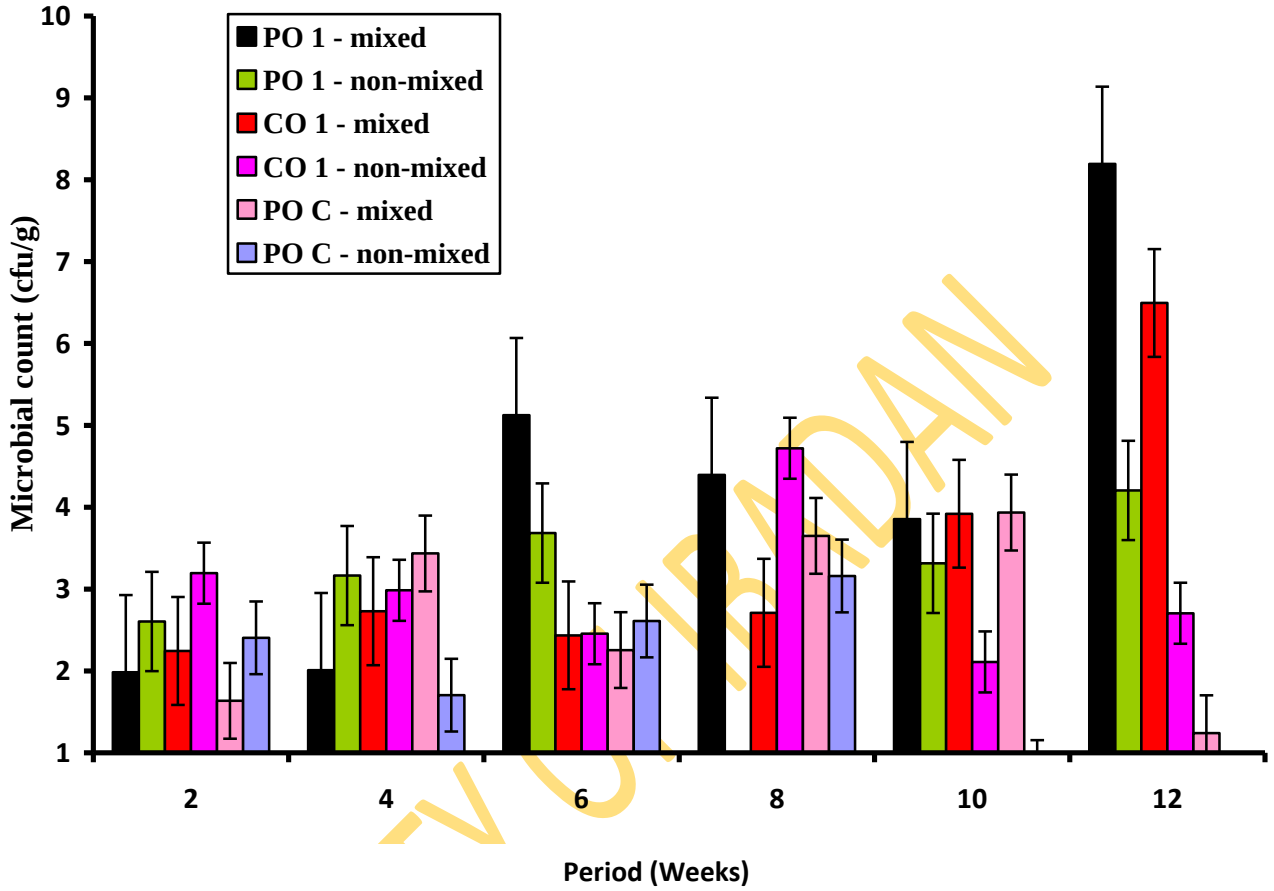

Fig.4.47: Total viable microbial count in sterile soil samples contaminated with palm oil with and without mixing

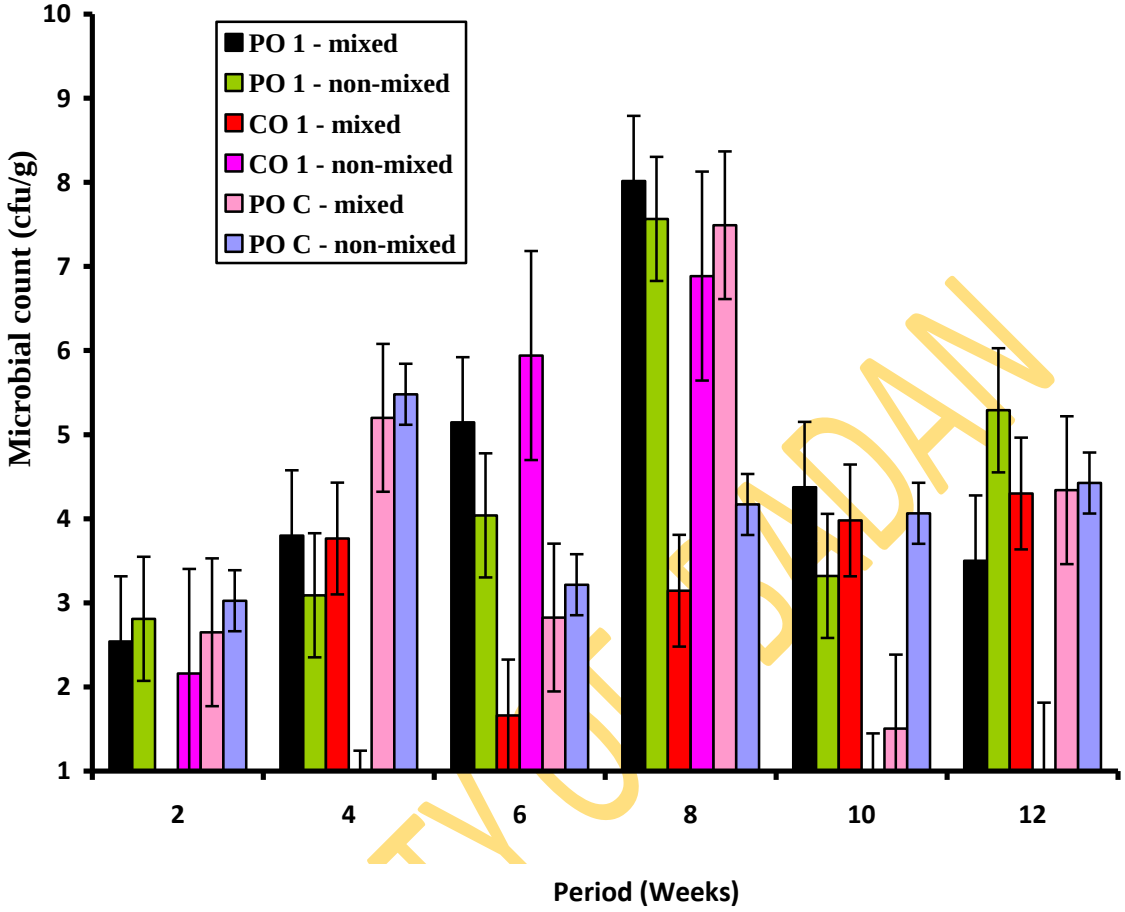

Fig.4.48: Total viable microbial count in non-sterile soil samples contaminated with palm oil with and without mixing

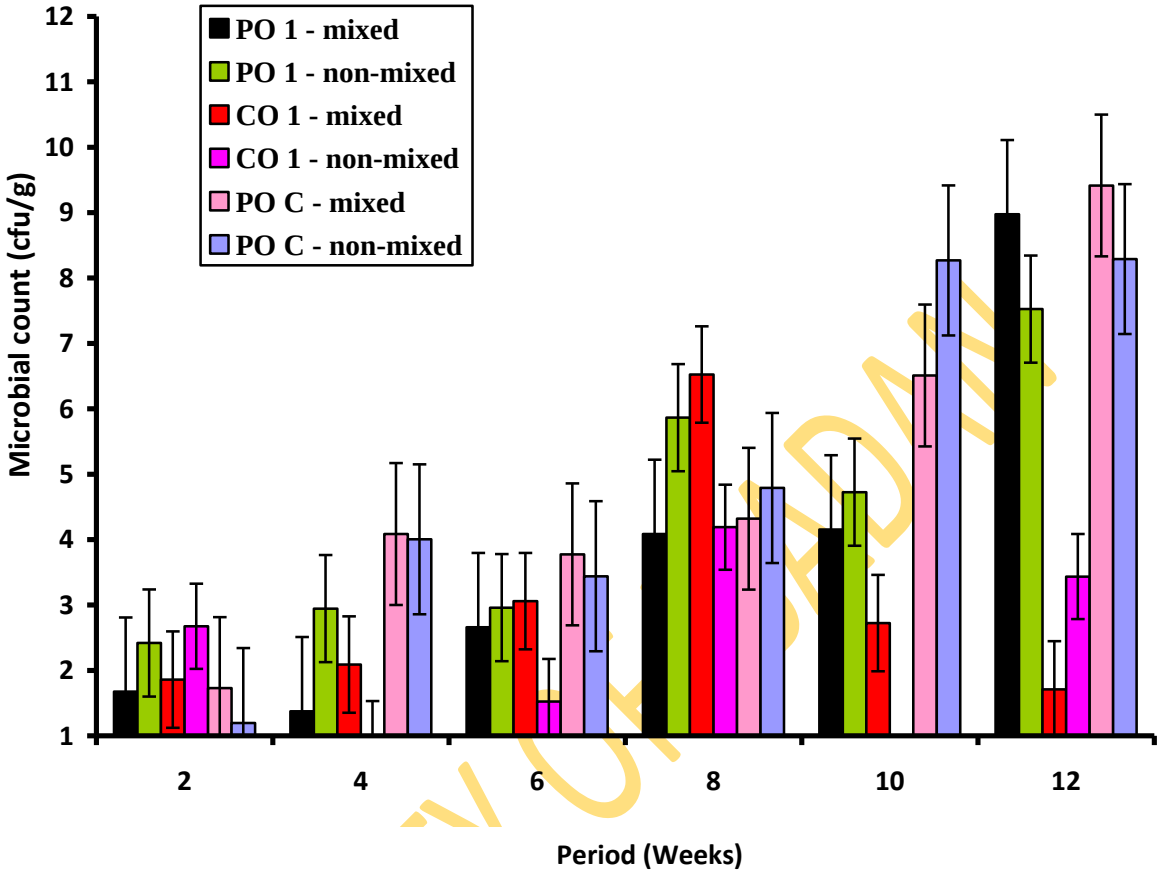

Fig.4.49: Total viable microbial count in sterile soil samples contaminated with palm kernel oil with and without mixing

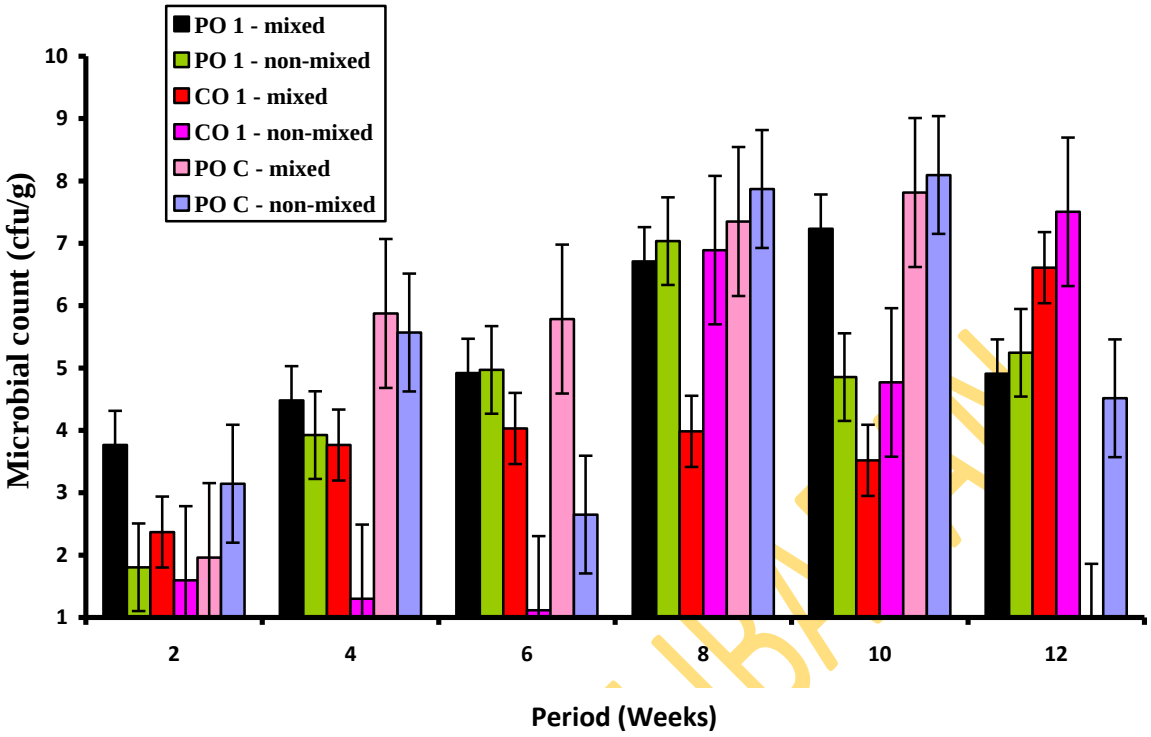

Fig.4.50: Total viable microbial count in non-sterile soil samples contaminated with palm kernel oil with and without mixing

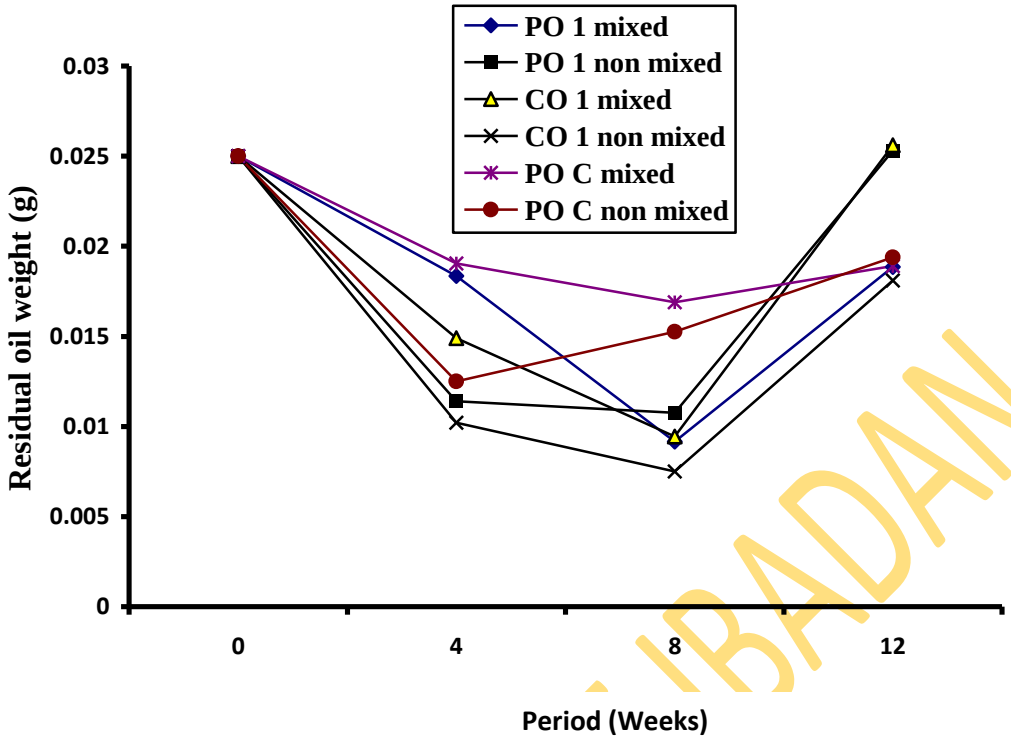

Fig.4.51: Total recoverable residual oil in sterile oil mill sample with and without mixing

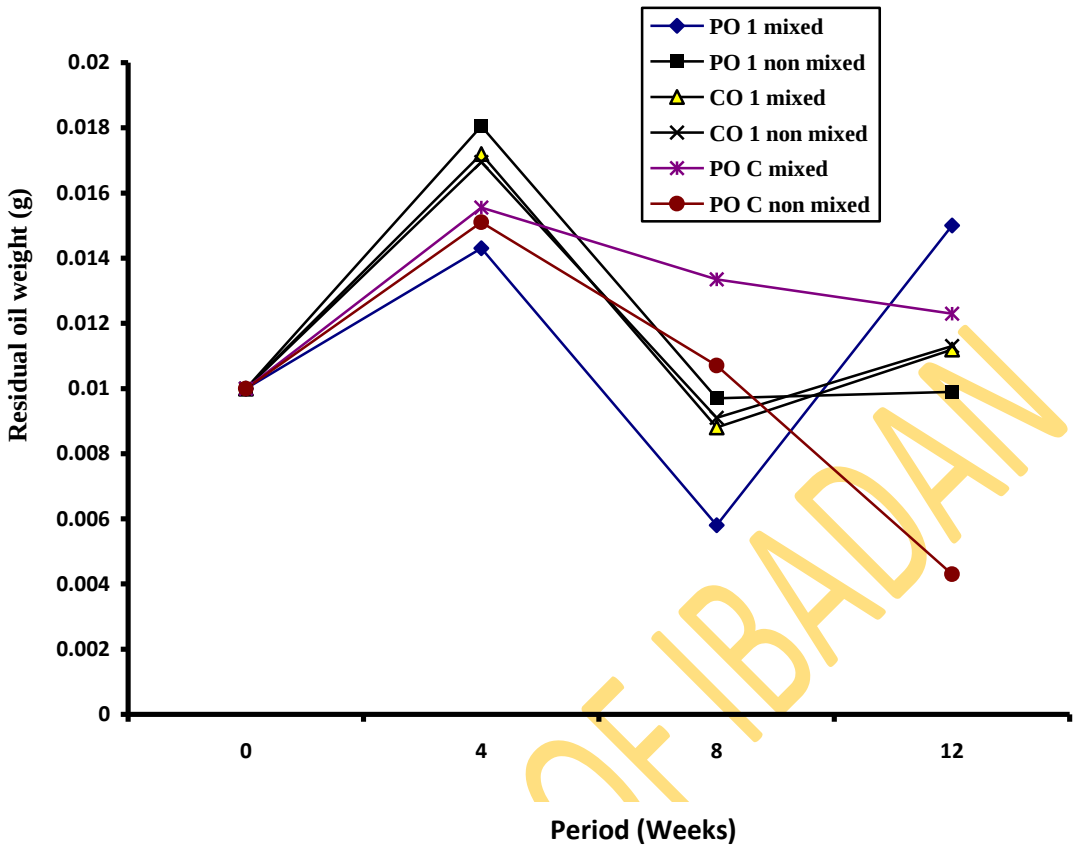

Fig.4.52: Total recoverable residual oil in non-sterile oil mill sample with and without mixing

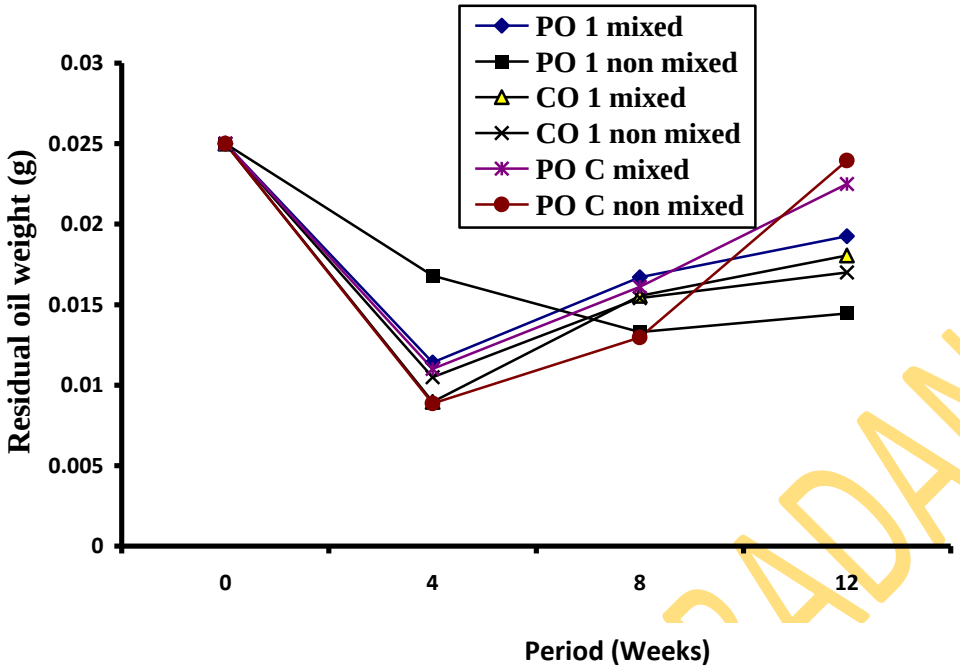

Fig.4.53: Total recoverable residual oil in sterile unpolluted sample with and without mixing

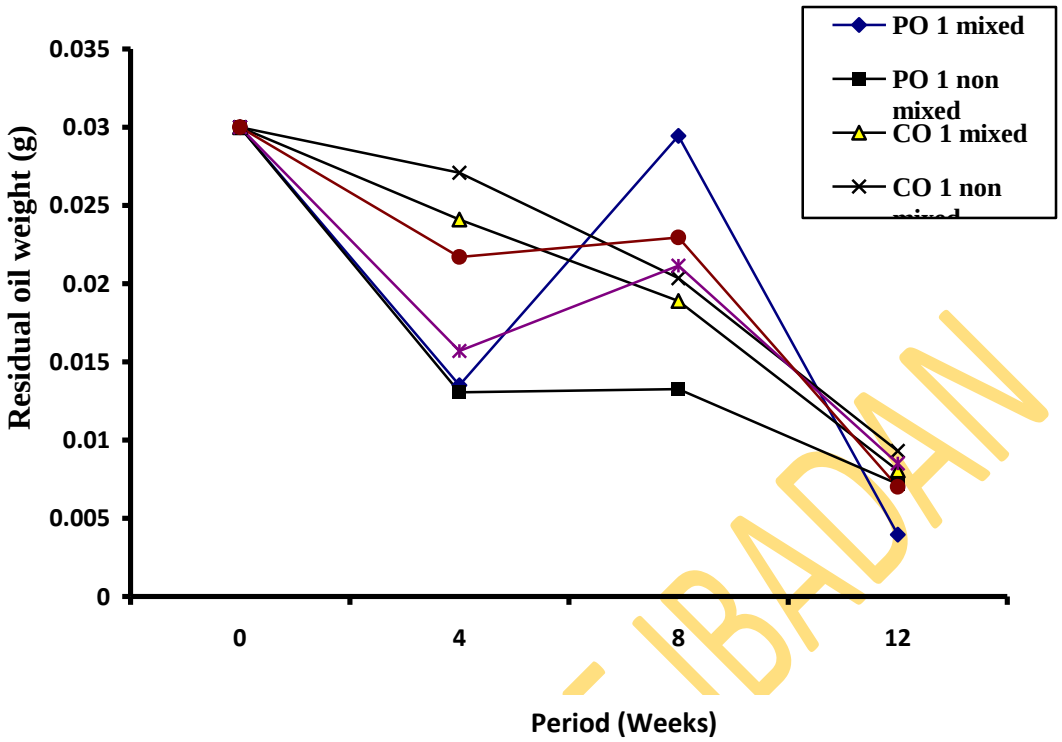

Fig.4.54: Total recoverable residual oil in non sterile unpolluted sample with and with out mixing

