Studies on lipase enzyme from *Pseudomonas fluorescens* NS2W

A
THESIS SUBMITTED TO THE
UNIVERSITY OF PUNE
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

BY
NEELIMA KULKARNI

CHEMICAL ENGINEERING DIVISION
NATIONAL CHEMICAL LABORATORY
PUNE 411 008 (INDIA)

FEBRUARY 2002

This thesis is dedicated to my parents…

CONTENTS

		Page
	Certificate	i
	Acknowledgement	ii
Chapter 1	**General Introduction**	1
	Introduction	2
	Lipase producing microorganisms and their occurrence	2
	Lipase production	7
	Mechanism of lipolysis	12
	Lipase assay methods	17
	Lipase purification	26
	Lipase classification	37
	Applications of lipases	44
	Scope of this thesis	50
Chapter 2	**Simple gas chromatography method for lipase assay : Optimization of assay conditions**	54
	Summary	55
	Introduction	55
	Materials and Methods	57
	Results and Discussion	60
Chapter 3	**Application of factorial designs for optimization of lipase production from *Pseudomonas fluorescens* NS2W**	66
	Summary	67
	Introduction	67
	Materials and Methods	68
	Results and Discussion	75
	Conclusions	89
Chapter 4	**Purification and characterization of alkaline lipase from *Pseudomonas fluorescens* NS2W**	90
	Summary	91
	Introduction	92
	Materials and Methods	92
	Results and Discussion	99
	Conclusions	116
Chapter 5	**Optimization of physicochemical parameters for downstream processing of alkaline lipase from *Pseudomonas fluorescens* NS2W using foam separation**	117
	Summary	118
	Introduction	118
	Materials and Methods	122
	Results and Discussion	126
	Conclusions	137

Chapter 6 **Fermentation studies on lipase production from *Pseudomonas fluorescens* NS2W** 138

Summary 139
Introduction 139
Materials and Methods 140
Results and Discussion 143
Conclusions 154

Chapter 7 **Product formulation and application studies: A. Preparation of detergent additive and immobilization of lipase B. Detergent compatibility and castor oil hydrolysis studies** 155

Summary 156
Introduction 156
Part A 158
Part B 164
Conclusions 170

Chapter 8 **General conclusions and future work** 171

Chapter 9 **References** 175

List of publications 213

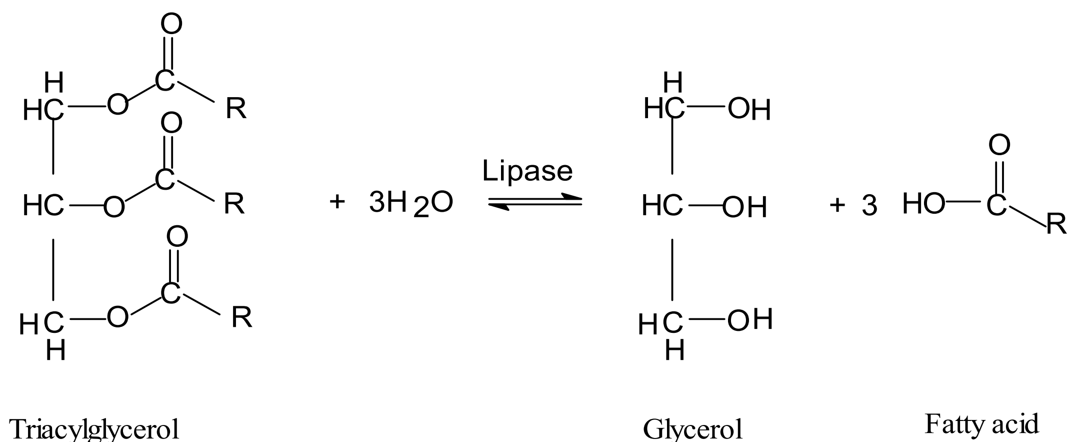
CHAPTER 1

General Introduction

Introduction

Lipases (E.C. 3.1.1.3) are produced by several microorganisms namely, bacteria, fungi, archea and eucarya, as well as by animals and plants (Olson *et al.* 1994). The present thesis will deal with the description of lipases from microbial sources, particularly bacterial lipases. A typical reaction catalyzed by lipases is shown in Figure 1.1.

