PRODUCTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR LIPASES FROM *Penicillium* sp.

by

Anwer Ali Ayad

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science

Department of Food Science and Agricultural Chemistry

McGill University

Montréal, Québec

September, 1999

ABSTRACT

Lipase extracts obtained from biomasses of *Penicillium camemberti* and *Penicillium roqueforti*, harvested after 5 and 4 days of fermentation, respectively, were partially purified and characterized. The lipase activity was assayed spectrophotometrically using *p*-nitrophenyl (PNP)-stearate as substrate. The extracellular lipase extract from *P. camemberti* exhibited optimal lipase activity at the two pH optima of 5.50 and 7.75, whereas that of *P. roqueforti* showed maximal enzyme activity at pH 5.25 and 8.75. The optimum temperature for lipase activity of the *P. camemberti* extract was at 45 and 30°C at pH 5.5 and 7.75, respectively, whereas that of the *P. roqueforti* extract was at 35 and 40°C at pH 8.75 and 5.25, respectively. The results demonstrated that the lipase activity of the *P. camemberti* extract indicated more preference, at pH 5.50, towards PNP-stearate as substrate, followed by PNP-palmitate; however, at pH 7.75, the enzyme activity showed a higher preference towards PNP-valerate, followed by PNP-stearate. In addition, the results showed that at pH 5.25, the lipase activity of the *P. roqueforti* extract exhibited a higher specificity towards PNP-palmitate, followed by PNP-valerate, while at pH 8.75, the enzyme showed a higher specificity towards PNP-valerate, followed by PNP-stearate. Using PNP-butyrate as substrate, the K_m value at pH 7.75 was two times higher than that obtained at pH 5.50 for the extracellular lipase activity of the *P. camemberti* extract, whereas that of the *P. roqueforti* extract showed similar K_m values at both pH. Using PNP-stearate, the corresponding K_m value for lipase biocatalysis of the *P. camemberti* extract, was determined to be much higher at pH 5.50 than that at pH 7.75, whereas the hydrolytic activity of the *P. roqueforti* extract, showed also a high K_m value at pH 8.75 than that at 5.25. The V_{max} values, using PNP-butyrate, for the enzyme activity of the *P. camemberti* extract at pH 5.50 and 7.75 were twice as high as those obtained with PNP-stearate. However, the lipase activity of the *P. roqueforti* extract showed similar V_{max} values with PNP-butyrate as substrate at both pH optima, whereas the V_{max} values, using PNP-stearate, for the enzyme activity of the *P. camemberti* extract at pH 5.50 and 7.75 were almost the same. In addition, the lipase activity of *P. roqueforti* extract using at pH 5.25 exhibited twice as less activity with PNP-stearate. Using olive oil and butterfat as substrates, the lipase extracts from *P. camemberti* and *P. roqueforti* exhibited a preference hydrolytic activity towards long-chain and short-chain fatty acid esters, respectively.

RÉSUMÉ

Des lipases obtenues de *Penicillium camemberti* et *Penicillium roqueforti* après 5 et 4 jours d'incubation, respectivement, ont été partiellement purifiées et caractérisées. L'activité lipolytique a été mesurée spectrophotométriquement en utilisant le *p*-nitrophényl (PNP)-stéarate comme substrat. L'extrait lipasique de *P. camemberti* a montré deux pH optima, à 5,50 et 7,75, de même que celui de *P. roqueforti* à 5,25 et 8,75. La température optimale de l'activité enzymatique de l'extrait lipasique de *P. camemberti* était de 45 et 30°C à pH 5,50 et 7,75, respectivement, alors que celle de *P. roqueforti* était de 35 et 40°C à pH 8,75 et 5,25, respectivement. En utilisant le PNP-butyrate comme substrat, les valeurs de K_m pour les extraits lipasiques de *P. camemberti* et *P. roqueforti*, à pH 5,50 et 5,25, ont été déterminées à 0,24 et 0,16 mM, respectivement, pH 7,75 et 8,75, à 0,56 et 0,19 mM, respectivement. En utilisant le PNP-stéarate les valeurs correspondantes de K_m pour les extraits lipasiques de *P. camemberti* et *P. roqueforti*, à pH 5,50 et 5,25, ont été déterminées à 0,16 et 0,08 mM, respectivement, et à pH 7,75 et 8,75, à 0,07 et 0,58 mM, respectivement. Les valeurs de V_{max} en utilisant le PNP-butyrate, pour *P. camemberti* à pH 5,50 et 7,75, étaient deux fois plus élevées que celles obtenues avec le PNP stéarate, alors que celles de la lipase extracellulaire de *P. roqueforti* à pH 5,25 et 8,75 étaient de 12,62 et 10,10, respectivement, et avec le PNP-stéarate, les valeurs de V_{max} à pH 5,25 et 8,75 étaient de 5,50 et 11,28, respectivement. Les résultats ont montré que l'extrait lipasique de *P. camemberti* avait une préférence, à pH 5,50 envers le PNP-stéarate comme substrat, suivi du PNP-palmitate; cependant, à pH 7,75 cet extrait a montré une préférence envers le PNP-valérate, suivi du PNP-palmitate et du PNP-stéarate. De plus, les resultats ont montré qu'à pH 5,25 l'activité lipasique de *P. roqueforti* présentait une spécificité plus élevée envers le PNP-palmitate et une spécificite plus élevée envers le PNP-valérate, alors qu' à pH 8.75, l'enzyme a montré une spécificité plus élevée envers le PNP-valérate, suivi PNP-stéarate. En utilisant le beurre et l'huile d'olive comme substrats, les extraits enzymatiques obtenus à partir de *P. camemberti* et *P. roqueforti* ont démontré une activité hydrolytique plus spécifique, respectivement, pour les esters des acides gras à longue chaîne et courte chaîne.

ACKNOWLEDGMENT

I would like to thank my supervisor, Dr. Selim Kermasha, for the guidance, advice, brotherhood and support that he offered me throughout my study.

I would like to extend my thanks to Dr. Barbara Bisakowski for her advice and help in achieving this work.

Special thanks to Rowaida for her help in the GC analysis and to Xavier for the translation of the Abstract. I would also like to thank Aliaa, Abzal and Wigdan for their help and friendship as well as to the rest of my colleagues in the laboratory.

I would like to extend my gratitude to my dear homeland Libya for having sponsored me throughout my program for which I am very thankful. I would also like to express my thanks to the Canadian Bureau for International Education (CBIE) staff, especially, the program manager, Ms. A. Mastelloto and to the Libyan representatives at the CBIE, Dr. M. Almahdi and Mr. M. Gargoom, for the administration of my scholarship.

Finally, I would like to express my heartfelt thanks to my dear, sincere and lovely wife, Zaineb, our beloved son, Mohammed, and a special thanks to my first teachers who taught me the knowledge of life, patience and wisdom, my dear parents Ali and Sheika Ayad.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerols of fats and oils into glycerols and free fatty acids at the oil-water interface used. Lipases are found in several sources including plants, animals and microorganisms; among these sources, a number of microorganisms have been investigated for their ability to produce lipases, including those from *Pseudomonas* (Pabai, 1997), *Geotrichum* (Veeraragavan *et al.*, 1990) and *Penicillium* (Mase *et al.*, 1995).

Lipases are used in many industrial processes such as cheese ripening, flavor production, additives to washing detergents and preparation of cocoa butter substitutes (Davranov, 1994). Fungal sources of lipase include *Aspergillus* (Sugihara *et al.*, 1988), *Geotrichum* (Veeraragavan *et al.*, 1990), *Penicillium* (Yamaguchi and Mase, 1991), *Mucor* (Gulyamova and Davranov, 1993) and *Candida* (Benjamin and Pandey, 1993).

Microbial lipases can be classified into two groups based on their hydrolytic activity towards triacylglycerols. Lipases can be specific toward the position of acyl group on the triacylglycerol molecule during the hydrolysis of fats e.g., lipases from *A. niger, M. javanicus, R. mucor*, and various *Rhizopus* spp. Lipases can be also be non specific so that they will randomly hydrolyze any of the three positions of the acyl group of triacylglycerols e.g., lipases from *C. cylinracea, C. acnes* and *S. aureus* (Macrae, 1983).

Lipase production in microorganisms can be stimulated by the addition of lipids or naturally occurring oils to the growth medium as carbon source; these include olive

oil, ground nut oil, cotton seed oil and fatty acids such as oleic acid (Macrae, 1983). Benjamin and Pandey (1995) indicated the inductive role of olive oil in the growth medium of *C. rugosa* for the production of lipases.

Microbial lipases have been used as biocatalysts for a variety of biotechnological applications including the hydrolysis of fats, the synthesis of glycerides and esters, and the modification of lipids; each of these applications requires a lipase activity with unique properties regarding its specificity, stability, temperature and pH dependence (Macrae, 1983).

The production and characterization of lipases from fungal sources are of great interest especially in the production of flavor esters for various foods (Mackay, 1993). They have been known to produce lipases; the nature of these enzymes is species dependent, in other wards the lipase could be specific for short-chain or long-chain fatty acid (Sztajer *et al.*, 1992).

Lipases produced by some *Penicillium* species have been well known in many dairy applications such as the use of *P. camemberti*, *P. caseicolum* and *P. roqueforti* in the manufacture of Camembert, Brie and Roquefort cheeses (Furtado *et al.*, 1984; Stöcklein *et al.*, 1992).

P. camemberti and *P. roqueforti* lipases are of particular interest to the cheese industry where they are used in the flavor development of specific types of cheese and in the acceleration of ripening processes of cheese or cheese-like products (Eitenmiller *et al.*, 1970; Furtado *et al.*, 1984). The role of the production, extraction and purification of

these enzymes may be vital in the creation of naturally synthesized sources of flavors from microbial source.

This study was aimed at the production, partial purification and characterization of lipase(s) from *Penicillium camemberti* and *Penicillium roqueforti*. The lipase activity of the partially purified extracts was characterized in terms of pH and temperature optima as well as substrate specificity. The specific objectives of this investigation were as follow:

1. To optimize the conditions of biomass production and the development of enriched enzymatic extracts of the selected fungal lipases.

2. To partially purify and characterize of the selected fungal lipases as a function of their activity, pH, kinetic parameters, activators, inhibitors, and substrate specificity.

3. To investigate the hydrolytic activity of the partially purified lipase extracts using selected edible oils including canola, fish, olive oils and butterfat.

2. LITERATURE REVIEW

2.1. Cheese Ripening and Flavor Production

Cheese is a desirable, nutritious food which can be made by the coagulation of milk protein with the use of enzymes or microorganisms. Cheese may be classified as either soft or hard cheese; the latter include Cheddar cheese, while the former one consists of Camembert and Limburger cheese. The ripening of hard cheese can be done through bacterial action such as that of *L. citrovorum* in Cheddar cheese whereas the ripening of soft cheese is carried out by yeasts, molds, or bacteria grown on the cheese surface such as Camembert and Limburger (Eskin, 1990).

Camembert, Brie, and blue-vein cheeses, including Gorgonzola, Roquefort, Stilton, are very popular and famous cheeses, made from the inoculation of milk or milk curd with the use of spores of *Penicillium* molds such as *P. roqueforti*, *P. camemberti*, and *P. glaucum* which germinate when the cheese is exposed to air (Stead, 1986).

Fungi are reported to play an important role in the formation of odorous compounds or flavors in cheese (Molimard and Spinnler, 1996). Cheese ripening includes a number of biochemical changes which happen in the cheese such as lipid and protein hydrolysis as result of microbial growth in the cheese (Eskin, 1990). The formation of flavors in cheeses is attributed to the occurrence of proteolysis and lipolysis in the cheese due to the presence of proteases and lipases, respectively (Adda *et al.*, 1982; Revah, 1989). In particular fungal lipases can accelerate the ripening of cheddar cheese, and improve the flavor of processed and blue cheeses (Macrae, 1983).

In addition, microbial lipases have the capability to modify cheese as certain fatty acids are enzymatically hydrolyzed in the milk are important in cheese ripening giving a characteristic flavor and promoted the process (Molimard and Splinnler, 1996). Kanawjia and Singh (1991) reported that when cheese was treated with high levels of lipase from *C. cylindracea* during the ripening period, excessive lipolysis occurred which resulted in the accumulation of a large amount of free fatty acids that consequently produced a highly rancid off flavor. A lipase extract from *P. roqueforti* also showed an inductive role in the production of the characteristic taste of Roqueforti and Blue cheese through the enzymatic hydrolysis of triacylglycerols which resulted in the liberation of fatty acids, used as precursors for the production of methyl ketones and secondary alcohols (Petrovic *et al.*, 1990).

2.2. Microorganisms

2.2.1. Penicillium camemberti

The *P. camemberti* species are members of the *Penicillium* genus known as Camembert-type cheese molds. These fungi are responsible for the production of soft cheeses, including Camembert, Brie and Neufchatel. Moreover, the shape of the colonies of *P. camemberti* are floccose, cottony, and deeply lanose; at the beginning of culture incubation, they appear pure white and then at maturity they either remain white or become pale gray green depending upon the species. The conidiophores, arising from the substratum or from the loose aerial felt, are variable in length up to 500 to 600 μm by 3.0 to 4.0 μm in diameter, with more or less roughened walls. At first, the conidia are elliptical, then they become subglobose, smooth-walled and comparatively large, about 4.0 to 5.0 μm in diameter at maturity. In addition, *P. camemberti* is asymmetric,

irregularly branched and variable in pattern, with metulae and sterigmata commonly arising at different levels, bearing conidia in tangled chains to form an irregular brush up to 100 to 150 μm in length (Raper and Thom, 1949).

2.2.2. Penicillium roqueforti

P. roqueforti is a mold involved in the production of Roquefort, Gorgonzola, and Stilton cheeses (Eskin, 1990). The mold is responsible for the synthesis of distinctive flavors through the formation of methyl ketones during cheese ripening of Blue, Roquefort, Stilton and Gorgonzola cheeses. *P. roqueforti* is also a member of the *Penicillium* genus. The fungus, known as Roquefort-type cheese mold, is characterized by streaks or "marbling" of green mold. The natural habitat of *P. roqueforti* is sheep milk. Different types of cheese are usually made with *P. roqueforti* by using different sources of milk, e.g. in southern France, cheese produced by *P. roqueforti* with sheep's milk is called Roquefort while that made from cow milk is called " formages bleus" or Blue cheese in central France or American Roquefort in the United States. These cheeses are characterized by their loose-texture and characteristic flavor and appearance which is due to the fungal action of lining up channels and cracks with their mass in the cheese (Eskin, 1990).

P. roqueforti usually appears broadly spread, soft, azonate, generally thin, with abundant short conidiophores arising from trailing hyphae or submerged hyphae just below the surface when grown in the agar; conidial areas are typically dark yellow-green shades, often showing a greenish to almost blackish shade in colony reverse. The conidiophores are clearly roughened or tuberculate in aerial portions, with cellular elements of the *Penicillus* similarly roughened. However, *P. roqueforti* is asymmetrical,

irregularly branched, bearing conidia in long tangled chains or adherent in loose columns. Which appear dark yellow-green (Raper and Thom, 1949).

2.3. Lipases

2.3.1. Definition of Lipase

Lipases are enzymes also known as carboxylic ester hydrolases or glycerol ester hydrolases. Lipases bear the international name of triacylglycerol acylhydrolase lipases (EC 3.1.1.3) (Taipa *et al.*, 1992). These enzymes are widely distributed in nature among plants, animals and microorganisms (Balcao and Malcata, 1998). They have been found in pancreas, blood plasma, saliva, pancreatic juice, milk, and in number of triglyceride-producing plants (e.g. soybean, castor bean, peanut), molds (e.g. *Rhizopus, Penicillium*) and bacteria (e.g. *Pseudomonas, Staphylococcus*) (Macrae, 1983; Kilara, 1985; Gulomova *et al.*, 1996). Figure 1 shows the bioconversion of triacylglycerols into mono- and diacylglycerols and the liberation of fatty acids by the hydrolytic activity of lipase.

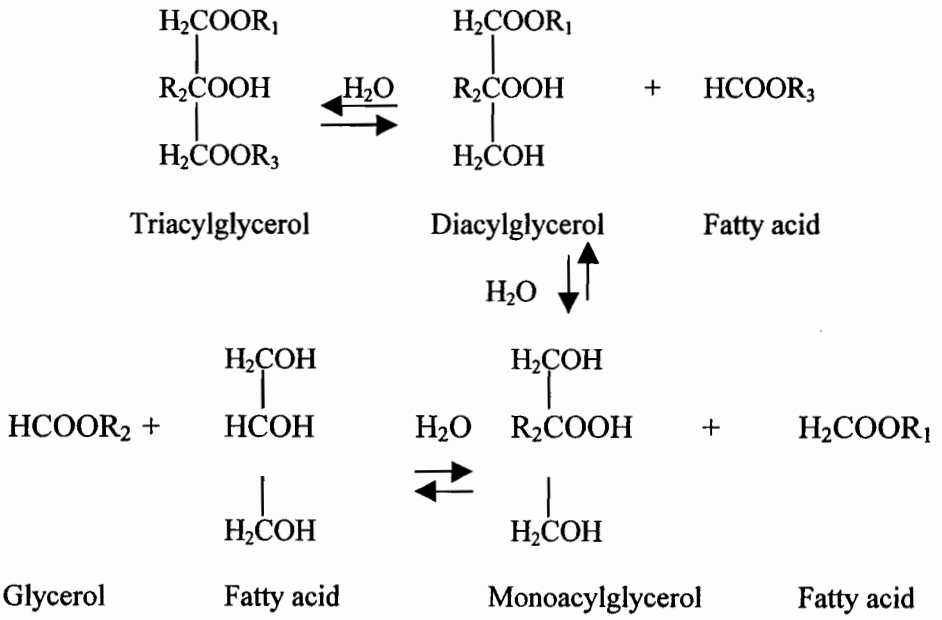


Figure 1. Hydrolytic activity of lipases towards triacylglycerol as substrate.

In addition, most microbial lipases are capable of catalyzing the hydrolysis of triacylglycerols into diacylglycerols and free fatty acids, as well as further hydrolysis of mono-and diacylglycerols; some lipases may also catalyze the interesterification of fatty acids present in the triacylglycerols of oil (Macrae, 1983; Pabai *et al.*, 1997). Lipases obtained from microbial origin have more potential applications in the food industry due to their diversified enzymatic properties and substrate specificities (Tanaka, 1993).

The hydrolytic activity of microbial lipase has drawn attention towards the importance of these enzymes in lipid metabolism and their role in different catalytic reactions including i.e., esterification, interesterification and transesterification; these reactions are also involved in the formation of modified fats and fatty acid esters (Macrae, 1983).

Lipases have received a great deal of attention in the food industry, especially the oil and fat industry since they present a good alternative to the use of chemical processes (Fischer *et al.*, 1992). In the food industry, lipases may be used to produce emulsifiers which are generally obtained by chemical interesterification of edible fats and oils at high temperatures (200°C) under harsh alkaline reaction conditions (Iwai *et al.*, 1964). Lipases can also be used in the dairy industry in the flavor development of specific cheeses or in the acceleration of cheese ripening. Due to the lipase activity present in microorganisms, the enzymatically hydrolyzed free fatty acids can act not only as flavor components but also as flavor precursors for the production of the characteristic flavor components of certain types of cheese, including the use of *P. camemberti* and *P. caseicolum* in the manufacture of Camembert and Brie cheeses respectively (Sztajer and Zboinska, 1988).

2.3.2. Sources of Lipase

Lipases are widely found to be present in nature in many sources including plants, animals and microorganisms. In the last few decades, many microbial species have been investigated as sources of lipase including bacteria and fungi, mainly due to their commercial availability and ease of improvement by way of genetic manipulation (Gilbert, 1993). In addition, microorganisms have shown the ability to produce lipases with a wide range of specificity. Moreover, they can be cultivated in a controlled environment; so that the yield can be increased (Macrae, 1983; Iwai and Tsujisaka, 1984). Microbial sources of lipases include bacterial sources such as *Pseudomonas*, *Staphylococcus*, yeast sources such as *Torulopsis, Candida*, and fungal sources such as *Rhizopus, Penicillium* and *Geotrichum* (Kilara, 1985).

Fungal lipases represent the main source of lipase used in biotechnological purposes, among other sources of lipases in particular, *Geotrichum*, *Aspergillus*, *Rhizopus*, *Mucor*, and *Penicillium* (Macrae, 1983; Iwai and Tsujisaka, 1984; Godtfredsen, 1990). Among the *Penicillium* strains, there are many sources of lipases such as *P. camemberti* U-150 (Mase *et al.*, 1995). *P. camemberti* and *P. roqueforti* are currently used as in many industrial applications such as cheese ripening and the preparation of Blue, Roquefort and Camembert cheeses.

2.3.3. Properties of Lipase

Microbial lipases are of great industrial value due to the diversity of their molecular properties and specificity in comparison (Iwai and Tsujisaka, 1984). The lipolytic activity of lipase was detected in microorganisms used in the production of cheeses such as *S. lactis*, *L. casei*, *P. roqueforti* (Sztajer and Zboinska, 1988). However,

the development and utilization of microbial lipases for specific applications involves the need to screen, purify and characterize lipase activity from different sources due to the wide variety of lipases available with respect to activity and specificity (Pabai, 1995). Table 1 shows the molecular properties of lipases from *Penicillium* species.

Table 1: Molecular properties of lipases from *Penicillium* species.