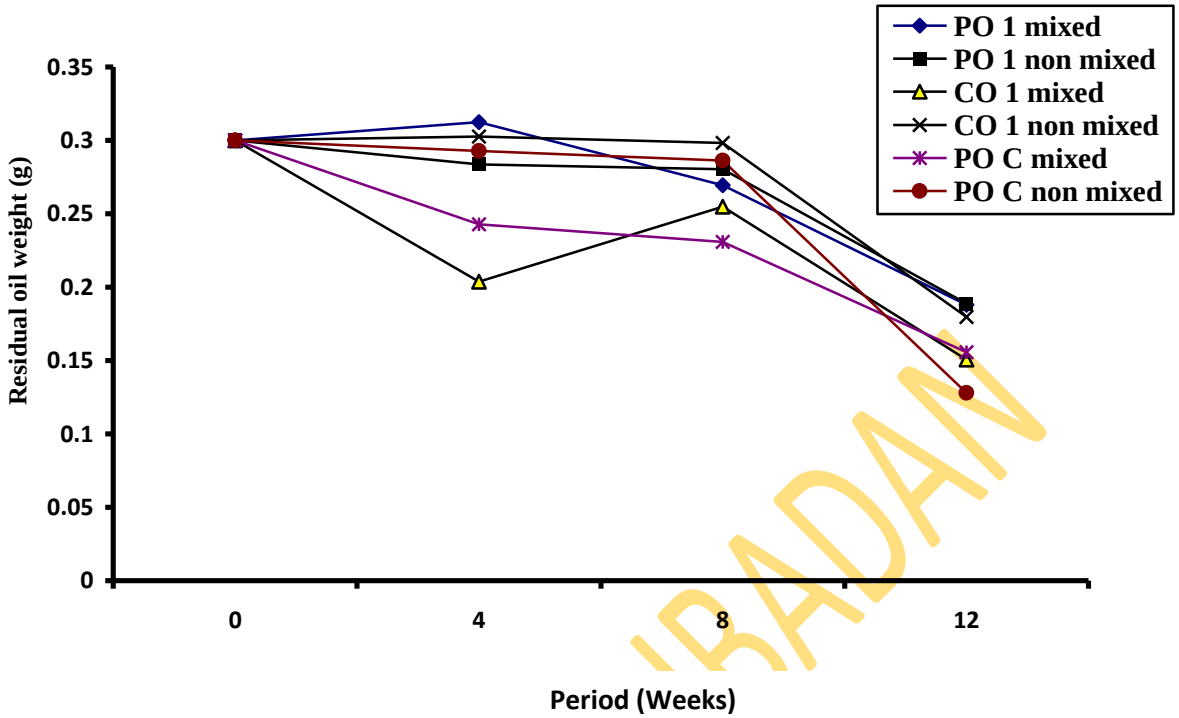

Fig.4.55: Total recoverable residual oil in sterile palm kernel oil contaminated sample with and without mixing

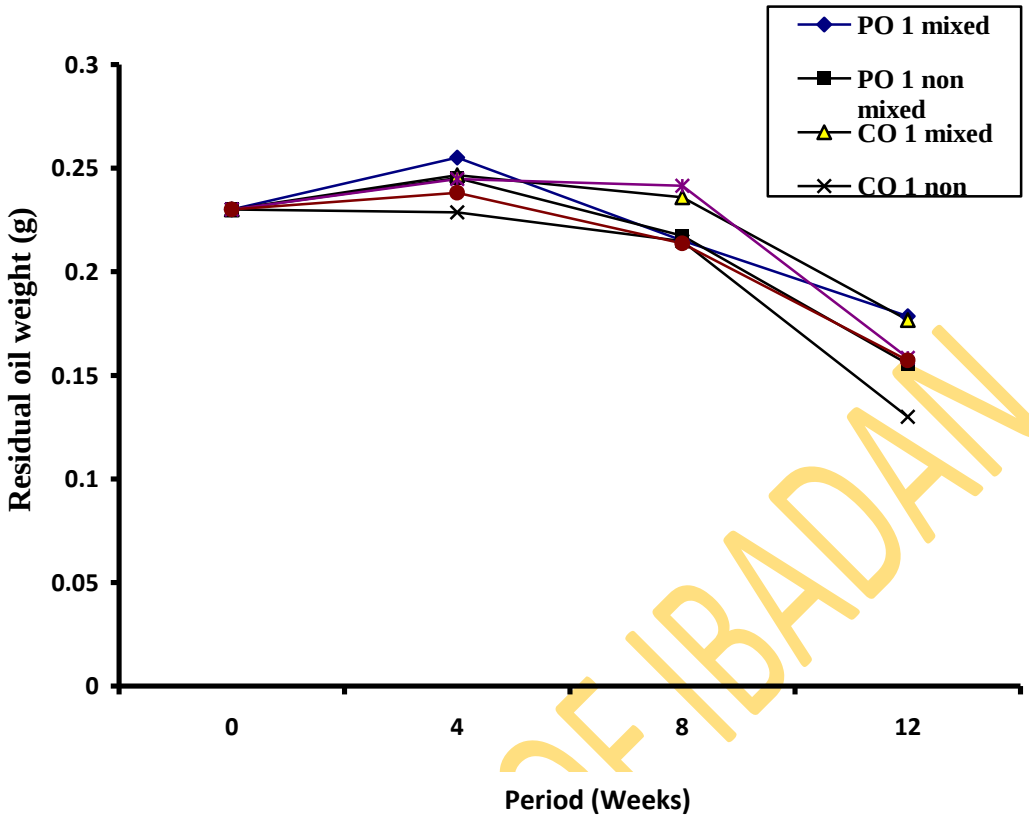

Fig.4.56: Total recoverable residual oil in non-sterile palm kernel oil contaminated sample with and without mixing

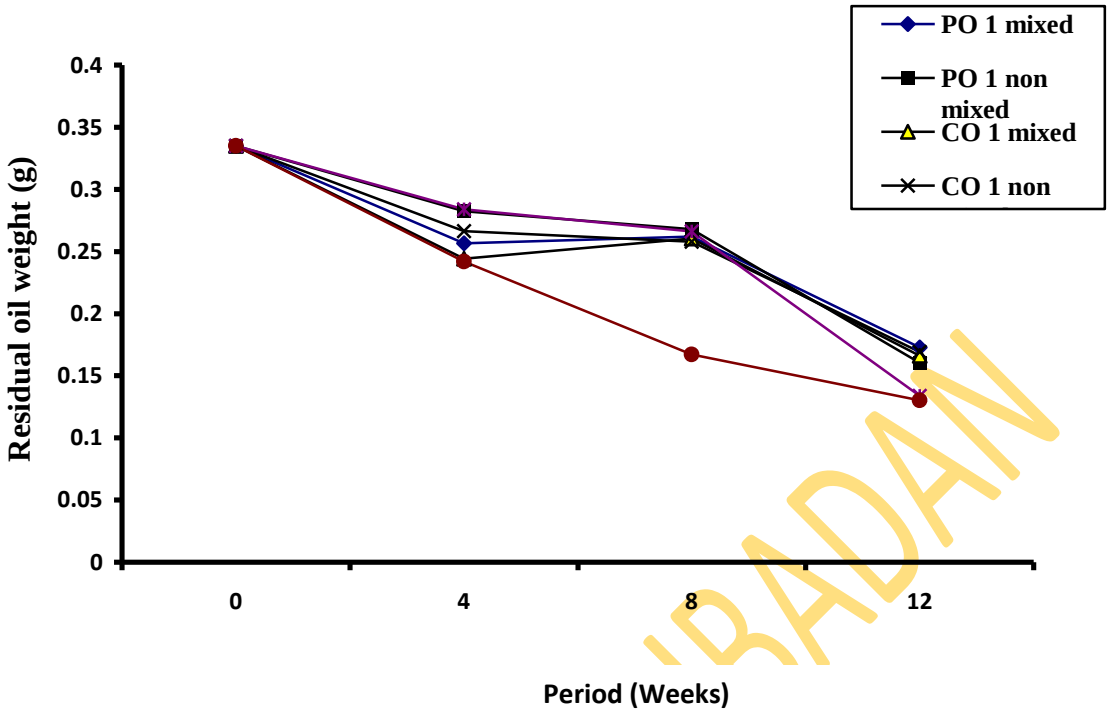

Fig.4.57: Total recoverable residual oil in sterile palm oil contaminated sample with and without mixing

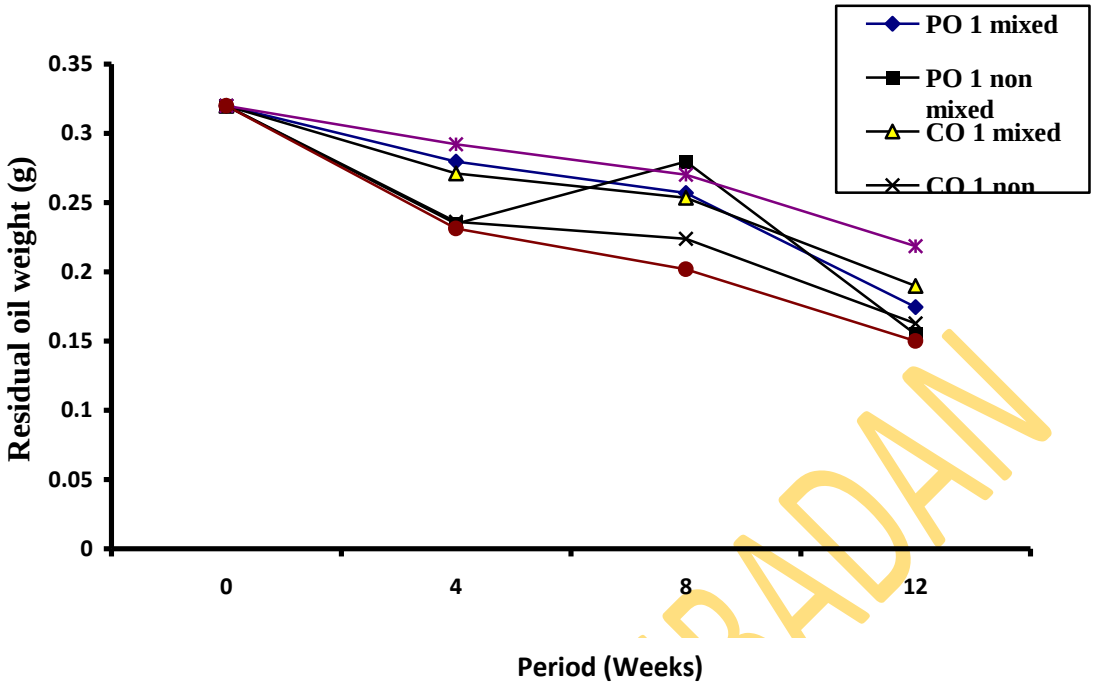

Fig.4.58: Total recoverable residual oil in non-sterile palm oil contaminated sample with and without mixing

CHAPTER FIVE

DISCUSSION

In the preliminary aspect of this research, 73 microorganisms belonging to the different groups were isolated from the different sources in bowls contaminated with the different types of edible oil and from the oil mill. Generally, the culture and biochemical properties of the isolates were used in their identification with reference to the Bergey's Manual of Systematic Bacteriology (Sneath, 1986).

The microbial isolates used in this study proved capable of lipase production. Indeed lipolytic activity has been observed for pure cultures of all the microbial genera used in this study (Sharma *et al.*, 2001; Saxena *et al.*, 2003; Chakraborty and Raj, 2008; Sharma *et al.*, 2011).

The formation of opaque zones of clearing around the colonies of the selected isolates is an indication of lipase production by the organisms. This was also reported by Sharma *et al.* (2001). Literatures have confirmed that under certain conditions, it is possible to isolate bacterial strains capable of degrading lipids by using selective medium containing required inducers (Loo *et al*., 2006). 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). Loo *et al.* (2006) used a selective medium, which contained Tween 80 or olive oil to screen through the appearances of clearing zones for the bacterial strain *Pseudomonas* sp.

Increase in microbial growth does not necessarily suggest increase in lipolytic activity as generally observed from this study. On the contrary, Becker *et al.* (1999), Keenan and Sabelnikov (2000) used microbial growth parameters to measure lipids degradation. However, according to Kramer (1971) and as seen in this study (physiological study of the selected organisms) an increase on biomass concentration

may not produce an increase in lipid matter hydrolysis, because lipase production is not a function of cell growth or concentration.

The obtained bacterial lipases were generally observed to work best in alkaline pH (Ece Yapasan, 2008). For instance, *Bacillus subtilis* produced maximum lipase activity at pH 7 (0.5 U/ml). In low and high medium pH tested, the lipase activity was less. This result is consistent with the report of Mohan *et al.* (2008). Who stated that the lipase activity of *Bacillus* sp was optimal at pH 7 during the 24 h culture period. Candida parapsilosis in this study showed lipase activity between pH 6 and 9.It has been well-documented that the optimum pH range of yeast lipases is generally between pH 5 and 8, with a few exceptions acting at low pH optima of 2.0 (Oishi *et al.*, 1999; Kakugawa *et al.*,2001; Vakhlu and Kour, 2006). It has also been shown that most of the yeast lipases are generally stable between pH 4.0 and 8.0.

In this study most of the bacteria isolated had their optimum growth temperature at 40^{o}C, whereas their optimum lipase activity was 27^{o}C. Ece Yapasan (2008) reported the optimum bacterial growth temperature to be 25^{o}C for *Pseudomonas* sp. with lipase enzyme showing activity above and below this temperature, although bacteria growth did not exceed 30^{o}C, enzyme works even above 30^{o}C. Temperature changes give rise to cleavage of hydrogen bonds between substrate and enzymes' active sites. Optimum temperature value promotes binding potential of enzyme and substrate (Ece Yapasan, 2008). However, in this study, temperature increase above known optimum tends most likely to denature the enzymes thereby reducing the enzyme activity.

The catalytic activities of the lipases in the current study were enhanced in the presence of Ca^{2+} ,Mg^{2+} and NH_3^{+}, but inhibited by Fe^{2+}, Zn^{2+} and Hg^{2+} . These results are in agreement with Chakraborty and Raj (2008). Ca^{2+} ions have been known to stimulate lipase activity in varying concentrations. It has been reported that in the presence of Ca^{2+}, lipase activity of *Bacillus licheniformis* strain H1 increased up to 120% (Khyami-Horani, 1996) while the activity of lipase from a *Pseudomonas* sp has been reported to be increased by 250% (Dong *et al.*, 1990). Ba^{2+} is also known to enhance lipase activity of lipase isolated from *Burkholderia* sp (Rathi *et al.*, 2001). Metal ions like Hg^{2+}, Zn^{2+} and Cu^{2+} have been reported to have inhibitory effect on *Pseudomonas* lipases (Iizumi *et al.*, 1990). *Pseudomonas* sp lipase has also been

reported to be inhibited in the presence of Al^{3+}, Mn^{2+}, Ni^{2+} and Fe^{3+} (Dong *et al.*, 1990).

In this study, agitation supported the growth of the experimental organisms, signifying that they are aerobic in nature and required large quantities of dissolved oxygen for their growth and multiplication. This is also affirmed by Chander *et al.* (1980). But on the contrary, Chander *et al.* (1980) noted that agitation improved lipase production while stationary cultures produced better lipolytic activity than agitated cultures. Further, Ebrahimpour *et al.* (2008) showed that shallow layer (static culture) where aeration is moderate produced much more lipase than shake cultures (high aeration). On the contrary *Bacillus licheniformis* and *Alcaligenes* sp., had increased lipase production at 120 rpm when compared with others. Increase in lipase production on increasing agitation could be due to increased oxygen transfer rate, increased surface area of contact with the media component or better dispersability of the carbon source. This is in agreement with Hala *et al.* (2010) who reported that at 150 rpm, *Fusarium oxyspirum* produced highest lipase activity compared with the static culture and even at 100 rpm and 200 rpm.

As observed in this research, maximum lipase activity for enzymes studied were obtained after 24 hrs of incubation, indicating that lipase was necessary for the first stages of growth, while minimum growth for majority of the organisms were detected after 48 hrs, an observation in agreement with Ginalska *et al.* (2007). Further, at longer incubation periods, and for all the isolates, the lipase activity decreased which might be due to the depletion of nutrients, accumulation of toxic end-products, the change in pH of the medium, or loss of moisture. Other investigators have reported a different incubation period for optimal lipase production. Maximum lipase activity was achieved after 48 hrs of incubation by UI – Haq *et al.* (2002) with *Rhizopus oryzae*. In another study, the maximum lipase activity by *Aspergillus niger* occurred after five days of incubation (Mahadik *et al.*, 2002). Benjamin and Pandey (1997) obtained maximum production of lipase by *Candida rugosa* after three days of incubation.

In the case of substrate hydrolysis, almost all the substrates tested were hydrolysed by the tested organisms. Most of the substrates have long carbon chains (olive oil has C18:1) which may take time to dissolve. Since lipases hydrolyse esters in emulsion and usually water-insoluble substrates, the organisms take up the substrate at

different concentration, form and time. Typically, triglycerides composed of long-chain fatty acids, whereas esterases preferentially hydrolyse "simple" esters and usually only tryglycerides bearing fatty acids shorter than six carbon chains (C6). Thus, these results strongly suggest that the enzymes used in this study show lipase activity. On the other hand, findings of Pogaku *et al.* (2010), olive oil supported good growth and increased lipase activity significantly. Olive oil in this case acted as an inducer of lipase production, for all the strains used, hence lipase production has been shown to be induced remarkably in the presence of lipidic carbon sources like oils and fatty acids. Glucose, however, was found to act as a repressor of lipase production because it caused a great repression though it supported growth.

Generally, peptone stimulated lipase production better than other nitrogen sources experimented with in this study. This is in agreement with the work of Tembhurkar *et al.* (2012). It is also in agreement with the finding of Sirisha *et al.* (2010) who recorded better lipase production by *Staphylococcus* when peptone was used in place of yeast extract and tryptone as nitrogen source. Gupta *et al.* (2004) generally observed that organic nitrogen source such as peptone and yeast extract is preferred by bacteria. Also, peptone and yeast extract has been used as nitrogen source for lipase production by various *Bacillus* spp., various *Pseudomonads* and *Staphylococcus haemolyticus*, respectively (Wang *et al.*, 1995; Khyami-Horani, 1996; Pabai *et al.*, 1995; Oh *et al.*, 1999; Ghanem *et al.*, 2000; Lanser *et al.*, 2002). Inorganic nitrogen sources such as ammonium chloride and di-ammonium hydrogen phosphate have also been reported to be effective in some microbes (Gilbert *et al.*, 1991; Bradoo *et al.*, 1999; Dong *et al.*, 1990; and Rathi *et al.*, 2001).

As also observed in this study, lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature, and the dissolved oxygen concentration (Elibol and Ozer, 2001).

The activity of lipase in synchrony with growth was found to be best at 0.1% concentration of $MgS0_4$ and $NaN0_3$ for enzymes from both *Candida parapsilosis* and *Pseudomonas fluorescens*. Islam *et al.* (2009) also found that higher concentration of Ca^{2+} decreased lipase activities. Similar results have been reported for *Pseudomonas* lipase by Ece Yapasan (2008). These metal ions may increase the binding affinity of enzyme to the substrate (Ece Yapasan, 2008).

Both enzymes (*Candida parapsilosis* and *Pseudomonas fluorescens*) had optimum lipase activity at substrate concentration of 1%. Similar results have been observed by Sugihara *et al.* (1991) who reported lipase production from *Bacillus* sp in the presence of 1% olive oil in the culture medium. Several other scholars have observed different percentages of olive oil in culture medium for lipase production by different enzymes. An example is Rajesh *et al.* (2010) who note lipase production by *Trichoderma reesei* at 2% concentration.

The inhibition of the synthesis of lipases at higher olive oil concentration could be due to poor oxygen transfer into the medium, low oxygen supplies can alter microbial metabolism and consequently, the production of lipase.

Temperature effect in this study showed that optimum *Pseudomonas* growth temperature was 25^{o}C, whereas enzymatic activity was best at 30^{o}C. This is in agreement with the report of Ece Yapasan (2008). Alteration of the optimum temperature resulted in gradual decrease in growth. This could be interpreted to mean increase in temperature alters the cell membrane composition and stimulates protein catabolism, thus causing cell death (Craig, 1985). In the case of lipase activity, temperature changes gave rise to cleavage of hydrogen bonds between substrates and enzyme active sides (Ece Yapasan, 2008). Optimum temperature value promotes binding potential of enzyme and substrates (Ece Yapasan, 2008).

Precipitations of enzymes were carried out by ammonium sulphate since it was highly soluble in water, cheap and had no deleterious effect on structure of protein (Manasi *et al.*, 2013). Both precipitation and dialysis showed increase in purification fold for both enzymes in this investigation. Sephadex G-75 and ion-exchange (CM) chromatography proved to be very effective for lipase activity increasing the purification folds to 3.60 and 8.01 times with specific activity reaching upto 1.43 U/mg and 3.18 U/mg, respectively in the case of *pseudomonas fluorescens* and to 5.14 and 5.95 times with specific activity reaching up to 1.56 U/mg and 1.81 U/mg respectively for *Candida parapsilosis* enzyme. These could be possible probably due to the removal of inhibitory and / or high molecular weight contaminants such as proteases and cellular debris that could decrease or mask the lipase activity during these purification stages. In view of lipase purification by a number of authors, the extracellular lipase by *Pseudomonas* sp Yo103 was purified using ammonium sulphate

fractionation, *DEAE* cellulose and Sephadex G-200 to 62 fold increase and a 3.7% yield (Kim *et al.,* 1997). In a similar study a lipase by *Pseudomonas* sp. KW1-56 was purified to 14-fold by acetone precipitation and gel filtration by HPLC giving a 4% yield (Iizumi *et al.*, 1990). Kojima *et al.* (1994) purified a lipase from *Pseudomonas fluorescens* by ultra filtration, ammonium sulphate, precipitation, DEAE- Toyopearl 650 M phenyl, Toyopearl 650 M to 6.1 fold and 42% yield. Also, similar to this study a lipase fraction from *P. fluorescens* (Lee *et al.*, 1995) is obtained with a 10-fold increase in purification.

Benjamin and Pandey (2001), isolated and characterised three distinct forms of lipases from *Candida rugosa* (DM-2031) produced in solid-state fermentation. Three distinct forms of extracellular lipase (Lip A, Lip B and Lip C) were purified by ammonium sulphate precipitation, dialysis, ultra filtration and gel filtration using sephadex -200 (Benjamin and Pandey, 2001). The purification was 43-fold with specific activity 64.35 mg/ml. The native lipase / acyltransferase from *Candida parapsilosis* was purified by hydropholic interaction chromatography. Through this purification step, lipase enzyme was purified 85-fold. The global yield and the final specific activity were 11% and 30 Umg^{-1} (Virgine *et al.*, 2002). Liu *et al.* (2008) purified a lipase from *Aureobasidium pullulans* by ammonium sulphate fractionation, gel filtration chromatography and anion-exchange chromatography to a 3.4-fold.

A purified and concentrated lipase preparation is essential before characterisation of an enzyme. An impure (crude) lipase preparation could pose undesirable and ambiguous results. In the last few years, emphases were given to purification and characterisation of bacterial lipases. Previously, different purification procedures were attempted to obtain homogenous lipase preparations (Reetz *et al.,* 1996). Most of the purification techniques involved fractionation by salting out using ammonium sulphate or use of an organic solvent (Sharma and Kanwar, 2012). Notably, ammonium sulphate method was relatively inexpensive, efficient, reliable and reversible and a general storage technique employed in an enzyme system (Sharma and Kanwar, 2012). The potential advantage of the ammonium sulphate precipitation over all other techniques is the increase in stabilisation of the protein (Sharma and Kanwar, 2012). A 2-3 M ammonium sulphate suspension of protein precipitates remain stable for years, thus it forms a normal packaging method for many enzymes

(Sharma and Kanwar, 2012). Also, high salt concentration prevents proteolysis and bacterial activation (Tan *et al.*, 2003).

This study shows that partially purified lipase of *Pseudomonas fluorescens* has an estimated molecular weight range of 35 to 50 based on the position of the protein marker. Aysun (2009) reported similar findings, strain KE38 of partially purified lipase of *Pseudomonas* sp was screened to be homogenous on SDS-PAGE gel and its molecular weight was estimated to be approximately 43 KDa based on the position of the protein marker. An extracellular lipase from *Pseudomonas aeruginosa* BN-1 has been reported to have an estimated molecular weight of 60 KDa (Syed *et al.*, 2010). Kojima and Shimizu, (2003) also reported the estimated molecular weight of lipase of *Pseudomonas fluorescens* HU 380 to be 64 KDa. On the other hand, partially purified lipase of *Candida parapsilosis* as obtained in this study showed a molecular weight of approximately 38 KDa. This is similar to the findings of Aloulou *et al.* (2007) that the molecular weight of the lipase from *Yarrowia lipolitica* was 38.48 KDa. However, a higher molecular weight was observed by Salihu *et al.* (2012), for *Candida cylindracea* which was shown to exhibit a single band with molecular mass of 58 KDa by SDS-PAGE and gel filtration.

