



**UNIVERSITI PUTRA MALAYSIA**

**CHARACTERIZATION OF LIPASE PRODUCED  
BY THERMOPHILIC FUNGI  
ISOLATED FROM POME**

**MOHD. YUSOPP BIN ABD. SAMAD**

**FSAS 1990 4**

**CHARACTERIZATION OF LIPASE PRODUCED  
BY THERMOPHILIC FUNGI  
ISOLATED FROM POME.**

**By**

**MOHD. YUSOFF BIN ABD. SAMAD**

**Thesis Submitted in Fulfillment of the Requirements for  
the Degree of Master of Science in the Faculty of  
Science and Environmental Studies,  
Universiti Pertanian Malaysia**

**October 1990**



**Dedicated To My Parents -**

**Abd. Samad & Siti Zainab, and**

**those who pray for my success ! ! !**



## ACKNOWLEDGEMENTS

All praise be to the Almighty Allah, the Merciful and the Benificent. Had it not been due to His will and favour, the completion of this study would not have been possible.

I wish to express my deep appreciation and gratitude to my supervisors, Associate Professor Dr. Abu Bakar Salleh and Dr. Che Nyonya Abd. Razak for their dedicated efforts, invaluable guidance, constant support and encouragement throughout the course of this study.

I am grateful to all staff members of the Department of Biochemistry and Microbiology and friends who have helped me in one way or another.

My sincere appreciation is extended to the C.A.B. Mycological Institute of Kew Garden Surrey, England for the identification of the isolated fungi for this study.

Finally, I am greatly indebted to the Ministry of Science Technology and Environment, Malaysia for the financial support through IRPA Project No. 2-07-05-05.



## TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS .....	iii
LIST OF TABLES .....	ix
LIST OF FIGURES .....	xi
LIST OF PLATES .....	xii
ABSTRACT .....	xiii
ABSTRAK .....	xv
 <b>CHAPTER</b>	
1 INTRODUCTION .....	1
2 LITERATURE REVIEW .....	6
Detection of Lipolytic Activity and Screening of Lipolytic Microorganism .....	6
Production of Microbial Lipases .....	8
Effects of Nitrogen Sources .....	9
Effects of Carbon Sources .....	11
Effects of Substrate Related Compounds, Natural Oils and Synthetic Triglycerides.....	13
Effects of Minerals .....	15
Effects of Physical Factors .....	17
Purification of Lipases .....	20



	Page
Properties of Purified Microbial Lipases .....	25
General Properties .....	25
Substrate Specificity and Mode of Action .....	28
The Application of Lipases .....	34
Development of Dairy Products .....	34
Interesterification of Oils and Fats .....	35
Esterification of Fatty Acids .....	36
Hydrolysis of Oils and Fats .....	39
Miscellaneous Applications .....	40
 3 MATERIALS AND METHODS .....	 42
Materials .....	42
Methods .....	48
Preparation of Plate Assay Medium .....	48
Source and Preparation of Enzyme for Development of Plate Assay .....	50
Assay of Lipase Activity .....	50
Preparation of Inoculum and Media for Cultural Studies .....	55



	Page
Purification of Lipase .....	58
Procedures for the Study on Properties of Partially Purified Lipase from <u>Rhizopus rhizopodiformis</u> .....	61
4 RESULTS .....	66
Plate Assay for Primary Screening of Lipolytic Activity .....	66
Development of Plate Assay .....	66
Effect of Enzyme Concentrations and Time of Incubation on the Diameter of the Zone of Intensification on a Selective Plate Assay .....	68
Effect of Known Lipolytic Microorganism on Plate Assay .....	69
Isolation and Screening of Thermophilic Fungi and Their Thermostability Lipases .....	76
Isolation and Screening .....	76
Screening of Lipase Production from Isolated Fungi in a Defined Medium .....	76
Thermostability Test for Lipase from <u>Rhizopus</u> <u>rhizopodiformis</u> .....	78
Study on the Cultural Conditions for the Production of Lipase from <u>Rhizopus rhizopodiformis</u> .....	81