	Optimum conditions for lipase activity			
Microorganism	pH	Temperature (°C)	Molecular weight (kDa)	Reference
P. albeanum	7.0 to 8.0	25 to 30	28	Sugihara *et al.* (1996)
P. camemberti	5.0 and 5.5	40 and 45	37 to 39	Isobe *et al.* (1992)
P. caseicolum	9.0	35	NA	Alhir *et al.* (1990)
P. citrinum	8.0	34 to 37	NA	Pimentel *et al.* (1994)
P. cyclobium	4.0 and 7.0	20 and 37	78 and 93	Druet *et al.* (1992)
P. cyclobium	5.8 and 7.5	35 and 40	27 and 36	Iwai &Tsujisaka (1984)
P. expansum	9.0	45	25	Stöcklein *et al.*(1993)
P. simplicissimum	5.0	37	56	Sztajer (1992)
P. roqueforti	6.0 to 7.0	35	25	Mase *et al.* (1995)
P. roqueforti	8.0	37	NA	Eitenmiller *et al.* (1970)
P. roqueforti	8.0	40	NA	Petrovic *et al.* (1986)

The wide range of substrate specificity of microbial lipases has drawn a considerable attention towards microorganisms as potential sources (Alford *et al.*, 1964).

Many studies have shown that the physico-chemical properties and substrate specificities of lipases from various sources are different from each other (Tsujisaka *et al.*, 1973). Many of the microbial lipases that have been reported so far can be classified as acidic or neutral/alkaline lipases with respect to their pH optimum, with molecular weights ranging from 20 kDa to 60 kDa (Macrae, 1983). The molecular weight of lipase from *P. camemberti* was reported to be between 37 to 39 kDa (Isobe, 1992), while that from *P. roqueforti* lipase was 25 kDa (Mase *et al.*, 1995). In addition, the purified lipase from *P. simplicissimum* showed a molecular weight of 56 kDa (Sztajer *et al.*, 1992).

2.3.4. Production of Lipase

Most lipases from fungal sources are produced as extracellular enzymes. In general, lipases are recovered from the supernatant of the culture incubation medium using simple techniques such as centrifugation, filtration, evaporation, ammonium sulfate precipitation. Fungi are therefore the preferred lipase source among microorganisms due to ease of recovery (Iwai and Tsujiska, 1984; Taipa *et al.,* 1992). The most productive sources species belong to the genera *Geotrichum*, *Aspergillus*, *Rhizpus*, *Mucor*, and *Penicillium* (Godtfredsen, 1990). However, lipases may also be present as intracellular enzymes which can be liberated by disintegration of the intact cell wall with the use of mechanical or physical methods; the homogenized enzyme extract is then subjected to either centrifugation or filtration for removal of the disintegrated cells from the culture medium so that the cell-free solution can be further concentrated by ultra filtration (Schuepp, 1995).

The production of lipases from fungal sources can be improved through the optimization of culture incubation conditions. The culture conditions used for biomass production influence the properties of the enzyme produced as well as the ratio of extracellular to intracellular lipases; parameters which may be optimized include the cultivation period, temperature, pH, carbon source, nitrogen source, concentration of inorganic salts and availability of oxygen. Lipase formation can also be induced by the addition of a lipid source such as olive oil, ground nut oil, and fatty acids including oleic acid to the culture medium (Iwai and Tsujiska, 1984).

The effect of carbon and nitrogen sources on the induction of extra- and intracellular lipases in fungal species was investigated by many workers. Benjamin and Pandey (1995) reported that there was a notable increase in lipase production by *C. rugosa* when cultivated in a medium containing olive oil. Iwai *et al.* (1973) stated that the *G. candidum* strain showed a substantial increase in the rate of lipase formation during incubation in culture medium containing lipids. Malizewska and Mastalerz (1992) found that olive oil and Tween 80 could stimulate lipase production from *P. citrinum*, whereas the presence of lauric acid showed an inhibitory effect on lipase production. Sztajer *et al.* (1992) reported that the highest lipase activity was obtained after 48 h of incubation of *P. simplicissimum* using triolein as an inducer. In addition, an increase in lipase activity from *Ps. mephitica* was observed by Kosugi and Kamibayashi (1971) after lipids including olive oil were added to the culture medium. However, some researchers have reported contradictory findings for the use of oils as lipase inducers. Eitenmiller *et al.* (1970) reported that olive oil inhibited lipase production by *P. roqueforti* by 5 %, while butter oil and corn oil inhibited lipase production

by 21 and 29 %, respectively. Petrovic *et al.* (1990) reported that the use of olive oil as a carbon source inhibited the growth of *P. roqueforti*.

Other growth parameters that affect lipase production include the pH of the culture incubation medium. The highest lipase activity was obtained when the biomass of *P. roqueforti* was cultivated at pH 5.5; however, when the pH of the culture medium was increased, the lipase activity decreased accordingly, followed by a corresponding decrease in mold growth (Eitenmiller *et al.*, 1970).

2.4. Purification of Lipases

Recent work performed on the purification of lipase crude extracts to homogeneity has made lipases more available for characterization and subsequent industrial applications. Furthermore, fungal sources of lipase are considered to be particularly invaluable as most fungal lipases are extracellular enzymes, thereby being readily easily separated and extracted from the mycelia by means of filtration or centrifugation (Iwai and Tsujisaka, 1984). The purification of the enzymatic extract can be achieved using a number of methods including precipitation or fractionation of proteins with the use of salt, including NaCl, $NH_4(SO)_4$, $MgSO_4$. The use of ammonium sulfate produces a high molarity at its saturation point which leads to the precipitation of most proteins. Organic solvents such as acetone, ethanol, methanol, and butanol can be also used for the precipitation of proteins; all of these organic solvents are miscible with water, but can yield considerable heat upon mixing which might denature the enzyme (Taipa *et al.*, 1992).

2.5. Characterization of Lipase

2.5.1. Determination of Lipase Activity

The hydrolytic rate of lipase activity can be determined by measuring either the rate of appearance of free fatty acids or the rate of consumption of triacylglycerols. The conditions required for lipase activity, i.e., temperature, pH, presence of cations and fatty acid receptors, provide the information required for the design of a suitable assay system (Jensen, 1983).

There are number of qualitative or quantitative assays being used for the detection of lipase activity. The Rhodamine-B plate test or the Tween 80 test are used as qualitative tests for the preliminary investigation of the existence and detection of lipase activity; these tests indirectly detect the presence of lipase activity by interacting with certain compounds in the reaction (Pabai, 1997). In the Rhodamine B plate, the presence of lipase activity is detected by the occurrence of an orange halo around the colonies due to the substrate (olive oil) hydrolysis (Kouker and Jaeger, 1987), while in the Tween-80 test, the presence of lipase activity is confirmed by the appearance of a visible halo around the colonies which is due to the formation of crystals of the calcium salt of the fatty acids liberated by lipases (Sierra, 1956). In quantitative methods for lipase activity which include the spectrophotometric (Winkler and Stuckman, 1979) and titrimetric assays (Kermasha *et al.*, 1986), the rate of the enzymatic reaction is followed by measurement of the free fatty acids released by lipase activity at a certain temperature for the reaction; however, the substrate used for the determination of enzyme activity is selected with respect to the substrate specificity of the lipase.

In order to increase the sensitivity and to rationalize the experimental time, a colorimetric procedure was developed using synthetic substrates (Lowry and Tinsley, 1976). The most common chromogenic substrates are the *p*-nitrophenyl derivatives of fatty acids (Winkler and Stuckmann, 1979; Becker *et al.*, 1997). Carrie *et al.* (1999) reported that the emulsification of *p*-nitrophenyl palmitate resulted in instable emulsion; the instability of the complex was attributed in part to the relatively long-chain (C16:0) hydrocarbon fatty acid of the *p*-nitrophenyl palmitate. However, Carrie *et al.* (1999) suggested the use of shorter chain such as *p*-nitrophenyl laurate (C12:0) which may increase the stability of the emulsion. In addition, Vorderwülbecke *et al.* (1992) reported that the lipases are capable to catalyze the hydrolysis of triolein as well as the *p*-nitrophenyl palmitate and *p*-nitrophenyl butyrate. Moreover, Brahimi-Horn *et al.* (1989) indicated that the lipase specificity of a wide range of triacylglycerols was depending on the chain length of fatty acids.

In the spectrophotometric assay, lipase is allowed to react with chromogenic substrates such as *p*-nitrophenyl stearate. A yellow color compound of *p*-nitrophenol is released due the hydrolysis of this chromogenic compound by the lipase. The intensity of this yellow-color compound can be attributed to the degree of lipase activity on the substrate. Lipase activity is detected by the occurrence of a yellow color at 410 nm (Winkler and Stuckman, 1979). Spectrophotometric is more sensitive, can measure small quantities of activity, more reliable and faster (Pimentel *et al.*, 1994).

While in the titrimetric assay, an emulsion of olive oil serves as the substrate and the enzymatically hydrolyzed free fatty acids are titrated with a pH-stat set to an end point with proper concentration of a base. However, determination of lipase activity using

this method is highly dependent on the composition of the substrate, the method used for preparation as well as the homogeneity of the emulsion (Peled and Krenzt, 1981). In addition, the substrate used in the lipase assay should be liquid at the temperature of the reaction due to the slow rate of hydrolysis of solid triglyceride.

Peled and Krenzt (1981) suggested that the use of triolein (trioleoyl glycerol) as substrate for the assay of lipase activity can be more useful and reproducible than olive oil; stable emulsions of triolein can be prepared by vigorous mechanical or ultrasonic mixing with a solution of an emulsion stabilizer consisting of gum arabic, polyvinyl, alcohol or criteria cellulose. In addition, it is essential for the hydrolyzed free fatty acids to remain ionized under the assay conditions used.

2.5.2. Factors Affecting Lipase Activity

2.5.2.1. Effect of pH and Temperature

The lipase activity from many *Penicillium* species has been investigated with respect to optimal temperature and pH. In general, fungal lipases exhibit pH optima in the range of 7 to 9. Malizewska and Mastalerz (1992) reported that optimal lipase activity from *P. citrinum* was obtained at pH 7.2 and 30°C; in addition, the enzyme activity was stable between pH 5.0 to 7.0 and in the temperature range of 10 to 40°C. A lipase from *A. niger*, *G. candidum*, *P. cyclopium* and *P. simplicissimum* showed maximal activity at the pH of 5.6, 8.0, 7.5 and 5.0, respectively (Table 1). Lipases are also active over a wide range of temperatures between -20 to 65°C with the optimum temperature being between 30 to 50°C.

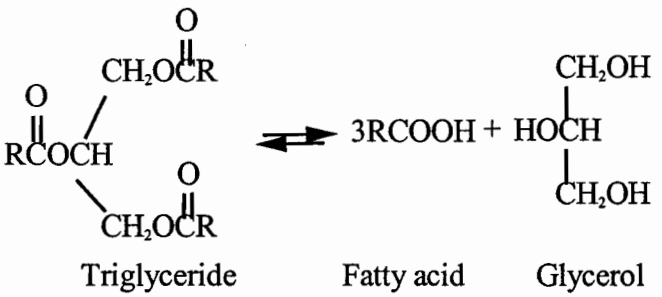
2.5.2.2. Substrate Specificity of Lipase

In general, enzymes are classified by the type of reaction they catalyze in terms of their specificity. The specificity of enzymes is controlled by three factors including the molecular properties of the enzyme, the structure of substrate and the factors affecting the binding of the enzyme to the substrate.

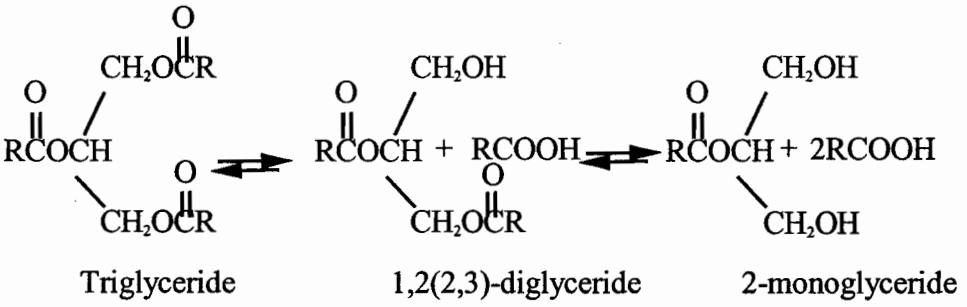
The rate of hydrolysis of the triacylglycerols is a function of the exposed surface area of the substrate at the oil-water interface in a reaction mixture. Recent work performed on the purification of crude lipase extracts to homogeneity has made characterization of these lipases possible for subsequent industrial applications. Moreover, the different sources of lipase is also a factor in the variation of the substrate specificity of the enzyme. Furthermore, fungal sources of lipase are particularly invaluable as most fungal lipases are extracellular enzymes and can therefore be readily separated and extracted from the mycelia by means of filtration or centrifugation (Iwai and Tsujisaka, 1984).

Lipases are capable of catalyzing the hydrolysis of fats, oils and synthetic mono-, di- and triacylglycerols (Davraov, 1994). The specificity of lipases towards different triacylglycerols in fat as substrates is also dependant on the chain length and saturation ratio, surface tension and viscosity of the associated fatty acids (Sztajer and Zboinska, 1988).

Lipases can be classified into two groups with respect to their positional specificity. The first lipase group is non-specific in that the enzyme can hydrolyze the fatty acids from any of three positions in the triacylglycerol molecule. *G. candidum* lipases represent an example of non specific group of lipases (Liu *et al.*, 1973). Figure 2 shows the reactions catalyzed by the two main groups of lipases.



non - specific lipases



1, 3 - specific lipases

Figure 2. Substrate specificity of lipases.

The second group consists of specific lipases which are capable of hydrolyzing the ester bond located at the 1 and 2 position of the triacylglycerols to produce free fatty acids and a mixture of mono- and diglycerides; this group includes lipases from *A. niger*, *R. delemar* (Okumura *et al.*, 1976), *M. miehei* and *P. fragi* (Alford *et al.*, 1964; Macrae, 1983). Table 2 reports the hydrolytic activity of selected microbial lipases, using milkfat as substrate.

Table 2. Hydrolytic activity of some microbial lipases towards the fatty acids of the triacylglycerols in milkfat (Sztajer and Zboinska, 1988).

	Liberated fatty acid (%)			
Fatty acid	*A. lipolyticum*	*G. candidum*	*A. niger*	*P. roqueforti*
Caprylic	1.9	1.3	1.0	3.1
Lauric	1.0	1.9	3.2	3.2
Palmitic	12.9	15.7	33.7	17.8
Stearic	8.0	1.1	14.0	9.0
Oleic	47.4	61.0	28.7	47.9
Linoleic	6.5	5.3	1.6	2.1

Yamaguchi and Mase (1991) reported that the substrate specificity of a lipase from *P. camemberti* showed more affinity towards mono- and diacylglycerols and little specificity toward triacylglycerols; the same authors reported that 22-29 % of the rate of hydrolysis was obtained with trihexanoyl-, trioctanoyl-and tridecanoylglycerols as substrate relative to that obtained with monoacylglycerols.

In addition, the lipase exhibited a greater positional specificity for hydrolysis of 1,3 monoacylglycerol than 2 monoacylglycerol and was therefore called a mono- and diacylglycerol lipase. Moreover, lipases from *P. cyclopium* M1, *P. cyclopium* Westling and *P. camemberti* showed a greater preference for the hydrolysis of mono- and diacylglycerols (Okumura *et al.*, 1980; Yamaguchi and Mase, 1991) in comparison to that obtained with triacylglycerols.

A *P. roqueforti* lipase showed a high specificity towards short-chain fatty acid esters such as butyrate (C 4:0) and caproate (C 6:0) compared to that obtained with long-chain fatty acids. A purified lipase from *P. simplicissimum* was reported to be nonspecific as it hydrolyzed each of the three bonds of triacylglycerols (Sztajer *et al.*, 1992).

2.5.2.3. Activation and Inhibition

The effect of many chemical substances on the activity of lipases from different sources has been extensively investigated (Iwai and Tsujisaka, 1984). The presence of cations such as Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}, Co^{2+} and Ni^{2+} showed a stimulating effect on lipase activity while that of Hg^{2+}, Al^{3+} and Fe^{2+} inhibited the activity. The addition of calcium ions to the reaction medium showed an activiatory effect on many microbial lipases which may be due to the stabilization of the lipase structure by Ca^{2+} or the formation of calcium salts with the free fatty acids which could then increase the rate of hydrolysis (Iwai and Tsujisaka, 1984; Whitaker, 1994). In addition, the presence of EDTA in the reaction medium produced an increase in lipase activity; these findings suggest that EDTA may chelate potential inhibitory metal ions present in the reaction medium (Iwai and Tsujisaka, 1984). Moreover, sodium taurocholate, a surface-active agent, is known as a lipase accelerator by acting as an emulsifier, thereby increasing the interfacial area between oil and water in the reaction mixture.

A variety of chemical substances are reported to have an inhibition effect on the enzyme activity. Lipase inhibitors have been used to characterize lipase activity, to investigate the active site on the enzyme and also to control enzyme activity. In general, enzyme inhibitors are classified as being either reversible or irreversible inhibitors.

Enzyme inhibitors are further divided into competitive, noncompetitive and uncompetitive; this classification is based on the effect of the inhibitor on enzyme activity as determined by the K_m and V_{max} values obtained from the best straight line from a Lineweaver-Burk plot (Lineweaver and Burk 1934; Segel, 1976). The inhibitory substances may act either directly on the active site or but inhibit lipase activity by changing the conformation of lipase or by interacting with the substrate and/or enzyme (Patkar and Björkling, 1994). Bile salts, i.e. cholic acid derivatives of cholesterol, are known to influence lipase activity by acting as either activators or inhibitors. As in the case of surfactants, bile salts show an activatory effect at low concentrations and an inhibitory effect at high concentrations. The inhibitory effect of bile salts was explained by two hypotheses: (A) bile salts accumulate at the surface of the substrate and inhibit lipase adsorption onto the interface; (B) bile salts form a complex with lipase, so that it has a low affinity towards the bile-salt-covered surface (Iwai and Tsujisaka, 1984).

Metal ions may bind to the enzyme, thereby altering its activity by either stabilization or destabilization of the protein conformation. Metal ions may also act as scavengers of free fatty acids at the interface, minimizing product inhibition of lipase activity by the free fatty acids. The inhibitory influence of iron ions on lipase activity has been reported in presence of 8 ppm iron in the reaction medium (Iwai and Tsujisaka, 1984); In addition, lipase activity was severely inhibited by metal ions such as Ag^{+}, Zn^{2+}, Cu^{2+}, and Hg^{2+}, with many lipases from microorganisms including *Pseudomonas* (Iizumi *et al.*, 1990), *Bacillus* (Sugihara *et al.*, 1988) and *Mucor* (Toida *et al.*, 1995).

2.6. Industrial Application of Lipase

Industrial applications of fungal lipases have been investigated by many researchers (Ratledge, 1984; Godtfredsen, 1990). The aim of these applications was to produce or enhance nutritional triacylglycerols and fatty acids by using lipase (Tanaka *et al.*, 1981; Macrae, 1983 and 1985; Macrae and How, 1986). Lipases have also found biotechnological applications in various industrial pharmaceutical areas such as being used as drugs for metabolic disorders of fats in the body (Seitz, 1973). Lipase can also be used to play role in the formation of flavors (Toida *et al.*, 1995) and the synthesis of triglycerides (Tanaka *et al.*, 1981; Jensen *et al.*, 1983; Gandhi, 1997).

In addition, microbial lipases have been studied extensively in the dairy industry due to the occurrence of rancidity in various food products as a result of lipase-hydrolyzed free fatty acids (Eskin, 1990). However, many *Penicillium* lipases have also been applied in dairy industry, in the process of flavor formation in cheese in order to increase the rate of cheese ripening and to produce cheese-like products resembling Brie, Camembert and Roquefort cheeses (Macrae, 1983; Sztajer *et al.*, 1992; Mase *et al.*, 1995).

Lipases can also be used to modify fats in bakery products such as bread, crackers etc. The use of modified lipases has been reported to improve the flavor, softness and structure of bread. Partial hydrolysis of beef fat by lipase activity has also showed an improvement in the properties of pet-food. Moreover, in leather industry, lipases are also used for the defatting of leather (Sztajer and Zbionska, 1988).

Chalier *et al.* (1993) reported the production of aroma compounds by *P. roqueforti* spores using copra oil and soybean oil as substrate; saturated and unsaturated methyl ketones were produced by the *P. roqueforti* spores at a concentration dependent on the degree of lipolysis by an exogenous lipase. Ghosh *et al.* (1995) have also investigated the formation of fatty acids and fatty acid esters with many commercial acid oils using microbial lipase biocatalysis.

3. MATERIALS AND METHODS

3.1. Chemicals

Potato dextrose agar (PDA) and Rhodamine B were obtained from BDH Chemical Co. (Darmstadt, Germany). Yeast extract and agar were purchased from Difco Laboratories (Detroit, MI). Sodium nitrate, potassium phosphate, magnesium sulfate, arabic gum and sodium deoxycholate were supplied by ACP Chemical Inc. (Montréal, QC). *p*-Nitrophenyl butyrate, *p*-nitrophenyl valerate, *p*-nitrophenyl palmitate, *p*-nitrophenyl stearate, olive oil and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St-Louis, MO).