Purified lipase from *Pseudomonas fluorescens* had exhibited maximum activity at pH 7.5, 27^{o}C, 0.05% ion concentration and at 100 revolutions per minutes while *Candida parapsilosis* purified lipase exhibited maximum activity at 6.5-7.5 pH, 35^{o}C, 0.05% ion concentration and 140 revolution per minute. Similar to what has been observed on pH range in the current study, several researchers have reported that most lipases produced by *Pseudomonas* sp have pH optimum around pH 7.0-9.5 (Lee and Rhee, 1993; Dong *et al.*, 1990). These include enzymes from *P. tolaasii* (Baral and Fox, 1997), *P. aeruginosa* to EF2 (Gilbert *et al.*, 1991) and *P. putida* (Lee and Rhee, 1993). Schuepp *et al.* (1997) reported similar findings for the lipase activity of the FII from *Pseudomonas fragi* CRDA 037, which had a pH optimum of 9.0. However, unlike the optimum temperature here at 27^{o}C, Schuepp *et al.* 1997 report an optimal temperature of 35^{o}C for lipase activity of F II from *P. fragi* CRDA 037. It has also been reported that, *P. aeruginosa* purified lipase I shows lipolytic activity over a wide range of temperature from 40 to 70^{o}C with an optimum temperature of 50^{o}C, which is in agreement with that of *P. aeriuginosa* EF2 and significantly higher than that of

Pseudomonas PACIR and psychrotrophic strain of *P. fluorescens* (Gilbert *et al.*, 1991; Yong and Rhee, 1993 and Lee *et al.*, 1995). On the other hand, purified lipase II showed an optimum temperature of 45^{o}C, which could be said to be close to the findings in this study. Purified lipase from *Candida rugosa* having three distinct forms of extra-cellular lipase (lipA, lipB, and lipC), all the three forms are similar to what was seen in *Candida paraspilosis* purified lipase in this study with optimal activity at 35-40^{o}C and pH 7-8.

The activities of purified lipases in this study were supported by increased agitation especially that of *Candida parapsilosis* unlike the crude extract of these lipases inhibited by agitation. This could be attributed to the removal of inhibitory materials during purification such as proteases, other enzymes, non-enzymic proteins and cellular debris that could compete with the even distribution of the substrate during agitation (aeration). However, high agitation speed reduced or inhibited lipase activity probably due to enzyme disruption.

Metal ions, though necessary for lipase activity, at increased concentration for the purified lipases they were seen to be inhibitory, suggesting that they were able to alter the enzyme conformation (Sharon *et al.*, 1998).

The lineweaver-Burke plots were linear and indicated that hydrolysis of the triglyceride esters by the lipases followed Michaelis-Menten kinetics (Guit *et al.*, 1991). The graphs gave two kinetic parameters, K_m and V_{max}, which show the substrate affinity of enzyme. The smaller K_m values indicate the higher enzyme affinity to the substrate. In this study, the Michaelis-Menten parameters K_m and V_{max} of purified *Pseudomonas* lipase was 0.7670 mg/ml and 0.702 µg/s respectively, whereas K_m and V_{max} of purified *Candida parapsilosis* lipase was 1.428 mg/ml and 1.095 µg/s respectively. These results revealed that the lipase from the bacterial deployed higher affinity for the substrate than the yeast lipase. In many cases, lipases appear to obey Michaelis-Menten kinetics (Guit *et al.*, 1991; Malcata *et al.*, 1992) Michaelis-Menten kinetics are characterised by two parameters, K_m, and V_{max}. The latter is the maximum rate of reaction and K_m is a measure of the affinity of an enzyme for a particular substrate. A low K_m value represents a high affinity. The K_m values of the enzyme range widely, but for most industrially relevant enzymes, K_m ranges from 10^{-1} to 10^{-5} M (Fullbrook, 1996).

Pabai *et al.* (1995) reported a similar result with that in this study, Michaelis-Menten parameters K_m and V_{max} of a purified lipase of *Pseudomonas fragi* CRDA 323 the K_m and V_{max} values where 0.7 mg/ml and 0.97 x 10^{-3} U/min, respectively. For a *Pseudomonas cepacia* lipase, Pancreac'h and Baratti (1996) reported K_m and V_{max} values of 12 mM and 30 µ mol/min, respectively when the substrate was P-nitrophenyl-palmitate.

Liu *et al.* (2008) reported K_m and V_{max} values of the yeast *Aureobasidium pullulans* HN2.3 enzyme for p-nitrophenyl-laurate to be 0.608 mM and 0.039 mM min, respectively. For in lipase of *R. glutinis*, the K_m values are 2.7 and 0.7 mM when the substrates are P-nitrophenyl-lityrate and p-nitrophenyl-laurate, respectively (Hatzinikolaou *et al.*, 1999).

From the biodegradation results obtained in this research it could be observed that concentration of residual olive oil in culture medium reduced (8.2%) within the period of degradation (25 days). Aluyor *et al.* (2009) reported results of tests carried out indicating that vegetable oils undergo about 70-100% biodegradation in a period of 28 days. Prasad and Manjunath (2011) also showed that wastewater samples having an average pH of 5, average BOD value of 3200 mg/L and average lipid content of 25,000 mg/L before the treatment. After treating with individual bacteria and the consortia, the amount of lipid content present in the samples were found to be reduced based on the degrading capacity of the organisms. According to Mongkolthanaruk and Dharmsthiti (2002), similar properties of kitchen waste-water with an average pH of 5.2, the reducing sugar content 719 mg/L, average BOD 3600 mg/L and the average lipid content 21,000 mg/L were determined.

It was observed especially with the use of medium 1 in this study that the oil content could not be reduced to values below 0.0031 mol/L (on the 15th day using *Pseudomonas fluorescens* for treatment) from the initial content of 0.0064 mol/L (Table 4.25). Other researchers have reported similar observation. The result of Kangala and Krystyna (2008) who studied the fate of lipids in activated sludge showed that the overall lipid content in the effluent could not be reduced to values below 300 mg/L from an initial content of 2000 mg/L. Keenan and Sabelnikov (2000) show that biological treatment could not reduce the content of lipids in waste-water to values lower than 305 mg/L. Similarly, Wakelin and Forster (1997) report that the content of

lipids in wastewater could not be reduced to values below 100 mg/L even when acclimatised microbial species (*Acinetobacter sp.)* was used for the degradation of lipids.

Results also show in this study that after initial decrease in oil weight and even the pH, there was subsequently an increase in the oil content. Researchers like Naidas *et al.* (2005) also observed similar increases in the content of lipids in biological treatment system. The different degradation efficiencies might be due to different reaction systems of lipase from each culture medium as indicated in the result of the different culture media.

Microbial population was observed to generally increase over the period of degradation. This corroborates the Al-Darbi *et al.* (2005) finding that the number of marine oil- degrading bacteria increased with time in the oil-contaminated samples. The increase in bacteria populations was more pronounced in both the seawater-containing nutrients and waste-water samples. Mudge *et al.* (1995) reported an increase in bacteria number due to the presence of oils. They equally observed that after a while, the growth rate decreased, possibly inferring substrate depletion.

The pH values in the culture media were reduced compared to the neutral pH value observed when biodegradation commenced. The reduction in pH could be as a result of fatty acid produced during fermentation (hydrolysis) which could probably bring down the pH of the culture medium. Apichat and Pailin (2011) carried out jatropha oil degradation using minimal salt media supplemented with 1% jatropha oil as sole carbon source. Free fatty acid formed during hydrolytic activity which resulted in the decrease of pH, and also retarded bacterial growth probably due to the toxicity of the fatty acids on the cells.

Monitoring the degradation pattern in the laboratory has shown that within 25 days lipase produced during fermentation could breakdown olive oil, which from this study had initial composition of oleic acid to be 76%. It has been reported that olive oil is the richest in monoenes fatty acids (Kolayli *et al*., 2011). It has unique double bonds the commonest of which are of the n-9 series, as oleic acid, probably the most common fatty acid (olive oil has a high content of this acid, about 60-70% (Al-Darbi *et al.,* 2005*).* Report also shows that oleic acid (C18:1), a mono-unsaturated omega 9 fatty acid makes up 55 to 83% olive oil (Oliveoilsource, 1998). Deferne and Pate

(1996) found olive oil to contain saturated stearic 16%, oleic acid 76% and mono-unsaturated linoleic 8%.

Olive oil contains more oleic acid and less linoleic and linolenic acids than other vegetable oils, as also noted in this study, that is, more mono-unsaturated (a single double bond) than poly-unsaturated (more than one double bond) fatty acids. This renders olive oil more resistant to oxidation because generally, the greater the number of double bonds in the fatty acid, the more unstable and easily broken down by heat, light, and other factors oil will be (Oliveoilsource, 1998). This infers that if olive oil could still be biodegradable as observed in this work, other vegetable oil could as well be easily broken.

These findings can be explained through an understanding of the effect of oil structure and composition on the degradation processes. The structure of a fatty acid molecule is characterised by the length of the carbon chain (number of carbon atoms), the number of double bonds and also the exact position of these double bonds, define and determine the biological reactivity of the fatty acid molecule and even of the lipid containing those fatty acids (Shanks, 2014).

Kizilaslan (2007) in reporting the treatment of vegetable oil-containing waste-water observes the removal of vegetable oil of about 77% to 95% during monitoring for 30 days, the most significant effect being observed at 18 days sludge age. This is similar to the result obtained in this study, whereby the best degradation in the two media used was observed on the 20th day except for sample degraded by *Pseudomonas fluorescence* in culture medium 2 over 25 days. Increasing sludge age did not improve the vegetable oil removal at 4% vegetable oil concentration but reduced the removal of the substrate as reported by Kizilaslan (2007). Such decrease was also seen in this study as there was decrease in oleic acid removal. Similarly, Mrinalini and Jayanth (2012) showed degradation of hydrocarbons present in diesel oil through observation of residual oil after 15 days of degradation.

During the experimental period of 25 days, the contents of all individual fatty acids in all the media treated with both *Pseudomonas fluorescens* and *Candida parapsilosis* underwent both increases and decreases in their percentage composition. This result suggests that these substrates were not consumed by microorganisms at the same rate. These are in agreement with Kangala and Krystyna (2008), who found

different utilisation rates of fatty acids by activated sludge microorganisms. Novak and Kraus (1973) also found that the utilisation rate of fatty acids by activated sludge microorganisms was different and depends on the length and degree of unsaturation of their carbon chains. Results of Dignac *et al* .(2000) also showed increases in contents of fatty acids (Palmitoleic, Stearic, Linoleic, Linolenic, Linoleic, Arachidic, Behenic and Lignoceric) in waste-water effluents after 24 hr of a biological treatment process using activated sludge. Pereira *et al.* (2002) reported the appearance of palmitic acid in waste-water after a biological treatment process. Because palmitic acid was absent from the waste-water at the beginning of the process, they concluded that it is a by-product of biodegradation of oleic acid initially added to the waste-water.

Many other researchers have reported that fatty acids accumulate in biological wastewater treatment systems (Beccari *et al.,* 1998; Salminen *et al*, 2000; Lalman and Bagley., 2001). Therefore, since biodegradation and biosynthesis of fatty acids occur inside microbial cells, the observed increases in contents of fatty acids showed that they were released into the waste-water as microbial by-products (Kunau *et al.*, 1995). At the same time, decreases in their contents show that fatty acids were subsequently consumed by the organisms as substrates.

The observed black colour of the oil mill polluted soil samples may be due to the chronic pollution of this site, as it has been known as site of vegetable oil production for over 12 years. This is evidenced from all the physicochemical parameters higher in the sample than all the unpolluted counterparts. Okwute and Isu (2007) also report the black with humus colouration of palm oil mill effluent (POME) in the soil. However, the observed dark brown colour of the unpolluted botanical garden soil may be due to high organic matter content of the soil, which has been reported by Lickacz and Penny (2001) to be a major component in the top soil of virgin soil.

The total nitrogen contents and organic carbon of the oil mill polluted soil was high than that of the unpolluted soil an observation which correlates with the findings of Wood (1977), Dolmat *et al.* (1987), Huan (1987), Acea and Carballas (1996), Okwute and Isu (2007). The higher organic carbon value for the oil mill polluted soil can be related to the constituents of raw and inadequate treatment of the palm kernel effluent released into the soil. It is possible that a slow decomposition of organic

matter in the palm kernel oil mill polluted soil, under water saturated conditions, particularly when mean soil temperatures are low (Batjes, 1996) contributed significantly to the higher organic carbon of the polluted soil.

According to Rhoades (1982), cation exchange capacity (CEC) usually expressed in milliequivalent / 100 grams of soil, is a measure of the quantity of readily exchangeable cations neutralising negative charges in the soil. The CEC values observed in this study (sodium and magnesium) when considered along with the exchangeable cations were higher in the polluted soil than the non polluted soil.

In a similar study by Oviasogie and Aghimien (2002), as well as Okwute and Isu (2007), the results showed an overall increase in the CEC of POME soils especially in the area close to its source which this study supports.

The polluted oil mill soil was observed to be richer in phosphorus than the non-polluted soil. This agrees with the findings of Wood (1977) and Huan (1987). There is ample evidence to suggest that phosphorus is the dominant element controlling carbon and nitrogen immobilisation (Paul and Clarke, 1989). The increase in the available phosphorus in the polluted soil suggests a possible high absorption in the soil or a possible precipitation of phosphate (Huan, 1987). This may be due to the gradual biodegradation of the oil mill polluted soil, which leads to a delayed effect on the soil.

The water holding capacity of the polluted soil was also higher than that of the un-polluted soil. Deiana *et al.* (1990) stated that organic matter content strongly affects the soil fertility by increasing the availability of plant nutrients, improving the soil structure and the water holding capacity and also acting as an accumulation phase for toxic, heavy metals in the soil environment. Okwute and Isu (2007) also reported increase in water holding capacity of POME sample.

It has been reported that when raw POME was discharged, the pH was acidic but seems to gradually increase to alkaline as biodegradation takes place (Hemming, 1977). This study followed this trend, as the soil at commencement had its pH near neutral (6.5), a value similar to Okwute and Isu (2007) (pH 6.59). The pH of the un-polluted soil was 7.7 hence the pH of both the polluted and unpolluted soils was in the near neutral range. The optimum pH range highlighted by Morgan and Watkinson (1989), Kings *et al.* (1998) as the pH values required for an optimum bioremediation

process also falls within this range. Thus, no pH adjustment was required for the biodegradation experiment.

In the case of hydrogen pollution all the physico-chemical parameters in the unpolluted soil were higher than those of the polluted soil (Brown and Donnelly, 1983; Morgan and Watkinson, 1989; Odokuma and Dickson, 2003).

In the bioremediation experiment, the total viable microbial count generally increased from the 2nd to the 12th week of bioremediation. This was probably due to the fact that untreated vegetable oil in the soil contained high concentrations of free fatty acids, starches, proteins and plant tissues (Bek-Nielsen *et al.,* 1999) and are also non-toxic (Ma and Ong, 1982; Ngan *et al.,* 1996). Organic matter plays an important role in soil productivity and the polluted soil samples are good sources of organic matter as observed in this work (Chan *et al.,* 1980). Numerous organisms invade and grow in palm oil mill effluent breaking down complicated molecules into simple ones. The high organic matter in the polluted soil sample which has been shown to be higher than control soil samples may have a major role in the proliferation of aerobic microorganisms.

In the sterile soil samples from oil mill whether mixed or unmixed, where averagely there was increase throughout the 12 weeks, in addition to the effect of organic matters on proliferation, sterilisation of the soil, removed the probable competition of other indigenous micro-flora naturally present in the soil. The competitive relationship of *Pseudomonas fluorescens* and *Candida parapsilosis* may account for the recorded growth averagely not being higher than when treated only with *Pseudomonas fluorescens*. From this treatment, it was observed that in the 2nd week microbial growth was better in the 'tilling' (mixed option) for all the treatment option but by the 12th week, it was observed that the "no tilling" option gave better growth. Normally and like Charles *et al.* (2000) observed, adequate oxygen supply was a necessary condition to enhance growth and metabolism in aerobic organisms and oil degraders. However, as revealed in the 12th week, anaerobic condition was favoured. Similar study by Apichat and Pailin (2011) on jatropha oil degradation by *Enterococcus faecalis* and *Burkholderia cenocepacia* W-1 under anaerobic condition recorded close results. Hence, the experimental result suggests that it may be possible to use anaerobic rather than aerobic condition as a treatment strategy.

In the non-sterile counterpart there was increase in the microbial count up till the 10th week beyond which there was decrease up till the 12th week. This may be due to unfavourable environmental conditions, generated by the production of certain metabolites on the microbial cells which agreed with the result of Watson *et al.* (1999).

The viable microbial count in the control soil samples shows gradual decrease from the 4th to the 8th week and then increased again from the 10th to the 12th week. This could be as a result of nutrient exhaustion by the 6th and 8th weeks. It could also be as result of unfavoured environmental condition generated by production of certain metabolites (Biosurfactants) which are amphipathic compounds excreted by microorganisms that exhibit surface activity (Llori *et al.,* 2005; Nitschke and Pastore, 2006). However, soil microbiological and biochemical properties respond rapidly to small changes that occur in soil, thereby providing immediate information on changes in soil quality. This is because soil microbial activity has a direct influence on soil fertility (Sims, 1990; Serna and Pomares, 1992; Smith and Papendick, 1993). Hence, in this study the complex molecules like glycolipids, peptides, phospholipids and fatty acids in the biosurfactants (excreted by the microorganisms) could be converted as substrates for the microbial cells again, thereby causing further increase in biomass again by the 10th to 12th week.

The residual edible oil present in the soil during bioremediation by spectrophotometric analysis showed that the weight recorded at the beginning of all the experiment indicated a high level of the different edible oil contamination in the soil. The high value recorded by the 4th week in most of the different treatments in the polluted soil sample was due to microbial degradation of the edible oil present in the soil. This is evident in the reduced quantity of residual oil obtained in the gravimetric analysis (especially in the sterile samples). The degradation of the vegetable oil into fatty acids and other simple inorganic chemical compounds may also be responsible for the reduction in the turbidity of the eluted substance in the solvent by the 4th week as also observed by Pirnik (1977). This probably contributed to the values of some of the recoverable oil and grease by the 4th week. The high molecular weight linear and branched chain hydrocarbon components such as the long-chain fatty acids left had low solubility in n-hexane, similar observations have been noted by Korda *et al.* (1997).

By the 8th week, high residual oil values were recorded in most of the soil samples. This could be as a result of biosurfactants excreted by the microorganisms which contain many complex molecules such as glycolipids, polysaccharide-protein complexes, peptides, phospholipids, lipopeptides and fatty acids. The release could be due to the acclimatisation of the inoculated organism and indigenous organisms (in the non-sterile soils) degrading most of the complex products into alcohols and acids. These products have been reported to be more soluble thus, increasing the amount of extractable materials in the n-hexane. Thus, the increased weight in some of the residual oil recorded and the total viable counts recorded for most of the soils in the 8th week.

Bharathi *et al.* (2012), in considering the coconut oil biodegradation per day observed that *Pseudomonas aeruginosa* had the highest biodegradation rate from 6 to 9 days. Further observation revealed that the rate of degradation by *Pseudomonas aeruginosa* decreased sharply with increase in the incubation period from the 4th day to the 12th day and then started to slow from the 24th day. When relating this trend to the increase in microbial cell count, it was reported that there was a rapid increase in the cell biomass of *Pseudomonas aeruginosa* within the first 12 days of incubation. Odokuma and Dickson (2003) also observed a similar result. The low residual oil values recorded again in the 12th week was due to a reduced biodegradation activity of the isolates, also resulted in the high total viable microbial count recorded. Some exceptional cases with high residual oil values were observed as in the case of edible oil of sterile oil mill sample without mixing in *Candila parapsilosis* where an initial increase was noticed in the 4th week as opposed to the general reduction in the 4th week. This could mean that normal hydrolysis was not favoured. Briand *et al*. (1995) observed that the lipases from *Candida parapsilosis* have been shown to catalyse transesterification in the presence of a large molar excess of water in the biphasic aqueous/lipid reactant medium with hydrolysis of ester being inhibited by a proposed competition of the acyl acceptor with water. Hence, it means these organisms actually acclimatised, degraded the oil to alcohol and other more soluble compounds, thus increasing amount of extractable materials in the n-hexane. However, transesterification most probably caused reduction in weight of residential oil recorded.

This generally could be the reason for low record of count especially in soil treated with *Candida parapasilosis* only.

Some of the samples in the treatment involving a mixture of *Pseudomonas fluorescens* and *Candida parapsilosis* also showed similar trend; for example, residual edible oil of sterile palm kernel oil sample without mixing. Possibly, antagonistic reaction of *Candida parapsilosis* with *Pseudomonas fluorescens* could also cause similar reactions. However, this was not observed in the oil mill sample, most likely due to the fact that natural degradation has been on in the sampling environment before this experiment.

CONCLUSION

Certain environmental factors such as pH, temperature, incubation time and aeration rate were observed to play a major role during enzyme production and metabolic activities. Such studies are aimed at assessing the potential microbial isolates which could be helpful in degrading lipid wastes from oil mills. In the near future, this would definitely help to organise a cleaner environment with waste utilisation by these microbial species.

Microbial species such as *Pseudomonas fluorescens* and *Candida parapsilosis*, among other microbiota, were found present in the oil mill-polluted site. This implies that with time, given favourable conditions, these microorganisms could naturally aid the degradation process in vegetable oil polluted soil. It was established that some of these microorganisms could metabolise complex triglyceride present in oil into fatty acids and glycerol which could be further mineralised into simple inorganic chemical compounds that might not be toxic to the environment. Effective bioremediation could be achieved within 2 to 8 weeks of bio-treatment after which an additional measure like additional inoculum application would be required for prolonged biodegradation process. This could be applicable in areas of need in Nigeria. Hence, these microorganisms (*Pseudomonas fluorescens* and *Candida parapsilosis*) could become potential candidates in the development of microbial bioaugmentation products for the treatment of vegetable oil mill effluents and soils, particularly in polluted areas and sites. Finally, a balanced process of microbial seeding and nutritional supplementation and tilling / non tilling would be required for an effective bioremediation programme.

There is need to produce lipases of *Pseudomonas fluorescens* and *Candida parapsilosis* with improved properties by protein engineering to further enhance usefulness of these enzymes.