	Page
Time Course of Lipase Production .....	81
Effect of Carbon Sources .....	81
Effect of Nitrogen Sources .....	81
Effect of Substrate Related Compounds, Natural Oils and Synthetic Triglycerides.....	85
Partial Purification of Lipase .....	87
Enzyme Purification .....	87
Purity of Enzyme .....	91
Properties of Lipase .....	91
Optimum pH for Lipase Activity .....	91
Optimum Temperature for Lipase Activity .....	91
pH Stability .....	95
Temperature Stability .....	95
Substrate Specificity .....	95
5 DISCUSSION .....	102
Development of Plate Assay .....	103
Isolation and Screening of Thermophilic and Lipolytic Fungi .....	105





	Page
Cultural Conditions for the Production of Lipase from <u>Rhizopus rhizopodiformis</u> .....	107
Partial Purification of Lipase from <u>Rhizopus rhizopodiformis</u> .....	111
Properties of Partially Purified Lipase .....	114
6 SUMMARY AND CONCLUSION .....	118
Summary .....	118
Implication and Suggestions .....	120
BIBLIOGRAPHY .....	123
APPENDIX .....	132
BIOGRAPHICAL SKETCH .....	154



## LIST OF TABLES

Table	Page
1	Physical Factors for Optimum Lipase Production by Lipase-producing Microorganisms ..... 19
2	Properties of Purified Lipase from Microbial Sources ..... 26
3	Medium Composition for Tween Agar ..... 49
4	Medium Composition for Tributyrin and Triolein Agar ..... 49
5	Effect of Tween-Chromogens and Other Substrate Systems for Detection of Lipase Activity ..... 67
6	Fungi Isolated and Screened by Tween 80 and Victoria Blue B Plate Assay..... 77
7	Activity of a 72 hr Broth Culture by Tween 80 and Victoria Blue B Plate and Batch Assay ..... 77
8	Ammonium Sulphate Fractionation of Crude Enzyme ..... 88
9	Acetone Fractionation of Crude Enzyme ..... 88
10	Partial Purification of Lipase ..... 89
11	Action of the Enzyme on Monoacid Triglycerides ..... 99
12	Action of the Enzyme on Esters ..... 99
13	Effect of Enzyme Concentrations and Time of Incubation on the Diameter of the Zone Intensification on Tween 80 Plate Assay ..... 133



Table	Page
14	Thermostability of Crude Enzyme from <u>Rhizopus rhizopodiformis</u> Strain S1 and Strain S2 for 30 min Incubation ..... 133
15	Thermostability of Crude Enzyme from <u>Rhizopus rhizopodiformis</u> Strain S1 and Strain S2 for 24 hr Incubation ..... 134
16	Time Course of Lipase Production by <u>Rhizopus rhizopodiformis</u> ..... 134
17	Effect of Carbon Sources on Lipase Production by <u>Rhizopus rhizopodiformis</u> ..... 135
18	Effect of Nitrogen Sources on Lipase Production by <u>Rhizopus rhizopodiformis</u> ..... 136
19	Effects of Some Substrate Related Compounds, Natural Oils and Synthetic Triglycerides on Lipase Production by <u>Rhizopus rhizopodiformis</u> ..... 137
20	pH Optimum for Lipase Activity ..... 138
21	Optimum Temperature for Lipase Activity ..... 138
22	pH Stability of Lipase ..... 139
23	Temperature Stability of Lipase ..... 139
24	Action of Lipase on Monoacid Triglycerides, Tweens and Methyl Esters ..... 140



## LIST OF FIGURES

Figure	Page
1 Reactions of Lipases .....	2
2 Effect of Enzyme Concentrations and Time of Incubation on the Diameter of Zone of Intensification .....	71
3 Thermostability of Lipases from <u>Rhizopus rhizopodiformis</u> S1 and S2 .....	79
4 Thermostability of Lipases from <u>Rhizopus rhizopodiformis</u> S1 and S2 .....	80
5 Time Course of Lipase Production .....	82
6 Effect of Carbon Sources on Lipase Production by <u>Rhizopus</u> <u>rhizopodiformis</u> .....	83
7 Effect of Nitrogen Sources on Lipase Production by <u>Rhizopus</u> <u>rhizopodiformis</u> .....	84
8 Effect of Substrate Related Compounds, Natural Oils and Synthetic Triglycerides on Lipase Production by <u>Rhizopus</u> <u>rhizopodiformis</u> .....	86
9 Elution Profile of Lipase Activity from a Sephadex G-100 Column .....	90
10 Optimum pH for Lipase Activity .....	93
11 Optimum Temperature for Lipase Activity .....	94
12 pH Stability of Lipase .....	96
13 Temperature Stability of Lipase .....	97
14 Standard Curve for the Determination of Protein .....	149