Anhydrous ethanol, acetone, hexane, hydrochloric acid (HCl), sodium chloride (NaCl), sodium hydroxide (NaOH), Tween 20 (polyoxyethylene-sorbitan monolaurate), calcium chloride ($CaCl_2$), potassium chloride (KCl), magnesium chloride ($MgCl_2$), citric acid, ammonium sulfate and ethylenediamine tetraacetic acid (EDTA) di-sodium salt were purchased from Fisher Scientific (Fair Lawn, NJ). Ammediol (2-amino-2-methyl-1,3-propandiol), ferrous chloride, Ellman's reagent (5'-5'-dithiobis-(2'-nitrobenzoic acid), Triton X-100 and *N*-bromosuccinimide were obtained from ICN Biochemicals Inc. (Aurora, OH).

Phenol reagent, isopropyl alcohol, sodium phosphate (monobasic and dibasic), citric acid monohydrate, and ferric chloride were purchased from ACP Chemicals Inc. (Montréal, QC). Sodium carbonate and sodium taurocholate were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Silver nitrate was purchased from Fisher

Scientific (Fair Lawn, N.J.). Butter, soybean oil, olive oil and canola oil were purchased from the local market.

3.2. Microorganisms

Two fungal strains of *Penicillium, P. camemberti* and *P. roqueforti* B, were selected as source of lipases. The two fungal strains were maintained on PDA slants at 4°C for further use.

3.3. Culture Conditions

3.3.1. Medium

The culture media used for the incubation of *P. camemberti* and *P. roqueforti* were prepared according to a slight modification of the method described by Yamaguchi and Mase (1991). The medium used for the cultivation of *P. camemberti* was composed as follows: (2.0 %, v/v) soybean oil, (0.5 %, w/v) yeast extract, (0.3 %, w/v) $NaNO_3$, (0.1 %, w/v) K_2HPO_4, (0.05 %, w/v) KCl, (0.05 %, w/v) $MgSO_4.7H_2O$, and (0.001 %, w/v) $FeSO_4$ 7 H_2O.

The growth medium used for *P. roqueforti* contained the same components as that for *P. camemberti* except that soybean oil was replaced by olive oil. The pH and temperature of both culture media were maintained at 6.0 and 25°C.

3.3.2. Inoculum Preparation

The liquid culture media were inoculated with a spore suspension prepared from PDA agar slant cultures. The suspension was standardized to 10^6 spores/ml and a 1ml

volume was inoculated into 50 ml of medium. The pH was adjusted to 6.0 using 4 N HCl and cultures were incubated at a temperature of 25°C on a rotary shaker at 110-rpm. Growth progress was monitored by measuring the cell weight at 0, 2, 4, 6, 7, 8 and 9 days of the culture incubation.

3.4. Screening for Lipases

The Rhodamine B agar plate test was carried out as a preliminary test (Hou and Johnston, 1992; Lee and Rhee, 1993) for the presence of lipase activity in both strains. The medium contained 39 g PDA, 31 ml olive oil and 0.001 % (w/v) Rhodamine B per liter of deionized water. The medium was autoclaved for 15 min at 121°C, and then 7-10 ml of the medium was poured into each petri-dish. To identify lipase activity, approx. 5 mm-diameter holes were punched into the agar and filled with 20 µl of fresh culture. The plates were then incubated at room temperature. Daily observation was carried out for lipase activity using a spectroline model CX-20 ultraviolet fluorescence analysis cabinet (Spectronics Corporation, Westbury, N.Y.). Lipase activity was detected by the presence of fluorescent halo around the colonies in the petri-dish.

In addition, a microscopic method was also carried out to confirm the presence of the lipase enzyme in the medium (Sierra, 1956). A peptone agar medium was prepared as follows: 10 g Bacto-peptone, 5.0 g NaCl, and 0.1 g $CaCl_2$. H_2O were dissolved in one liter of deionized water at pH 7.4. The culture medium and Tween 80 were sterilized separately by autoclaving for 20 min at 120°C. Tween 80 (0.5 ml) was then added to the medium to obtain a final concentration of 1 %. The medium was poured into plates and a small plug of fresh culture was inoculated. The plates were then incubated at 25°C for 3-5

days. The lipase activity was exhibited by the formation of calcium crystals visualized under microscope.

3.4.1. Protein Determination

Protein concentration was determined using a modification of the Lowry method as described by Hartree (1972). Bovine serum albumin at different concentrations was to establish the standard curve.

3.4.2. Lipase Activity Assay

3.4.2.1. Spectrophotometric Method

Lipase activity was monitored by the formation of *p*-nitrophenol measured colorimetrically according to the method described by Winkler and Stuckmann (1979). This spectrophotometric method measures the release of *p*-nitrophenol from a fatty acid ester as a result of enzymatic hydrolysis. Moreover, the released compound is yellow whose intensity is directly related to the rate of hydrolysis of the enzyme. *p*-Nitrophenyl stearate was used as the fatty acid ester substrate for the enzymatic assays.

The reaction medium consisted of solution A, containing 0.111 g gum arabic and 0.230 g sodium deoxycholate in 100 ml of sodium phosphate buffer solution (0.05 M, pH 8.0), which was then mixed with solution B, composed of 30 mg of substrate dissolved in 10 ml of isopropyl alcohol. However, when low pH buffer solutions were used for the enzyme assays, sodium deoxycholate was replaced by sodium taurocholate due to its limited solubility. The enzymatic reaction was initiated by the addition of an enzyme suspension volume of 0.1 to 2.4 ml aliquots of the reaction medium. The enzyme assays were incubated at 37°C for 60 min with continuous shaking at 110 osc/min. A control,

containing all the components except the enzyme suspension, was used as a blank for the assay. The optical density of the reaction medium was measured at a wavelength of 410 nm, using a Beckman DU-650 spectrophotometer (Beckman Instrument, San Ramon, CA) against an enzyme-free control. The absorbance of the *p*-nitrophenol standard at a wide range of concentrations was determined to establish a standard curve used for the estimation of lipase activity. The specific activity of lipase was defined as µmol of *p*-nitrophenol per ml per min.

3.4.2.2. Titrimetric Method

The lipase activity was determined using the titrimetric method according to a slight modification of the procedure described by Kermasha *et al.* (1986). The assay measured the free fatty acids liberated by the enzymatic hydrolysis of the substrate. The reaction medium was prepared by suspending 0.5 ml of 0.1 M tributyrin in 10 ml of an emulsion solution containing 0.1ml of 0.5 M KCl, 1 ml of 0.005 M NaCl, 5 ml of 0.01 M phosphate buffer solution (0.05 M, pH 8.0) and 0.1 ml of Tween-20. A volume of 0.1 ml of the partially purified lipase suspension (10-30 mg protein) was added to the Erlenmeyer flasks (50 ml) containing the reaction medium. The final volume of the reaction medium was adjusted to 10 ml using deionized water (Millipore Corp., Bedford, MA). The enzyme assays were incubated at 37°C for 30 min at 110 osc/min in a reciprocal shaking water bath (Precision Scientific Inc., Chicago, IL). Three control solution flasks, containing all the components of the reaction mixture except the enzyme, were used as blanks for the assay. The reaction was halted by the addition of 10 ml of a mixture of ethanol/acetone (1:1, v/v). The liberated fatty acids were measured by titrating the reaction medium with a standardized solution of NaOH (0.01 M NaOH) using the DL

53 automatic titrator (Metler Toledo, AG, Switzerland). The specific activity of lipase was defined as micromoles of fatty acid equivalent released per mg protein per minute.

3.5. Extraction and Enzymatic Recovery

3.5.1. P. camemberti

The extraction of the crude enzyme from the culture medium of *P. camemberti* was performed by filtration of the medium through several layers of cheesecloth. The resulting supernatant was then filtered using an ultrafiltration unit (cut-off 10,000 Da, Millipore Corp., Bedford, MA). The filtrate, considered to be the crude enzyme extract (FI), was lyophilized at -50°C for 24 hours. The lyophilized crude extract was stored at -80°C for further use.

3.5.2. P. roqueforti

The extraction of the crude enzyme from the culture medium of *P. roqueforti* was obtained using the same procedure as that described for *P. camemberti.* In addition, the lyophilized extract was further defatted and extracted according to a slight modification of the procedure reported by Kermasha *et al.* (1986). The lyophilized extract was defatted with cold acetone (-30°C) to remove the lipids bound to the enzyme thereby eliminating their interference in the proceeding steps of purification. The defatted enzyme extract was then suspended (1:10, w/v) in sodium phosphate buffer solution (0.05 M, pH 7.0) and subjected to mechanical stirring for 16 h. The suspension was then filtered through Whatman #1 paper and the resulting filtrate was suspended in acetone (1:20, w/v) with slow stirring for 15-20 min. The suspension was again filtered through Whatman #1 paper and the precipitate was subsequently dried under vacuum for 6 hrs to eliminate any

traces of remaining acetone. The dry filtrate, considered to be the crude enzymatic extract (FI), was stored at -80°C for further use.

3.6. Partial Purification of the Enzymatic Extracts

3.6.1. P. camemberti

The crude extract (FI) of *P. camemberti* was suspended (1:10 w/v) in sodium phosphate buffer solution (0.05 M, pH 8.0) and stirred for 30 min. All purification steps were carried out at 4°C, unless otherwise noted. The crude enzyme suspension was then centrifuged (12,096 x g, 15 min) using a flow-through centrifuge, Avanti-J25 I (Beckman Instrument). The precipitate was discarded whereas the supernatant was subjected to partial purification.

The enzyme suspension was subjected to ammonium sulfate precipitation at 0-60 % of saturation with continuous stirring; the resulting suspension was left to settle for 30 min and then centrifuged (12,096 xg, 15 min). The precipitate, fraction FIIa, was removed and the resulting supernatant was subjected to further purification by ammonium sulfate precipitation at 60-80 and subsequently at 80-100 % of saturation to obtain the respective precipitates FIIb and FIIc.

The partially purified fractions FIIa, FIIb and FIIc were resuspended in a minimum amount of sodium phosphate buffer solution (0.05 M, pH 8.0) and subjected to dialysis using a diluted sodium phosphate buffer solution (0.005 M, pH 8.0). The fractions were then lyophilized and stored at -80°C.

3.6.2. P. roqueforti

The crude enzymatic extract (FI) of *P. roqueforti* was suspended (1:10, w/v) in sodium phosphate buffer solution (0.05 M, pH 7.0) and stirred for 30 min. The suspension was then centrifuged (12,096 xg, 15 min) to eliminate insoluble cellular debris. The resulting supernatant was subjected to ammonium sulfate precipitation at a saturation of 0-20 %.

The suspension was allowed to stand for 30 min, and then centrifuged (12,096 xg, 30 min). The precipitate, fraction (FIIa), was recovered whereas the subsequent supernatant was further saturated at 20-40, 40-60 and 80-100 % of ammonium sulfate in order to obtain the respective precipitates, fractions FIIb, FIIc and FIId. The enzyme fractions were resuspended in a minimum amount of sodium phosphate buffer solution (0.05 M, pH 7.0) and dialyzed against a diluted sodium phosphate buffer solution (0.005 M, pH 7.0) for a period of 12 h. The desalted enzymatic fractions were then lyophilized and stored at -80°C.

3.7. Characterization of the Partially Purified Lipase Extracts

3.7.1. Effect of pH on Lipase Activity

The effect of pH on the lipase activity of the enzymatic extracts of *Penicillium* sp was investigated using reaction mixtures which varied in the pH range of 4.5-11.0. The buffers used were citrate-phosphate (pH 4.0-7.0), tris (hydroxymethyl) amino methane (pH 7.0-9.0), ammediol-HCl (2-amino-2-methyl-1,3-propandiol) (pH 9.0-10.0), sodium borate (pH 10.0-11.0) and phosphate-NaOH (pH 11.0-11.5).

3.7.2. Effect of Temperature on Lipase Activity

The optimum temperature for lipase activity of the enzyme extracts of the *Penicillium* sp. was also studied. The enzyme assays were incubated over a temperature range of 20 to 70°C, at the two pH optima for lipase activity obtained for both strains.

3.7.3. Substrate Specificity

3.7.3.1. Hydrolysis of Model Substrates

The substrate specificity of lipase activity of the partially purified fractions of both strains was investigated with various lipid fatty acid ester substrates including *p*-nitrophenyl butyrate (C4:0), *p*-nitrophenyl valerate (C5:0), *p*-nitrophenyl palmitate (C16:0) and *p*-nitrophenyl stearate (C18:0). The enzyme assays were performed at the two pH optima obtained for maximal lipase activity of the two fungal strains. The specific activity was calculated as μmol of *p*-nitrophenol per mg protein per min.

3.7.3.2. Hydrolysis of Edible Oils

Hydrolysis of free fatty acids from edible oils, including canola, fish and olive oils and butter fat, by the lipase activity of the partially purified extracts of *P. camemberti* and *P. roqueforti* was performed according to the titrimetric method described by Kermasha *et al.* (1986). The reaction mixture consisted of 200 mg of substrate oil suspended in 10 ml of emulsion solution composed of 0.1 ml of 0.05 M KCl, 1 ml of 0.005 M NaCl, 5 ml of 0.01 M of buffer solution and 0.1 ml of Tween-20 in Erlenmeyer flasks (50 ml). The enzymatic extract (10 mg) was suspended in buffer solution and shaken well. The final volume of the reaction mixture after the addition of the enzyme

was adjusted to 10 ml using deionized water (Millipore Corp.). The reaction was started by adding 0.1 ml of the partially purified lipase extract to the Erlenmeyer flasks (50 ml) which were then incubated at 37°C for 30 min with shaking at 110 osc/min in a reciprocal shaking water bath (Precision Scientific Inc., Chicago, IL). The reaction was then stopped by adding 10 ml of hexane.

3.7.3.3. Gas Chromatographic Analysis of Free Fatty Acids

Solvent extraction of fatty acids was conducted in order to separate the hexane layer containing the free fatty acids. The hexane extract was then filtered and concentrated using a speed Vac (Savant Instruments, Inc., Holbrook, N.Y.) and further dried under a gentle flow of nitrogen gas. Methylation of the free fatty acids was done following a procedure described by Badings and De Jong (1983): 1 ml of a HCl/ methanol mixture (20:80, v/v) was added to the dried extract which was then incubated at 85°C with continuous shaking for 15 min. The reaction mixture was subsequently centrifuged (3000 xg, 5 min) and the recovered supernatants were subjected to GC analysis.

Gas chromatographic analysis of the methylated free fatty acids was performed using a Varian Model 3400 gas chromatograph (Varian Associates, Sunnyvale, CA). An Omegawax 320 capillary column (30 m x 0.32 mm i.d., Supelco, Oakville, ON) coated with polyetheylene glycol was used. Injector and detector temperatures were set at 230 and 200°C, respectively. The column was maintained at 40°C for 5 minutes, followed by an increase in temperature from 40 to 200°C at a rate of 8°C/min. The flow rate of the carrier gas helium was 1.8 ml/min whereas those of air and hydrogen were at 45 and 260 ml /min, respectively.

3.7.4. Effect of Substrate Concentration on Lipase Activity

Two selected fatty acid esters, including *p*-nitrophenyl butyrate and *p*-nitrophenyl stearate, were used to determine the kinetic parameters for lipase biocatalysis of the two *Penicillium* sp. The assays were performed at the two pH optima obtained for lipase activity of both strains in order to investigate the influence of substrate concentration on enzyme activity in the reaction medium. Substrate concentration was varied from 1 to 10 mM.

3.7.5. Effect of Selected Chemical Reagents on Lipase Activity

The effect of various compounds, including ferrous chloride, ferric chloride, Ellman's reagent, *N*-bromosuccinimide, calcium chloride, magnesium chloride, Triton X-100 and EDTA on enzyme activity was investigated. The lipase activity of the partially purified fractions of both strains was assayed using the spectrophotometric method. The effect of the chemical compounds on lipase activity was investigated at two concentrations and at the two pH optima.

4. RESULTS AND DISCUSSION

4.1. Production of Extracellular Lipases from *Penicillium* Sp.

Optimization of the culture incubation conditions for the two biomasses production of the fungal strains was performed to obtain the highest lipase activity. Following optimization, a biomass of both strains was produced and the crude enzymatic extract was subsequently harvested.

The results (Figure 3) show the effect of culture incubation time on lipase activity of *Penicillium* sp. The results show that lipase activity increased with respect to time of culture incubation as indicated by the appearance of an orange fluorescent halo when the Rhodamine B agar plates were irradiated with UV light at 350 nm; the results also show that the diameters of the halos increased with time of incubation of the microorganisms, in tandem with increase in lipase activity measured spectrophtometrically. However, the formation of an orange fluorescent halo around the mycelial colonies could be a direct indication of lipase production by the fermented microorganism. Carrie *et al.* (1999) reported that the dimension of the diameter of the halo zone is correlated with the lipase hydrolytic activity. The appearance of fluorescent compounds may be due to the formation of a complex between cationic Rhodamine B and the uranyl-fatty acid ions (MacKenzie *et al.*, 1967; Kouker and Jaeger, 1987).

Kouker and Jaeger (1987) indicated that the Rhodamine B method is considered to be a sensitive and reliable method with respect to the lowest detection limit (1 nkat) compared to that of the titrimetric method (20 nkat); these authors indicated that the

fluorescent halo was formed as result of the generation of excited dimers of Rhodamine B, which fluoresce at longer wavelengths than that of the excited monomer.

Kouker and Jaeger (1987) also reported that the logarithm of lipase activity was linearly related to the zone diameter of the orange florescent halo. In addition, Pabai (1997) reported that lipases obtained from *Pseudomonas fragi* CRDA (halo diameter of 9.5 mm) and *Pseudomonas putida* ATCC 795 (halo diameter of 2.3 mm) were considered good and week lipase producers, respectively, with respect to their fluorescent halo diameters.

Figure 3 also shows that maximal lipase activity was obtained after 5 and 4-5 days of culture incubation of *P. camemberti* and *P. roqueforti*, respectively; however, further incubation showed a decrease in lipase activity. The results also demonstrate that the biomass production, determined as dry weight, showed a similar trend to that obtained for lipase activity. The concomitant increase in dry weight with that of the activity of extracellular lipase, detected in the media, could be considered as a direct indication of the capability of both mycelia to use the two carbon sources to produce lipase (Pabai, 1997). Watanabe *et al.* (1977) stated that the production of lipase is concomitant with the growth of microorganisms.

Shelley *et al.* (1987) suggested that there are three factors that should work together if a lipase-positive microorganism is to be considered as source of lipase: the organism must grow; the microorganism should produce lipase under the optimized conditions; and the detection method must be of sufficient sensitivity. In addition, the production of the enzyme can be detected from microorganisms when the nutrients in the

medium become limiting or during the late logarithmic phase or early stationary phase (Lee and Rhee, 1993).

In addition, the results show that there were slight variations in the pH of the culture media of *Penicillium* sp. during the fermentation time; the experimental findings indicate that the pH of the *P. camemberti* medium increased slightly from 6.0 to 6.5 during the entire time of incubation and reached a maximum which coincided with the highest lipase activity, while that of *P. roqueforti* slightly increased from 6.0 to 6.5 after six days and then became more acidic.

The observation of an acidic pH at the end of the microbial growth might be attributed in part to the formation of acidic lipases in the culture medium, since some fungal species such as *P. roqueforti* produce more than one type of lipase (Mase *et al.*, 1995). Lee and Rhee (1993) reported that maximal lipase production after 16 h of incubation of *Pseudomonas putida* 3SK at the late logarithmic phase. Stöcklein *et al.* (1992) indicated that maximum lipase activity of *Penicillium expansum* was obtained in the late logarithmic phase of growth after 4 days of incubation using olive oil as a carbon source. Long *et al.* (1996) studied the growth of *Aspergillus flavus* in a medium containing corn oil and yeast extract as carbon and organic nitrogen sources, respectively.

Druet *et al.* (1992) reported similar results for the culture incubation of *P. cyclopium* where maximum lipase activity was detected after five days of incubation. Mase *et al.* (1995) reported that maximum lipase formation by *P. roqueforti* was observed after 72 h of incubation whereas, Eitenmiller *et al.* (1970) reported that the enzyme activity was detected at the end of seven days.