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APPENDIX I

Media

Preliminary screening for lipolytivc activity (Mineral Salt Medium)

KH_2PO_4	7.584 g
K_2HPO_4	0.80 g
$MgS)_4.7H_20$	0.80 g
$CaCl_2$	0.16 g
$(NH_4)_2N0_3$	0.80
$FeS0_4$	0.16 g
Agar agar	20 g
Olive oil	2%

Mineral salt medium 2

KH_2PO_4	7.584 g
K_2HPO_4	0.80 g
$MgS)_4.7H_20$	0.80 g
NaCl	0.16 g
$(NH_4)_2N0_3$	0.80g
$Fe_2(S0_4)_3$	0.16 g
Olive oil	0.08 g
Agar agar	20 g

Simmon's Citrate Medium

NaCl	5.0 g
$MgS0_4$	0.20 g
$NH_4H_2P0_4$	1.0 g
KH_2P0_4	1.0 g
$Na_3C_6H_50_7.2H_20$	5.0 g
Agar	15.0 g
Bromothymol blue 0.2%	40.0 ml
Distilled water	1.0 L

Indole Test Reagent (Kovac's)

Paradimethyl amino benzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Concentrated Hydrochloric acid	25.0 ml

Screening for Lipolytic Activity

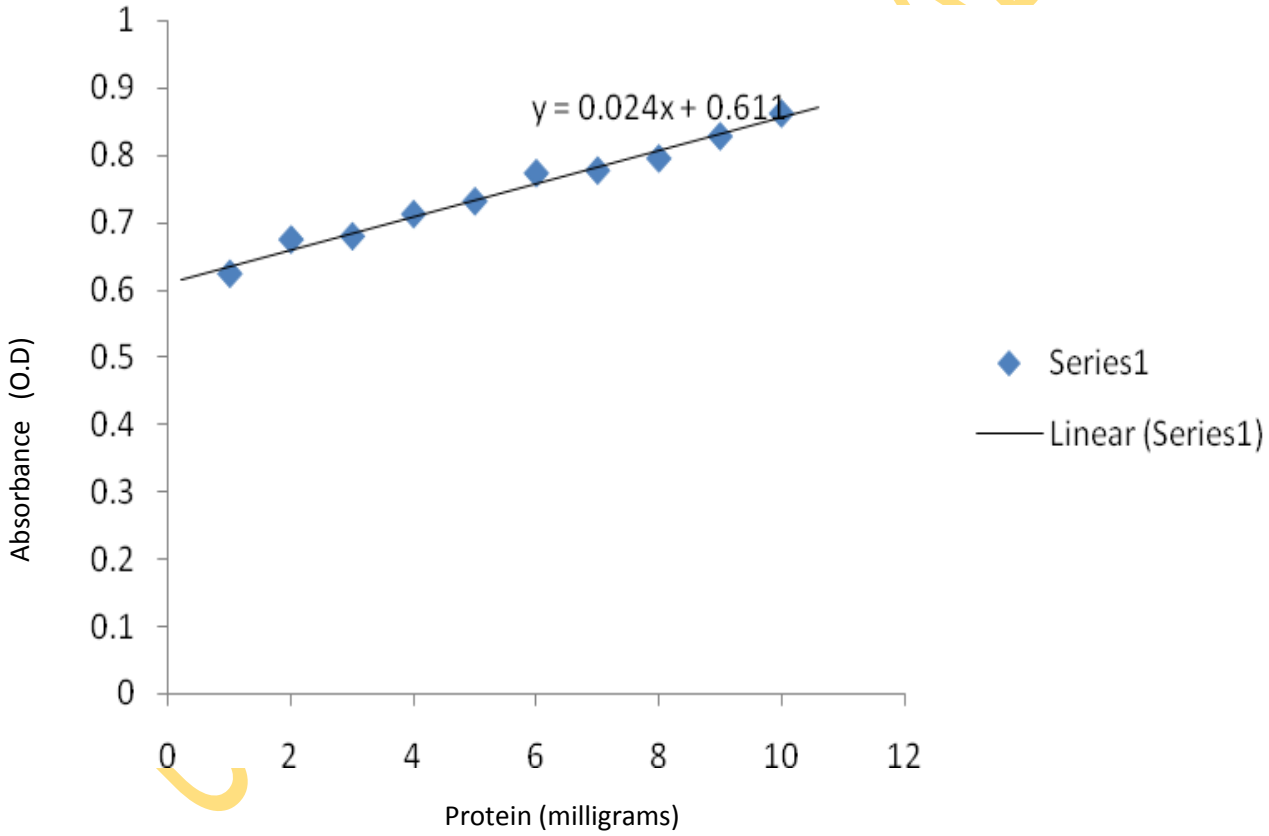
Olive Oil Medium

Peptone	10 g
NaCl	5 g
$CaCl_2$	0.1 g
Olive oil	1%
Agar	20 g
Aniline blue	0.5 g

Protein Estimation (Lowry *et al.*, 1951)

Reagent I

Na_2C0_3	2%	50 mls
Na0H	0.1 M	
Reagent II		
$CuS0_4.5H_20$	0.5%	1 ml
Sodium potassium tartarate	1%	
Folin reagent diluted twice		



Standard protein curve

Weight of residual oil in culture medium (1) degraded by individual soil microorganism over a period of 25 days

	Degradation period (days) / Residual oil weight (g)				
	5th Day	10th Day	15th Day	20th Day	25th Day
Pseudomonas fluorescens	1.8769±0.074[a]	1.7645±0.180[a]	1.6704±0.114[a]	1.6691±0.046[a]	1.6726±0.061[a]
Candida parapsilosis	1.6383±0.097[a]	1.5850±0.134[a]	1.6801±0.152[a]	1.4850±0.211[a]	1.3179±0.030[a]

Means with different superscript across the rows are significantly different at P≥ 0.05

Weight of residual oil in culture medium (2) degraded by individual soil microorganism over a period of 25 days

	Degradation period (days) / Residual oil weight (g)				
	5th Day	10th Day	15th Day	20th Day	25th Day
Pseudomonas fluorescens	1.8906±0.007[a]	1.8093±0.081[a]	1.8522±0.020[a]	1.8088±0.029[a]	1.7681±0.066[a]
Candida parapsilosis	1.9326±0.071[a]	1.7710±0.063[a]	1.9265±0.094[a]	1.5315±0.240[a]	1.7393±0.027[a]

Means with different superscript across the rows are significantly different at P≤ 0.05 using Duncan's Multiple Range Test

APPENDIX II

Fatty acids

FGN1 Caprylic Acid Methyl Ester (C8:0)

FGN2 Capric Acid Methyl Ester (C10:0)

FGN3 Lauric Acid Methyl Ester (C12:0)

FOO1 Myristic Acid Methyl Ester (C14:0)

FOO2 Palmitic Acid Methyl Ester (C16:0)

FOO3 Palmitoleic Acid Methyl Ester (C16:1)

FCS1 Margaric Acid Methyl Ester (C17:0)

FCS2 Stearic Acid Methyl Ester (C18:0)

FCS3 Oleic Acid Methyl Ester (C18:1)

FCS4 Linoleic Acid Methyl Ester (C18:2)

FSF1 Linolenic Acid Methyl Ester (C18:3)

FSF2 Arachidic Acid Methyl Ester (C20:0)

FSF3 Arachidonic Acid Methyl Ester (C20:4)

FPO1 Behenic Acid Methyl Ester (C22:0)

FPO2 Erucic Acid Methyl Ester (C22:1)

Lignoceric Acid Methyl Ester (C24:0)

Analysis Method : C:\HPCHEM\1\METHODS\F-DEGOIL.M
Last changed : 11/10/2011 12:43:36 PM
FATTY ACID METHYL ESTER ANALYSIS

Normalized Percent Report

Sorted By : Retention Time
Calib. Data Modified : 11/10/2011 12:43:25 PM
Multiplier : 1.0000
Dilution : 1.0000

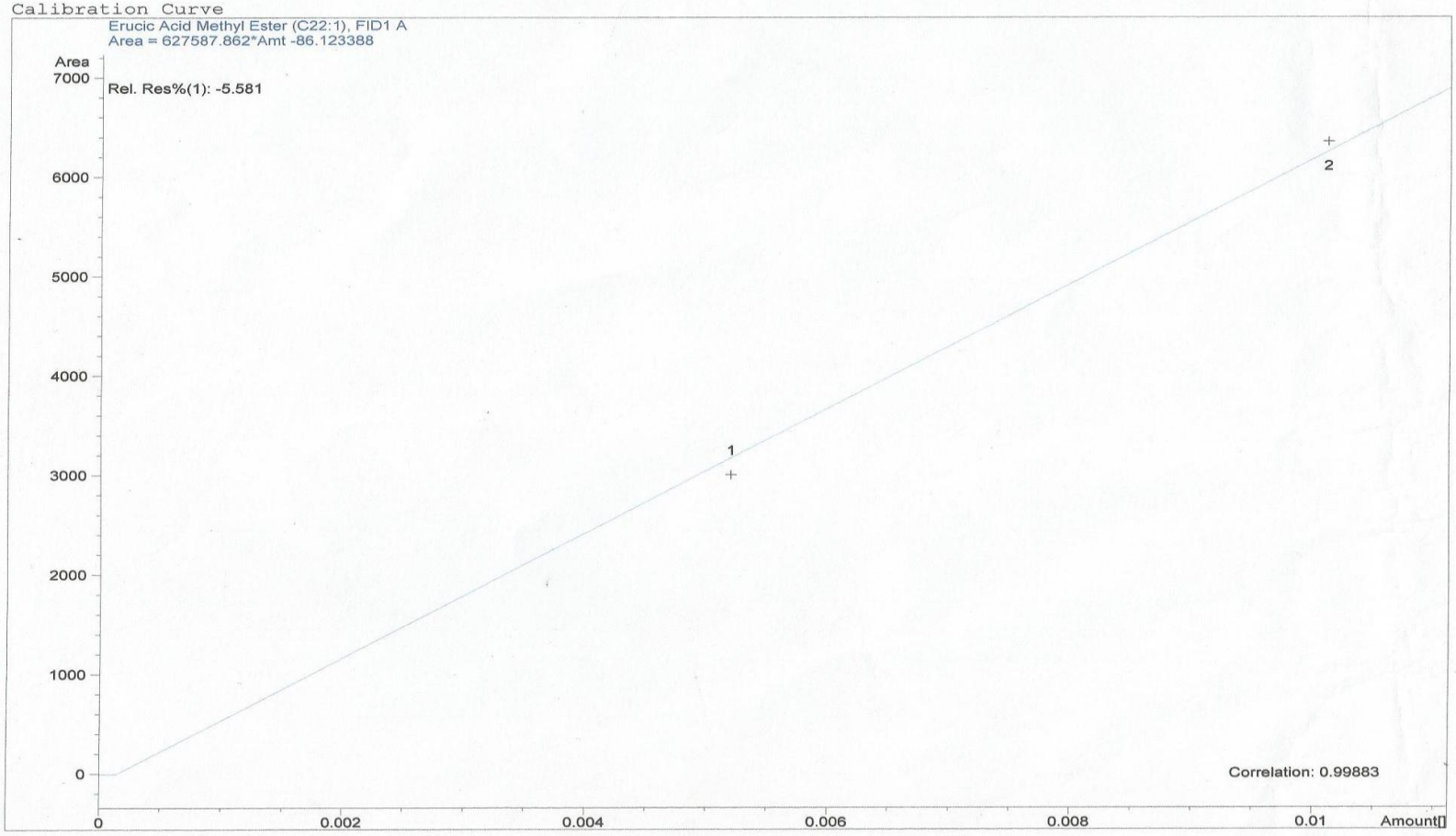
Signal 1: FID1 A,

RetTime [min]	Sig	Type	Area [pA*s]	Amt/Area	Norm %	Grp	Name
8.571	1	VV	39.66898	0.00000	0.000000		Caprylic Acid Methyl Ester (C8:0)
10.329	1	VV	53.22193	0.00000	0.000000		Capric Acid Methyl Ester (C10:0)
12.096	1	VV	55.74408	0.00000	0.000000		Lauric Acid Methyl ester (C12:0)
13.418	1	VV	71.52041	6.93339e-6	0.365792		Myristic Acid Methyl Ester (C14:0)
14.835	1	VV	58.30087	3.24951e-4	13.974994		Palmitic Acid Methyl Ester (C16:0)
16.137	1	VV	44.14641	0.00000	0.000000		Palmitoleic Acid Methyl Ester (C16:1)
17.092	1	VV	134.34036	0.00000	0.000000		Margaric Acid Methyl Ester (C17:0)
18.052	1	VV	211.42328	3.52825e-5	5.502634		Stearic Acid Methyl Ester (C18:0)
19.519	1	VV	196.07762	5.28257e-4	76.406805		Oleic Acid Methyl Ester (C18:1)
20.788	1	VV	182.25481	2.51212e-5	3.377362		Linoleic Acid Methyl Ester (C18:2)
21.821	1	VV	148.00012	1.22954e-6	0.134234		Linolenic Acid Methyl Ester (C18:3)
22.230	1	VV	111.44493	0.00000	0.000000		Arachidic Acid Methyl Ester (C20:0)
23.117	1	VV	80.33804	0.00000	0.000000		Arachidonic Acid Methyl Ester (C20:4)
23.968	1	VV	107.26530	1.24701e-6	0.098671		Behenic Acid Methyl Ester (C22:0)
24.792	1	VV	36.41836	0.00000	0.000000		Erucic Acid Methyl Ester (C22:1)
25.366	1	VV	56.87317	3.32529e-6	0.139507		Lignoceric Acid Methyl Ester (C24:0)
Totals :					100.000000		

Print of window 66: Calibration Curve

APPENDIX I

Calibration Curve

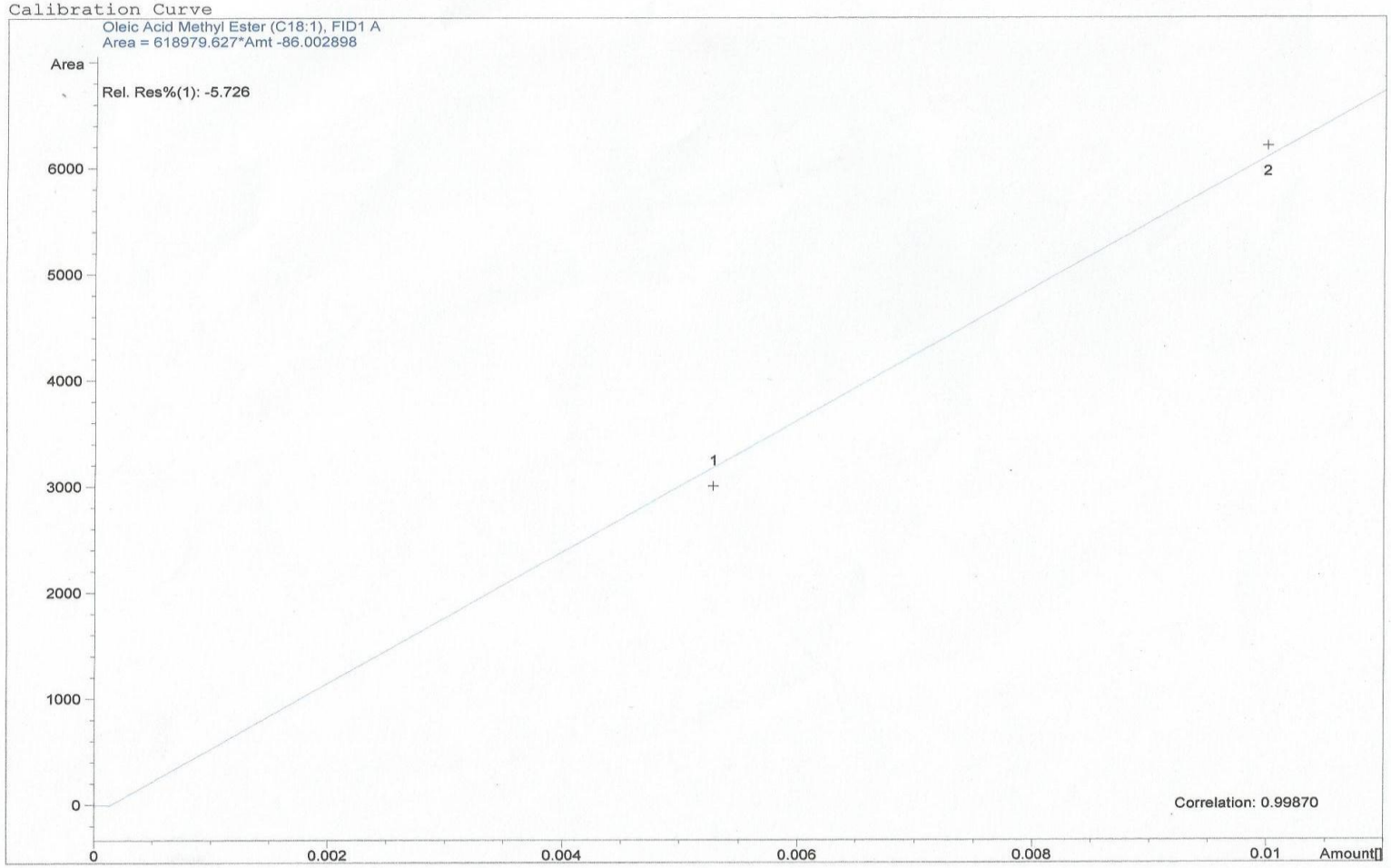


Instrument 1 8/5/2011 2:35:01 AM

Page 1 of 1

Print of window 66: Calibration Curve

Calibration Curve

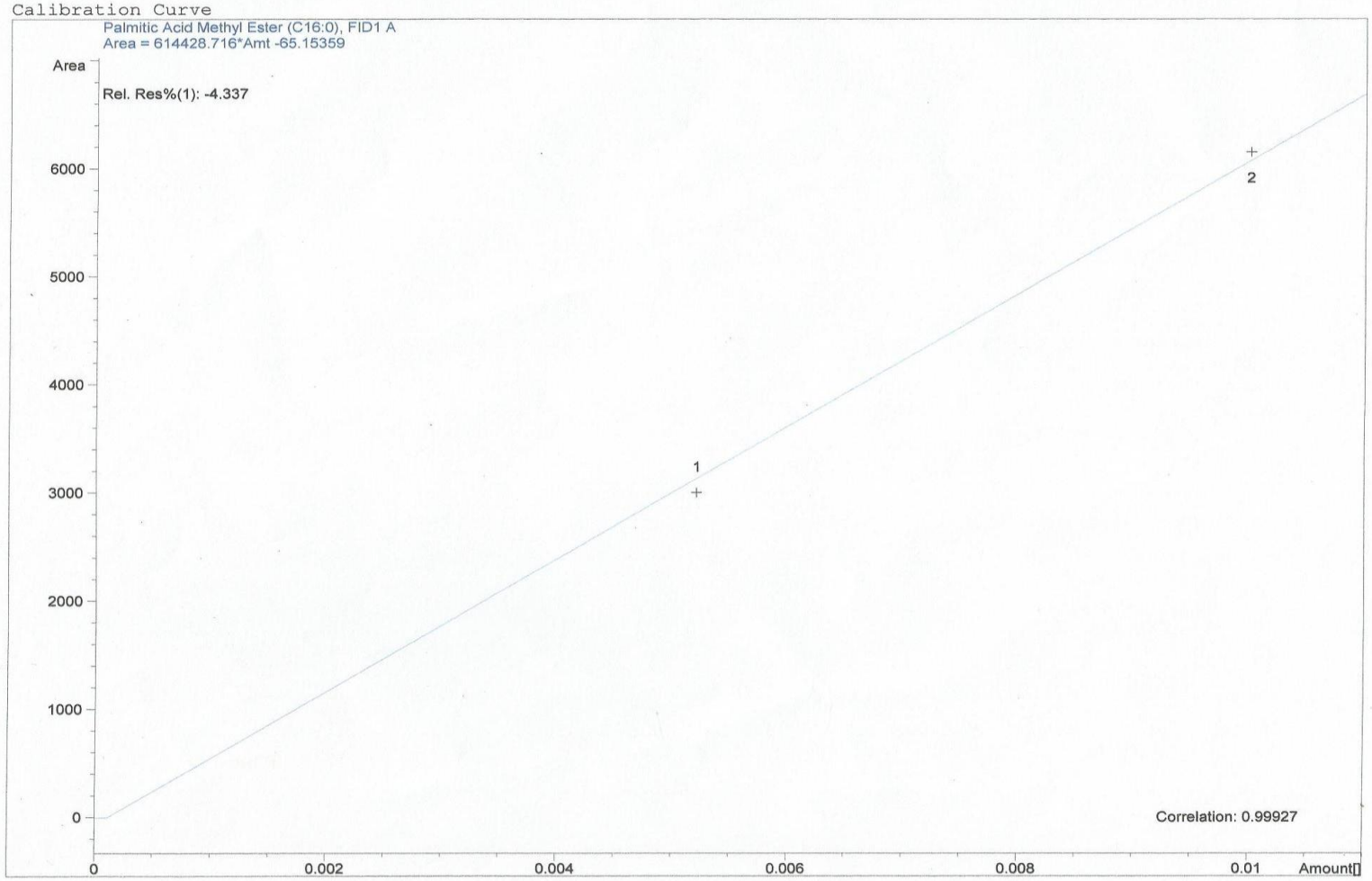


Instrument 1 8/5/2011 2:34:38 AM

Page 1 of 1

Print of window 66: Calibration Curve

Calibration Curve

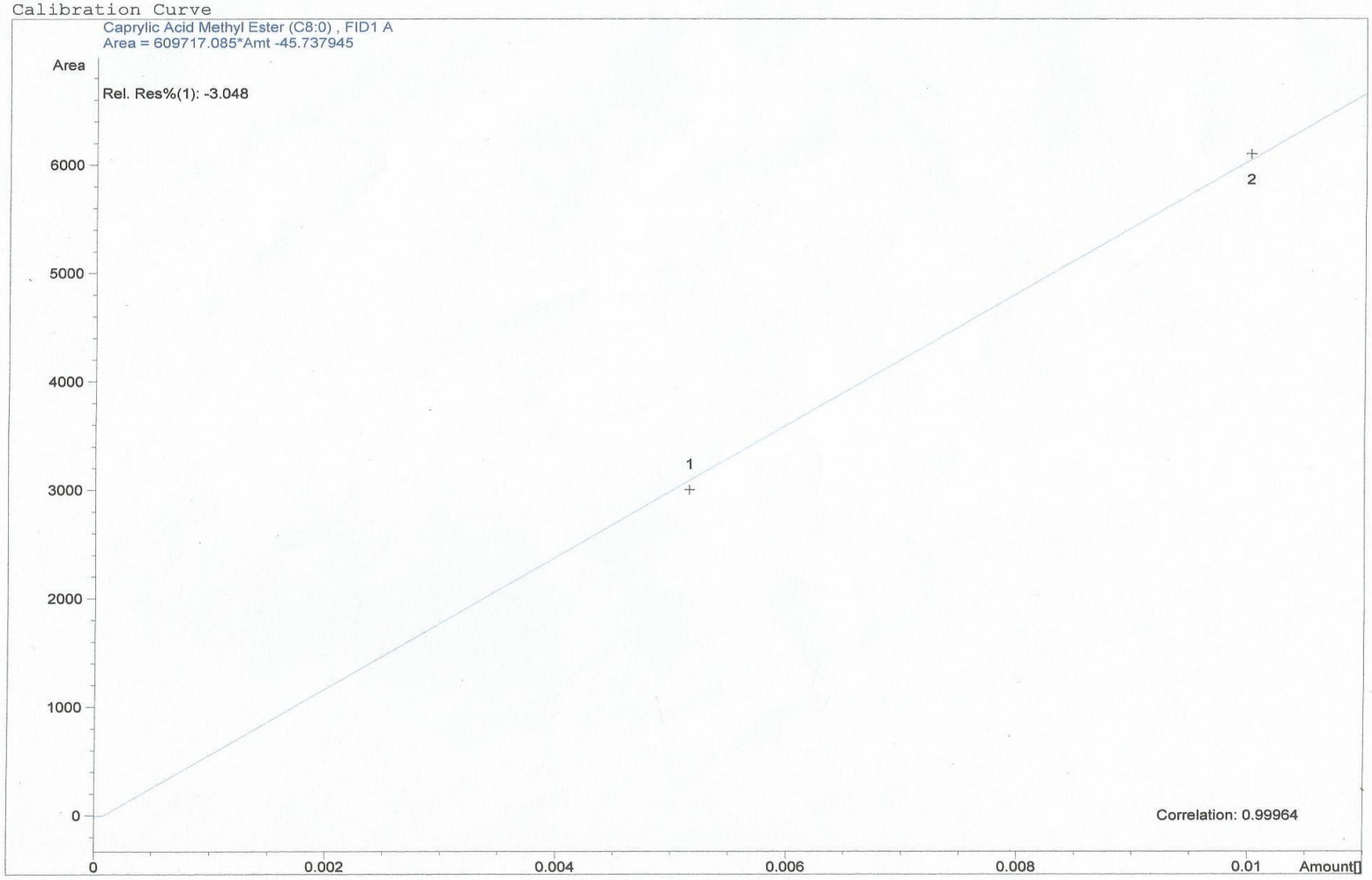


Instrument 1 8/5/2011 2:34:27 AM

Page 1 of 1

Print of window 66: Calibration Curve

Calibration Curve



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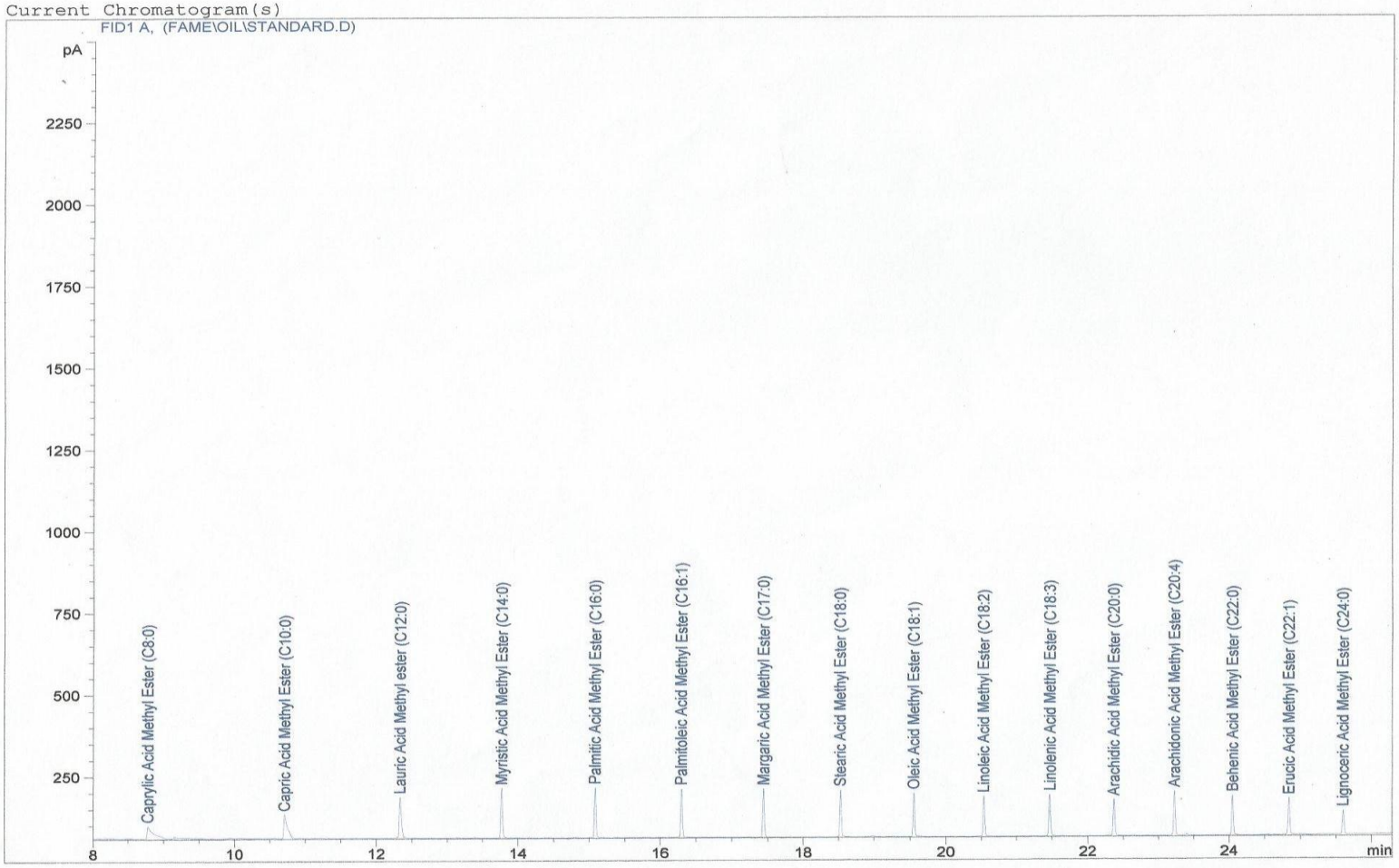
Page 1 of 1