Figure 1.1 Hydrolysis of triacylglycerol by lipase



The interest in lipases arises due to the ability of these enzymes to catalyze the hydrolysis as well as synthesis of fatty acid esters. Lipases act on a variety of substrates including natural oils, synthetic triglycerides and esters of fatty acids. Many lipases are resistant to solvents and hence find use in the synthesis of chiral drugs. They show varied substrate and positional specificities and find use in various industries like, food, chemical, pharmaceutical, cosmetic, leather and detergent.

Lipase producing microorganisms and their occurrence

Lipase production from a variety of bacteria, fungi and actinomycetes has been reported (Sztajer *et al.*1988, Rapp and Backhaus 1992). The presence of lipases in bacteria had

been observed as early as 1901 A.D. for *Bacillus prodigiosus*, *Bacillus pyocyneus*, and *Bacillus fluorescens* (Jaeger *et al*. 1994) which represent some of today's best studied lipase producers, now named, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively. Lipase producers have been isolated mainly from soil, or spoiled food material that contain vegetable oils. Lipases also represent important virulence factor of many plant and animal pathogens. Lipases with novel properties have been discovered from microorganisms isolated from antarctic ocean (Feller *et al.* 1990), hot springs (Gowland *et al*. 1987, Lee *et al*. 1999), compost heaps (Gowland *et al*. 1987, Rathi *et al*. 2000) and highly salty or sugary environments (Elwan *et al*. 1985, Ghanem *et al*. 2000). Lipase producers have been reported to grow at varied pH and temperatures. The fungi are usually reported to require acidic pH for growth and lipase production (Arima *et al*. 1772, Pokorny *et al*. 1994). Many bacteria are found to prefer neutral pH but there are reports of alkalophic (Gao *et al*. 2000. Ghanem *et al.* 2000) bacteria also. Psychrophilic and thermophilic organisms, as well as organisms having different oxygen demand (aerobic, microaerophilic and anaerobic) are reported to produce lipases. The occurrence of fungal and bacterial lipases is listed in Table 1.1 and 1.2.

Table 1.1 Lipase producing bacteria and actinomycetes

Organism	Reference
Achromobacter lipolyticum	Scholefield *et al*. 1978
Acinetobacter baumannii, *A. calcoaceticus* 69-V, *A. radioresistens*	Hostacka 2000, Haferburg and Kleber 1983, Wang and Chen 1998
Aeromonas hydrophila MCC-2*, *A. sobria LP004*	Chuang *et al*. 1997, Lotrakul and Dharmsthiti 1997a
Alcaligenes sp. strain No. 679, *A. denitrificans**	Kokusho *et al*. 1982, Odera *et al*. 1986
*Alteromonas**	Saimoku *et al*. 1999
Anerovibrio lipolytica	Henderson 1971