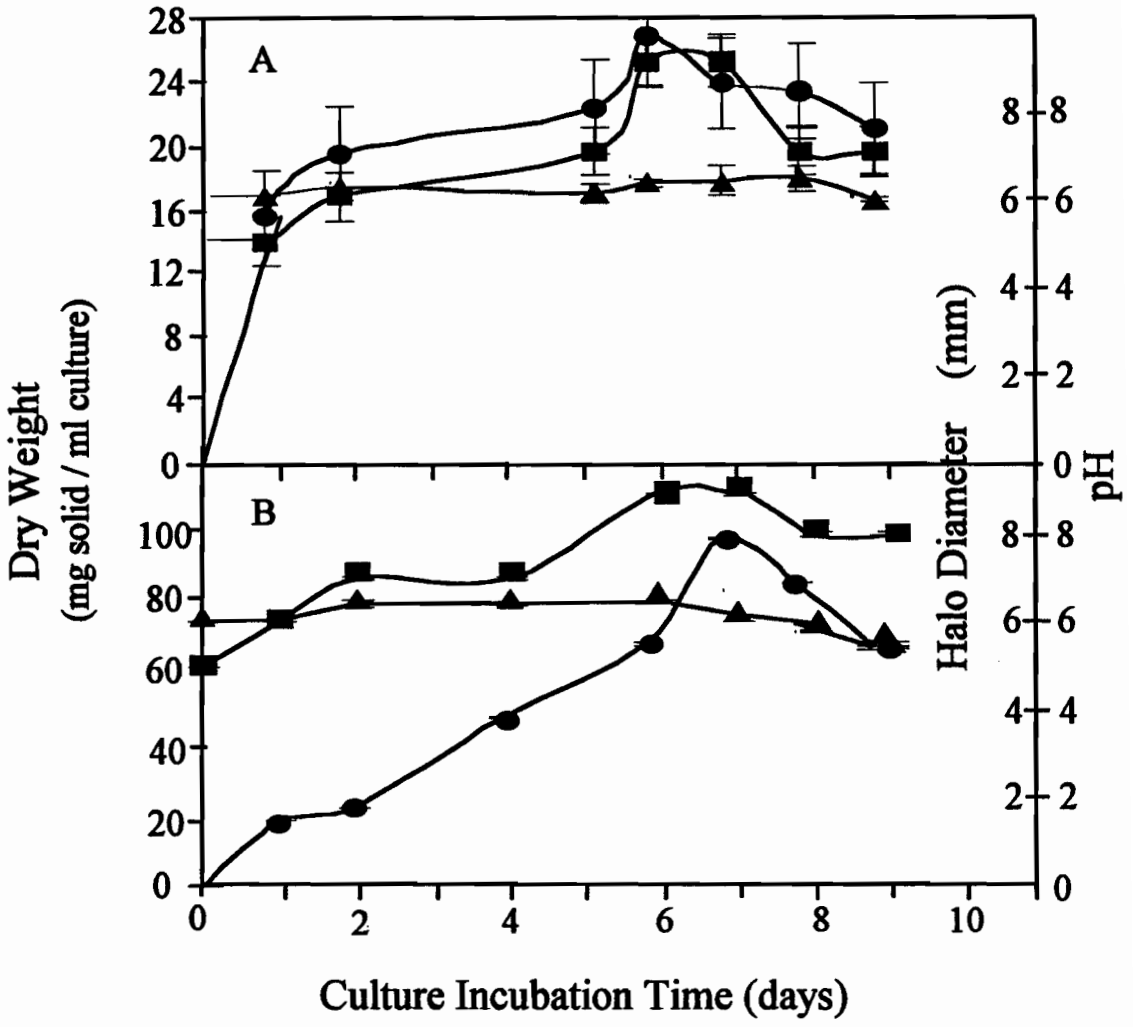


Figure 3. Culture incubation of (A) *P. camemberti* and (B) *P. roqueforti* determined by dry weight (—●—), pH (—▲—) and the Rhodamine test (—■—).

The results (not shown) also indicated the presence of lipase activity in both *Penicillium* strains, as confirmed under light microscope by the detection of a visible halo around colonies, incubated on peptone agar medium containing Tween 80. The formation of the halo was due to the presence of crystals of calcium salts of fatty acids liberated by lipase activity. This method can be used in studies of substrate specificity to show that lipases can have a preference toward Tween 80 as substrate (Sierra, 1956).

The effect of olive and soybean oils, as a carbon source for both strains, was investigated. The results (Fig. 4) show that the incubation of *P. camemberti* in culture medium containing soybean oil produced a slightly higher lipase activity compared to that obtained with the same medium but with olive oil.

Figure 4 also shows that a maximal lipase activity of 2.4 µmol *p*-nitrophenol/ml/min was obtained after 4-5 days of incubation with the medium containing soybean oil as opposed to an enzyme activity of 1.6 µmol *p*-nitrophenol/ml/min with that containing olive oil. In addition, Figure 4 indicates that the protein concentration of the medium supplemented with either soybean or olive oil increased with incubation time reaching a maximum at two days; however, the results also show that a dramatic decrease in protein concentration was observed in the latter medium with an increase in incubation time while in the former one, it remained stable.

In contrast, the results (Fig. 5) show that the highest lipase activity from *P. roqueforti* was obtained after 4-5 days of incubation with the culture medium containing olive oil as opposed to soybean as a carbon source. In addition, the results show that the extracellular protein content was slightly higher in the medium containing olive oil.

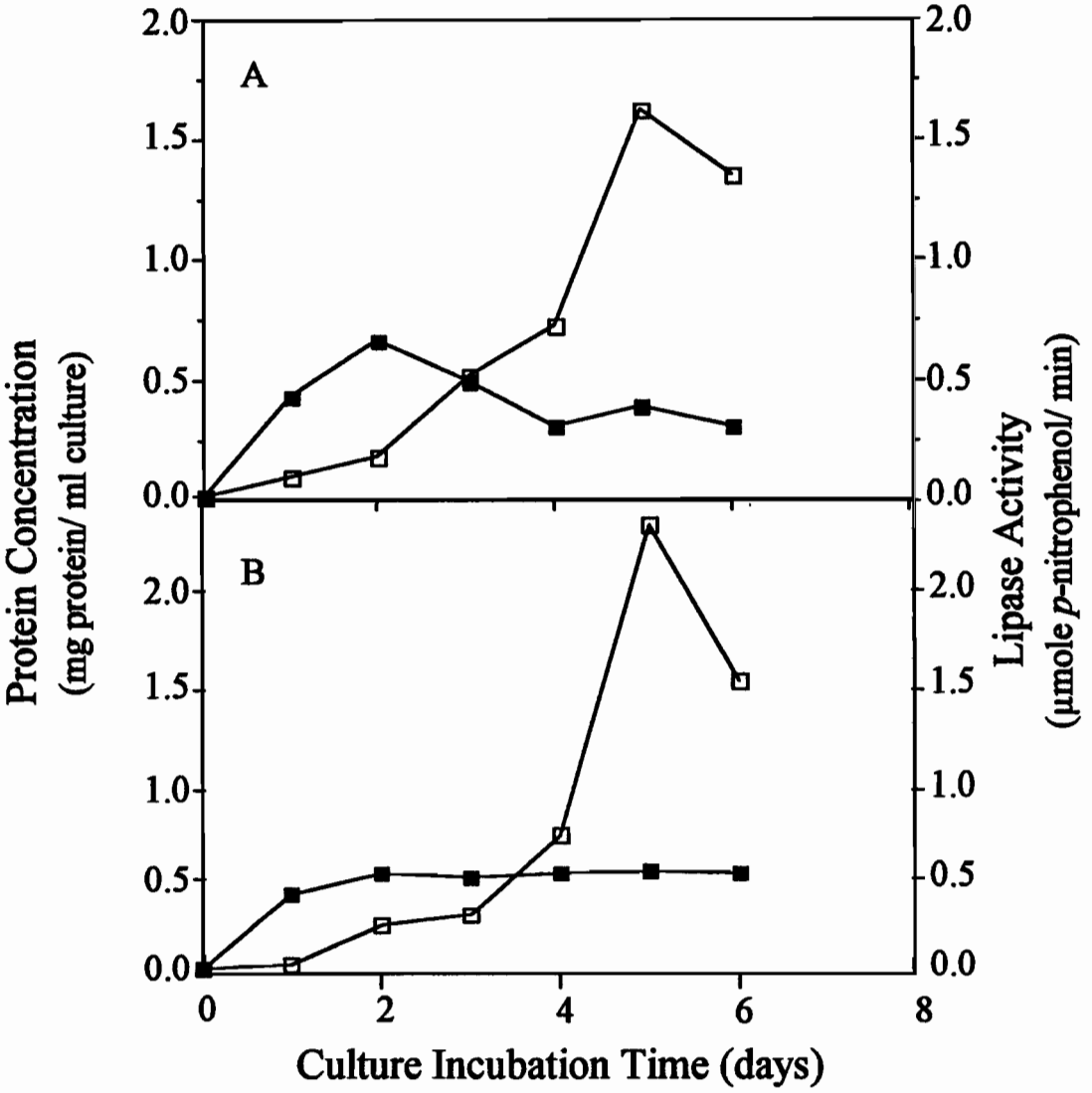


Figure 4. Effect of (A) olive oil and (B) soybean oil on the lipase activity (□) and protein concentration (■) during the culture incubation of *P. camemberti*.

The overall findings demonstrate the important role of oils as inducers of lipase production in microorganisms.

The decrease in lipase activity of *P. camemberti*, observed at the end of the incubation time, may be due to the substantial residual enzyme that remained attached to the cell walls (Macrae, 1983). In addition, the drop in lipase activity could also be due to the presence of both lipases and proteases in the culture medium (Iwai and Tsujisaka, 1984).

Davaranov (1994) stated that the production of lipase in microorganisms may be a response to the presence of inducers added to the culture medium; in addition, the same author stated that the quantity of the induced enzyme produced is related to the type of substrate added as well as its concentration. Davranov (1994) reported that microorganisms are characterized by the fact that they can synthesize extracellular lipases, whose activity is many times greater than the intercellular level of activity. In addition, lipases can be inducible, in some cases, by the introduction of lipid substrates in growth media. In addition, Macrae (1983) reported that the addition of lipids such as olive oil, groundnut oil and cottonseed oil, and fatty acids such as oleic acid can be effective in the stimulation of lipase production.

Miranda *et al.* (1999) reported that a lipase extract from *Penicillium citrinum* was produced after 24 h incubation in a medium containing a vegetable oil refinery residue as a carbon source; these authors indicated that the lipase activity produced by *P. citrinum*, with olive oil as carbon source, was less than that produced with the oil refinery residue.

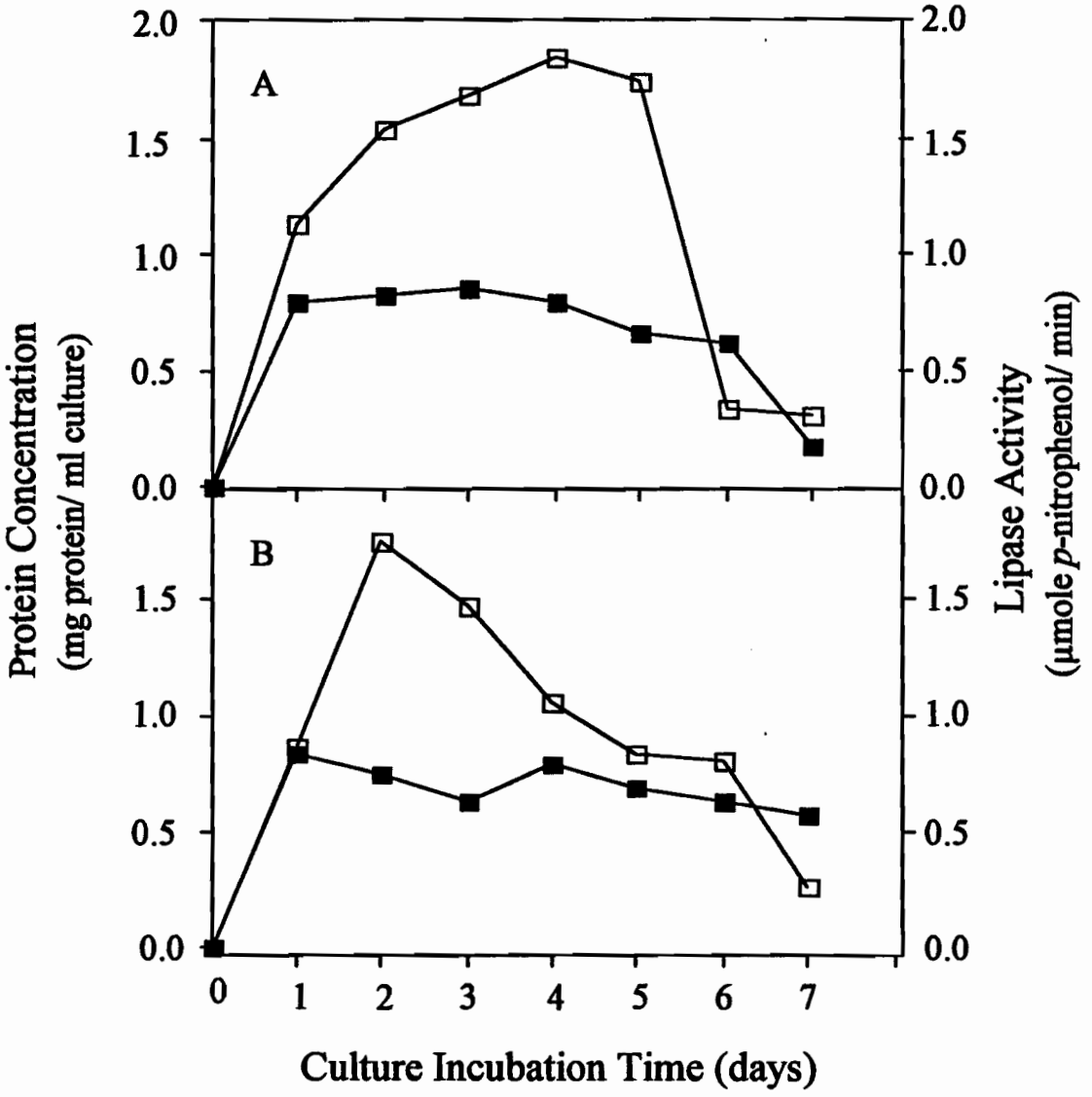


Figure 5. Effect of (A) olive oil and (B) soybean oil on the lipase activity (□) and protein concentration (■) during the culture incubation of *P. roqueforti*.

Pimentel *et al.* (1997) stated that a lipase from a brazilian strain *P. citrinum* cultured in a simple and inexpensive medium containing 0.75% ammonium sulfate complemented with minerals salts instead of yeast extract and using soybean oil and corn oil, resulted in a lower activity compared to that obtained with olive oil. In addition, Baillargeon and McCarthy (1991) found that the highest lipase activity was obtained from *G. candidum* NRRL Y-553 cultured in a medium containing soybean oil.

Delatorre *et al.* (1996) reported that a *Penicillium candidum* was incubated in a culture medium supplemented with 0.2% olive oil and produced lipase. Mozaffar and Weete (1993) reported that after 6 days of incubation in a medium containing 2 ml soybean oil, an extracellular lipase was produced from a filamentous fungous *Pythium ultimum*. Yamaguchi and Mase (1991) reported that the production of a novel lipase by *P. camemberti* U-150, using a culture medium which contained soybean oil as a carbon source. A strain of *Penicillium cyclopium* was also found to be capable of producing lipase in a culture medium containing 1% commercial olive oil (Ibrik *et al.*, 1998). Freire *et al.* (1997) reported that a lipase from *Penicillium restrictum* showed a high capability of producing lipase in a medium containing olive oil; however, low extracellular lipase activity was observed using glucose as a carbon source

Benjamin and Pandey (1995) reported that the inclusion of olive oil in the culture medium of *C. rugosa* gave a high yield of lipase. In contrast, Eitenmiller *et al.* (1970) stated that lipase activity might be inhibited by the presence of butteroil, corn oil and olive oil in the culture medium. Moreover, Petrovic *et al.* (1990) also reported that no measurable lipase activity was detected when olive oil was used as a carbon source for the culture incubation of *P. roqueforti*.

4.2. Partial Purification of Enzymatic Extracts

Table 3 shows the partial purification of the crude extracellular lipase extract of *P. camemberti* by ammonium sulfate precipitation. The results show that fraction (FIIa), precipitated at 0-60 % of ammonium sulfate saturation, exhibited the highest specific activity (44.83 µmol/mg protein/min) in comparison to those obtained for fraction (FIIb) (11.96 µmol/mg protein/min) and fraction (FIIc) (7.07 µmol/mg protein/min). In addition, fraction (FIIa) showed a 6.9-fold increase in purification over the crude extract (FI) while fractions (FIIb) and (FIIc) showed only a 2.60-and 1.09-fold increase in purification, respectively, whereas fractions (FIIb) and (FIIc) showed considerably lower recoveries of 1.50 and 2.00, respectively. Based on these findings, fraction (FIIa) was selected as the partially purified lipase extract for further characterization. However, the results (Tables 3 and 4) show that the specific lipase activity was improved and the elimination of interfering compounds was achieved. Consequently the enzyme extract was subjected to further steps of chromatographic purification which could also improve the affinity of the enzyme toward the substrate (Schuepp, 1995; Pabai, 1997; Bisakowski and Kermasha, 1998).

In addition, the partial purification of the crude extract was performed in order to eliminate the interference and also removal of the debris that may mask the lipase activity, although the addition of sodium sulfate salt at high concentrations may lead to some loss in lipase activity (Kermasha and Metche, 1986; Pabai, 1997; Abdul Wahab, 1999). Sztajer *et al.* (1992) reported that the partial purification of an extracellular

Table 3. Scheme of partial purification of the extracellular lipase extract from *Penicillium camemberti.*

Fraction	Total protein (mg)	Specific activity[a]	Total activity[b]	Purification (fold)	Recovery (%)
Crude extract (FI)[c]	900.0	6.5	5850.0	1.0	100.0
Ammonium sulfate saturation (FII)[d]					
0-60 % (FIIa)	29.2	44.8	1308.6	6.9	22.4
60-80 % (FIIb)	5.0	17.0	86.0	2.6	1.5
80-100 % (FIIc)	16.2	7.0	114.6	1.1	2.0

[a]Specific activity was expressed as μmol *p*-nitrophenol per mg protein per min.

[b]Total activity was expressed as μmol *p*-nitrophenol per min.

[c]Crude extracellular lipase extract.

[d]Partially purified enzyme extract fractionated by ammonium sulfate.

lipase from *P. simplicissimum* resulted in a 3-fold increase in specific activity and a recovery of 60 %.In addition, a lipase extract from *P. camemberti* U150 was partially purified at 60 % of ammonium sulfate saturation which resulted in 66-fold increase in purification and a recovery yield of 65 % (Yamaguchi and Mase, 1991).

Table 4 shows the partial purification of the crude extract of *P. roqueforti*. The results show that fractions FIIa and FIIb, obtained at 0-20 and 20-40 % of saturation, respectively, showed the highest total activity compared to fractions (FIIc), (FIId) and (FIIe) obtained at 40-60, 60-80 and 80-100 % of ammonium sulfate saturation. The results also showed that fractions (FIIa) and (FIIb) demonstrated an overall combined high purification fold and recovery yield of 4.7 and 3.6, respectively, in comparison to that obtained with fractions (FIIc), (FIId) and (FIIe). Based on these results, the crude extract of *P. roqueforti* was partially purified at 0-40 % of ammonium sulfate saturation. The lower enzymatic recovery might be due to the presence of debris and non-enzymatic proteins in the crude extract (Kermasha and Metche, 1986).

Mase *et al.* (1995) reported that a crude lipase extract from *P. roqueforti* was fractionated using ammonium sulfate at 0-40 % saturation which produced an increase in purification of 1.6-fold and a recovery yield of 32 %.

The purification of the crude extract of a lipase produced by *Pseudomonas putida* has been resulted in 21-fold increase with a yield of 5.3 % (Lee and Rhee, 1993). Veeraragavan *et al.* (1990) reported that the purification of a crude lipase extract produced by *Geotricum candidum*, by ethanol precipitation resulted in a 3-fold increase in purification.

Table 4. Scheme of partial purification of the extracellular lipase extract from *Penicillium roqueforti.*

Fraction	Total protein (mg)	Specific activity[a]	Total activity[b]	Purification (fold)	Recovery (%)
Crude extract (FI)[c]	2000.0	1.0	2026.0	1.0	100.0
Ammonium sulfate saturation (FII)[d]					
0-20 % (FIIa)	6.4	1.6	25.8	1.6	1.3
20-40 % (FIIb)	14.5	3.2	46.0	3.1	2.3
40-60 % (FIIc)	5.2	1.8	9.1	1.7	0.5
60-80 % (FIId)	6.2	1.3	7.8	1.3	0.4
80-100 % (FIIe)	9.8	1.3	12.6	1.3	0.6

[a]Specific activity expressed as μmol *p*-nitrophenol per mg protein per min.

[b]Total activity expressed as μmol *p*-nitrophenol per min.

[c]Crude extracellular lipase extract.

[d]Partially purified enzyme extract fractionated by ammonium sulfate.

Ibrik *et al.* (1998) reported that the ammonium sulfate precipitation of a crude lipase extract produced by *Penicillium cyclopium*, resulted in 2-fold increase in purification and yield of 71%. Schuepp *et al.* (1995) indicated that the highest lipase activity of an extracellular lipase from *Pseudomonas fragi* was obtained with ammonium sulfate precipitation at 20-40% of saturation; Pabai (1997) also reported that the partial purification of an extracellular lipase extract produced by *Pseudomnas fragi*, by ammonium sulfate precipitation at 20-40%, resulted in 19-fold increase in purification and a recovery of 53%.

Eitenmiller *et al.* (1970) reported that a 7-fold increase in specific activity was obtained when the lipase extract from *P. roqueforti* was partially purified at 30-50 % of ammonium sulfate precipitation. A lipase from *P. citrinum* was also partially purified with ammonium sulfate at 40-60 % saturation, which resulted in a 11-fold increase in purification and a recovery of 71.2 % (Pimentel *et al.,* 1994).