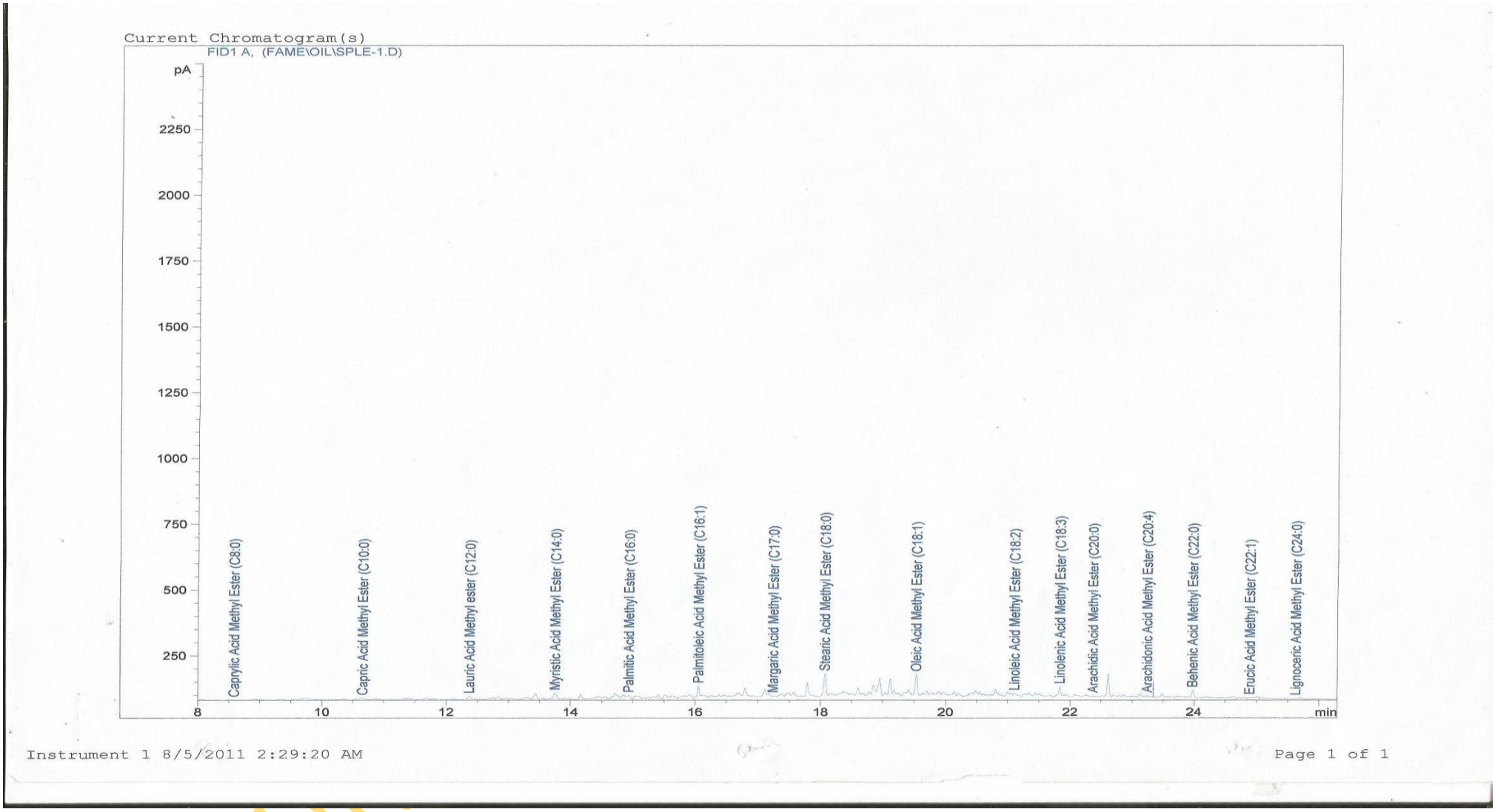
Print of window 38: Current Chromatogram(s)

Current Chromatogram(s)



Instrument 1 8/5/2011 2:02:40 AM

Page 1 of 1


Current Chromatogram(s)
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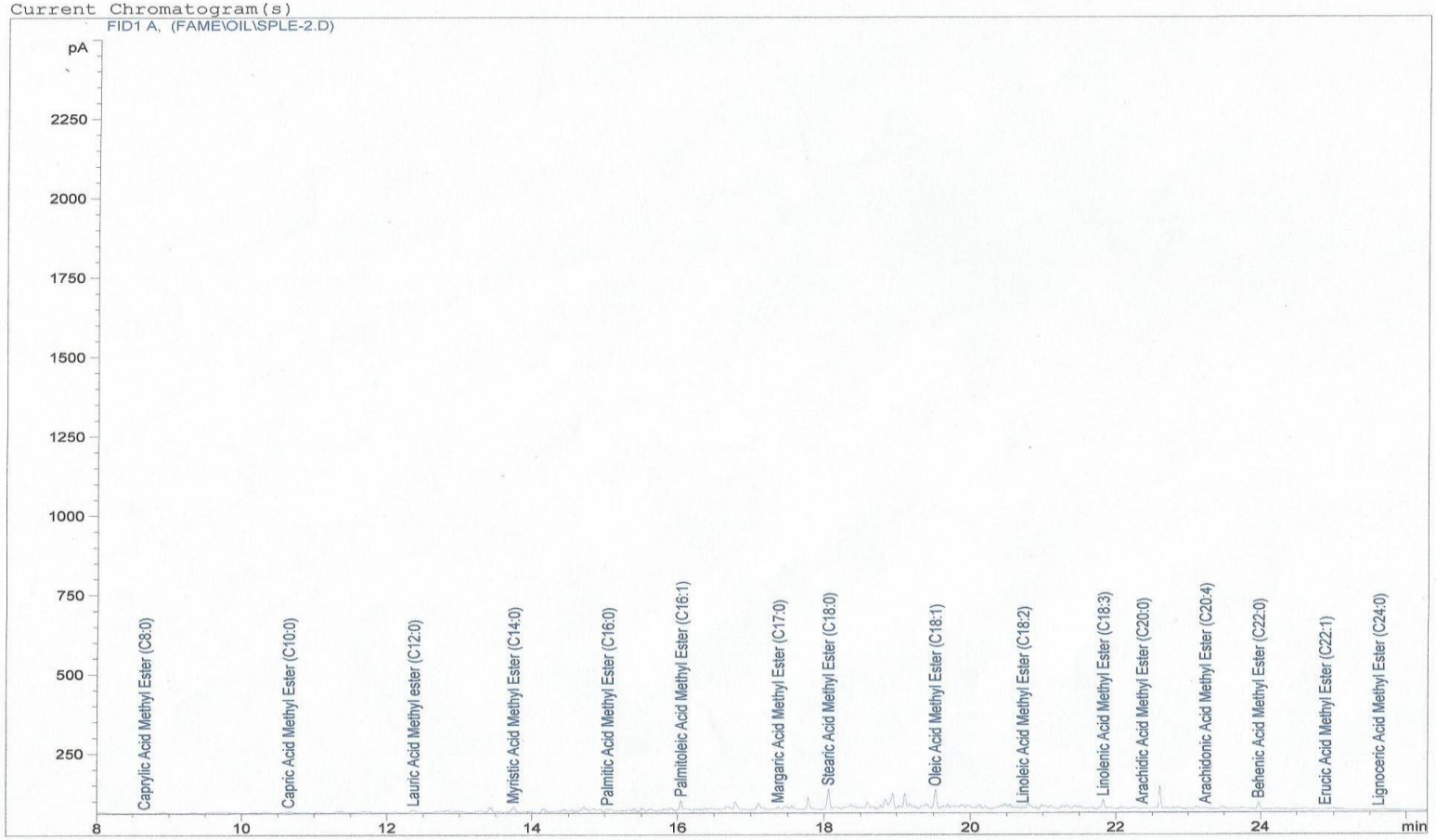
pA
2250
2000
1750
1500
1250
1000
750
500
250
Caprylic Acid Methyl Ester (C8:0)
Capric Acid Methyl Ester (C10:0)
Lauric Acid Methyl ester (C12:0)
Myristic Acid Methyl Ester (C14:0)
Palmitic Acid Methyl Ester (C16:0)
Palmitoleic Acid Methyl Ester (C16:1)
Margaric Acid Methyl Ester (C17:0)
Stearic Acid Methyl Ester (C18:0)
Oleic Acid Methyl Ester (C18:1)
Linoleic Acid Methyl Ester (C18:2)
Linolenic Acid Methyl Ester (C18:3)
Arachidic Acid Methyl Ester (C20:0)
Arachidonic Acid Methyl Ester (C20:4)
Behenic Acid Methyl Ester (C22:0)
Erucic Acid Methyl Ester (C22:1)
Lignoceric Acid Methyl Ester (C24:0)
8
10
12
14
16
18
20
22
24
min
Instrument 1 8/5/2011 2:29:20 AM
Page 1 of 1

Print of window 38: Current Chromatogram(s)

Current Chromatogram(s)



Instrument 1 8/5/2011 2:19:32 AM

Page 1 of 1

Current Chromatogram(s)

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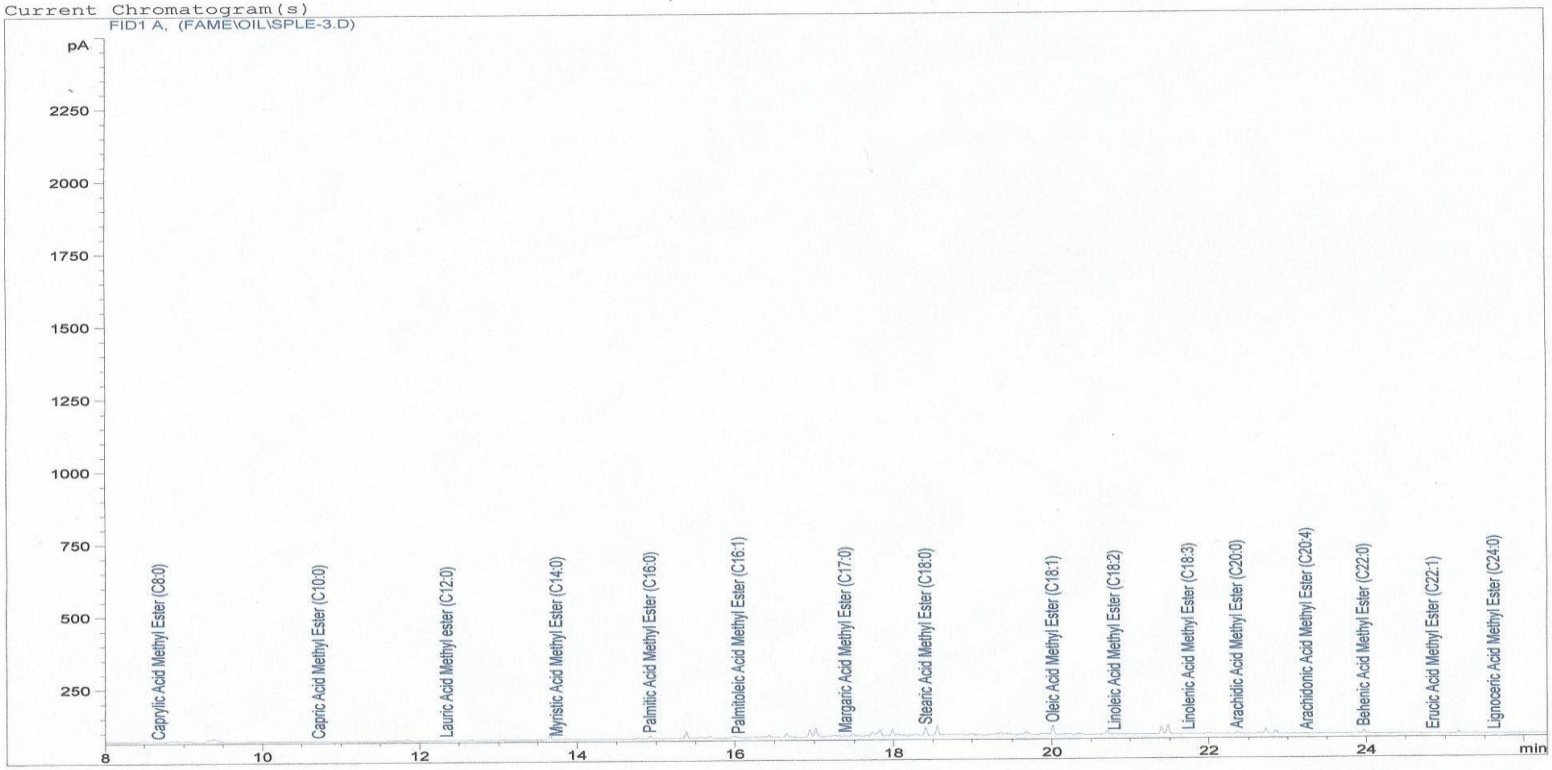

Instrument 1 8/5/2011 2:21:25 AM

Page 1 of 1

Print of window 38: Current Chromatogram(s)

Current Chromatogram(s)

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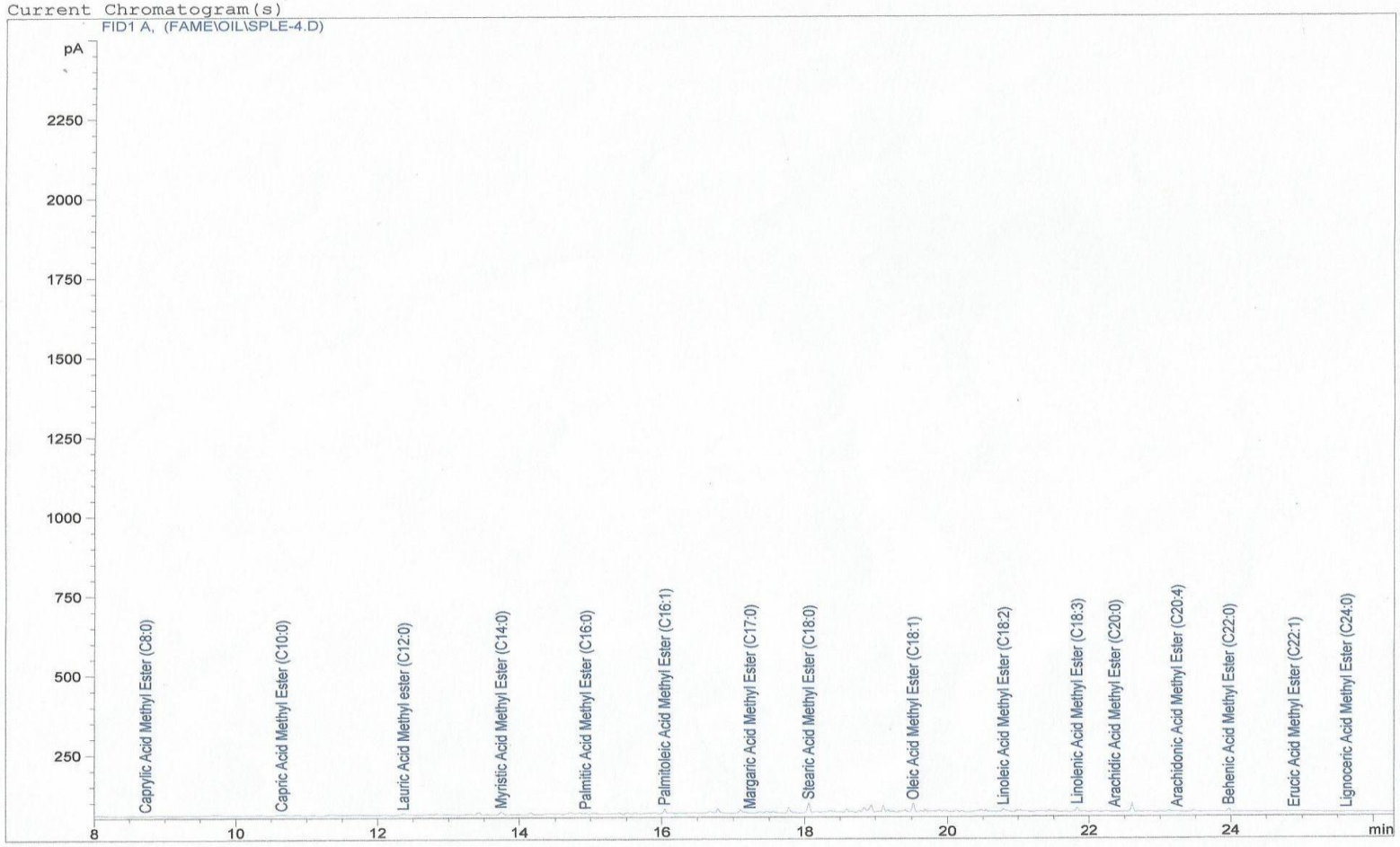


Instrument 1 8/5/2011 2:22:28 AM

Page 1 of 1

Print of window 38: Current Chromatogram(s)

Current Chromatogram(s)

Instrument 1 8/5/2011 2:23:16 AM

Page 1 of 1

Print of window 38: Current Chromatogram(s)

Current Chromatogram(s)

Instrument 1 8/5/2011 2:24:11 AM

Page 1 of 1

Print of window 38: Current Chromatogram(s)

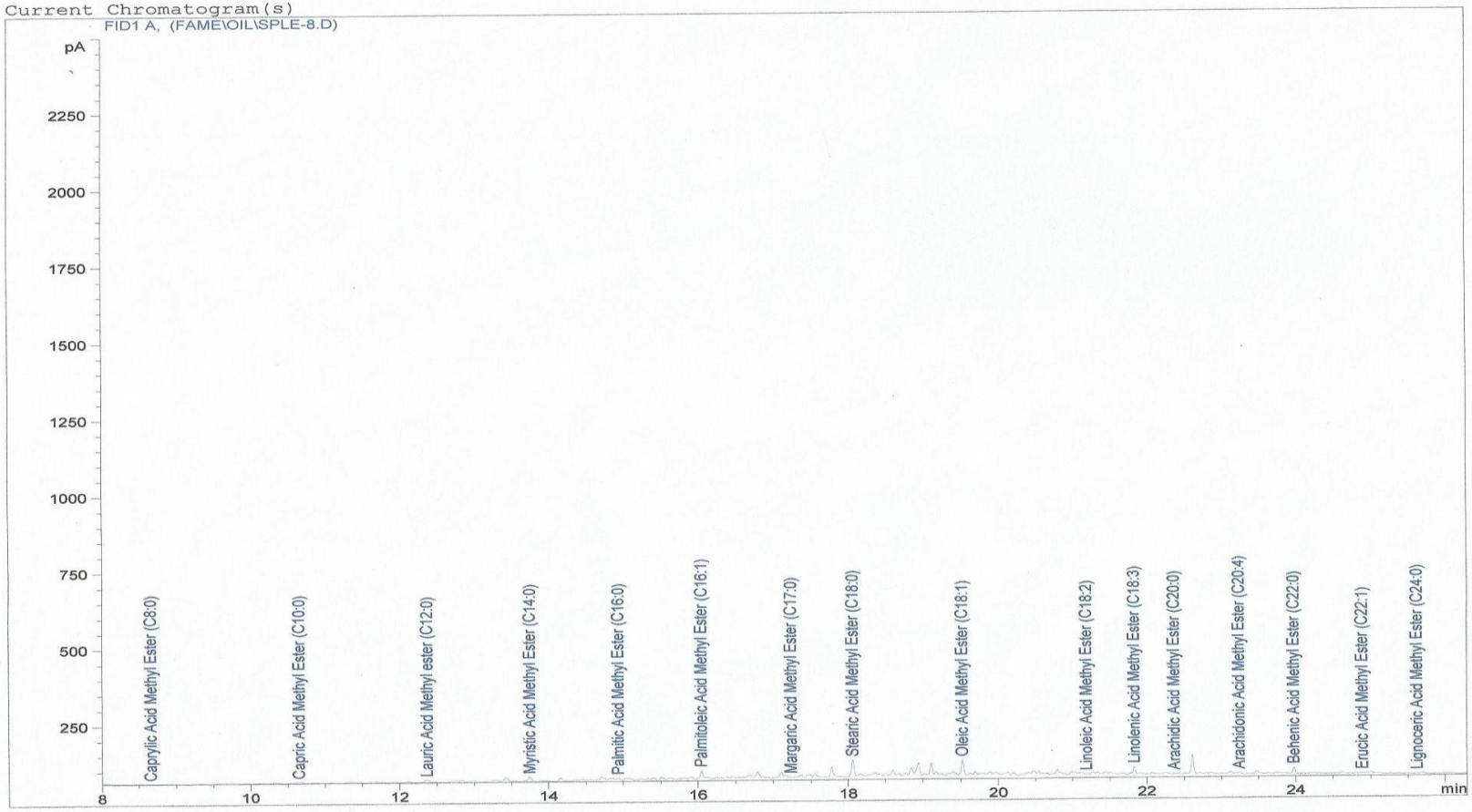
Current Chromatogram(s)

Instrument 1 8/5/2011 2:25:18 AM

Page 1 of 1

Print of window 38: Current Chromatogram(s)

Current Chromatogram(s)