Bacillus acidocaldarius, *B. alcalophilus*, *B. atrophaeus* SB-2, *Bacillus stearothermophilus* SB-1, *B. licheniformis* SB-3, *B. circulans*, *B. pumilus*, *B. subtilis*, *Bacillus thermocatenulatus**, *B. thermoleovorans ID-1**	Manco *et al*. 1998, Ghanem *et al*. 2000, Bradoo *et al*. 1999, Elwan 1985, Mourey 1981, Kennedy and Lennarz 1979, Rua 1998, Cho 2000
Brevibacterium linens	Adamitsch and Hampel 2000
Brochothrix thermosphacta	Papon and Talon 1988
Burkholderia cepacia, *B. glumae*, *B. pseudomallei*	Ishii 2001, El Khattabi 2000, DeShazer *et al*. 1999
Campylobacter jejuni, *C. coli*, *C. lari*	Colomina *et al*. 1997
Chromobacterium viscosum, C. viscosum pararipoliticum	Horiuti and Imamura 1977, Sagai *et al*. 1889
Corynebacterium sp.	Ping and Omar 1993
Escherichia coli	Nantel *et al*. 1978
Flavobacterium odoratum	Labuschagne *et al*. 1997
Lactobacillus casei-subsp-*casei* LLG, *L. casei* subsp. *pseudoplantarum* LE2, *L. plantarum* 2739, *L. casei* 2756, *L. fermentum* DT41, *L. acidophilus* A2, *L. sanfranciscensis*	Lee and Lee 1990, Lee and Lee 1989, El-Sawah 1995, Gobbetti *et al*. 1996, De Angelis *et al*. 1999
Lactococcus helveticus	Carrasco *et al*. 1995
Leuconostoc citrovorum	Belov and Umanskii 1977
Micrococcus varians CAS4	**Zahran 1998**
Moraxella TA144*	Feller *et al*. 1990
Mycobacterium rubrum	Lebedeva 1977
Pediococcus pentosaceus SV61	Oestdal *et al*. 1996
Propionibacterium arabinosum ATCC 4965, *P. shermanii* ATCC 6915, *P. acnes, Propionibacterium freudenreichii subsp. freudenreichii.*	Merilainen and Uusi-Rauva 1976, Ingham *et al*. 1981, Dupuis *et al.* 1993
Proteus vulgaris K80	Kim and Oh. 1998
Pseudomonas aeruginosa, *P. alcaligenes*, *P. cepacia**, *P. fragi* *, *P. fluorenscens*, *P. glumae*, *P. mendocina* 3121-1, *P. mephitica var. lipolytica*, *P. plantarii*, *P. pseudoalcaligenes*, *P. putida* 3SK, *P. stutzeri*, *P. solanacearum*, *P. tolaasii*, *Pseudomonas wisconsinensis*	Jaeger and Winkler 1984, Holmes 1990, Dünhaupt *et al.* 1992, Mencher and Alford. 1967, Makhzoum *et al*. 1995, Deveer *et al*. 1991, Bachmatova *et al*. 1995, Bycroft *et al*. 1990, Lin *et al*. 1996, Lee and Rhee 1993, Piao *et al*. 1998, Kotsuka *et al*. 1996, Baral and Fox 1997, Charmoille *et al*. 1997
Selenomonas lipolytica	Dighe *et al*. 1998
Serratia liquefaciens, *S. marcescens* 345	Zou *et al*. 1996, Bashkatova and Severina 1980

Streptococcus cremoris, S. diacetilactis S. faecalis, S. lactis, S. thermophilus	DeMoraes and Chandan 1982, Kamaly *et al*. 1990, Chander *et al*. 1979, Belov and Umanskii 1977
*Streptomyces exfoliates** M11*, S. cinnamomeus*, *S. parvulus*, *S. clavuligerus*, *S. coelicolor, S. rimosus*	Servin-Gonzalez *et al.* 1997, Sommer *et al*. 1997, El-Shirbiny and Ghaly 1992, Large *et al*. 1999
Staphylococcus aureus, S. epidermidis, S. haemolyticus, S. hyicus, S. carnosus*, S. warneri and S. xylosus*	Bisignano 1980, Farrell *et al*. 1993 Oh *et al*. 1999, Goetz *et al*. 1985, Voit *et al*. 1991, Talon *et al*. 1996
Thermoactinomyces vulgaris	Elwan *et al*. 1978
Thermus sp.	Silva *et al*. 1991
Thermosyntropha lipolytica gen. nov., sp. nov.	Svetlitshnyi *et al*. 1996
Vibrio cholerae El	Ogierman *et al*. 1997
Xanthomonas campestris pathovar *sesami*	Sheela *et al*. 1996
Yersinia	Kuznetsov and Bagryantsev 1992

* indicates recombinant strain

Table 1.2 Lipase producing yeasts and fungi

Actinomucor taiwanensis	Chou *et al*. 1988
Alternaria alternata	Osman *et al*. 1988
Aspergillus awamori, *A. carneus*, *A. flavus* , *A. flavipes*, *A. foetidus*, *A. fumigatus*, *A. japonicus*, *A. niger*, *A. oryzae*, *A. repens*, *A. saitoi*, *A. sydowi*, *A. tamari*, *A. wentii*	Svetlov *et al*. 1984, Parmar 1998, Shash 1998, Savitha and Ratledge 1992, Nair and Bone. 1987, Hamed 1996, Vora *et al*. 1988, Pokorny *et al*. 1994, Toida *et al*. 2000, Kunimoto *et al*. 1996, Tanaka and Imamura 1986, Elwan *et al*. 1986, Saad 1995, Chopra *et al*. 1980
Basidiobolus	Okafor and Gugnani 1990
Botryosphaeria	Hirayama and Halkier 1996
Botrytis cinerea	Commenil *et al*. 1995
Byssochlamys fulva	Chander and Klostermeyer 1983
Candida antarctica, *C. albicans*, *C. curvata*, *C. cylindracea*, *C. deformans*, *C. entomophila*, *C. lipolytica*, *C. parapsilosis*, *C. paralipolytica*, *C. rugosa*, *Candida rugosa**	Svendsen *et al*. 1994, Hube *et al*. 2000, Montet *et al*. 1985, Lee and Choo 1989, Muderhwa *et al*. 1985, Fujimoto *et al*. 1996, Padmini *et al*. 1990, Briand *et al*. 1995, Vecozola and Bekers 1978, Valero *et al*. 1988, Fusetti *et al*. 1996