4.3. Characterization of Extracellular Lipases

4.3.1. Effect of pH and Temperature on Enzymatic Activity

The effect of pH on the lipase activity of the partially purified extracellular lipase extracts was investigated using a number of buffer solutions ranging from pH 4.5 to 11.0. The results (Fig. 6) show that the lipase activity of the partially purified *P. camemberti* extract exhibited two pH optima at 5.50 and 7.75, whereas that of the *P. roqueforti* extract showed maximal activity at pH 5.25 and 8.75. Figure 6 also shows that the enzymatic extracts from *P. camemberti* and *P. roqueforti* exhibited approximately 20-30 %

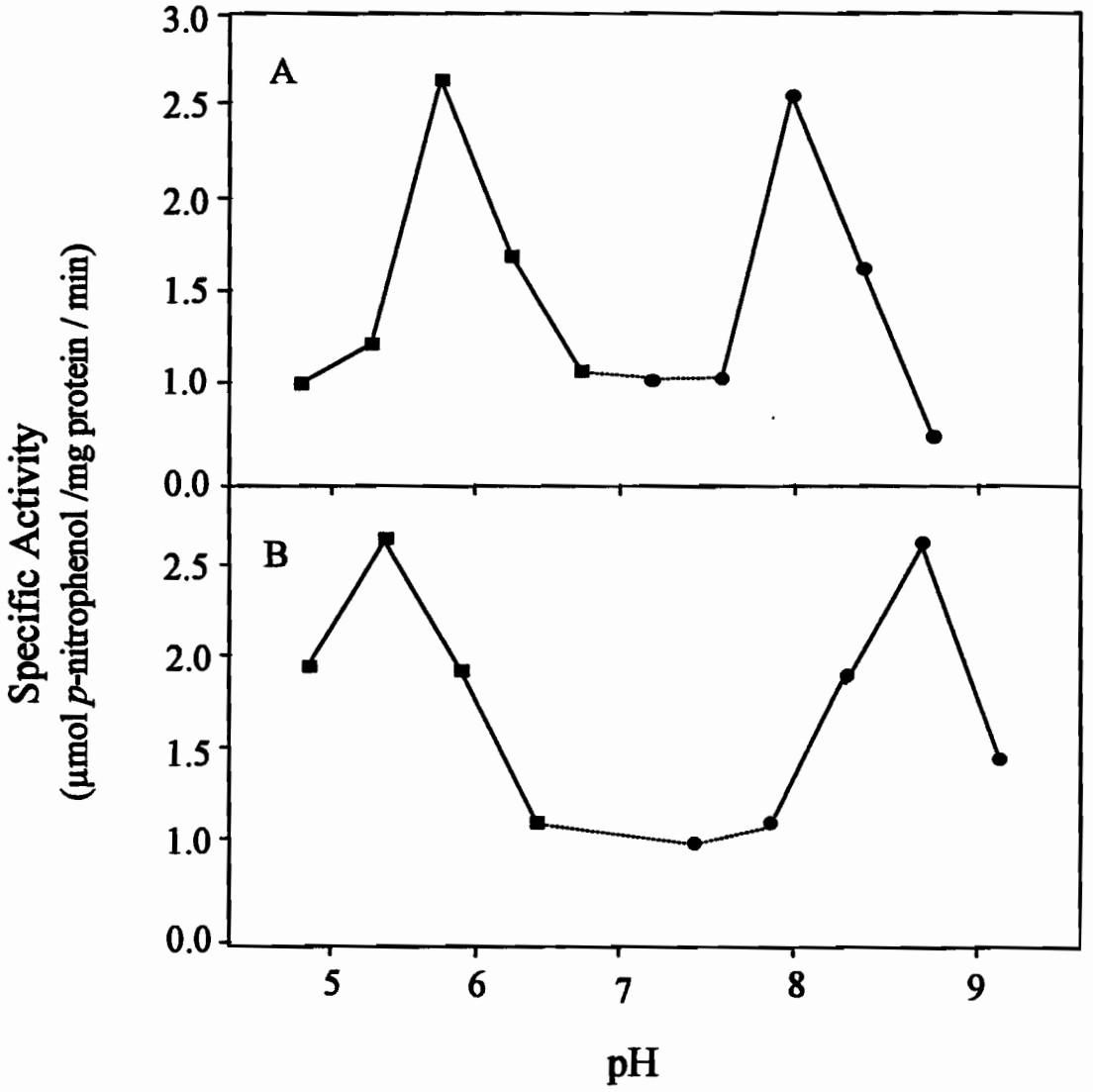


Figure 6. The effect of pH on the lipase activity from *P. camemberti* (A) and *P. roqueforti* (B).

of the maximal activity in the neutral pH range of 6.5-7.5 and 6.0-7.5, respectively; however, this low activity level could be due to incomplete ionization of the free fatty acids rather than to the absence of activity in the lipase fractions (Macrae, 1983; Abdul Wahab, 1999). An extract from *P. camemberti* showed maximum lipase activity at the two pH optima of 5.5 and 5.0 (Yamaguchi and Mase, 1991).

Most microbial lipases have their optimum activity in the pH range of 7.0 to 9.0 (Pimentel *et al.*, 1994). However, the occurrence of acidic lipases has also been reported by many researchers (Macrae, 1983). Eitenmiller *et al.* (1970) and Iwai *et al.* (1973) found that optimal lipase activity of *P. cyclopium* extract was obtained at pH 5.8 and 7.0, respectively, whereas Pimentel *et al.* (1994) stated that a lipase extract from *P. citrinum* showed maximum activity at pH 8.0. However, these variations in pH values for optimal lipase activity may be attributed to many factors such as incubation time, temperature, nature and concentration of buffer, ionic strength of the medium, as well as the purity of the enzyme preparation (Whitaker, 1972).

Mase *et al.* (1995) reported that *P. roqueforti* produces two extracellular lipases, an acidic and an alkaline lipase. Veerargavan *et al.* (1990) separated two forms of lipases (I and II) from a strain of *G. candidum* whose respective pH optima were 6.0 and 6.8. A lipase from *P. camemberti* was purified into four active fractions (Isobe *et al.*, 1992). Two extracellular lipases (A- and B-lipase) were identified, isolated and characterized from a strain of *P. cyclopium* (Druet *et al.*, 1992). An extracellular lipase was obtained from a strain of *Pseudomonas putida* and indicated an optimum pH range of 8.0–9.0 for activity (Lee and Rhee, 1993). Ibrik *et al.* (1998) indicated that a lipase from *Penicillium cyclopium* exhibited an optimum activity in the pH range of 8-10. Maximum lipase

activity from an extract of *Penicillium caseicolum* was observed at pH 9.0 (Alhir *et al.*, 1990). The presence of two pH optima for lipase activity could be partially due to the presence of multiple forms of the enzyme (Veeraragavan *et al.*, 1990; Druet *et al.*, 1992; Larsen and Jensen, 1999). In addition, the differences in the molecular properties of lipases may also be due to other factors, including the type of strain, the degree of enzyme production, the purification method and the enzymatic assay (Pimentel *et al.*, 1994).

In addition, variations in pH values for maximum lipase activity may differ due to the occurrence of multiple forms of lipase obtained after purification of the crude lipase extracts and to the presence of different carbohydrate structure that could be bound to the enzyme (Isobe *et al.*, 1992). The optimum temperature for lipase activity of the *P. camemberti* and *P. roqueforti* extracts was also investigated at the two pH optima.

The results (Fig. 7) show that the optimum temperature for lipase activity of the *P. camemberti* extract was at 45 and 30°C at pH 5.50 and 7.75 respectively, while that of the *P. roqueforti* extract was at 35 and 40°C at pH 8.75 and 5.25, respectively. These findings are similar to those reported for optimal lipase activity in the literature. Isobe *et al.* (1992) reported that the optimum temperature for lipase activity of a *P. camemberti* extract was determined to be at 40 and 45°C at pH 5.0 and 5.5, respectively. In addition, Mase *et al.* (1995) reported that the optimum temperature for enzyme activity of a lipase extract from *P. roqueforti* was at 35°C and pH 7.0. Moreover, Petrovic *et al.* (1990) stated that the optimum temperature for lipase activity of a *P. roqueforti* extract was at

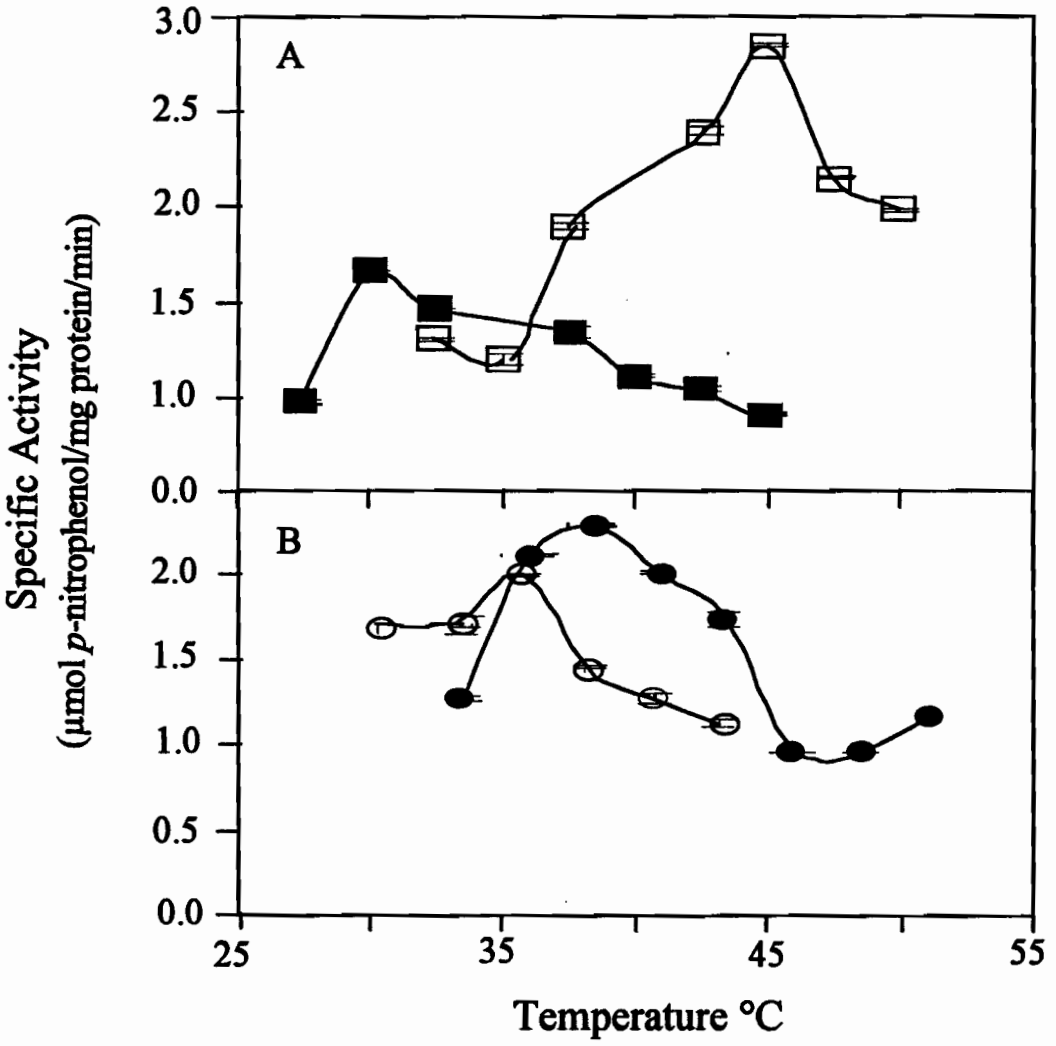


Figure 7. Effect of temperature on the activity of extracellular lipases of *P. camemberti* (A) at pH 7.75 (—■—) and pH 5.5 (—□—) and *P. roqueforti* (B) at pH 8.75 (—○—) and pH 5.25 (—●—).

30°C and pH 8.0. An optimum temperature of 45°C was reported for a lipase produced by a strain of *Penicillium wormanii* (Costa and Peralta, 1999). Also, a lipase from *Penicillium cyclopium* showed maximum lipase activity at 35°C whereas that from *Penicillium albeanum* exhibited optimal activity in the temperature range of 25-30°C at pH 7-8 (Sugihara *et al.*, 1996). These differences in the properties of the lipases, obtained from different sources, may be explained by many factors such as the type of strain used, the degree of enzyme production, the methodology used for purification, and the reaction conditions used to determine lipase activity (Pimentel *et al.*, 1994).

4.3.2. Substrate Specificity

The substrate specificity of the partially purified extracacellular lipase extracts (Fig. 8) was investigated using four selected fatty acid esters of *p*-nitrophenol at the two pH optima. Figure 8 A and Table 5 indicate that at pH 5.50, the lipase activity of the partially purified extract of *P. camemberti* showed a preference towards *p*-nitrophenyl stearate as substrate, followed by *p*-nitrophenyl palmitate, whereas at pH 7.75, a higher affinity was exhibited towards *p*-nitrophenyl valerate followed by *p*-nitrophenyl palmitate and *p*-nitrophenyl stearate. These findings suggest that the lipase activity from *P. camemberti* may be more specific towards long-chain fatty acid esters at acidic pH of 5.50, whereas at the alkaline pH, is slightly more specific towards short-chain fatty acid esters than long chain fatty acid esters. These findings are in agreement with these reported previously by other researchers.

Lipase extracts from *G. candidum* NRRL Y-553 have shown two types of specificity, one towards oleic acid (18:1) and the other one towards palmitic acid (16:0)

(Baillargeon and McCarthy, 1991). Gulomova *et al.* (1996) reported that the affinity of lipase towards the substrate was affected by the surface pressure of the interface. *Penicillium* sp. have shown to possess a high content of fatty acids with different chain lengths. *P. roqueforti* was found to be rich in short chain fatty acids, whereas *P. camemberti* was more rich in long chain fatty acids; the variation in the fatty acid content of these strains might be a factor that affects the affinity of the lipase activity of the extracts from both strains towards a specific chain length fatty acid (Lomascolo *et al.*, 1994). The difference in affinity of lipases towards mono-, di- and triacylglycerols was also attributed to the level of surface pressure (Laurent *et al.*, 1994). Yamaguchi and Mase (1991) suggested that the preference of a lipase, produced by *P. camemberti*, towards mono- and diacylglycerols was dependant on the type of acyl glycerol group.

Druet *et al.* (1992) reported that the extracellular lipase, from *Penicillium cyclopium*, showed a preferential specificity towards short-chain homogeneous triacylglycerols. A lipase activity from *P. caseicolum* was also found to be more specific toward short-chain fatty acids than those long-chain ones (Alhir *et al.*, 1990).

Yamaguchi and Mase (1991) reported that the purified lipase obtained from *P. camemberti* was highly specific towards glycerides possessing palmitic and stearic acids. The results obtained by Lomascolo *et al.* (1994) also showed that the high content of saturated and unsaturated long-chain fatty acid esters, such as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) in the cells of *P. camemberti*, could explain the preference of lipases from *P. camemberti* towards long-

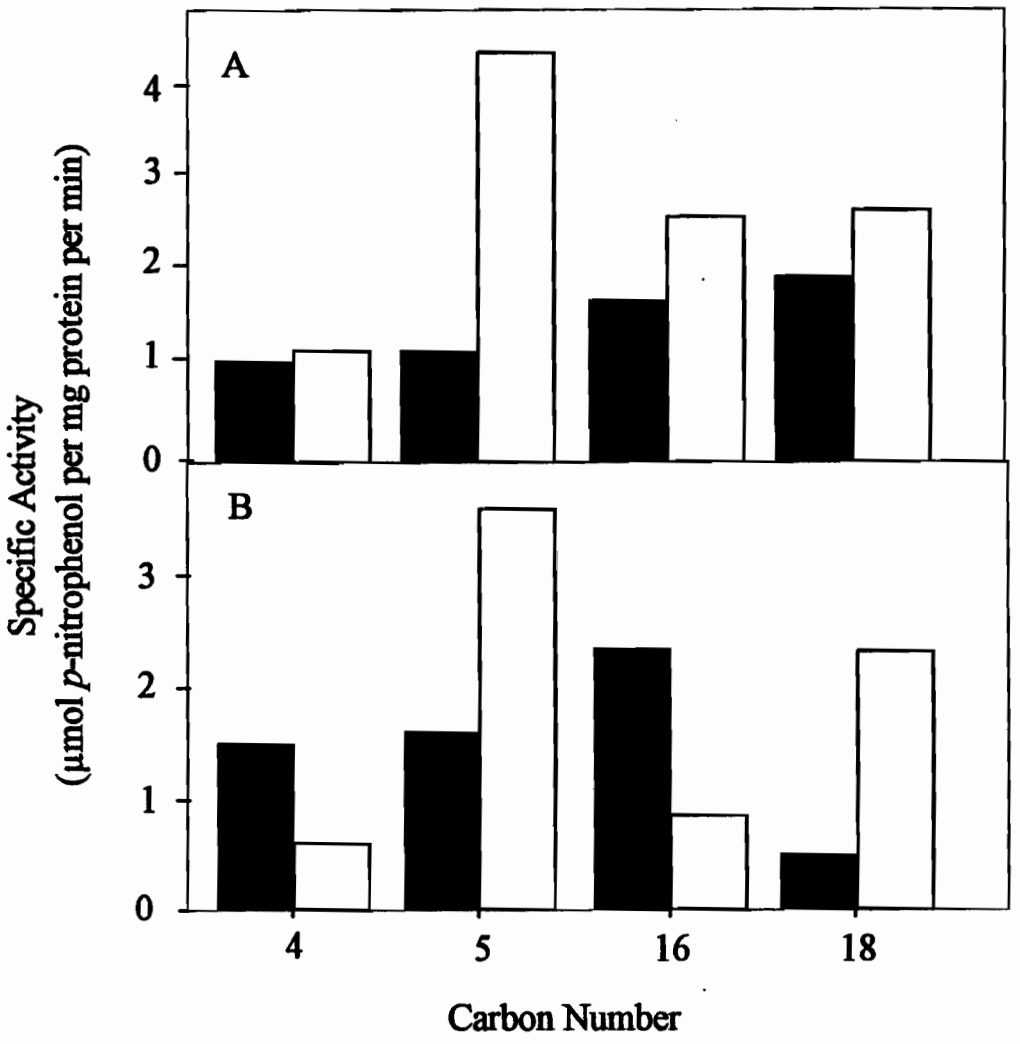


Figure 8. Specific activity of extracellular lipases from *P. camemberti* (A) at pH 5.50 (■) and 7.75 (□) and *P. roqueforti* (B) at pH 5.25 (■) and 8.75 (□), using *p*-nitrophenyl (PNP) butyrate (C4:0), PNP-valerate (C5:0), PNP-palmitate (C16:0) and PNP-stearate (C18:0).

Table 5. Specific activity of the extracellular lipase extracts from *Penicillium* sp. using model substrates.

		Specific activity[a]			
		pH			
Microorganism	Substrate	5.25	5.50	7.75	8.75
P. camemberti					
	p-Nitrophenyl butyrate	-	1.40	1.50	-
	p-Nitrophenyl valerate	-	1.50	5.50	-
	p-Nitrophenyl palmitate	-	2.17	3.32	-
	p-Nitrophenyl stearate	-	2.52	3.40	-
P. roqueforti					
	p-Nitrophenyl butyrate	1.50	-	-	0.60
	p-Nitrophenyl valerate	1.60	-	-	3.60
	p-Nitrophenyl palmitate	2.33	-	-	0.85
	p-Nitrophenyl stearate	0.50	-	-	2.30

[a]Specific activity was expressed as μmol *p*-nitrophenol per mg protein per min.

chain fatty acids. A lipase activity of a *P. simplicissimum* extract was also reported to hydrolyze *p*-nitrophenyl palmitate (Sztajer *et al.*, 1992).

An extracellular lipase from *P. fragi* CRDA 037 showed a wide range of specificity towards triacylglycerols of different fatty acid chain lengths as substrates. Schuepp (1995) stated that an extracellular lipase produced from *P. fragi* CRDA 037 indicated the highest lipase activity with trimyrstin as substrate followed by triacetin then tributyrin. In addition, lipases produced by a strain of *P. fluorescens* BW96CC1, exhibited preferential specificity for triacylglycerols of long-chain fatty acids (Pabai, 1997). Lipases produced from *Geotrichum candidum* NRRL Y-553 were interesting with respect to their unique specificity towards *cis*-9-unsaturated fatty acids in comparison to that exhibited towards both stearic and palmitic acids (Baillargeon and McCarthy, 1991). Davranov (1994) reported that when using milk fat as substrate, a lipase from *Mucor miechei* exhibited the highest activity toward oleic acid followed by palmitic acid and stearic acid. Stöcklein *et al.* (1992) reported that using a wide range of substrates, a lipase from *Penicillium expansum* exhibited the highest lipase activity toward stearic followed by caproic, oleic, lauric and palmitic methyl acid esters.

Figure 8 B and Table 5 also show that the lipase activity of the partially purified *P. roqueforti* extract exhibited a different preference towards the fatty acid ester substrates than that of the *P. camemberti* extract. The results show that at pH 5.25, a maximal lipase activity was exhibited towards short-chain fatty acid esters, including *p*-nitrophenyl palmitate, *p*-nitrophenyl valerate and

substrate specificity of the *P. roqueforti* extracellular lipase towards short-chain fatty acids is similar to that reported in the literature. Mase *et al.* (1995) reported that the lipase activity of *P. roqueforti* IAM 7268 showed a preference towards short-chain fatty acids including *p*-nitrophenyl esters and triglycerides. A lipase from *P. cyclopium* was also reported capable of hydrolyzing short-chain fatty acid glycerides more rapidly than long-chain ones (Druet *et al.*, 1992). Eitenmiller *et al.* (1970) also observed that the lipase activity of the *P. roqueforti* extract showed a certain affinity towards short-chain fatty acids; the enzyme hydrolyzed mostly tricaprylin, tributyrin, tricaproin, triolein and triacetin in decreasing order.

Alford and Pierce (1961) and Wiclox *et al.* (1955) also reported that the lipase from the *P. roqueforti* extract preferentially hydrolyzed short-chain fatty acids from butter fat as opposed to the long-chain fatty acids. Moreover, Lomascolo *et al.* (1994) indicated that *P. roqueforti* strain was rich in short-chain fatty acids compared to *P. camemberti*; these findings may explain the difference in the substrate specificity of the lipase extracts towards a certain chain length group of fatty acids.