## LIST OF PLATES

Plate	Page
1 Agar Plate with Triolein (0.078%) and Victoria Blue B as Indicator .....	70
2 Agar Plate with Triolein (0.078%) and Rhodamine B as Indicator .....	72
3 Agar Plate with Tween 80 as Substrate and Victoria Blue B as Indicator .....	73
4 Agar Plate with Tween 80 and Victoria Blue B with the Fungus, <u>Rhizopus oryzea</u> Grown on it .....	74
5 Agar Plate with Tween 80 and Victoria Blue B with the Bacteria of <u>Pseudomonas sp</u> Grown on it .....	75
6 Disc-gel Electrophoresis Pattern of Lyophilized Enzyme and Partially Purified Enzyme.....	92
7 Positional Specificity of Lipase .....	101
8 A Sephadex G-100 Column Chromatography Apparatus .....	142
9 Disc-Electrophoresis Apparatus .....	143
10 The Growth of <u>Rhizopus</u> <span style="margin-left: 150px;">o</span> <u>rhizopodiformis</u> on PDA at 45 C .....	144



Abstract of thesis submitted to the Senate of Universiti  
Pertanian Malaysia in fulfillment of the requirements for the  
degree of Master of Science.

**CHARACTERIZATION OF LIPASE PRODUCED  
BY THERMOPHILIC FUNGI ISOLATED FROM POME**

By

**MOHD. YUSOFF BIN ABD. SAMAD**

October 1990

Supervisor : Assoc. Prof. Hj. Abu Bakar Salleh, Ph.D.

Co-supervisor : Hjh. Che Nyonya Abd. Razak, Ph.D

Faculty : Science and Environmental Studies

A plate assay to determine lipase activity was developed. Tween 80, used as a substrate with Victoria Blue B as the indicator seemed to be a reliable technique. Lipolytic activity was determined by the formation of the zone of intensification of the indicator colour after 24 hr. Intensity of the colour developed was greater than that of triolein dye system and clearer than the hydrolysis zone of tributyrin plate. Test using a commercial enzyme and growth media containing lipolytic activity showed that the zone of intensification increased with increased lipolytic activity. A linear relationship could be seen when log enzyme concentration was plotted against the diameter of zone of intensification. Using this technique primary



screening of lipolytic microorganisms could be conducted using the formation of zone of intensification around the colonies and mycelia.

Five fungi showing lipolytic activity on plate were isolated using plate assay. However, using broth culture only one fungus was found to produce lipase. The fungus was identified as Rhizopus rhizopodiformis, (strain S1 and S2). Strain S1 was found to produce maximum lipase yield in the basal medium containing of peptone and glycerol.

The enzyme was partially purified by means of acetone precipitation and chromatography on Sephadex G-100. The enzyme was purified about 9.7-fold and yielded 37%. The enzyme showed two bands on disc polyacrylamide gel electrophoresis. The optimum pH of the enzyme activity was 6.0 and the optimum temperature was 45 °C. The enzyme was most stable at pH 7.0 and could withstand temperature of up to 50 °C. The enzyme had a greater affinity to triglycerides with short chain fatty acids and it was shown to have 1, 3-positional specificity.



Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi syarat untuk mendapat Ijazah Master

**PENCIRIAN LIPASE YANG DIHASILKAN OLEH  
KULAT TERMOFILIK YANG DIPENCIL DARI  
SISA BUANGAN KELAPA SAWIT (POME)**

Oleh

**MOHD. YUSOFF BIN ABD. SAMAD**

Oktober 1990

Penyelia : Prof. Madya Hj. Dr. Abu Bakar Salleh

Penyelia Bersama : Dr. Hjh. Che Nyonya Abd. Razak

Fakulti : Sains dan Pengajian Alam Sekitar

Kaedah plat asai untuk menentukan aktiviti lipase telah dihasilkan. Kaedah ini menggunakan Tween 80 sebagai substrat dan Victoria Blue B sebagai penunjuk. Teknik ini didapati boleh dipercayai. Aktiviti lipolitik ditentukan dengan pembentukan zon keamatan penunjuk warna selepas pengeraman plat selama 24 jam. Keamatan warna yang diperolehi adalah lebih tinggi daripada sistem pewarna triolein dan lebih terang daripada zon hidrolisis plat tributirin. Ujian menggunakan enzim komersial dan media pertumbuhan yang mengandungi aktiviti lipolitik menunjukkan zon keamatan bertambah dengan pertambahan aktiviti lipolitik. Hubungan yang linear boleh didapati bila log kepekatan enzim diplotkan berbanding dengan zon garispusat keamatan.