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Page 1 of 1

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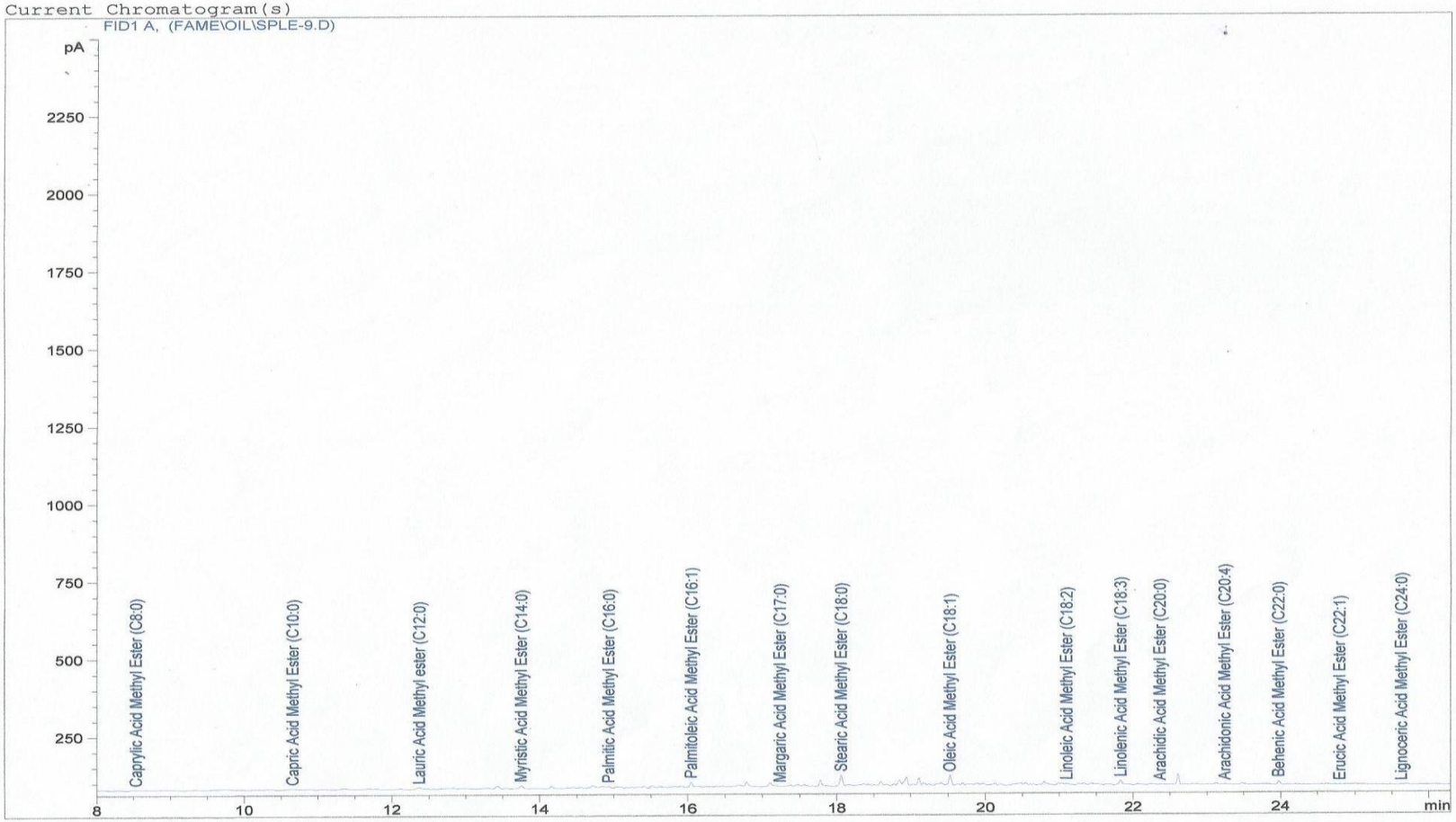
Current Chromatogram(s)



Instrument 1 8/5/2011 2:25:49 AM

Page 1 of 1

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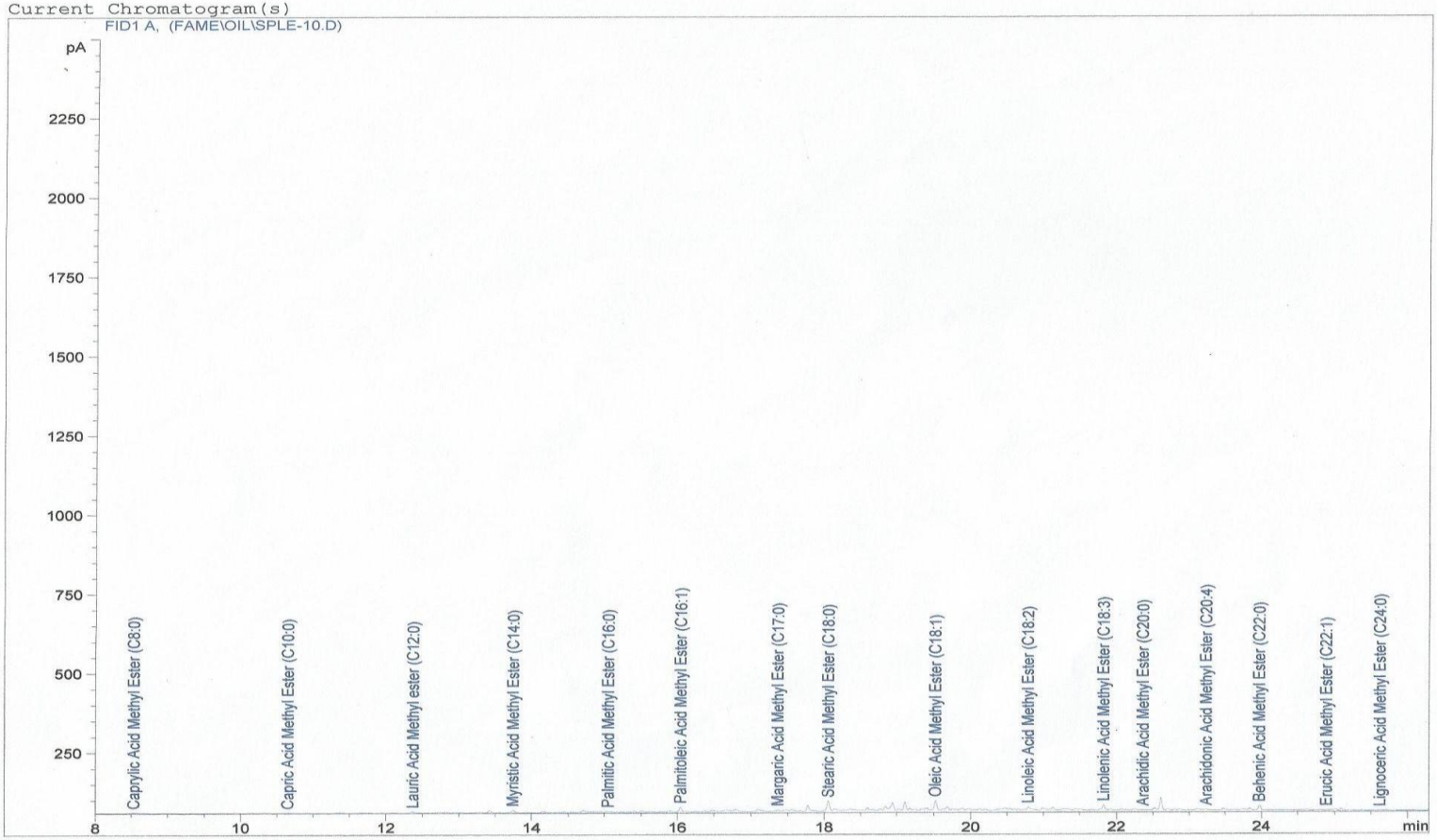
Current Chromatogram(s)



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Page 1 of 1

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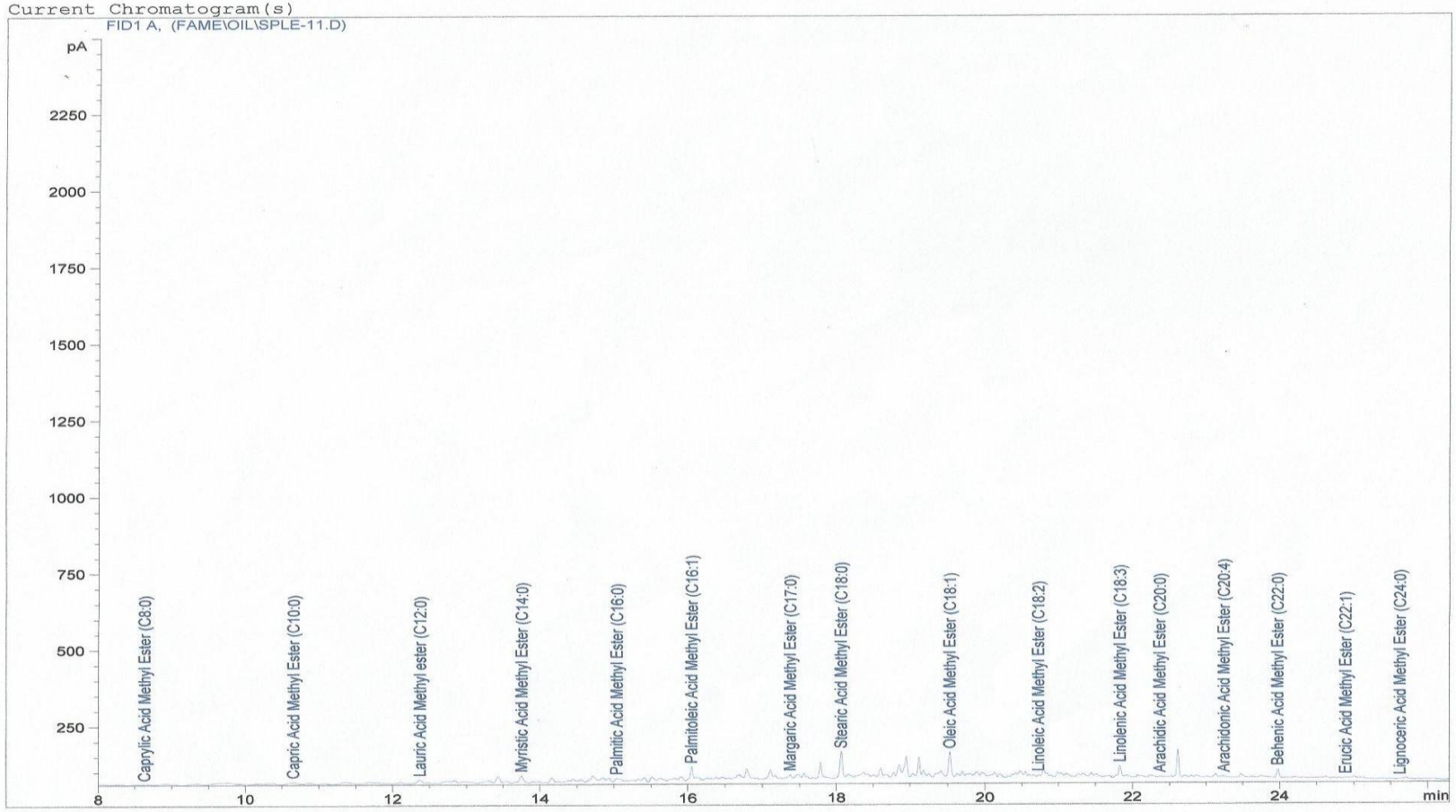
Current Chromatogram(s)



Instrument 1 8/5/2011 2:14:12 AM

Page 1 of 1

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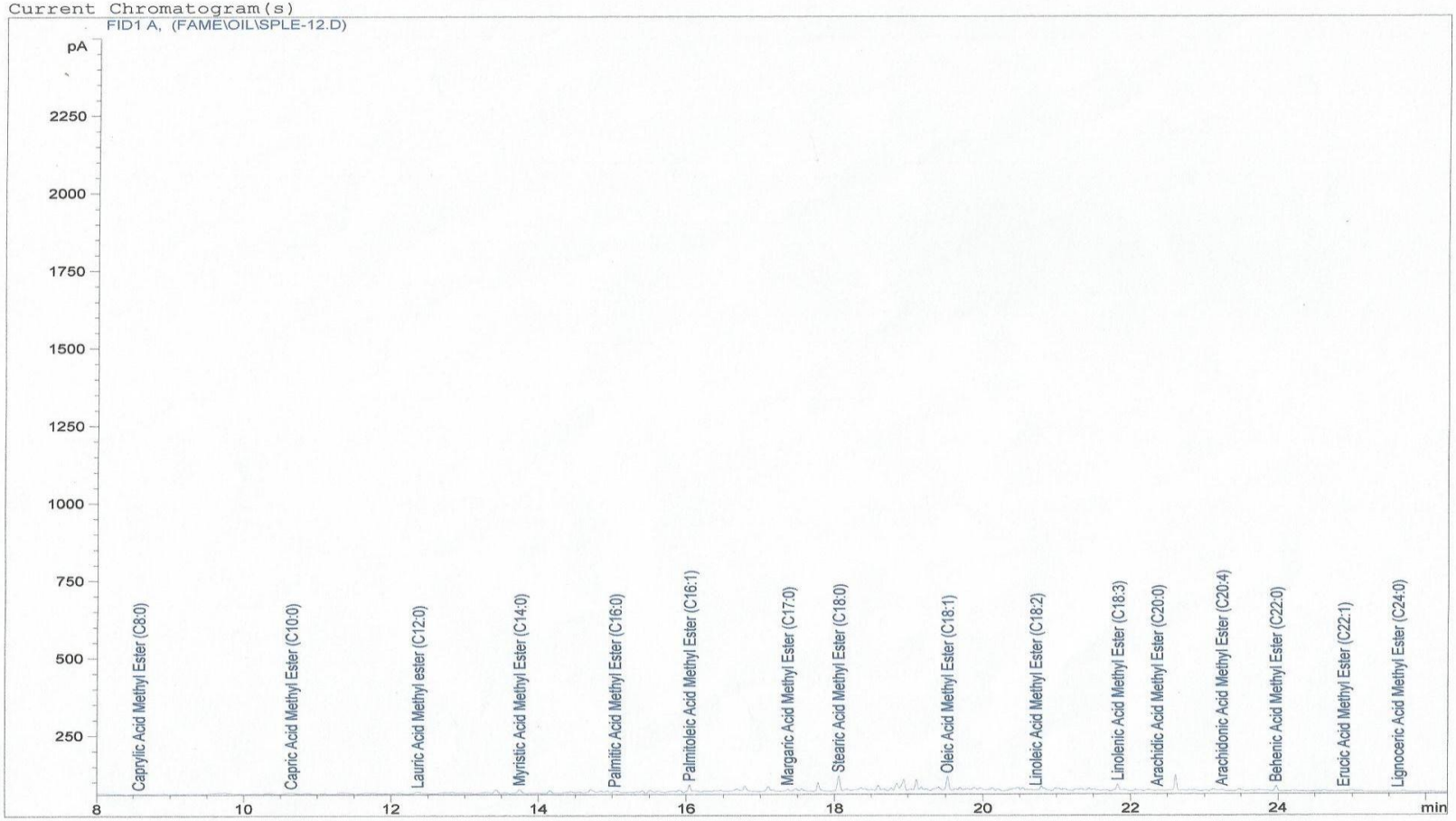
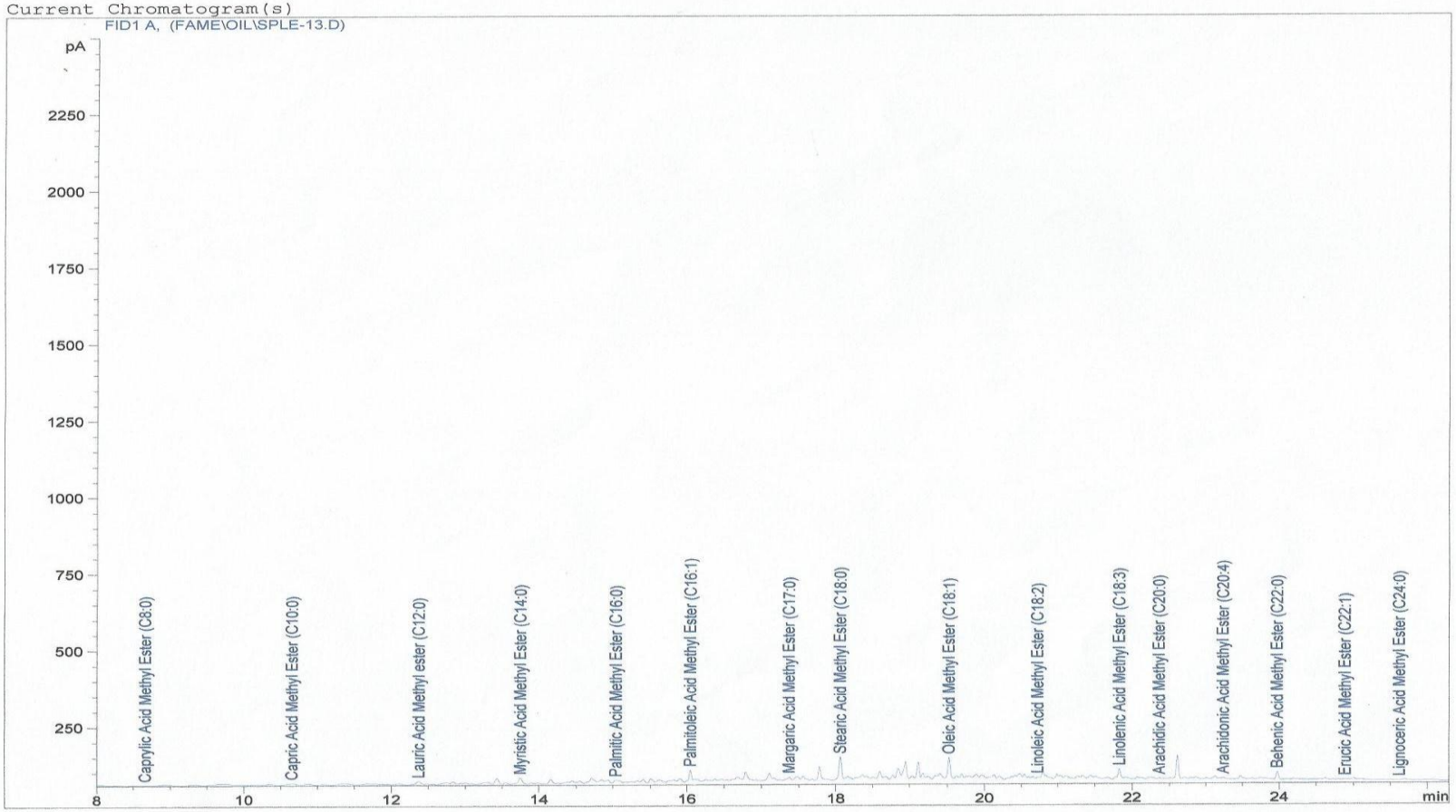


Instrument 1 8/5/2011 2:14:57 AM

Page 1 of 1

Print of window 38: Current Chromatogram(s)

Current Chromatogram(s)



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Page 1 of 1

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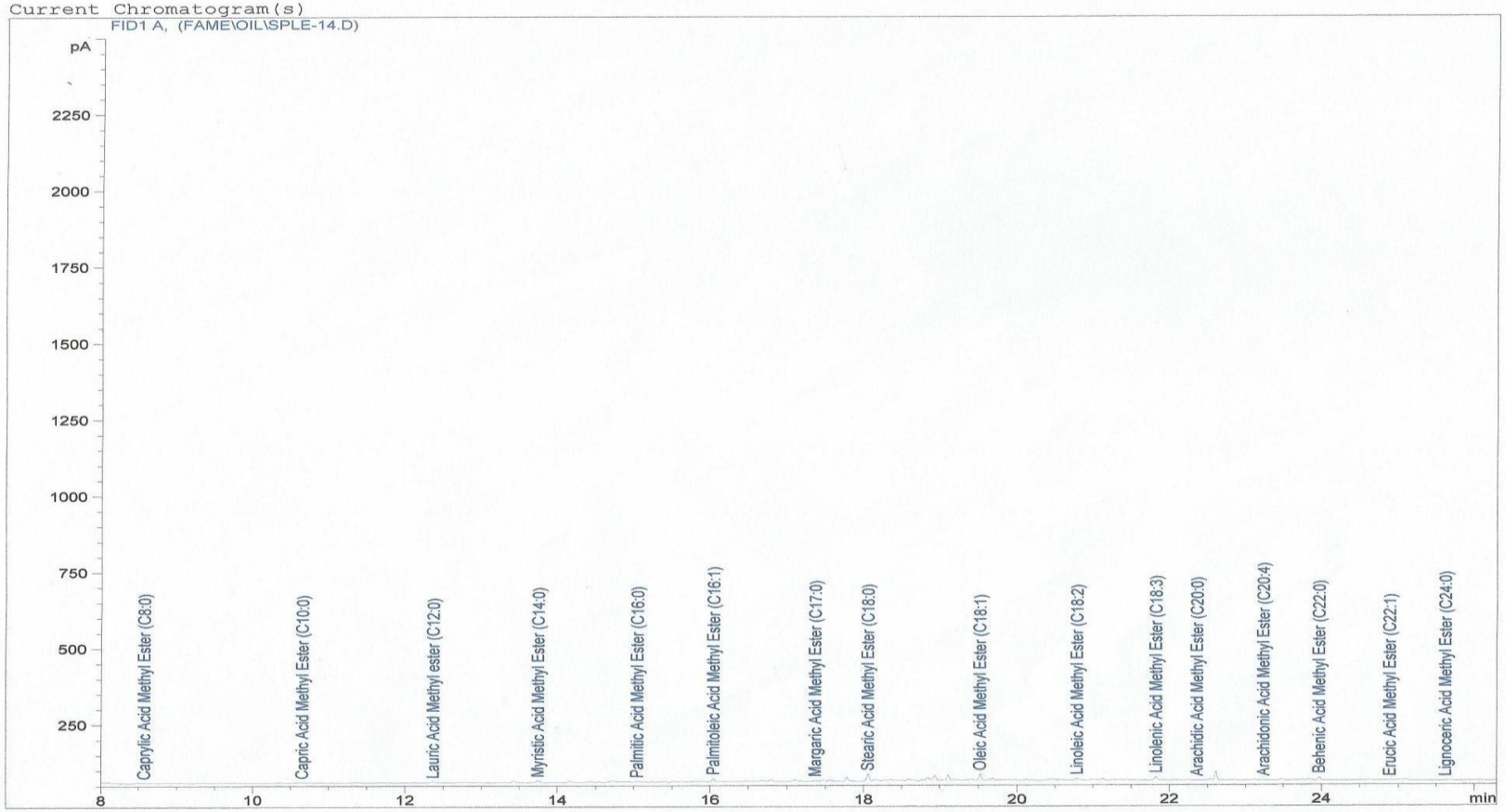
Current Chromatogram(s)



Instrument 1 8/5/2011 2:15:36 AM

Page 1 of 1

Print of window 38: Current Chromatogram(s)

Current Chromatogram(s)

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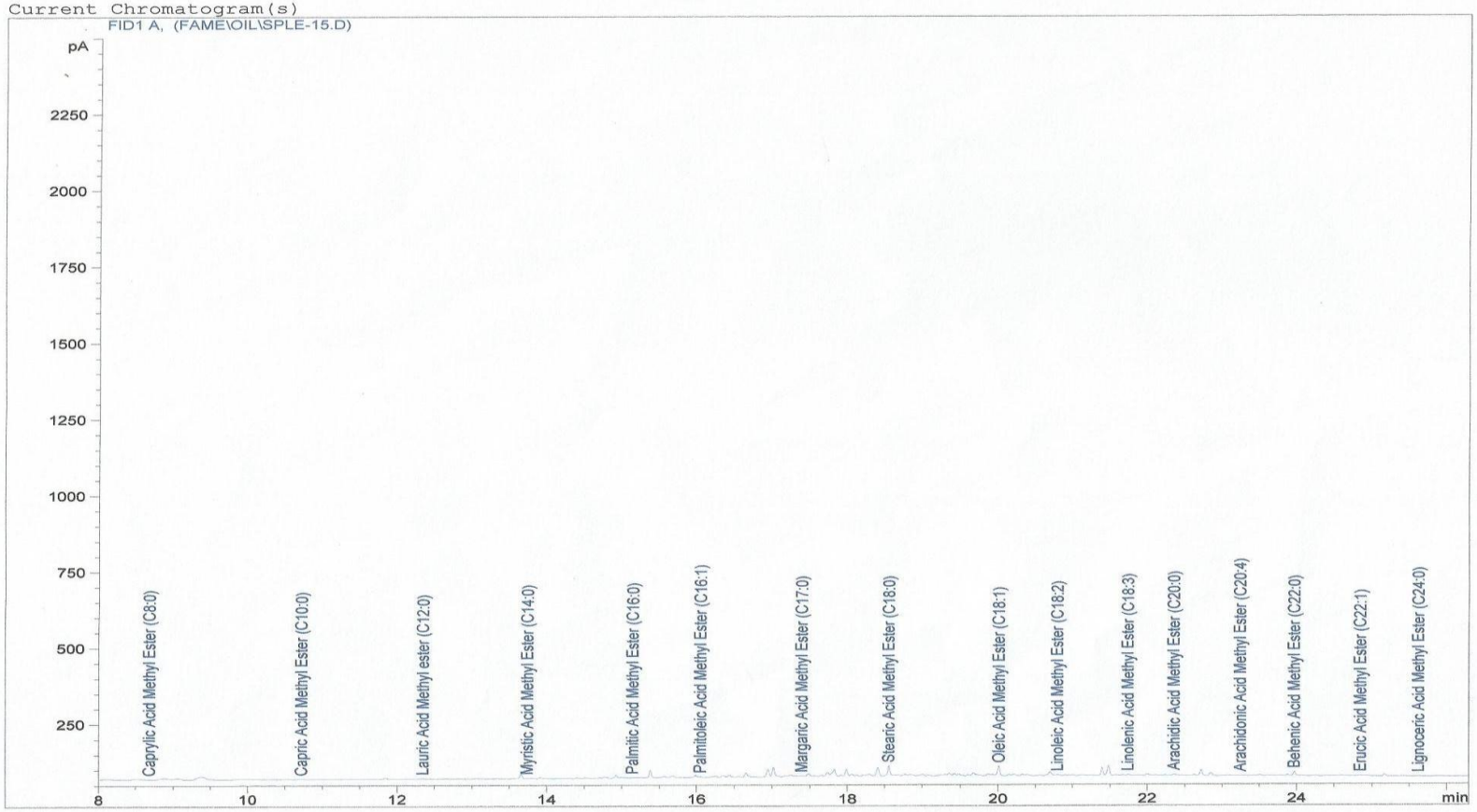