4.3.3. Determination of Kinetic Parameters

Figures 9 and 10 show the kinetic study of lipase biocatalysis using the partially purified extracellular lipase extracts from *P. camemberti* and *P. roqueforti* with substrate models at the two pH optima for enzyme activity. The kinetic parameters were calculated from the best straight line using the Lineweaver-Burk reciprocal plots of $1/v$ versus $1/[S]$ (Lineweaver and Burk, 1934). The results (Table 6) show that the lipase activity from *P. camemberti* showed a higher affinity towards *p*-nitrophenyl butyrate at pH 5.5 compared to

pH 7.75 as indicated by the corresponding K_m values of 0.24 and 0.56 mM. The results also show that the at pH 5.50 and 7.75, extracellular lipase activity showed a higher preference, as indicated by the K_m values, toward *p*-nitrophenyl stearate as substrate than *p*-nitrophenyl butyrate. However, the V_{max} values for lipase biocatalysis with *p*-nitrophenyl butyrate as substrate were approximately twice as high as the V_{max} values obtained with *p*-nitrophenyl stearate at pH 5.50 and 7.75. On the other hand, the results indicate that the lipase activity from the *P. roqueforti* extract showed the highest affinity towards *p*-nitrophenyl stearate, at pH 5.25, followed by *p*-nitrophenyl butyrate, at pH 5.25 and 8.75, and then by *p*-nitrophenyl stearate as indicated by the respective K_m values of 0.08, 0.16, 0.19 and 0.58 mM. However, the results also indicate that the highest activity was obtained with the substrates *p*-nitrophenyl stearate, at pH 8.75, followed by *p*-nitrophenyl butyrate, at pH 5.25 and 8.75 and then by *p*-nitrophenyl stearate at pH 5.25, as indicated by the V_{max} values. followed by *p*-nitrophenyl butyrate, at pH 5.25 and 8.75 and then by *p*-nitrophenyl stearate at pH 5.25, as indicated by the V_{max} values. The overall findings demonstrate that the lipase activity from *P. camemberti* showed a higher catalytic efficiency, with *p*-nitrophenyl stearate as substrate compared to *p*-nitrophenyl butyrate while that from *P. roqueforti* showed higher catalytic efficiency with *p*-nitrophenyl butyrate than with *p*-nitrophenyl stearate.

However, Pimentel *et al.* (1994) suggested that the lipase activity was not affected by substrate concentration but rather by the interfacial area. Schuepp *et al.* (1997) reported that, using tributyrin as substrate, the lipase activity of the partially purified extract from *Pseudomonas fragi* CRDA 037 showed a K_m value of 7.1 mM whereas Abdul Wahab (1999) indicated a K_m value of 8.057 mM. In addition, Gobbetti *et al.* (1996) reported that,

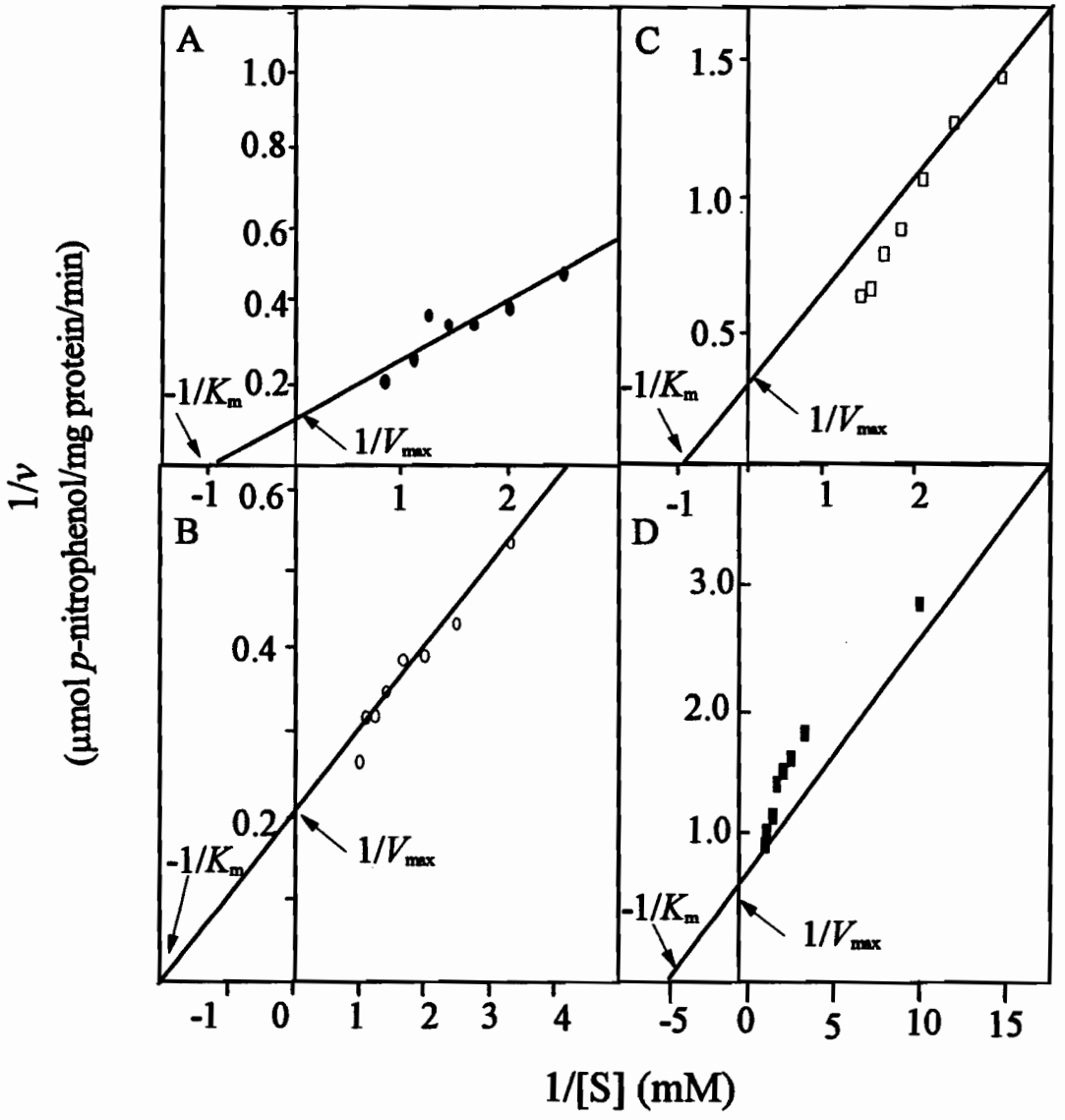


Figure 9. Lineweaver - Burk double reciprocal plots for extracellular lipases of *P. camemberti* at (A) pH 5.5 (•) and (B) pH 7.7 (o) and *P. roqueforti* at (C) pH 5.25 (▫) and (D) pH 8.75 (▪) using *p*-nitrophenyl butyrate as substrate.

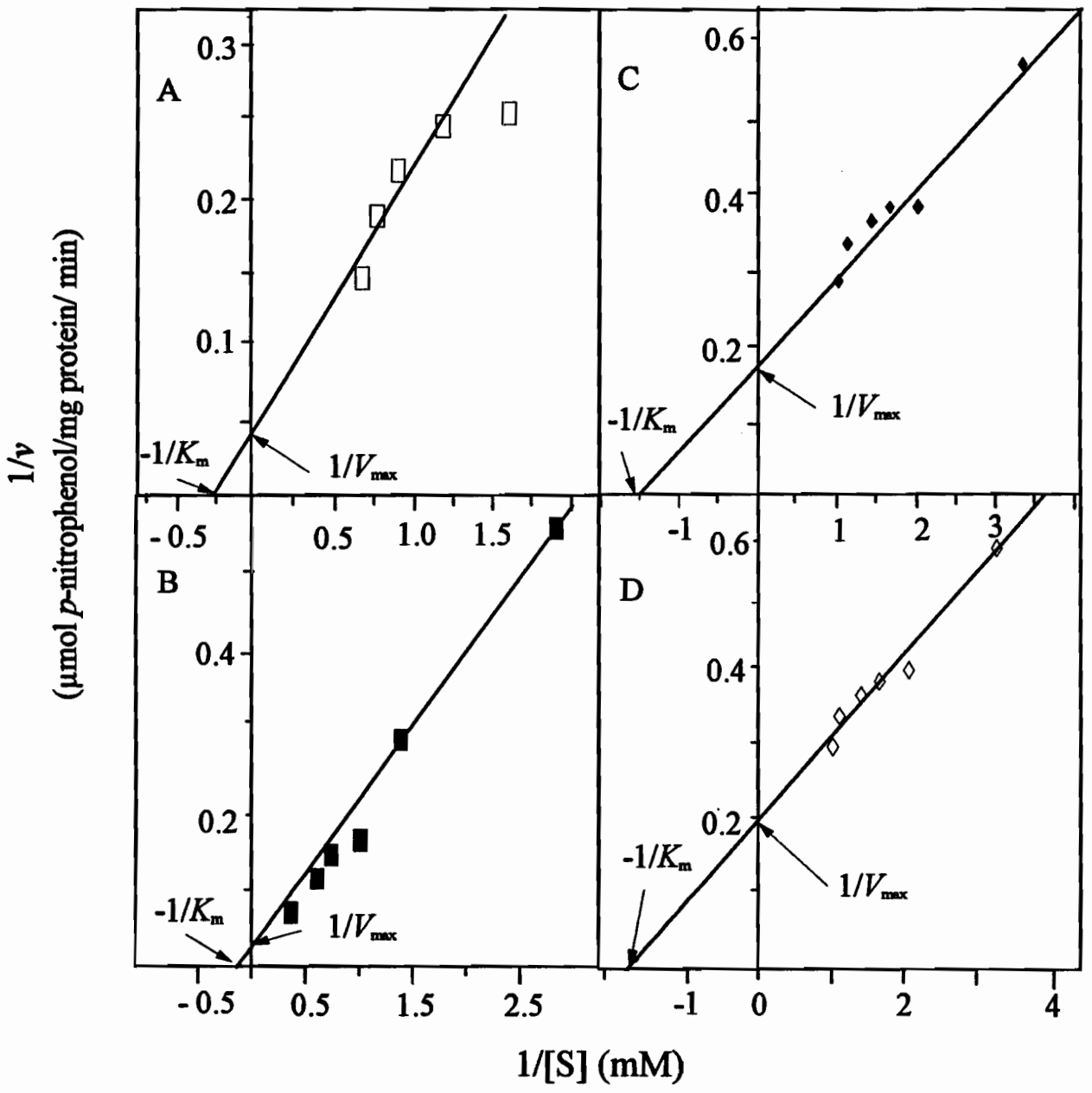


Figure 10. Lineweaver - Burk double reciprocal plots for extracellular lipases of *P. camemberti* at (A) pH 5.5 (□) and (B) pH 7.75 (■) and *P. roqueforti* at (C) pH 5.25 (♦) and (D) pH 8.75 (◇) using *p*-nitrophenyl stearate as substrate.

Table 6. Kinetic parameters of the lipase activity from the *Penicillium* sp. using model substrates.

		P. camemberti			*P. roqueforti*		
Substrate	pH	K_m[a]	V_{max}[b]	Catalytic efficiency[c]	K_m[a]	V_{max}[b]	Catalytic efficiency[c]
p-Nitrophenyl butyrate	5.25	-[d]	-[d]	-[d]	0.16	2.02	12.62
	5.50	0.24	5.30	22.08	-[d]	-[d]	-[d]
	7.75	0.56	5.68	10.14	-[d]	-[d]	-[d]
	8.75	-[d]	-[d]	-[d]	0.19	1.92	10.10
p-Nitrophenyl stearate	5.25	-[d]	-[d]	-[d]	0.08	0.44	5.50
	5.50	0.16	2.76	17.25	-[d]	-[d]	-[d]
	7.75	0.07	2.54	36.28	-[d]	-[d]	-[d]
	8.75	-[d]	-[d]	-[d]	0.58	6.54	11.28

[a]The K_m value was defined as mM of substrate.
[b]The V_{max} value was defined as μmol of *p*-nitrophenol per mg protein per min.
[c]The catalytic efficiency was defined as the ratio of V_{max} to K_m.
[d]Not determined.

using tributyrin as a substrate, a lipase activity from an extract from *Lactobacillus plantarum* showed a K_m value of 2.31 mM.

4.3.4. Effect of Selected Chemical Reagents on Lipase Activity

The effect of selected chemical reagents including ferrous chloride, ferric chloride, Ellman's reagent, *N*-bromosuccinimide, calcium chloride, magnesium chloride and ethylenediamine tetraacetic acid and Triton X-100 on the lipase activity of the partially purified extract of *P. camemberti* and *P. roqueforti* at both pH optima, was investigated.

4.3.4.1. Calcium and Magnesium Chlorides

The effect of magnesium and calcium ions on the lipase activity from *P. camemberti* is shown in table 7. The results (Table 7) indicate that the enzyme activity at pH 7.75 increased by 42 % after the addition of 10 mM of magnesium chloride, whereas at pH 5.5, a decrease in activity of 40 % was observed. The addition of calcium chloride at concentration of 1 mM to the reaction medium at pH 5.5 and 7.75 also resulted in a decrease in the lipase activity of the *P. camemberti* extract by 12 and 21 %; however, the results show that there was an increase in enzyme activity by 4 and 21 % in the presence of 10 mM $CaCl_2$ at pH 5.50 and 7.75, respectively.

Table 8 shows the effect of magnesium and calcium ions on the lipase activity from *P. roqueforti*. The results show that the presence of magnesium chloride at a concentration of 10 mM in the reaction medium produced an activation of 35 % of the lipase activity of *P. roqueforti* extract at pH 5.25, while at pH 8.75, a slightly lower

Table 7. Effect of selected chemical reagents on the lipase activity of extracellular extract from *Penicillium camemberti*.

Substance	Concentration (mM)	Relative activity (%)[a] pH 5.50	Relative activity (%)[a] pH 7.75
Blank	0.0	100	100
$FeCl_2$	1.0	58	107
	10.0	44	41
$FeCl_3$	1.0	80	77
	10.0	53	0
$CaCl_2$	1.0	88	79
	10.0	104	121
Ellman's reagent	1.0	79	91
	10.0	66	0
N-Bromosuccinimide	1.0	34	3
	10.0	20	0
$MgCl_2$	1.0	87	94
	10.0	59	142
Ethylene diamine-tetraacetic acid (EDTA)	1.0	81	150
	10.0	137	131
Triton X-100	1.0	23	43
	10.0	97	129

[a]Percentage relative activity is expressed as the activity obtained with the addition of the chemical reagent compared to that obtained in the absence of the chemical reagent, multiplied by 100.

activatory effect was exhibited. The results also indicate that calcium chloride enhanced the lipase activity of the *P. roqueforti* extract by approximately 60 % at pH 8.75, while at pH 5.25, an inhibitory effect of 20-30 % on enzyme activity was observed.

These overall findings suggest that the activation effect of calcium and magnesium salts on lipase activity might be due to the formation of calcium or magnesium salts with the liberated fatty acids which would thereby enhance enzyme activity by minimizing the fatty acid inhibitory effect on the active site of the enzyme (Iwai and Tsujisaka, 1984; Abdul Wahab, 1999).

In addition, Iwai and Tsujisaka (1964) reported that the activatory effect of calcium ions on lipase could be due to an improved emulsified state of the reaction mixture as a result of the formation of the calcium soap of the hydrolyzed fatty acids. Petrovic *et al.* (1990) reported that magnesium and calcium ions at a concentration of 10 and 1 mM, respectively, produced an activation effect on the lipase activity of the *P. roqueforti* extract. A lipase activity from an *A. niger* extract also increased in the presence of Ca^{++} ions with olive oil as substrate (Iwai and Tsujisaka, 1964).

Stöcklein *et al.* (1993) reported that Ca^{++} ions had an activation effect on the enzyme activity from *P. expansum.* However, the divalent salts of Ca^{++} and Mg^{++} ions at concentrations of more than 50 mM showed an inhibitory effect on the lipase activity from *G. candidum* (Veerargavan *et al.*, 1990).

4.3.4.2. Ethlyene Diamine Tetraacetic Acid

Table 7 shows that the addition of EDTA to the reaction mixture at a concentration of 10 mM produced an increase of 37 and 31 %, respectively, in the lipase activity from *P. camemberti* at pH 5.5 and 7.75. The results also show that at both pH, Triton X-100 produced an inhibitory effect on the enzymatic activity of the *P. camemberti* extract at 1 mM concentration whereas at the higher concentration of 10 mM there was a little or slightly activatory effect on the lipase activity.

In contrast, table 8 shows that the addition of EDTA at a concentration of 10mM to the reaction medium resulted in a decrease in the lipase activity of the *P. roqueforti* extract at pH 5.25 and 8.75. An inhibitory effect of Triton X-100 on the lipase activity of the *P. roqueforti* extract was also observed at pH 5.25 and 8.75. Stöcklein, *et al.* (1992) reported that in the presence of Triton X-100 in the reaction medium with a lipase from *P. expansum*, only 1% activity was observed.

4.3.4.3. Ferrous and Ferric Chlorides

Table 7 shows that the addition of ferric and ferrous chlorides to the reaction medium produced an inhibition of the lipase activity of the *P. camemberti* extract. The results show that total inhibition was observed after the addition of 10 mM of $FeCl_3$ at pH 7.75 and about 50 % of the activity was lost with the same concentration at pH 5.50. The presence of ferrous chloride ($FeCl_2$) at a concentration of 10 mM also inhibited enzyme activity by approximately 60 % at pH 5.50 and 7.75.

Moreover, Table 8 indicates that ferrous chloride at concentration of 10 mM also caused an inhibitory effect on lipase activity of about 20 and 50 % at pH 5.25 and 8.75, respectively, when added to the reaction medium containing the *P. roqueforti* extracellular lipase extract.

Table 8 also indicates that ferric chloride acted as an inhibitor of the lipase activity of the *P. roqueforti* extract; the results show that at a concentration 10 mM approximately 60 and 80 % inhibition of lipase activity was observed at pH 5.25 and 8.75, respectively. Iwai and Tsujisaka, (1984) reported previously that the hydrolytic activity of lipase was inhibited more by the presence of by iron ions than that observed by other metal ions. An irreversible type of inhibition of a lipase activity from an *A. niger* extract was reported when the enzymatic extract was exposed to a low iron concentration (Iwai and Tsujisaka, 1984). Yamaguchi and Mase (1991) also reported that a 1 mM concentration of ferric ions inhibited lipase activity from a *P. camemberti* extract by 87 %; however, the lipase activity was not affected by the presence of metal ions such as Ca^{++}, Mg^{++} and EDTA. In addition, a lipase activity from a *Pseudomonas fragi* extract was strongly inhibited by the presence of ferric and ferrous ions (Schuepp, 1997).

4.3.4.4. N-Bromosuccinimide

Table 7 shows that *N*-bromosuccinimide at a concentration of 1 and 10 mM showed a strong inhibitory effect on lipase activity of the *P. camemberti* extract at pH 5.50 and 7.75. Ellman's reagent a concentration of 10 mM also inhibited the activity of the *P. camemberti* extract by 40 and 100 % at pH 5.5 and 7.75, respectively. In addition, table 8 shows that at pH 5.25 and 8.75, the lipase activity of the *P. roqueforti* extract was

Table 8. Effect of selected chemical reagents on the lipase activity of extracellular lipase extract from *Penicillium roqueforti*.

		Relative activity (%)[a]	
		pH	
Substance	Concentration (mM)	5.25	8.75
Blank	0.0	100	100
$FeCl_2$	1.0	90	62
	10.0	78	51
$FeCl_3$	1.0	73	63
	10.0	42	19
$CaCl_2$	1.0	72	156
	10.0	83	163
Ellman's reagent	1.0	93	107
	10.0	79	76
N-Bromosuccinimide	1.0	76	86
	10.0	67	65
$MgCl_2$	1.0	90	129
	10.0	135	116
Ethylene diamine-	1.0	108	105
tetraacetic acid (EDTA)	10.0	95	46
Triton X-100	1.0	29	66
	10.0	79	51

[a]Percentage relative activity is expressed as the activity obtained with the addition of the chemical reagent compared to that obtained in the absence of the chemical reagent, multiplied by 100.

inhibited approximately 30 % in the presence of *N*-bromosuccinimide at concentration of 10 mM. The results also show that the presence of Ellman's reagent on the lipase activity of the *P. roqueforti* extract produced a small decrease in activity which was further inhibited when the concentration of the reagent was increased to 10 mM, at pH 5.25 and 8.75.

4.3.5. Hydrolysis of Edible Oils by Extracellular Lipases

The hydrolytic activity of the partially purified lipase extract of *P. camemberti* and *P. roqueforti* at both pH optima was investigated with edible oils including canola, fish, and olive oils and butterfat. The results (Figures 11 and 12) show that the extracellular lipase activity of the *P. camemberti* and *P. roqueforti* extracts was capable of catalyzing the hydrolysis of the edible oils and butterfat at different rates.