Penyaringan primer mikroorganisma-mikroorganisma lipolitik dengan menggunakan teknik ini boleh dijalankan melalui pembentukan zon keamatan di sekeliling koloni bakteria dan miselia kulat.

Dengan teknik plat asai ini lima jenis kulat dengan aktiviti lipolitik telah dipencilkan. Walau bagaimanapun, hanya dua jenis sahaja yang mengeluarkan lipase dalam kultur kaldu. Kulat-kulat ini telah dikenalpasti sebagai Rhizopus rhizopodiformis strain S1 dan strain S2. Strain S1 ini telah didapati mengeluarkan enzim lipase yang maksimum dalam media asas yang mengandungi pepton dan gliserol.

Enzim ini telah diseparatulkan dengan menggunakan pemendakan aseton dan selanjutnya dengan proses kromatografi menggunakan gel Sephadex G-100. Enzim ini telah dituliskan sebanyak 9.7 kali ganda dengan penghasilan sebanyak 37%. Dua jalur protein telah didapati pada cakra gel poliakrilamid. Enzim ini menunjukkan pH dan suhu optimum bagi aktiviti pada 6.0 dan 45 C.

Enzim ini juga didapati sangat stabil pada pH 7.0 dan suhu sehingga 50 C. Ia juga menunjukkan keafinan substrat yang tinggi kepada trigliserida yang mempunyai rantai asid lemak yang pendek. Kespesifikan kedudukan enzim ini menunjukkan ianya adalah pada kedudukan 1 dan 3 pada trigliserida.

## CHAPTER 1

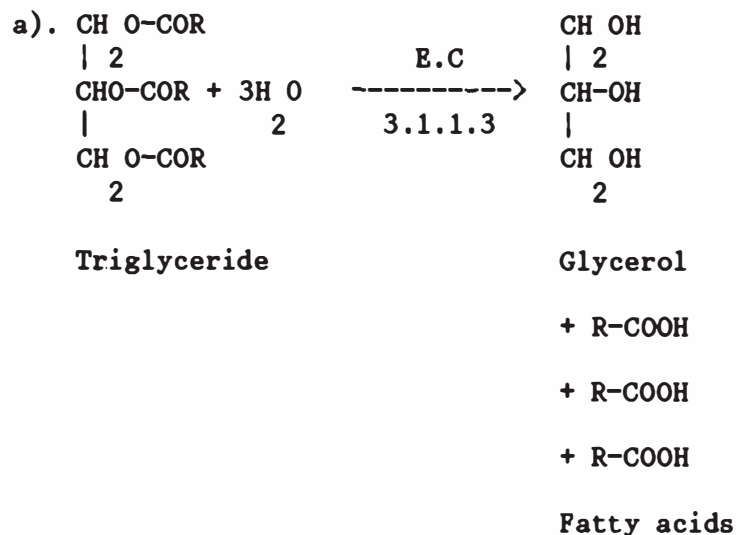
### INTRODUCTION

Lipolytic enzymes are indispensable for the biological turnover of lipids. These digestive enzymes are responsible for the transfer of lipid from one organism to another, such as from animal to plant or from one animal to the other. Within the organisms, they play a role in the deposition and mobilization of fats as well as in the metabolism of intracellular lipids. Therefore lipolytic enzymes are essential to the functioning of biological membranes (Brockerhoff and Jensen, 1974). Lipases, a major group of lipolytic enzymes, have recently received much attention from the academic and industrial sectors. In fact, lipases which are distributed in microorganisms such as yeasts, bacteria and fungi are potentially very important in industrial applications.