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Page 1 of 1

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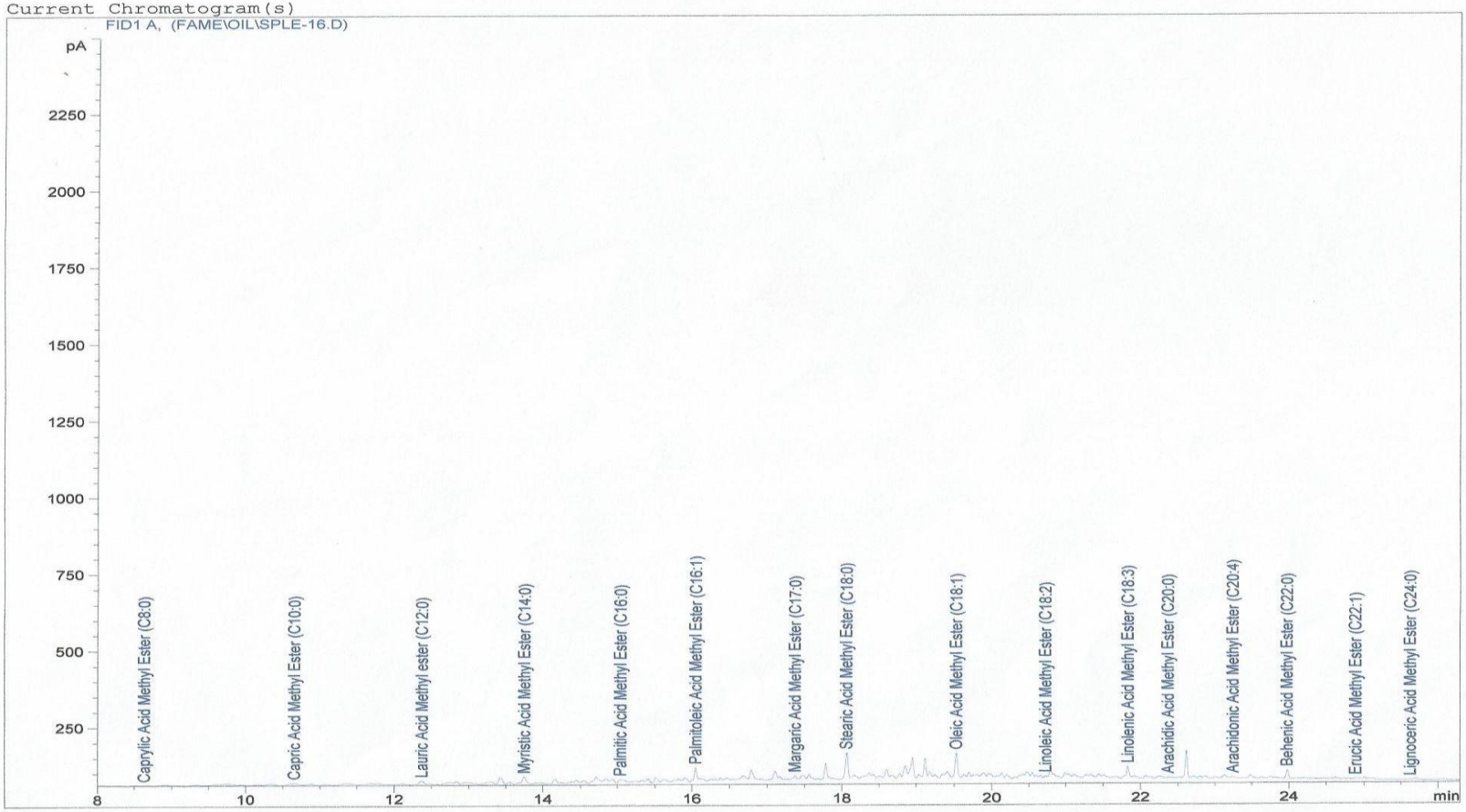
Current Chromatogram(s)



Instrument 1 8/5/2011 2:17:46 AM

Page 1 of 1

Print of window 38: Current Chromatogram(s)

Current Chromatogram(s)

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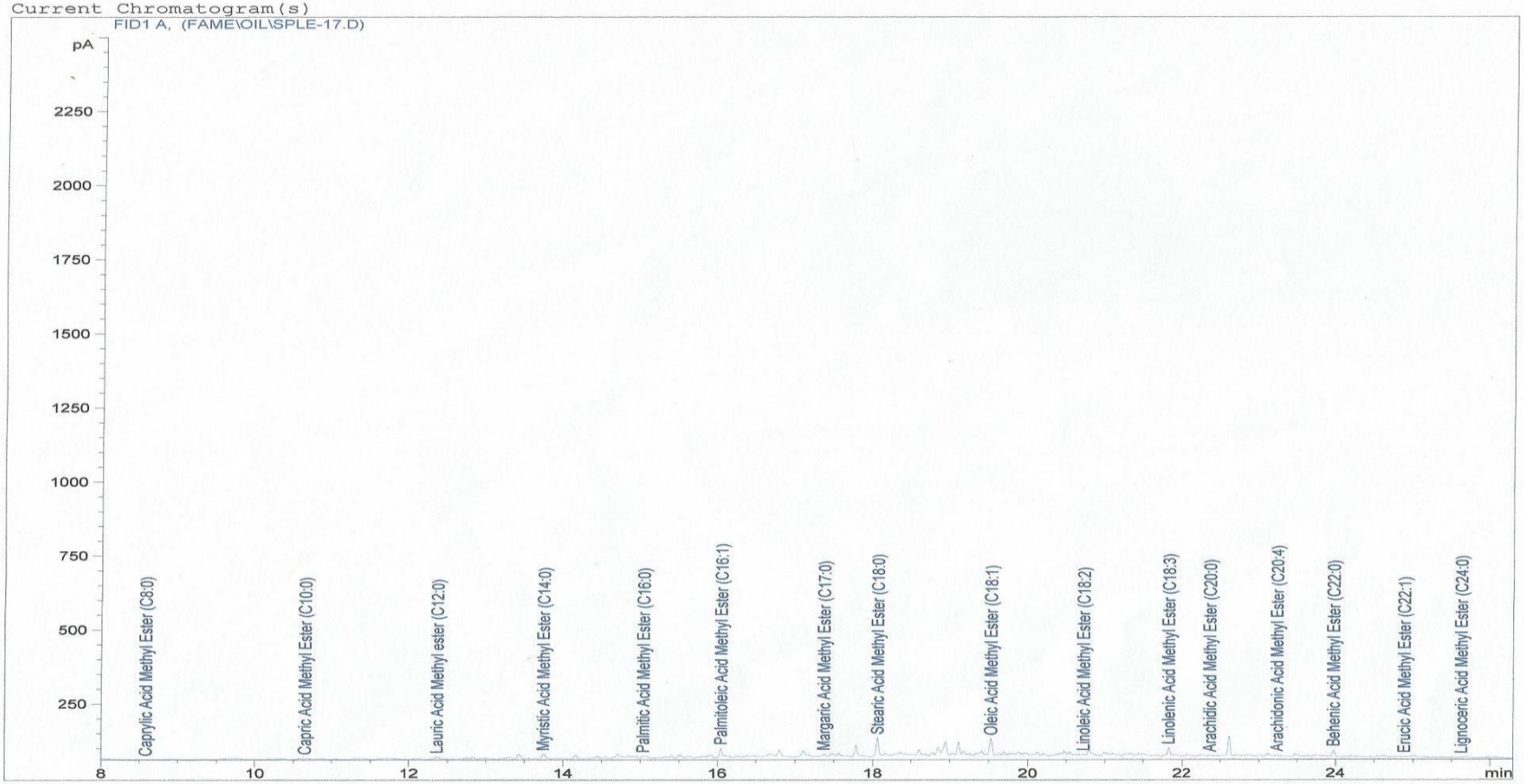

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Page 1 of 1

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Current Chromatogram(s)

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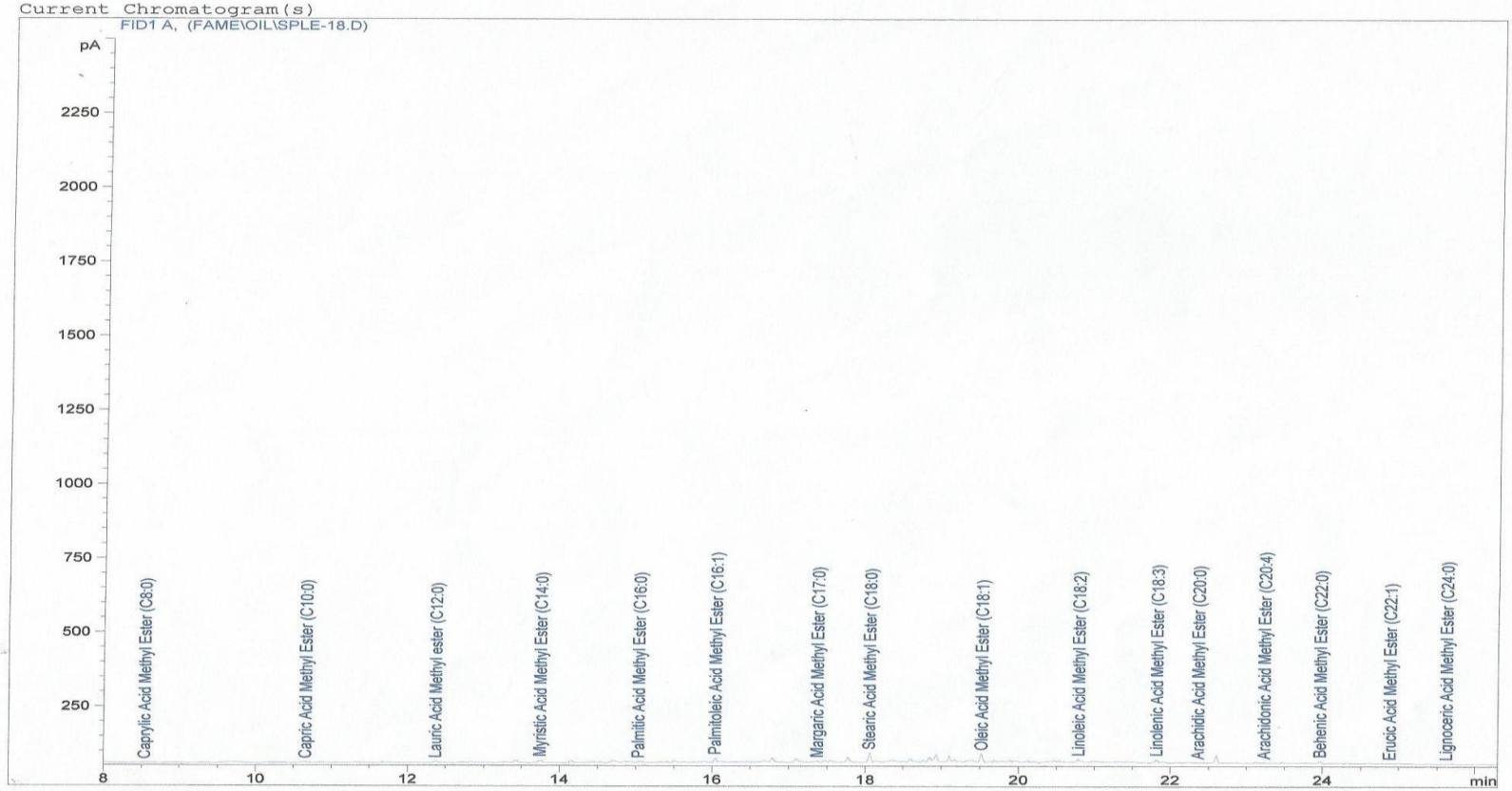

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Page 1 of 1

Print of window 38: Current Chromatogram(s)

Current Chromatogram(s)

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Instrument 1 8/5/2011 2:19:05 AM

Page 1 of 1

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Current Chromatogram(s)

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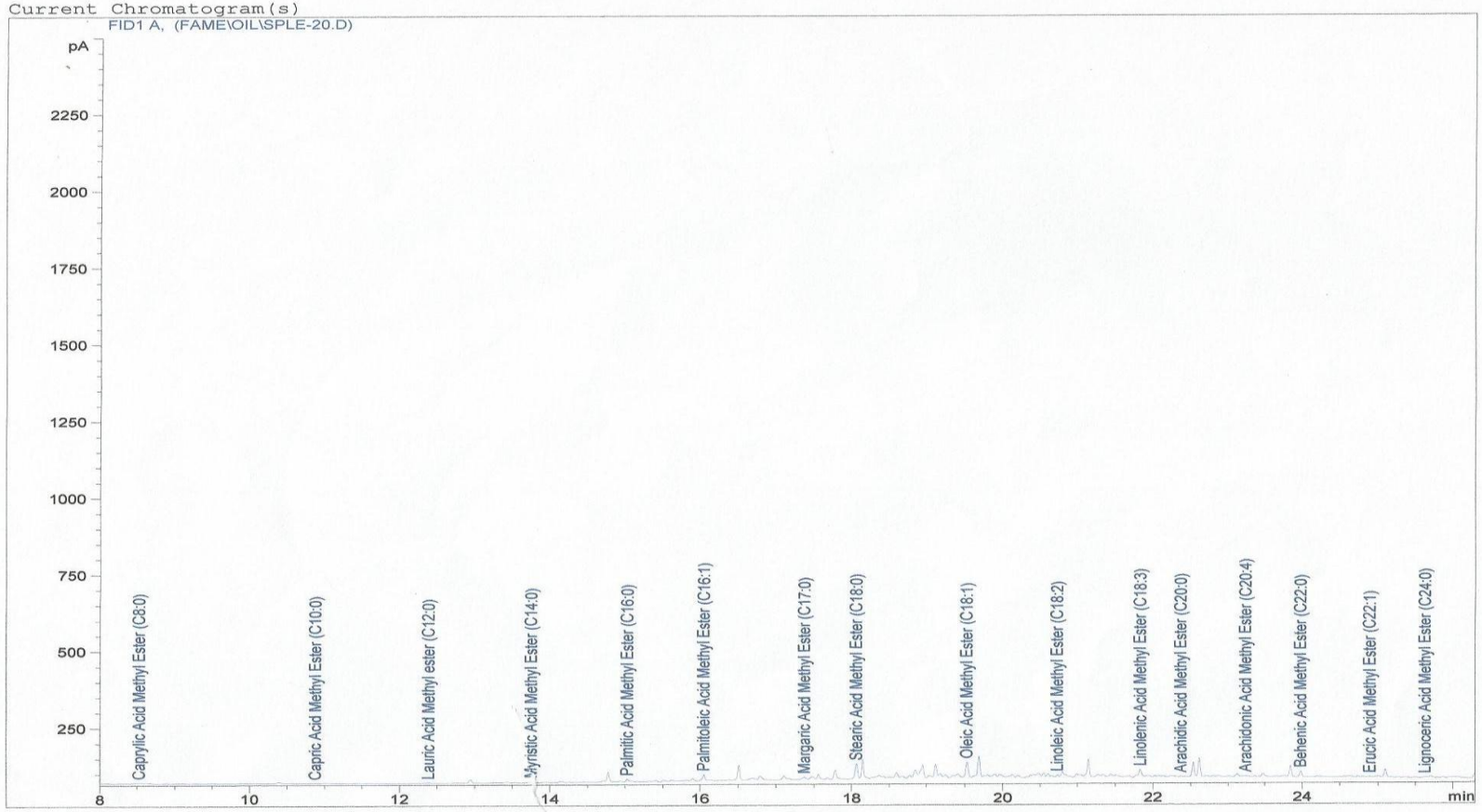
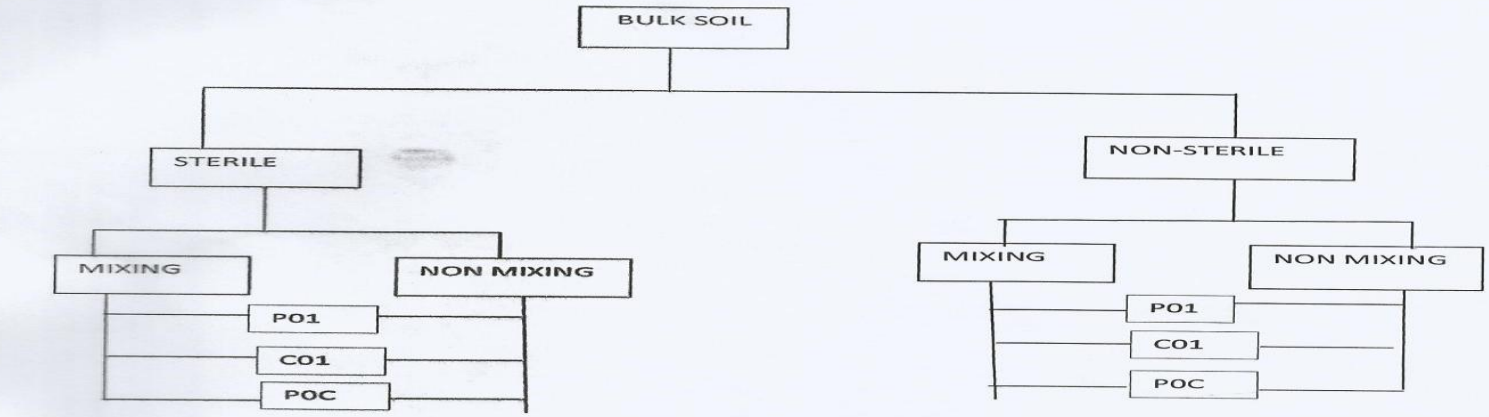


Instrument 1 8/5/2011 2:20:06 AM

Page 1 of 1

APPENDIX III

EXPERIMENTAL DESIGN



Key:

P01 – *Pseudomonas* ***fluorescens***

C01 – *Candida* ***parapsilosis***

POC – *P. fluorescens* + ***C. parapsilosis***

APPENDIX IV

Microbial count in soil samples from oil mill (cfu/g)

	STERILE MIXING (MS) PERIODS (WEEKS)						STERILE NON-MIXING (NMS) PERIODS (WEEKS)						NON-STERILE MIXING (MN/S) PERIODS (WEEKS)						NON-STERILE NON-MIXING (N/MN/S) PERIOD (WEEKS)					
SAMPLE	2	4	6	8	10	12	2	4	6	8	10	12	2	4	6	8	10	12	2	4	6	8	10	12
PO 1	2.315 ± 0.075	2.335± 1.855	6.790± 0.180	6.990± 0.230	7.665± 0.055	7.955± 0.175	1.415± 0.395	1.365± 0.305	4.190± 0.380	4.000± 0.820	0.750± 0.420	8.890± 0.140	1.295± 0.225	4.550± 0.340	2.615± 1.205	5.595± 0.695	6.210± 0.790	0.570± 0.570	3.000± 0.610	2.595± 0.195	2.225± 1.595	4.955± 0.055	5.485± 1.505	0.730± 0.730
CO 1	3.625 ± 0.375	2.265± 0.455	3.375 ± 0.515	1.455± 0.655	0.750± 0.420	3.115± 0.705	2.775± 0.085	3.600± 0.200	2.270± 2.270	4.450± 0.730	3.780± 1.670	5.290± 1.470	3.405± 0.135	0.965± 0.965	1.520± 0.090	0.000± 0.000	0.910± 0.000	1.015± 0.305	2.720± 0.730	2.720± 0.730	3.150± 2.270	1.605± 1.055	0.000± 0.000	0.185± 0.185
PO C	2.195 ± 0.195	2.010± 0.480	2.090 ± 1.800	2.225± 0.185	7.660± 0.050	8.115± 0.355	1.395± 0.115	3.370± 0.110	2.450± 0.340	1.910± 0.500	7.550± 0.660	8.525± 0.065	3.085± 0.805	3.570± 0.240	2.490± 1.860	3.065± 0.355	6.560± 1.000	4.620± 0.150	2.335± 0.055	5.095± 0.085	4.230± 0.410	3.655± 0.075	4.225± 0.245	3.140± 0.130

Total viable microbial count in unpolluted soil sample (cfu/g)

	STERILE MIXING (MS) PERIODS (WEEKS)						STERILE NON-MIXING (NMS) PERIODS (WEEKS)						NON-STERILE MIXING (MN/S) PERIODS (WEEKS)						NON-STERILE NON-MIXING (N/MN/S) PERIOD (WEEKS)					
SAMPLE	2	4	6	8	10	12	2	4	6	8	10	12	2	4	6	8	10	12	2	4	6	8	10	12
PO 1	3.140± 0.130	1.930± 0.120	4.390± 0.180	2.785± 2.105	4.645± 2.575	8.295± 0.515	2.030± 0.040	3.675± 0.065	3.675± 0.135	2.375± 0.965	3.965± 1.255	9.465± 0.025	3.035± 0.445	4.020 ±0.480	4.475± 0.415	3.840± 3.470	4.700±0.410	4.670± 0.940	3.375± 0.035	2.110± 0.680	4.190± 2.560	4.110± 2.940	5.480± 0.480	7.920± 0.370
CO 1	3.445± 0.335	3.270± 0.550	2.770± 0.720	2.395± 0.135	4.885± 1.435	3.610± 3.610	2.525± 0.165	2.630± 0.640	2.260± 0.330	3.160± 1.050	2.175± 1.005	0.870± 0.160	3.240± 0.340	0.000± 0.000	3.735± 0.875	1.670± 1.670	6.230± 0.000	6.810± 0.050	2.385± 0.145	1.495± 0.435	2.315± 1.295	0.000± 0.000	4.860± 0.040	6.285± 0.055
PO C	3.105± 0.145	3.705± 0.095	1.015± 0.215	6.415± 0.095	0.515± 0.515	8.975± 0.055	2.355± 0.365	3.890± 0.030	1.965± 1.165	3.315± 0.135	4.115± 3.405	8.430± 0.650	4.075± 0.595	2.765± 0.365	2.045± 1.135	2.055± 1.025	4.850± 2.810	5.650± 0.640	4.005± 0.085	4.920± 0.030	2.150± 0.740	1.710± 0.130	8.245± 0.215	5.865± 0.205

Total viable microbial count in soil samples contaminated with palm oil (cfu/g)