The results indicate that at pH 7.75, the lipase activity of the *P. camemberti* extract had more preference towards butter fat as substrate whereas that of the *P. roqueforti* extract, at pH 8.75, showed a greater affinity towards olive oil. In addition, at pH 5.5, the hydrolytic activity of the *P. camemberti* extract showed preference towards butter fat and olive oil, while that of the *P. roqueforti* extract, at pH 5.25, showed a greater specificity towards olive oil followed by butter fat. The results also show that the hydrolytic activity of both the *P. camemberti* and *P. roqueforti* extracts was considerably less using fish and canola oils as substrates in comparison to that obtained with butter fat and olive oil. In addition, these results indicate that the hydrolytic activity of the *P. camemberti* extract showed a greater specificity towards butter fat thereby suggesting that the lipase activity was more specific toward long-chain fatty acids as butter fat contains

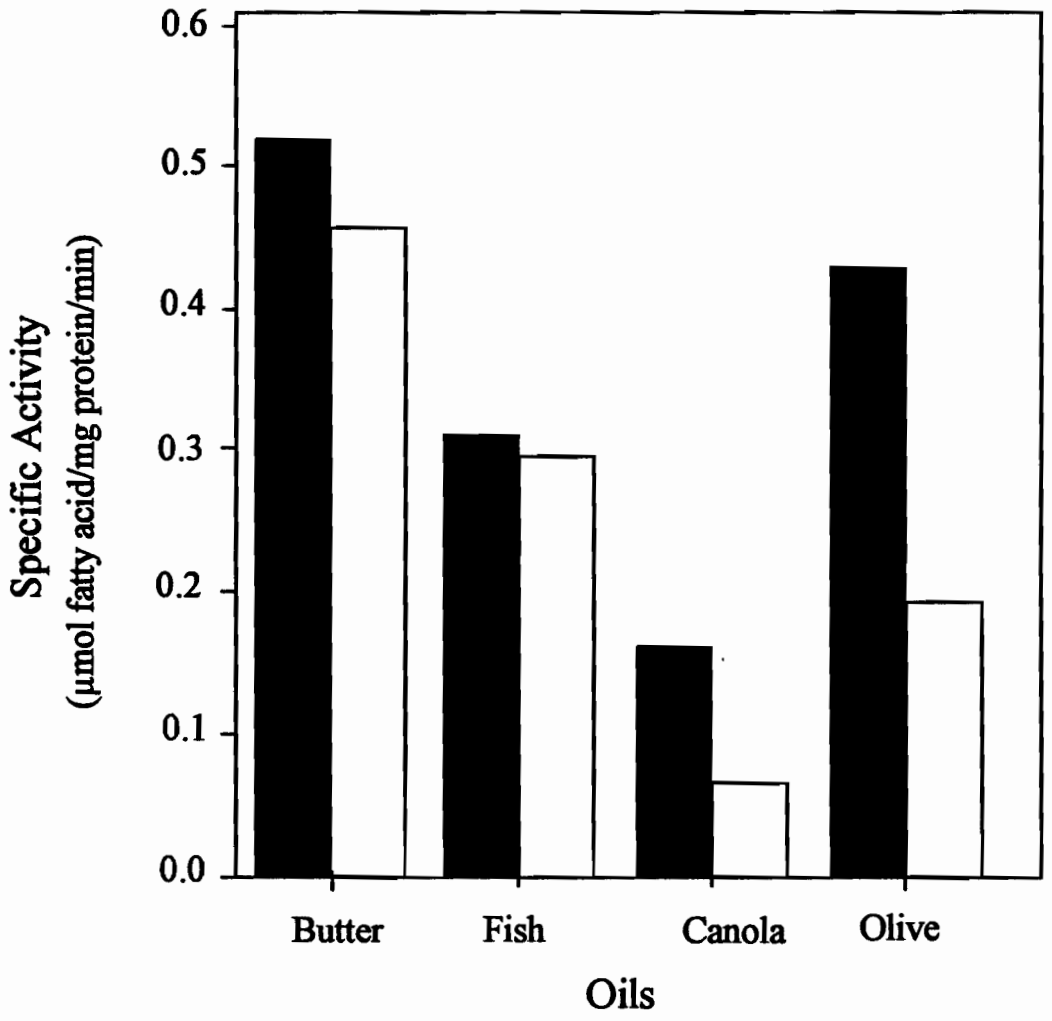


Figure 11. Specific activity of the extracellular lipase from *P. camemberti* using different oils and butter fat as substrates at pH 5.50 (■) and pH 7.75 (□).

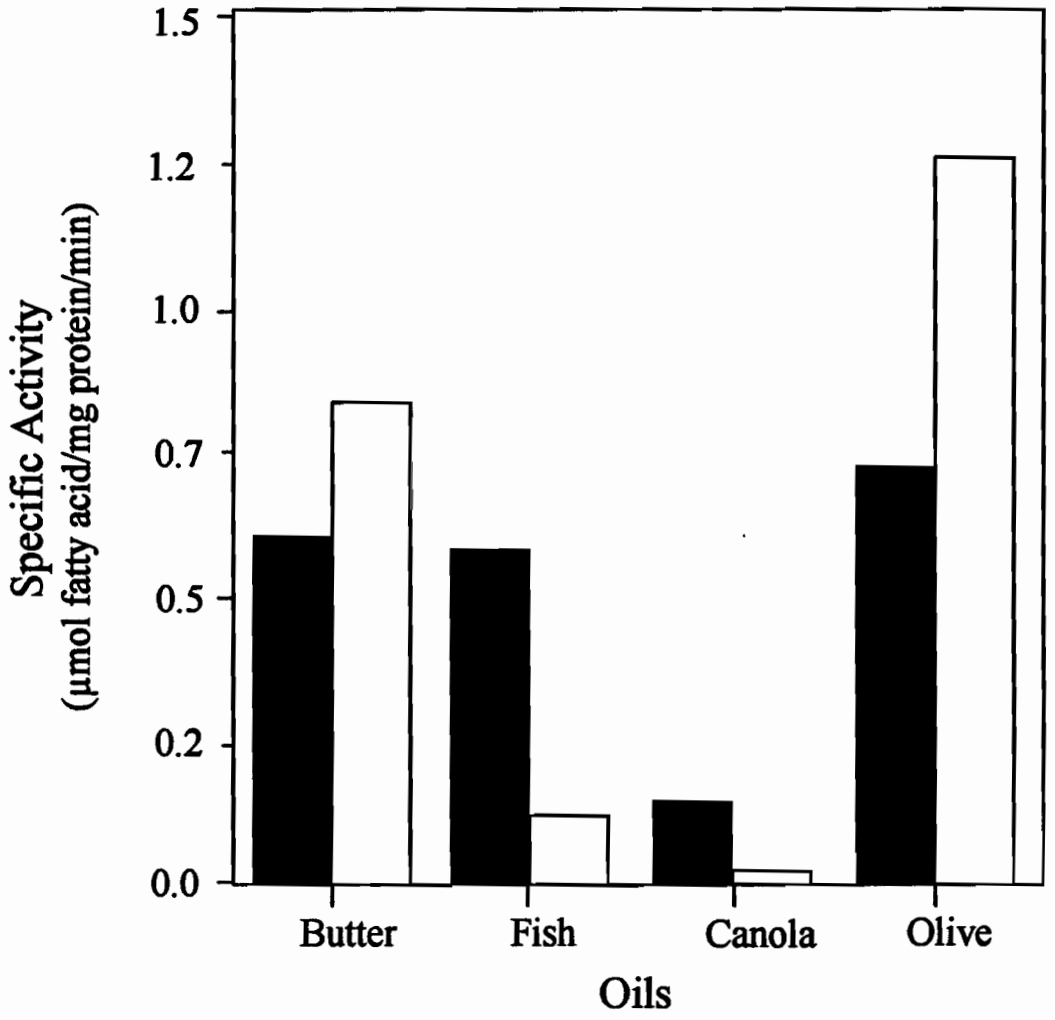


Figure 12. Specific activity of the extracellular lipase from *P. roqueforti* using edible oils and butter fat as substrates at pH 5.25 (■) and pH 8.75 (□).

26 and 28 % of palmitic and oleic acids, respectively (Gurr, 1992). These findings also indicate that the hydrolytic activity of the *P. roqueforti* extract was more specific toward olive oil which contains high amounts of long-chain fatty acids (60-70 % of oleic acid) (Eskin, 1990). However, the lipase activity of the *P. camemberti and P. roqueforti* extracts edible oils as substrates was similar to that reported in the literature including the hydrolysis of olive oil by *P. expansum* extract (Stöcklein *et al.*, 1993), and fish oil and butter oil by the *Candida rugosa* extract (Hoshino, *et al.*, 1990; Garcia *et al.*, 1992).

An extracellular lipase from *Galactomyces geotricum* was reported to liberate 90-100% of the unsaturated fatty acids and 10-50% of the saturated ones when edible oils were used as substrates (Phillips and Pretorius, 1991).A lipase from *Geotricum candidum* showed more preference toward long chain fatty acids containing a *cis*-9 double bond (Baillargeon and McCarthy, 1991).

Lencki, *et al.* (1998) mentioned that a lipase from *P. roqueforti* was able to catalyze the hydrolysis of short-chain fatty acids of butterfat used as substrate. The same authors reported that when a short-chain triglyceride fraction was used as substrate, as opposed to whole butterfat, the amount of desirable short-chain free fatty acids (FFA) to undesirable medium-chain fatty acids increased. However, accumulation of the fatty acids eventually led to lipase inhibition and limited the total amount of triglyceride hydrolysis.

4.3.5.1. Gas-Chromatographic Analysis of Free Fatty Acids

The gas chromatographic analysis of the liberated free fatty acids of the edible oils and butter fat by the extracellular lipase activity of the *P. camemberti* and *P.*

roqueforti extracts is shown in Tables 9 and 10, respectively. The results (Table 9) indicate that the enzyme activity of the *P. camemberti* extract exhibited the capability to catalyze the hydrolysis of the three edible oils, including olive, canola and fish oils and butterfat.

The results (Table 9) show that at pH 5.50 the lipase activity of the *P. camemberti* showed a high specificity towards linoleic acid (C18:2) with all four substrates followed by palmitoleic acid (C16:1) for butterfat, butyric acid (C4:0) for olive oil, and oleic acid oleic acid (C18:1) for both canola and fish oils. However, the results also indicate that at pH 7.75, the highest rate of hydrolytic activity of the *P. camemberti* extract was exhibited towards linoleic acid (C18:2), followed by oleic acid (C18:1) and butyric acid (C4:0) using butterfat as substrate, whereas with olive oil, a high preference was demonstrated towards the liberation of palmitoleic acid (C16:1), followed by linoelic acid (C18:2) and stearic acid (C18:0). Moreover, Table 9 shows that at pH 7.75 the lipase activity exhibited similar specificites with canola and fish oils as substrates, as both linolenic acid (C18:3) and linoleic acid (C18:2) were hydrolyzed. These findings suggest that the lipase activity from the *P. camemberti* extract was more active toward medium and long-chain fatty acids.

Table 10 indicates that the lipase activity of the *P. roqueforti* extract was capable of catalyzing the hydrolysis of edible oils at different rates. The results show that at both pH 5.25 and 8.75, the lipase activity exhibited a similar affinity towards the hydrolysis of linoleic acid (C18:2) and oleic acid (C18:1) using butterfat as substrate. The results also show that at pH 5.25, a high specificity was observed towards the liberation of linoleic

Table 9. Gas chromatographic analysis of the free fatty acids liberated by the hydrolysis of edible oils and butterfat using the extracellular lipase extract from *Penicillium camemberti.*

		Free fatty acid (%)[a]													
Oil	pH	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0
Butter															
	5.50	-	-	-	-	-	-	-	0.8 (49.0)[b]	33.1 (35.7)[b]	5.3 (27.5)[b]	10.1 (26.0)[b]	41.3 (20.9)[b]	4.9 (21.9)[b]	5.3 (26.6)[b]
	7.75	22.0(40.2)[b]	-	-	-	-	-	-	-	5.9 (32.5)[b]	-	25.9 (8.4)[b]	46.2 (14.5)[b]	-	-
Olive															
	5.50	24.7 (8.6)[b]	-	-	-	-	-	-	-	8.2 (30.7)[b]	-	19.3 (2.1)[b]	38.7 (0.4)[b]	4.0 (1.0)[b]	5.0 (2.4)[b]
	7.75	9.1 (63.6)[b]	-	-	-	-	-	-	2.5 (37.7)[b]	53.2 (39.7)[b]	10.6 (76.5)[b]	4.4 (83.4)[b]	20.6 (65.8)[b]	2.4 (85.8))[b]	2.2 (85.0)[b]
Canola															
	5.50	-	-	-	-	-	-	-	-	15.0 (16.4)[b]	2.5 (35.3)[b]	19.4 (17.9)[b]	50.7 (0.2)[b]	5.7 (3.3)[b]	6.7 (2.2)[b]
	7.75	-	-	-	-	-	-	-	-	-	-	-	35.8 (1.0)[b]	64.2 (0.6)[b]	-
Fish															
	5.50	-	-	-	-	-	-	-	-	14.2 (12.9)[b]	-	23.4 (5.9)[b]	50.3 (0.8)[b]	5.4 (1.3)[b]	6.6 (0.4)[b]
	7.75	-	-	-	-	-	-	-	-	-	-	-	36.5 (2.7)[b]	63.5 (1.6)[b]	-

[a] Relative percentage of each free fatty acid (FFA) calculated as a percentage of peak area to the total peak area of all FFA.

[b] Relative standard deviation.

Table 10. Gas chromatographic analysis of the free fatty acids liberated by the hydrolysis of edible oils and butterfat using the extracellular lipase extract from *Penicillium roqueforti.*

		Free fatty acid (%)[a]													
Oil	pH	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0
Butter															
	5.25	-	-	-	-	-	-	-	-	-	10.8 (48.9)[b]	17.7 (21.7)[b]	58.0 (2.9)[b]	6.4 (8.5)[b]	7.1 (4.3)[b]
	8.75	-	-	-	-	-	-	-	-	-	-	45.9 (4.0)[b]	54.1 (3.4)[b]	-	-
Olive															
	5.25	-	-	-	-	-	-	-	-	-	-	43.9 (2.8)[b]	56.1(2.2)[b]	-	-
	8.75	31.6 (49.5)[b]	-	-	-	-	-	-	-	-	-	26.2 (8.9)[b]	42.3 (22.3)[b]	-	-
Canola															
	5.25	15.4 (60.2)[b]	-	-	-	-	-	-	-	-	-	17.2 (102.4)[b]	35.9 (55.4)[b]	25.0 (116.0)[b]	6.5 (12.1)[b]
	8.75	49.8 (16.2)[b]	-	-	-	-	-	-	-	-	-	23.3 (15.7)[b]	27.0 (16.3)[b]	-	-
Fish															
	5.25	12.4 (7.4)[b]	-	-	-	-	-	-	-	-	-	36.4 (2.0)[b]	51.1 (0.4)[b]	-	-
	8.75	79.4 (21.4)[b]	-	-	-	-	-	-	-	-	-	8.3 (81.5)[b]	12.1 (77.6)[b]	-	-

[a] Relative percentage of each free fatty acid (FFA) calculated as a percentage of peak area to the total peak area of all FFA.

[b] Relative standard deviation.

acid (C18:2), followed by oleic acid (C18:1) with olive oil as substrate, and of linoleic acid (C18:2) followed by linolenic acid (C18:3) and linoleic acid (C18:2) with canola oil; however, at pH 8.75, the lipase activity exhibited a strong preference towards the hydrolysis of butyric acid (C4:0), in addition to oleic acid (C18:1) and linoleic acid (C18:2) with both olive and canola oils. Moreover, Table 10 shows that at pH 5.25, the hydrolytic activity of the *P. roqueforti* extract catalyzed mainly the hydrolysis of linoleic acid (C18:2) and oleic acid (C18:1) with fish as substrate while at pH 8.75, the enzyme activity was capable of catalyzing mainly the liberation of butyric acid (C4:0). These findings indicate that the lipase activity from the *P. roqueforti* extract possessed as a certain specificity towards short-chain fatty acids.

Comparatively, the above mentioned results are similar to those reported by other researchers; the ability of some *Penicillium* species capable of catalyzing the hydrolysis of oils into long-chain fatty acids was reported including the hydrolysis of fish oil by lipases of *P. expansum* (Stöcklein *et al.*, 1993) and *P. abeanum* (Sugihara *et al.*, 1996) extracts.

In addition, Lomascolo *et al.* (1994) indicated high content of saturated and unsaturated long-chain fatty acid esters such as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) in the cells of *P. camemberti*, which could explain the preference of lipases from *P. camemberti* towards long-chain fatty acids. In addition, Alford and Pierce (1961) and Wiclox *et al.* (1955) reported that a lipase activity from a *P. roqueforti* extract preferentially hydrolyzed short-chain fatty acids from butter fat as opposed to long-chain fatty acids. Lomascolo *et al.* (1994) also indicated that the *P. roqueforti* strain was rich in short-chain fatty acids compared to *P.*

camemberti; these findings may explain the difference in the substrate specificity of the lipase activity in the extracts towards a certain chain length group of fatty acids.

5. CONCLUSION

The results obtained in this study showed the presence of lipase activity in the biomasses of *Penicillium camemberti* and *Penicillium roqueforti*. The production of active lipase fractions was carried out and the enzyme activity was characterized with respect to source and substrate specificity; the highest activity from *P. camemberti* was obtained using soybean as a carbon source while that from *P. roqueforti* was obtained with olive oil.

The results indicated that, using model substrates, the lipase activity of the *P. camemberti* extract demonstrated a greater preference towards long-chain fatty acid esters whereas that of the *P. roqueforti* has more affinity towards short-chain fatty acid esters.

Using selected edible oils, the *P. camemberti* and *P. roqueforti* extracellular lipases were also capable of hydrolyzing edible oils at different levels of specificity. The gas chromatographic analysis of the free fatty acids, liberated by the hydrolytic activity of the lipase extracts of the *Penicillium* sp. demonstrated a selected preference towards the release of long-chain and short-fatty acids present in the edible oils. Using butterfat and olive oil as substrates, the lipase extracts from *P. camemberti* and *P. roqueforti* exhibited a preference hydrolytic activity towards long-chain and short-chain fatty acid esters, respectively.

The overall results indicated the potential biotechnological use of the selected *Penicillium* sp. for the recovery of free fatty acids from a wide range of model substrates and edible oils.

6. REFERENCES

Abdul Wahab, A. (1999). In *Characterization of Purified Extracellular Lipase Fractions from Pseudomonas fragi CRDA 037*, M.Sc. Thesis, McGill University, Montreal, Canada.

Adda, J., Grippon, J. and Vassal, L. (1982). The chemistry of flavor and texture generation in cheese. *Food Chem.* **9**, 115-129.

Alford, J.A. and Pierce, D.A. (1961). Lipolytic activities of microorganisms at low and intermediate temperatures. III. Activity of microbial lipases at temperatures below 0°C. *J. Food Sci.* **26**, 518-524.

Alford, J.A., Pierce, D.A. and Suggs, F.G. (1964). Activity of microbial lipases on natural fats and synthetic triglycerides. *J. Lipid Res.* **5**, 390-394.

Alhir, S., Markakis, P. and Chandan, R. (1990). Lipase of *Penicillium caseicolum. J. Agric. Food Chem.* **38**, 598-601.

Alhir, S., Markakis, P. and Chandan, R.C (1990). Lipase of *Penicillium caseicolum. J. Agric. Food Chem.* **38**, 598-601.

Badings, H.T. and De-Jong, C. (1983). Capillary gas chromatography of fatty acid methyl esters: A study of conditions for the quantitative analysis of short- and long chain fatty acids in lipids. *J. Chromatogr.* **270**, 493-506.

Baillargeon, M.W. and McCarthy, S.G. (1991). *Geotrichum candidum* NRRL Y-553 lipase: purification, characterization and fatty acid specificity. *Lipids* **26,** 831-836.

Balcao, V.M. and Malcata, F.X. (1998). Lipase catalyzed modification of milkfat. *Biotechnol. Advances* **16**, 309-341.

Becker, P., Abu-Reesh, I., Markossian, S., Antranikian, G. and Märkl, H. (1997). Determination of the kinetic parameters during continuous cultivation of the lipase producing thermophile Bacillus sp. on olive. *Appl. Microbiol. Biotechnol.* **48**, 184-190.

Benjamin, S. and Pandey, A. (1995). Optimization of liquid media for lipase production by *Candida rugosa. Bioresource Technol.* **55**, 167-169.

Brahimi-Horn, M-C., Guglielmino, M.L., Elling, L. and Sparrow, L.G. (1989). The esterase profile of a lipase from *Candida cylindracea. Biochim. Biophys. Acta* **1042**, 51-54.

Bisakowski, B. and Kermasha, S. (1998). Characterization of purified lipoxygenase extracts from *Fusarium proliferatum. J. Agric. Food Chem.* **46**, 2382-2388.

Brokerhoff, H. and Jensen, R.G. (1974). Lipases. In *Lipolytic Enzymes*, Brockerhoff, H. and Jensen, R.G. (Eds.), Academic Press Inc., New York, N.Y., pp. 25-34.

Carrie, A., Thomson, P., Delaquis, J. and Mazza, G. (1999). Detection and measurement of microbial lipase activity: a review. *Crit. Rev. Food Sci. Nutri.* **39**, 165-187.

Chalier, P. and Crouzet, J. (1993). Aroma compounds production by *Penicillium roqueforti.* In *Food-flavors, Ingredients and Composition*, Developments in Food Science Series, Vol. 32,Charalambous, G. (Ed.), Elsevier, New York, N.Y., pp. 205-219.

Costa, M.A.F. and Peralta, R.M. (1999). Production of lipase by soil fungi and partial characterization of lipase from a selected strain *Penicillium wortmanii. J. Basic Microbiol.* **39**, 11-15, 1999.

Davranov, K. (1994). Microbial lipases in biotechnology. *Appl. Biochem. Microbiol.* **30**, 427-432.