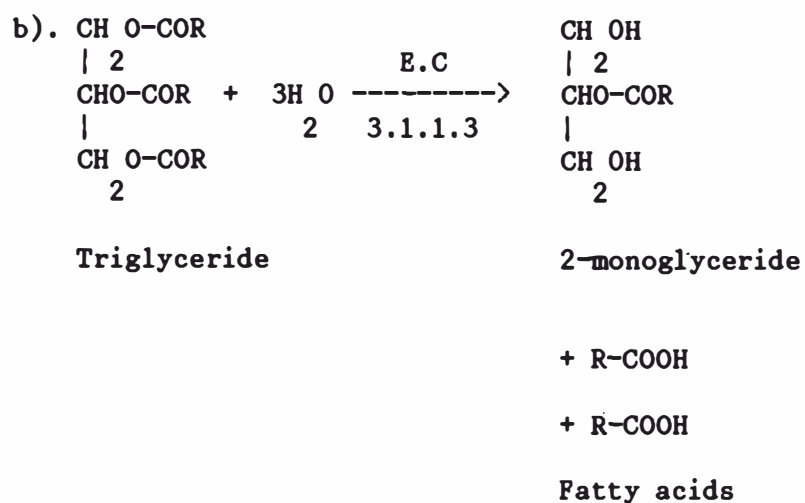
By definition, lipases are glycerol-ester-hydrolases (E.C.3.1.1.3) in the narrowest sense, i.e., long chain triglyceride acylhydrolases. They may or may not have positional specificity for the primary ester bonds. Usually the enzymes also hydrolyze di- and monoglyceride (Brockerhoff and Jensen, 1974). The action of lipases with water-insoluble substrates only occurs at water-oil interface. But this definition now becomes complicated as it is also known that some lipases can hydrolyze water-soluble substrates. However lipases can be



looked upon as the enzymes with a high activity towards water insoluble substrates. A complete reaction catalyzed by lipases is shown in Figure 1 below:



Non-specific reaction of lipase.



1,3 - positional specificity of lipase

Figure 1: Reactions of Lipases

Lipases are widely distributed in animals, plants and microorganisms. Many strains of bacteria, yeasts and fungi such as Staphylococcus sp (Vadehra and Harmon, 1969), Pseudomonas fragi (Nishio et al., 1987a), Chromobacterium (Yamaguchi et al., 1973), Alcaligenes (Kokusho et al., 1982), Geotrichum candidum (Iwai et al., 1973), Rhizopus delemar (Iwai et al., 1966), Aspergillus niger (Pal et al., 1978), Humicola lanuginosa (Arima et al., 1972, Ibrahim et al., 1987a; Morinaga et al., 1986), Candida cyclindracea (Ota et al., 1968) and some other yeasts, molds or bacteria have been reported to be lipase-producing microorganisms.

Microbial lipases have always received more attention when compared to lipases from other sources. Microbial lipases can be produced in large quantity and microbes have short growth periods compared to plant and animal cells. Production of lipases in certain microbes can also be induced by addition of lipids and certain compounds. Based on a recent report, many microbial lipases have been successfully studied from academic and industrial viewpoints (Kilara, 1985). These microbial lipases were also successfully purified to homogeneity with the recent technical advances in the isolation of these enzymes through chromatographic and electrophoretical methods. With these preparations, the properties and reaction mechanisms of lipolysis are being steadily elucidated (Borgstrom and Brockman, 1984).



Fundamental studies for obtaining the optimum cultural condition for lipase production were carried out by several investigators (Chander et al., 1977, 1980, 1981; Chopra and Chander, 1983; Iwai et al., 1966). Some findings reported that the formation and synthesis of lipase are under feedback control of mono- and disaccharides and glycerol in the cultivation medium. Some microbial lipases are glycoprotein where the sugar moiety is thought to facilitate the passage of the enzyme through the microbial cell wall and into the growth medium (Kilara, 1985).

Previously, little studies on lipases have been carried out compared with the other hydrolytic enzymes such as amylases and proteases. However, recently, in view of increasing interest in biotechnology of oils and fats and their use in oleochemical industry, lipase-catalyzed hydrolysis or synthesis of lipid has become more than just an academic interest (Macrae, 1983a). Also, some other reactions catalyzed by lipase, such as synthesis of terpene alcohol esters (Iwai et al., 1980), interesterification of oils and fats (Macrae, 1983b), synthesis of glycerides (Tsujiisaka et al., 1977) and optical resolution of racemic compounds (Kilara, 1985) have recently attracted much attention of both academic and industrial researchers. These bioconversions are expected to produce various value-added products from fats, oils, fatty acids and their related compounds.