	STERILE MIXING (MS) PERIODS (WEEKS)						STERILE NON-MIXING (NMS) PERIODS (WEEKS)						NON-STERILE MIXING (MN/S) PERIODS (WEEKS)						NON-STERILE NON-MIXING (N/MN/S) PERIOD (WEEKS)					
SAMPLE	2	4	6	8	10	12	2	4	6	8	10	12	2	4	6	8	10	12	2	4	6	8	10	12
PO 1	1.985± 0.085	2.010± 0.300	5.125± 0.125	4.395± 1.055	3.855± 0.965	8.195± 0.135	2.605± 1.095	3.165± 1.355	3.685± 1.195	0.000± 0.000	3.315± 0.135	4.205± 3.425	2.540± 0.050	3.800± 0.260	5.145± 0.855	8.015± 0.525	4.375± 0.395	3.500± 3.500	2.810± 0.360	3.090± 2.190	4.040± 1.140	7.565± 0.565	3.320± 0.100	5.290± 1.000
CO 1	2.245± 0.655	2.730± 0.070	2.435± 2.435	2.710± 2.710	3.920± 1.810	6.495± 0.265	3.195± 0.665	2.985± 0.025	2.455± 1.775	4.720± 0.050	2.110± 0.000	2.705± 0.195	0.105± 0.105	3.765± 0.905	1.660± 0.760	3.145± 0.435	3.980± 0.000	4.390± 0.080	2.160± 0.250	0.000± 0.000	5.940± 0.020	6.885± 0.025	0.205± 0.205	0.570± 0.570
PO C	1.635± 0.755	3.345± 0.175	2.255± 0.145	3.650± 1.240	3.93± 0.355	1.240± 0.370	2.405± 1.205	1.705± 0.195	2.610± 2.610	3.160± 3.160	0.710± 0.600	0.435± 0.435	2.650± 0.840	5.200± 0.440	2.825± 0.715	7.490± 0.060	1.505± 1.505	4.340± 3.930	3.025± 0.975	5.480± 0.300	3.215± 3.215	4.170± 3.460	4.065± 4.065	4.425± 3.285

Total viable microbial count in soil samples contaminated with palm kernel oil (cfu/g)

	STERILE MIXING (MS) PERIODS (WEEKS)						STERILE NON-MIXING (NMS) PERIODS (WEEKS)						NON-STERILE MIXING (MN/S) PERIODS (WEEKS)						NON-STERILE NON-MIXING (N/MN/S) PERIOD (WEEKS)					
SAMPLE	2	4	6	8	10	12	2	4	6	8	10	12	2	4	6	8	10	12	2	4	6	8	10	12
PO 1	1.675± 1.105	1.375± 0.155	2.660± 0.130	4.085± 0.265	4.155± 0.135	8.975± 0.055	2.420± 0.690	2.945± 0.085	2.960± 0.170	5.865± 0.635	4.725± 0.175	7.525± 1.505	3.765± 0.155	4.480± 1.350	4.920± 0.670	6.710± 0.420	7.235± 1.215	4.910± 0.140	1.805± 0.585	3.925± 1.525	4.970± 0.620	7.035± 0.035	4.855± 0.565	5.245± 0.195
CO 1	1.860± 1.040	2.090± 1.520	3.060± 0.200	6.525± 0.315	2.725± 0.615	1.710± 0.130	2.675± 0.225	0.880± 0.000	1.525± 0.645	4.190± 0.380	0.000± 0.000	3.435± 3.435	2.370± 1.690	3.765± 0.905	4.030± 2.260	3.985± 2.405	3.520± 0.100	6.610± 0.380	1.595± 0.215	1.3000± 0.750	1.115± 1.115	6.890± 0.600	4.770± 0.000	7.505± 0.345
PO C	1.730± 0.580	4.085± 0.105	3.755± 0.435	4.320± 0.500	6.510± 0.220	9.415± 0.125	1.195± 1.195	4.005± 0.085	3.440± 0.100	4.790± 0.440	8.270± 0.140	8.29 ± 0.160	1.960± 0.430	5.875± 0.045	5.785± 1.435	7.350± 0.680	7.815± 0.035	0.665± 0.205	3.145± 0.465	5.570± 0.120	2.650± 2.650	7.870± 0.260	8.095± 0.035	4.515± 4.515

Total recoverable residual oil from unpolluted soil samples (g)

	STERILE MIXING (MS) PERIODS (WEEKS)				STERILE NON-MIXING (NMS) PERIODS (WEEKS)				NON-STERILE MIXING (MN/S) PERIODS (WEEKS)				NON-STERILE NON- MIXING (N/MN/S) PERIODS (WEEKS)			
SAMPLE	0	4	8	12	0	4	8	12	0	4	8	12	0	4	8	12
PO 1	0.0250 ±0.0050	0.0114± 0.0012	0.0167± 0.0023	0.0192± 0.0016	0.0250± 0.0050	0.0168± 0.0033	0.0133± 0.0026	0.0144± 0.0036	0.0030± 0.0000	0.0135± 0.0114	0.0294± 0.0071	0.0039± 0.0019	0.0300± 0.0000	0.0131± 0.0043	0.0133± 0.0041	0.0072± 0.0017
CO 1	0.0250± 0.0050	0.0090± 0.0009	0.0155± 0.0075	0.0181± 0.0035	0.0250± 0.0050	0.0105± 0.0011	0.0154± 0.0019	0.0170± 0.0042	0.0030± 0.0000	0.0241± 0.0035	0.0189± 0.0023	0.0080± 0.0065	0.0300± 0.0000	0.0271± 0.0026	0.0220± 0.01165	0.0093± 0.0012
PO C	0.0250± 0.0050	0.0110± 0.0038	0.0161± 0.0043	0.0225± 0.0051	0.0250± 0.0050	0.0088± 0.0037	0.0129± 0.0089	0.0239± 0.0093	0.0030± 0.0000	0.0157± 0.0057	0.0211± 0.0070	0.0085± 0.0024	0.0300± 0.0000	0.0217± 0.0034	0.0229± 0.0048	0.0070± 0.0002

Total recoverable residual oil from oil mill soil samples (g)

	STERILE MIXING (MS) PERIODS (WEEKS)				STERILE NON-MIXING (NMS) PERIODS (WEEKS)				NON-STERILE MIXING (MN/S) PERIODS (WEEKS)				NON-STERILE NON- MIXING (N/MN/S) PERIODS (WEEKS)			
SAMPLE	0	4	8	12	0	4	8	12	0	4	8	12	0	4	8	12
PO 1	0.0250± 0.0050	0.01835± 0.0000	0.00915± 0.0074	0.01885± 0.0001	0.0250± 0.0050	0.01140± 0.0030	0.01075± 0.0033	0.02530± 0.0059	0.01000± 0.0000	0.01805± 0.0028	0.00970± 0.0021	0.00990± 0.0000	0.0100± 0.0000	0.01400± 0.0028	0.00945± 0.0006	0.02560± 0.0030
CO 1	0.0250± 0.0050	0.0102± 0.0042	0.0075± 0.0024	0.0181± 0.0022	0.0250± 0.0050	0.0102± 0.0042	0.00750± 0.0024	0.0181± 0.0022	0.01000± 0.0000	0.01720± 0.0013	0.00880± 0.0025	0.01120± 0.0037	0.0100± 0.0000	0.01695± 0.00001	0.00910± 0.0011	0.01130± 0.0004
PO C	0.0250± 0.0050	0.01905± 0.0021	0.01690± 0.0025	0.01890± 0.0005	0.0250± 0.0050	0.0125± 0.0003	0.01525± 0.0043	0.01938± 0.0021	0.01000± 0.0000	0.01555± 0.0016	0.01335± 0.0018	0.01230± 0.0003	0.0100± 0.0000	0.01510± 0.0003	0.01070± 0.0063	0.00430± 0.0034

Total recoverable residual oil from palm kernel oil contaminated soil samples (g)

	STERILE MIXING (MS) PERIODS (WEEKS)				STERILE NON-MIXING (NMS) PERIODS (WEEKS)				NON-STERILE MIXING (MN/S) PERIODS (WEEKS)				NON-STERILE NON- MIXING (N/MN/S) PERIODS (WEEKS)			
SAMPLE	0	4	8	12	0	4	8	12	0	4	8	12	0	4	8	12
PO 1	0.3000± 0.0000	0.1124±0.0399	0.26935±0.009	0.1880± 0.0054	0.0300± 0.0000	0.28355±0.034	0.28025±0.003	0.18890±0.009	0.23000±0.000	0.25515±0.0127	0.21510±0.008	0.17855±0.029	0.2300± 0.0000	0.24525±0.002	0.21745±0.059	0.15550±0.004
CO 1	0.3000± 0.0000	0.20370±0.039	0.25470± 0.0096	0.15055± 0.0099	0.0300± 0.0000	0.30265± 0.0294	0.29820± 0.0280	0.17965± 0.0017	0.23000± 0.0000	0.24665± 0.0152	0.23595± 0.0309	0.17665± 0.0361	0.2300± 0.0000	0.22875± 0.0127	0.21470± 0.0132	0.13000±0.019
PO C	0.3000± 0.0000	0.24285± 0.0056	0.2308± 0.0244	0.15560± 0.0032	0.0300± 0.0000	0.29270± 0.0134	0.28862± 0.0247	0.12785± 0.0095	0.23000± 0.0000	0.24470± 0.0350	0.24145± 0.0350	0.15850± 0.0102	0.2300± 0.0000	0.23810± 0.0204	0.21365± 0.0282	0.15725±0.007

Total recoverable residual oil from palm oil contaminated soil samples (g)

	STERILE MIXING (MS) PERIODS (WEEKS)				STERILE NON-MIXING (NMS) PERIODS (WEEKS)				NON-STERILE MIXING (MN/S) PERIODS (WEEKS)				NON-STERILE NON- MIXING (N/MN/S) PERIODS (WEEKS)			
SAMPLE	0	4	8	12	0	4	8	12	0	4	8	12	0	4	8	12
PO 1	0.33500±00	0.25660±0243	0.26320±0.005	0.17290±0.009	0.33500±0.000	0.28245±0.024	0.26790±0.000	0.16035±0.004	0.32000±0.000	0.27960±0.001	0.25690±0.012	0.17450±0.015	0.32000±0.000	0.23475±0.002	0.27970±0.035	0.15485±0.017
CO 1	0.33500±0.000	0.24420±0.028	0.26045±0.006	0.16600±0.002	0.33500±0.000	0.26640±0.006	0.25780±0.020	0.16920±0.023	0.32000±0.000	0.27115±0.014	0.25345±0.04	0.18985±0.004	0.32000±0.000	0.23610±0.000	0.22390±0.000	0.16280±0.000
PO C	0.33500±0.000	0.28390±0.014	0.26605±0.018	0.13380±0.016	0.33500±0.000	0.24190±0.032	0.16792±0.018	0.13025±0.003	0.32000±0.000	0.29205±0.020	0.27010±0.024	0.21855±0.017	0.32000±0.000	0.23130±0.025	0.20185±0.025	0.15000±0.022

Residual oil from unpolluted soil samples (OD)

	STERILE MIXING (MS) PERIODS (WEEKS)				STERILE NON-MIXING (NMS) PERIODS (WEEKS)				NON-STERILE MIXING (MN/S) PERIODS (WEEKS)				NON-STERILE NON- MIXING (N/MN/S) PERIODS (WEEKS)			
SAMPLE	0	4	8	12	0	4	8	12	0	4	8	12	0	4	8	12
PO 1	0.0595± 0.043	0.0187± 0.0005	0.014± 0.005	0.009± 0.003	0.0595± 0.043	0.038± 0.032	0.009± 0.006	0.011± 0.004	0.0972± 0.000	0.038± 0.025	0.059± 0.000	0.000± 0.000	0.0972± 0.000	0.0256± 0.0064	0.0343 ±0.0015	0.000± 0.000
CO 1	0.0595± 0.043	0.084± 0.0351	0.006± 0.004	0.013± 0.004	0.0595± 0.043	0.061± 0.030	0.011± 0.000	0.009± 0.002	0.0972± 0.000	0.021± 0.010	0.027± 0.003	0.000± 0.000	0.0972± 0.000	0.0149± 0.0047	0.0300± 0.0060	0.000± 0.000
PO C	0.0595± 0.043	0.098± 0.0009	0.010± 0.001	0.008± 0.001	0.0595± 0.043	0.119± 0.001	0.006± 0.003	0.009± 0.003	0.0972± 0.000	0.021± 0.010	0.027± 0.003	0.000± 0.000	0.0972± 0.000	0.0149± 0.0047	0.0300± 0.0060	0.000± 0.000

Residual oil from oil mill soil samples (OD)

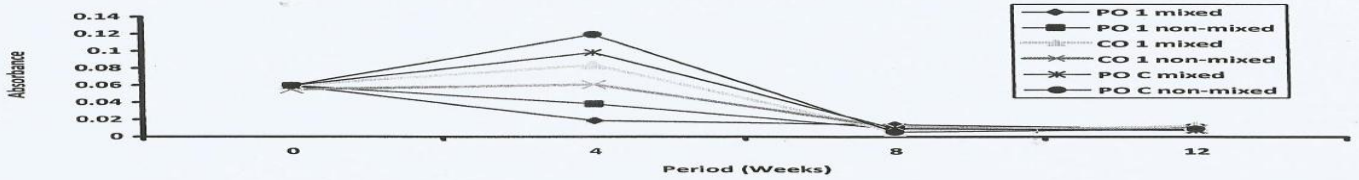
	STERILE MIXING (MS) PERIODS (WEEKS)				STERILE NON-MIXING (NMS) PERIODS (WEEKS)				NON-STERILE MIXING (MN/S) PERIODS (WEEKS)				NON-STERILE NON- MIXING (N/MN/S) PERIODS (WEEKS)			
SAMPLE	0	4	8	12	0	4	8	12	0	4	8	12	0	4	8	12
PO 1	0.0498± 0.000	0.0402± 0.0005	0.017± 0.0015	0.000± 0.000	0.0498± 0.000	0.0397± 0.0008	0.0175±0.0005	0.000± 0.000	0.0430±0.000	0.002950.0012	0.00550±0.0035	0.000± 0.000	0.0430±0.000	0.0148±0.0011	0.0115±0.0025	0.000± 0.000
CO 1	0.0498±0.000	0.0482± 0.0072	0.008± 0.0025	0.000± 0.000	0.0498±0.000	0.0837± 0.0254	0.0110±0.0090	0.000± 0.000	0.0430±0.000	0.019250.0037	0.01450±0.0155	0.000± 0.000	0.0430±0.000	0.0122±0.0026	0.0170±0.0020	0.000± 0.000
PO C	0.0498±0.000	0.0517± 0.0068	0.020± 0.0030	0.000± 0.000	0.0498± 0.000	0.0364± 0.0216	0.0220± 0.0210	0.000± 0.000	0.0430±0.000	0.005250.0038	0.01250±0.0035	0.000± 0.000	0.0430±0.000	0.0110±0.0050	0.0070±0.0020	0.000± 0.000

Residual oil from palm kernel oil contaminated soil samples (OD)

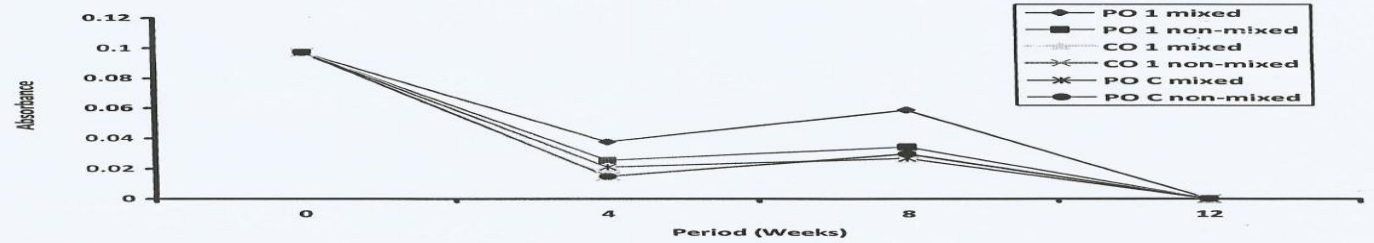
	STERILE MIXING (MS) PERIODS (WEEKS)				STERILE NON-MIXING (NMS) PERIODS (WEEKS)				NON-STERILE MIXING (MN/S) PERIODS (WEEKS)				NON-STERILE NON- MIXING (N/MN/S) PERIODS (WEEKS)			
SAMPLE	0	4	8	12	0	4	8	12	0	4	8	12	0	4	8	12
PO 1	0.6145±0.0064	0.3337± 0.1081	1.6045± 0.0745	0.1505± 0.1075	0.6145±0.0064	0.03334± 0.07665	0.7580±0.1660	0.0650± 0.025	0.24885±0.0013	1.6347±0.0991	0.8660±0.4800	1.5650± 0.0545	0.24885±0.0013	0.7221±0.1952	0.3270±0.0670	0.7250± 0.0125
CO 1	0.6145±0.0064	0.6232± 0.2037	1.0460± 0.0260	0.1090± 0.6540	0.6145±0.0064	0.24665± 0.0666	0.8945±0.0445	0.4285± 0.256	0.24885±0.0013	1.3359±0.4266	1.5445±0.4395	0.0705± 0.0075	0.24885±0.0013	0.29875±0.1018	0.3240±0.0160	0.0605± 0.0165
PO C	0.6145±0.0064	2.2535± 0.7545	0.6715± 0.0165	0.5290± 0.1190	0.6145±0.0064	1.704115± 0.04515	0.6540± 0.1650	0.2785± 0.1065	0.24885±0.0013	1.75335±0.2662	1.5220±0.2510	0.0995± 0.0205	0.24885±0.0013	0.81730±0.0900	0.39550±0.1725	0.0735± 0.0175

Residual oil from palm oil contaminated soil samples (OD)

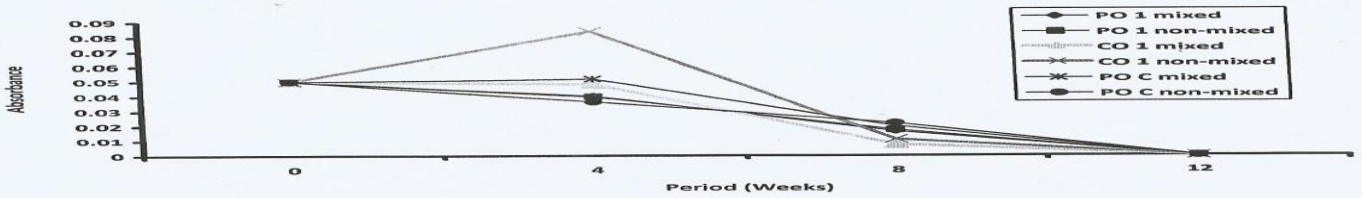
	STERILE MIXING (MS) PERIODS (WEEKS)				STERILE NON-MIXING (NMS) PERIODS (WEEKS)				NON-STERILE MIXING (MN/S) PERIODS (WEEKS)				NON-STERILE NON- MIXING (N/MN/S) PERIODS (WEEKS)			
SAMPLE	0	4	8	12	0	4	8	12	0	4	8	12	0	4	8	12
PO 1	1.8333± 0.2118	2.3247± 0.3442	0.5695± 0.2315	0.2675± 0.0085	1.8333±0.2118	1.2152 ± 0.5843	0.3280±0.0660	0.2810± 0.073	1.2675±0.02255	1.3764±0.0386	0.3255±0.0085	0.5900± 0.007	1.2677±0.02255	0.92040±0.39960	0.29000±0.0190	0.1510± 0.0620
CO 1	1.8333± 0.2118	0.12960± 0.0307	0.4690± 0.2680	0.2805±0± 0.0785	1.8333±0.2118	1.06694± 0.24015	0.57350±0.3215	0.2190± 0.0300	1.2675±0.02255	0.70375±0.09375	0.3230±0.0510	0.2730± 0.0130	1.2677±0.02255	0.6062±0.0000	0.3145±0.0025	0.1925±0± 0.0050
PO C	1.8333± 0.2118	2.09915± 0.4346	0.7560± 0.4900	0.3510±0± 0.0980	1.8333±0.2118	0.99110± 0.21510	0.33650± 0.0485	0.2465± 0.0515	1.2675±0.02255	0.94145±0.08665	0.81150±0.26350	0.20150± 0.0315	1.2677±0.03135	0.69380±0.15240	0.28350±0.0175	0.1680± 0.0070



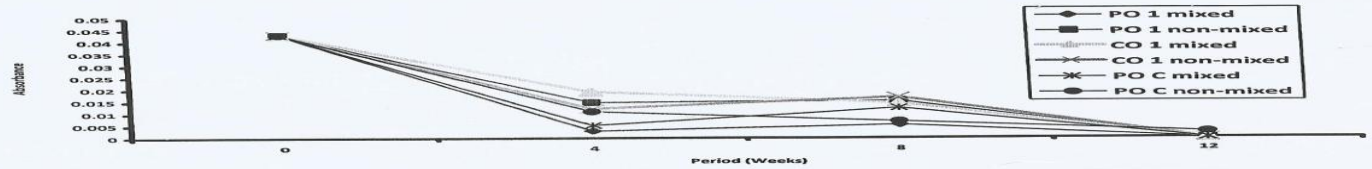
Residual edible oil in sterile unpolluted soil sample with and without mixing



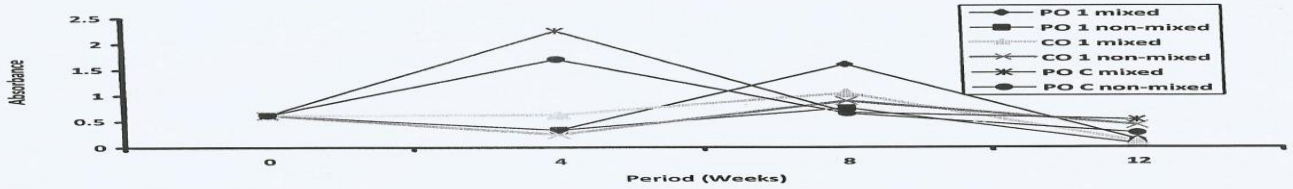
Residual edible oil in non-sterile unpolluted soil sample with and without mixing



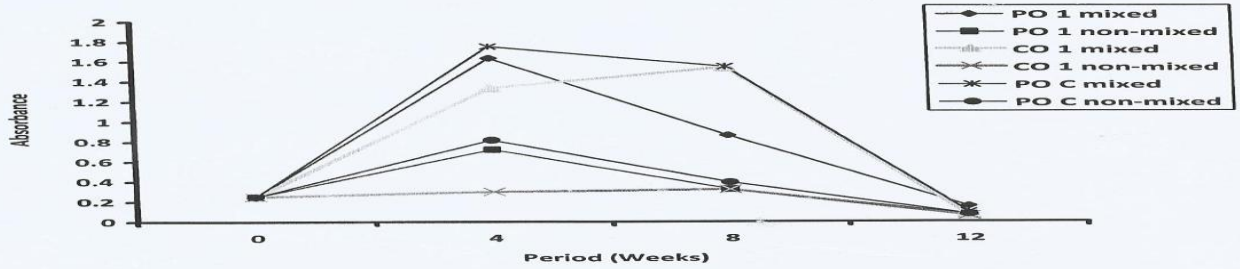
Residual edible oil in sterile soil sample from oil mill with and without mixing



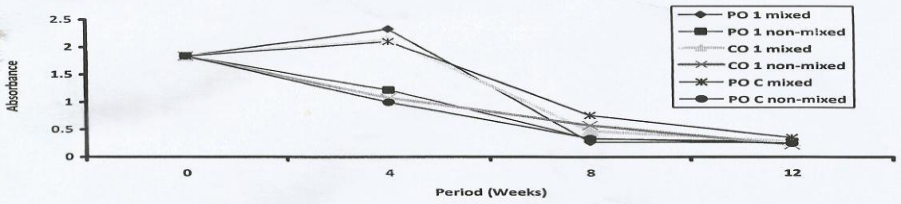
Residual edible oil in non-sterile soil sample from oil mill with and without mixing



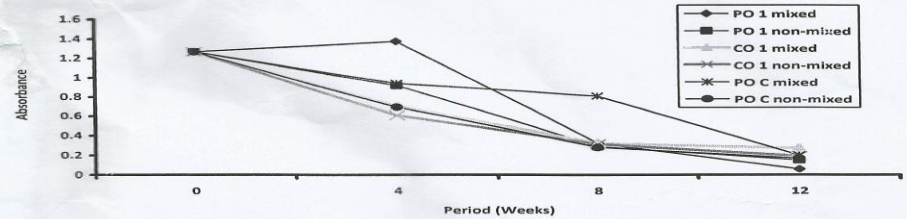
Residual oil in sterile soil sample contaminated with palm kernel oil with and without mixing



Residual oil in non-sterile soil sample contaminated with palm kernel oil with and without mixing



Residual oil in sterile soil sample contaminated with palm oil with and without mixing



Residual oil in non-sterile soil sample contaminated palm oil with and without mixing