Delatorre, M.E. and Diaz, A., Ruiz, B., Farres, A., Aramburo, C. and Sanchez, S. (1996). Physiology of lipase formation in *Penicillium candidum. J. Indust. Microbiol.* **17**, 73-76.

Druet, D., El Abbadi, N. and Comeau, L. (1992). Purification and characterization of the extracellular and cell-bound lipases from a *Penicillium cyclopium* variety. *Appl. Microb. Biotechnol.* **31**, 745-749.

Eitenmiller, R.R., Vakil, J.R. and Shahan, K.M. (1970). Production and properties of *Penicillium roqueforti* lipases. *J. Food Sci.* **35**, 130-133.

Englard, S. and Seifter, S. (1990). Precipitation techniques. In *Methods in Enzymology: Guide to Protein Purification,* Vol. 182, Deutscher, M.P. (Ed.), Academic Press, Inc., San Diego, CA, pp. 285-300.

Eskin, N.A.M. (1990). Biochemistry of food spoilage: Off favors in milk. In *Biochemistry of Foods* (2nd), Academic Press Inc., San Diego, CA, pp. 437-502.

Freire, D.M., Teles, E.M.F., Bon, E.P.S. and Sant'Anna, G.L.Jr. (1997). Lipase production by *Penicillium restrictum* in a bench-scale fermenter; Effect of carbon and nitrogen nutrition, agitation, and aeration. *Appl. Biochem. Biotechnol.* **63**, 409-421.

Furtado, M., Chandan, R. and Wishnetsky, T. (1984). Characterization of cheese curd ripened with *Penicillium casecolum* for producing a flavor concentrate. *J. Dairy Sci.* **67**, 2850-2855.

Gandhi, N. (1997). Applications of lipase. *J. Am. Oil Chem. Soc.* **74**, 621-634.

Garcia, H.S., Malcata, F.X., Hill-Jr, C.G. and Amundson, C.H. (1992). Use of *Candida rugosa* immobilized in a spiral wound membrane reactor hydrolysis of milkfat. *Enzyme Microb. Technol.* **14**, 535-545.

Gatfield, I.L. (1988). Enzymatic generation of flavor and aroma components. In *Food Biotechnology*, King, R.D. and Cheetham, P.S.J. (Eds.), Elsevier Applied Science, London, UK, pp. 59-69.

Ghosh, S. and Bhattacharyya, D.K. (1995). Utilization of acid oils in making valuable fatty products by microbial lipase technology. *J. Am. Oil. Chem. Soc.* **72**, 1541-1544.

Gilbert, E.J. (1993). *Pseudomonas* lipases: Biochemical properties and molecular cloning. *Enzyme Microb. Technol.* **15**, 634-644.

Gilbert, E.J., Cornish, A. and Jones, C.W. (1991). Purification and properties of extracellular lipase from *Pseudomonas aeruginosa* EF2. *J. Gen. Micrbiol.* **137**, 2223-2229.

Gobbetti, M., Fox, P., Smacchi, E., Stepaniak, L. and Damiani, P. (1996). Purification and characterization of a lipase from *Lactobacillus plantarium* 2739. *J. Food Biochem.* **20**, 227-246.

Godtfredsen, S.E. (1990). Microbial lipases. In *Microbial Enzymes and Biotechnology*, 2nd ed., Fogart, W.M. and Kelly, C.T. (Eds.), Elsevier Applied Science, Essex, London, pp. 225-274.

Gulomova, K., Ziomek, E., Schrag, J.D., Davranov, K. and Cygler, M. (1996). Purification and characterization of a *Penicillium* sp. lipase which discriminates against diglycerides. *Lipids* **31**, 379-384.

Gulyamova, K.A. and Davranov, K.D. (1993). Effect of cultivation conditions on the production of extra- and intracellular lipases by the fungus *Mucor miehei. Prikl. Biokhim Mikrobiol.* **29**, 706-711.

Gurr, M.I. (1992). Fats in foods. In *Role of Fats in Food and Nutrition*, Gurr, M.I. (Ed.), (2nd), Elsevier Applied Science, London, UK, pp. 32-39.

Hartree, E.F. (1972). Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48**, 422-427.

Hoshino, T., Yamane, T. and Shimizu, S. (1990). Bioreactor for enzymatic reaction of fat and fatty acid derivatives. Part XII selective hydrolysis of fish oil by lipase to concentrate n-3 polyunsaturated fatty acids (n-3 PUFA). *Agric. Biol. Chem.* **54**, 1459-1467.

Hou, C.T. and Jonstone, T.M. (1992). Screening of lipase activities with cultures from agricultural research service collection. *J. Am. Oil Chem. Soc.* **69**, 1088-1097.

Ibrik, A., Chachinian, H., Rugani, N., Sarda, L. and Comeau, L.C. (1998). Biochemical and structural characterization of triacylglycerol lipase from *Penicillium cyclopium*. *Lipids* **33**, 377-384.

Iizumi, T., Nakamura, K. and Fukase, T. (1990). Purification and characterization of a thermostable lipase from newly isolated *Pseudomonas* sp. KWI-56. *Agric. Biol. Chem.* **54**, 1253-1258.

Isobe, K., Nokihara, K., Yamaguchi, S., Mase, T. and Schmid, R.D. (1992). Crystallization and characterization of monoacylglycerol and diacylglycerol lipase from *Penicillium camemberti*. *Eur. J. Biochem.* **203,** 233-237.

Iwai, M. and Tsujisaka, Y. (1984). Fungal lipase. In *Lipases*, Borgstroem, B. and Brockman, H.L. (Eds.), Elsevier Applied Science, Amsterdam, The Netherlands, pp. 443-469.

Iwai, M., Tsujisaka, Y., Okumura, S. and Fukumoto, J. (1964). Studies on lipase III. Hydrolytic and esterifying actions of crystalline lipase of *Aspergillus niger*. *J. Gen. Appl. Microbiol.* **10**, 13-22.

Iwai, M., Tsujisaka, Y., Okumura, S. and Fukumoto, J. (1973). Lipid requirement for the lipase production by *Geotrichum candidum* link. *Agric. Biol. Chem.*, **37**, 929-931.

Jaegar, K., Ransac, S., Dijkstra, B., Colson, C., Heuvel, M. and Misset, O. (1994). Bacterial lipases. *FEMS Microbiol. Rev.* **15**, 29-63.

Jensen, R.G., Dejong, F.A and Clark, R.M. (1983). Determination of lipase activity. *Lipids* **18**, 239-251.

Kanawjia, S.K. and Singh, S. (1991). Flavor and free fatty acid changes in buffalo milk Cheddar cheese by lipase (*Candida cylindracea*) during ripening. *Asian J. Dairy Res.* **10**, 153-157.

Kermasha, S. and Metche, M. (1986). Characterization of seed lipooxygenase of *Phaseolus vulgaris* cv. Haricot. *J. Food Sci.* **50**, 88-92.

Kermasha, S., Safari, M. and Bisakowski, B. (1998). Characterization of purified lipase fractions from *Rhizopus niveus*. *J. Agric. Food Chem.* **46**, 4451-4456.

Kermasha, S., van de Voort, F.R. and Metche, M. (1986). Characterization of French beans (*Phaseolus vulgaris*) seed lipase. *Can. Inst. Food Sci. Technol. J.* **19**, 23-27.

Kilara, A. (1985). Enzyme-modified lipid food ingredients. *Process Biochem.* **20**, 149-157.

Kosugi, Y. and Kamibayashi, A. (1971). Thermostable lipase from *Pseudomonas* species: Culture conditions and properties of the crude enzyme. *J. Ferment. Technol.* **49**, 968-980.

Kouker, G. and Jaeger, K. (1987). Specific and sensitive plate assay for bacterial lipases. *Appl. Environ. Microbiol.* **53**, 211-213.

Krieger, N., Taipa, M.A., Melo, E.H.M., Lima-Filho, J.L., Aires-Barros, M.R. and Cabral, J.M.S. (1999). Purification of a *Penicillium citrinum* lipase by chromatographic processes. *Bioprocess Engineer.* **20**, 59-65.

Laurent, S., Ivanova, M.G., Pioch, D., Graille, J. and Verger, R. (1994). Interactions between ß-Cyclodextrin and insoluble glyceride monomolecular films at the argon/water interface: applications to lipase kinetics. *Chem. Phys. Lipids* **70**, 35-42.

Ledingham, W.M. and Lima, F.J.L. (1994). Lipase from a Brazilian strain of *Penicillium citrinum*. *Appl. Biochem. Biotechnol.* **49**, 59-74.

Lee, S.Y. and Rhee, J.S. (1993). Production and partial purification of a lipase from *Pseudomonas putida* 35K. *Enzyme Microb. Technol.* **15**, 617-623.

Lee, S.Y. and Rhee, J.S. (1993). Production and partial purification of a lipase from *Pseudomonas putida* 3SK. *Enzyme Microb. Technol.* **15**, 617-623.

Lencki, R. W., Smink, N. and Sneltin, H.(1998). Increasing short-chain fatty acid yield during lipase hydrolysis of a butterfat fraction with periodic aqueous extraction. *J. Am. Oil Chem. Soc.* **75**, 1195-200.

Lencki, R.W., Smink, N. and Sneltin, H. (1998). Increasing short-chain fatty acid yield during lipase hydrolysis of a butterfat fraction with periodic aqueous extraction. *J. Am. Oil Chem. Soc.* **75**, 1195-1200.

Lineweaver, H. and Burk, D. (1934). The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**, 658-666.

Linfield, W.M., O'Brien, D.J., Serata, S. and Barauskas, R.A. (1984). Lipid lipase interactions. Fat splitting with lipase from *Candida rugosa*. *J. Am. Oil Chem. Soc.* **61**, 1067-1071.

Liu, W.A., Beppu, T. and Amira, K. (1977). The chemical modification of the lipase of *Humicola lanuginosa* by *N*-bromosuccinimide in urea solution. *Agric. Biol. Chem.* **41**,131-135.

Lomascolo, A., Dubreucq, E., Perrier, V. and Galzy, P. (1994). Mycelial lipid composition of three *Penicillium* strains. *J. Dairy Sci.* **77**, 2160-2167.

Long, K., Ghazali, H.M., Ariff, A., Ampon, K. and Bucke, C. (1996). Mycelium-bound lipase from a locally isolated strain of *Aspergillus flavus* link-pattern and factors involved in its production. *J. Chem. Technol. Biotechnol.* **67**, 157-163.

Mackay, A.M. (1993). Microbial carboxylic ester hydrolases (EC 3.1.1) in food biotechnology. *Lett. Appl. Microbiol.* **16**, 1-6.

Mackenzie, R.D., Blohm, T.R., Auxier, E.M. and Luther, A.C. (1967). Rapid colorimetric micro method for free fatty acids. *J. Lipids Res.* **8**, 589-597.

Macrae, A.R. (1983). Extracellular microbial lipases. In *Microbial Enzymes and Biotechnology*, Fogarty, W.M. and Kelly, C.T. (Eds.), Elsevier Applied Science, New York, N.Y., pp. 225-250.

Macrae, A.R. (1985). Interesterfication of fats and oils. In *Biocatalysts in Organic Synthesis*, Tramper, J., van der Plas, H.C. and Linko, P. (Eds.), Elsevier Acadmic Press, Amsterdam, The Netherlands, pp. 195-208.

Macrae, A.R. and How, P. (1986). United states patent No. 4, 719,178.

Malizewska, I. and Mastalerz, P. (1992). Production and some properties of lipase from *Penicillium citrinum*. *Enzyme Microb. Technol.* **14**, 190-193.

Mase, T., Matsumiya, Y. and Matsuura, A. (1995). Purification and characterization of *Penicillium roqueforti* IAM 7268 lipase. *Biosci. Biotech. Biochem.* **59**, 329-330.

Miranda, O.A., Salgueiro, A.A., Pimentel, M.C.B., Lima Filho, J.L., Melo, E.H.M. and Durán, N. (1999). Lipase production by a Brazilian strain of *Penicillium citrinum* using industrial residue. *Bioresource Technol.* **69**, 145-147.

Molimard, P. and Spinnler, H.E. (1996). Review: Compounds involved in the favor of surface mold-ripened cheeses: origins and properties. *J. Dairy Sci.* **79**, 169-184.

Mozaffar, Z. and Weete, J.D. (1993). Purification and properties of an extracellular lipase from *Phythium ultimum*. *Lipids* **28**, 377-382.

Okumura, S., Iwai, M. and Tsujisaka, Y. (1976). Positional specificites of four kinds of microbial lipases. *Agric. Biol. Chem.* **40**, 655-660.

Okumura, S., Iwai, M. and Tsujisaka, Y. (1980). Purification and properties of partial glyceride hydrolase of *Penicillium cyclopium* M 1. *J. Biochem.* **87**, 205-211.

Omar, I.C., Nishio, N. and Nagai, S. 1987. Production of a thermostable lipase by *Humicola lanuginosa* grown on sorbitol-corn steep liquor medium. *Agric. Biol. Chem.* **51**, 2145-2151.

Pabai, F. (1997). In *Production, Purification, Characterization of Selected Microbial Lipases and Their Application for Interesterification of Butter Fat*, Ph.D. Thesis, McGill University, Montreal, Canada.

Pabai, F., Kermasha, S. and Morin, A. (1995). Lipase from *Pseudomonas fragi* CRDA 323: partial purification, characterization and interesterification of butter fat. *Appl. Microbiol. Biotechnol.* **43**, 42-51.

Patkar, S. and Björkling, F. (1994). Lipase inhibitors. In *Lipases: Their Structure, Biochemistry and Application*, Woolley, P. and Petersen, S.B. (Eds.), Cambridge University Press, London, UK, pp. 207-224.

Peled, N. and Kenzt, M.C. (1981). A new assay of microbial lipases with emulsified trioleylglycerol. *Anal. Biochem.* **112**, 219-222.

Petrovic, S.E., Skrinjar, M., Becarevic, A., Vujicic, I.F. and Banka, L. (1990). Effect of various carbon sources on microbial lipases biosynthesis. *Biotchnol. Lett.* **12**, 299-304.

Phillips, A. and Pretorius, G.H.J. (1991). Purification and characterization of an extracellular lipase of *Galactomyces geotricum*. *Biotechnol. Lett.* **13**, 833-838.

Pimentel, M.C.B., Krieger, N., Coelho, L.C.C.B., Fontana, J.O., Melo, E.H.M., Ledingham, W.M. and Lima, F.J.L. (1994). Lipase from a Brazilian strain of *Penicillium citrinum*. *Mycopathol.* **133**, 119-121.

Pimentel, M.D.B., Melo, E.H.M., Lima, J.L., Ledingham, W.M. and Duran, N. (1997). Lipase from a Brazilian strain *Penicillium citrinum* cultured in a simple and inexpensive medium-heat-denaturation, kinetics, and pH stability source. *Appl. Biochem. Biotechnol.* **66**, 185-195.

Rajal, V.B., Carrillo, L. and Cuevas, C.M. (1996). Exocellular hydrolases from *Penicillium ulaiense. World J. Microbiol. Biotechnol.* **12**, 413-414.

Raper, K.B. and Thom, C. (1949). Asymmetrica. In *A Manual of the Penicillia*, Raper K.B., Thom C. (Eds.), The Williams and Wilkins, Baltimore, MD, pp. 419-556.

Rapp, P. and Backhaus, S. (1992). Formation of extracellular lipases by filamentous fungi, yeasts, and bacteria. *Enzyme Microb. Technol.* **14**, 938-943.

Ratledge, C. (1984). Biotechnology as applied to the oils and fats industry. *Fette Seinfen Austrichm* **86**, 379-389.

Revah, S. and Lebeault, J.M. (1989). Accelerated production of blue cheese flavors by fermentation on granular curds with lipase addition. *Lait* **69**, 281-289.

Rivera-Munoz, G., Tinoco-Valencia, J.R., Sanchez, S. and Farres, A. (1991). Production of microbial lipases in a solid state fermentation system. *Biotechnol. Lett.* **13**, 277-280.

Schuepp, C. (1995). In *Partial Purification and Characterization of Lipases from Pseudomonas fragi*, M.Sc. Thesis, McGill University, Montreal, Canada.

Schuepp, C., Kermasha, S., Morin, A. and Michaliski, M.C. (1997). Partial purification and characterization of lipases from *Pseudomonas fragi* CRDA 037. *Process Biochem.* **32**, 225-232.

Segal, I.H. (1976). Enzymes. In *Biochemical Calculations* (2^{nd}). John Wiley and Sons Inc., New York, N.Y., pp. 208-319.

Seitz, E.W. (1973). Industrial application of microbial lipases. *J. Am. Oil Chem. Soc.* **51**, 12-16.

Shelley, A.W., Deeth, H.C. and Macrae, I.C. (1987). Review of methods of enumeration, detection and isolation of lipolytic microorganisms with special reference to dairy applications. *J. Microbiol. Methods* **6**, 123-137.

Shimada, Y., Sugihara, A., Nagao, T. and Tominaga, Y. (1992). Induction of *Geotrichum candidum* lipase by long-chain fatty acids. *J. Ferment. Bioengineer.* **74**, 77-80.

Sierra, G. (1956). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie Van Leeuwenhoek J. Microbiol. Serol.* **23,** 15-22.

Stead, D. (1986). Microbial lipases: their characteristics, role in food spoilage and industrial uses. *J. Dairy Res.* **53**, 481-505.

Stöcklein, W., Sztajer, H., Menge, U. and Schmid, R.D. (1993). Purification and properties of a lipase from *Penicillium expansum. Biochim. Biophys. Acta* **1168**, 181-189.

Sugihara, A., Shimada, Y., Takada, N. and Tominaga, Y. (1988). Purification characterization of *Aspergillus niger* lipase. *Agric. Biol. Chem.* **52**, 1591-1592.

Sugihara, A., Shimada, Y., Takada, N., Nagao, T. and Tominaga, Y. (1996). *Penicillium abeanum* lipase: Purification, characterization and its use for docosahexanoic acid enrichment of tuna oil. *J. Ferment. Bioengineer.* **82**, 498-501.

Sztajer, H. and Zboinska, E. (1988). Microbial lipases in biotechnology. *Acta Biotechnol.* **2**, 169-175.

Sztajer, H., Lüensdorf, H., Erdmann, H., Menge, U. and Schmid, R. (1992). Purification and properties of lipase from *Penicillium simplicissimum*. *Biochim. Biophys. Acta* **1124**, 253-261.

Taipa, M.A., Aires-Barros, M.R. and Cabral, J.M.S. (1992). Purification of lipases. *J. Biotechnol.* **26**,111-142.

Tanaka, T., Ono, E., Ishihara, M., Yamanaka, K. and Takinami, K. (1981). Enzymatic acyl exchange of triglyceride in n-hexane. *Agric. Biol. Chem.* **45**, 2387-2389.

Tanaka, Y., Funada, T., Hirano, J. and Hashizume, R. (1993). Triglyceride specificity of *Candida cylindracea* lipase: effect of docosahexanoic acid on resistance of triglyceride to lipase. *J. Am. Oil Chem. Soc.* **70**, 1031-1034.

Toida, J., Kondoh, K., Fukuzawa, M., Ohnishi, K. and Sekiguchi, J. (1995). Purification and Characterization of a Lipase from *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* **59**, 1199-1203.

Tomasini, A., Bustillo, G. and Lebeault, J.M. (1995). Production of blue cheese flavor concentrates from different substrates supplemented with lipolyzed cream. *Internal. Dairy J.* **5**, 247-257.

Vagina, O.N., Davranov, K.D. and Khalameizer, V.B. (1995). Synthesis activity of *Penicillium sp.* lipase in reversed micelles AOT in benzene. *Biokhimya* **60**, 263-269.

Veeraragavan, K., Colpitts, T. and Gibbbs, B.F (1990). Purification and characterization of two distinct lipases from *Geotrichum candidum*. *Biochim. Biophys. Acta* **15**, 26-33.

Ventkata-Rao, P., Jayaraman, K. and Lakshmanan, C. M. (1993). Production of lipase by *Candida rugosa* in solid state fermentation 2: Medium optimization and effect of aeration. *Process Biochem.* **28**, 391-395.

Vorderwülbecke, T., Kieslich, K. and Erdmann, H. (1992). Comparison of lipases by different assays. *Enzyme Microb. Technol.* **14**, 631-39.

Watanabe, N., Ota, Y., Minoda, Y. and Yamada, K. (1977). Isolation and identification of alkaline lipase producing microorganisms, cultural conditions and some properties of crude enzymes. *Agric. Biol. Chem.* **41**, 1353-1358.

Whitaker, J. R. (1972). Active sites and factors responsible for enzyme catalysis. In *Principles of Enzymology for the Food Science*, Marcel Dekker, New York, N.Y., pp. 119-143.

Wiclox, J.C., Nelson, W.O. and Wood, W.A. (1955). The selective release of volatile acids from butterfat by microbial lipases. *J. Dairy Sci.* **38**, 775-781.

Winkler, U.K. and Stuckmann, M. (1979). Gylycogene, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J. Bacteriol.* **138**, 663-670.

Yamaguchi, S. and Mase, T. (1991). Purification and characterization of mono- and diacylglycerol lipase isolated from *Penicillium camemberti* U-150. *Appl. Microbiol. Biotechnol.* **34**, 720-725.

Yamaguchi, S., Takeuchi, K., Mase, T., Oikawa, K., Mcmullen, T., Derewenda, U., Mcelhaney, R.N., Kay, C.M. and Derewenda, Z.S. (1996). The consequences of engineering an extra disulfide bond in the *Penicillium camemberti* mono- and diglyceride specific lipase. *Protein Engineer.* **9**, 789-795.