In view of the potential biotechnological applications of lipases in the oleochemical industry, the exploitation of these enzymes will lead to the manufacturing of some useful products in the oil industry, especially in the oil palm modification in obtaining a valuable product such as cocoa butter substitute. In addition, the use of these enzymes in the modification of some essential oils by interesterification process will be very useful. Therefore this research was undertaken with the following objectives:

- a) To develop a rapid and suitable plate assay media for detection of lipase activity as well as for screening lipase-producing microorganisms.
- b) To screen and isolate thermostable lipase-producing microorganisms.
- c) To study the effect of growth substrates for optimal lipase production.
- d) To partially purify lipase from culture medium.
- e) To characterize the partially purified lipase.

## CHAPTER 2

### LITERATURE REVIEW

#### Detection of Lipolytic Activity and Screening of Lipolytic Microorganism

Numerous reports regarding the methods for detection and screening of microorganisms with lipolytic activities have been presented by many researchers (Fryer et al.,1966; Karnetova et al.,1984; Oterholm and Ordal,1965). Substrates from triglycerides and polyoxysorbitan have always been employed for the detection of lipase activity. Sometimes the incorporation of chromogenic substances with the substrates gives easier detection as the zone of intensification or clearing that indicates lipolytic activity can be clearly seen.

Several substrates such as oils, triolein and tributyrin have been used in the agar after being emulsified to a certain extent. For example, Fryer et al. (1966) developed two double-layer techniques for detection of lipases in microorganisms. The two double-layer agar plates were developed in which the organisms grown on nutrient agar plate were overlaid with tributyrin agar or a thin layer of milk fat saturated with Victoria Blue. These techniques offer advantages such as the colonies can be isolated after detection, lipolysis can be followed and there is no toxicity due to the dye.



Many investigators (Adriana and Wouters, 1976; Jacks and Kircher, 1967) have employed chromogenic substrates such as fatty acid esters of 4-methylcoumarin (umbelliferone) for detection of lipase activity. Esterases or lipases released an alcohol (phenol, eosin, umbelliferon naphtol, etc.) from these compounds which were directly determined by fluorimetry. The oleic acid ester of 4-methyl umbelliferon was found to be a suitable substrate for the fluorimetric assay of the lipase produced by Geotrichum candidum (Adriana and Wouters, 1976). However, the above method was only suitable for detection of isolated lipase and not suitable for screening of lipolytic organisms.

Karnetova and coworkers (1984) proposed a diffusion plate method. The substrate Tween 80 and indicators such as methylene blue, bromothymol blue and Nile blue (0.5-2.5%) were incorporated into the agar. Lipase activity was detected by the formation of light green zone on a dark blue background. A linear relationship between the logarithm of lipase activity and the diameter of the zone was found. In this method, the enzyme was placed in a well (8.5 mm diameter) and incubated at 28 C for 20 hr.

Using tributyrin as a substrate sometimes creates a problem as zone of clearing due to lipase activity is difficult to be seen. Thus, Oterholm and Ordal (1965) have improved the method for isolation of lipolytic microorganisms as well as detection of lipolytic activity. The method using plating medium consisting





of 0.3% tributyrin and 1% agar was found to be a reliable technique for the detection of lipase activity as well as for screening for lipolytic microorganisms.

However, Mourey and Kilbertus (1976) developed a simple medium containing stabilized tributyrin for demonstrating lipolytic bacteria in foods and soils. The medium containing nutrient, tributyrin and polivinyl alcohol was emulsified then solidified with agar. Using this medium, isolation of microorganism with lipolytic activity shown by the clearance zone was easily done.

Detection of lipolytic activity and lipolytic microorganisms on agar plates is always based on using either tributyrin or Tween as a substrate. These substrates are considered to be unsuitable for detection of true lipase since they are also hydrolyzed by esterases. For that reason, Kouker and Jaeger (1987) developed a specific and sensitive plate for detection of bacterial lipases by adapting the method proposed by Hofelmann et al. (1983). They made use of nutrient with 2.5% olive oil and 0.001% Rhodamine B. The mixture was homogenised to an emulsion. Quantification of lipase activity was determined by the diameter of zone of fluorescence.

#### **Production of Microbial Lipases**

Many studies on cultural conditions for lipase production have been reported (Saiki et al., 1969; Alford and Smith, 1965;