Conidiobolus	Okafor and Gugnani 1990
Cryptococcus sp.	Kamini *et al*. 2000
Cunninghamella echinulata.	Hoong and Omar 1993
*Fusarium culmorum, F. heterosporum**, F. moniliforme, F. oxysporum, F. oxysporum f. sp. Vasinfectum, F. solani, Microdochium nivale (syn. Fusarium nivale).*	Oxenboell *et al*. 1996, Nagao *et al*. 1995, Hamed 1997, Hoshino *et al*. 1991, Rapp 1995, Hoshino *et al*. 1996
Fusidium sp. BX-1.	Ohno and Matsuno 1997
Galactomyces geotrichum	Phillips and Pretorius 1991
Geotrichum asteroids, *Geotrichum candidum, Geotrichum candidum**	Selezneva and Kazanina 1986, Tahoun *et al*. 1982, Vandamme *et al*. 1987
Humicola insolens DSM 1800, *Humicola lanuginosa**	Sandal *et al*. 1996, Liu *et al*. 1977, Boel *et al*. 1989
*Mucor mucedo, M. phillipovi, M. circinelloides, M. pusillus, M. fragilis, M. racemosus, M. javanicus, M. hiemalis, M. miehei**	Chander and Klostermeyer 1982a, Mirza *et al*. 1979, Akhtar *et al*. 1976, Chopra *et al*. 1981, Akhtar *et al*. 1980, Deploey *et al*. 1981, Gaskin *et al*. 1994
Neurospora crassa	Kundu *et al*. 1987
Oospora fragrans, Oospora lactis	Ruban *et al*. 1978, Shchelokova *et al*. 1977
*Penicillium camembertii**, *P. caseicolum*, *P. candidum*, *P. chrysogenum*, *P. citrinum*, *P. cyclopium* M1, *P. expansum*, *P. funiculosum*, *P. restrictum*, *P. roqueforti*, *P. simplicissimum*, *P. solitum*, *P. verrucosum var. cyclopium,*	Yamaguchi *et al*. 1997, Alhir *et al*. 1990, Kornacki *et al*. 1980, Sztajer and Maliszewska 1989, Chander *et al*. 1977, Okumura *et al*. 1980, Dahot and Memon 1989, Lamberet and Menassa 1983, Hamed 1996, Freire *et al*. 1997, Sztajer *et al*. 1991a, Selezneva and Kazanina 1986, Glenza and Ben Jaballah, 1985
Pichia burtonii	Sugihara *et al*. 1995
Pythium ultimum	Mozaffar and Weete 1993
Rhizomucor miehei	Boel *et al*. 1988
Rhizopus arrhizus, R. boreas, R. chinensis, *R. circinans*, *R. cohnii sp.*, *R. delemar*, *R. fusiformis*, *R. javanicus*, *R. japonicus*, *R. microsporus*, *R. nigricans*, *R. niveus**, *R. oligosporus*, *R. oryzae*, *R. rhizopodiformis*, *R. stolonifer*, *R. thermosus, R. usamii*	Ozer and Elibol 2000, Rapp and Backhaus 1992,Yasuda *et al*. 1999, Nahas and De Assis 1988, Tsereteil *et al*. 1994, Chander *et al*. 1981a, Chander *et al*. 1981b, Szell *et al*. 1977, Samad *et al*. 1990, Martinez *et al*. 1993, Kugimiya *et al*. 1989, Uyttenbroeck *et al*. 1993, Aisaka and Terada 1980

Rhodotorula rubra, Rhodotorula glutinis	Rapp and Backhaus 1992, Papaparaskevas *et al*. 1992
Saccharomycopsis lipolytica	Ruschen and Winkler 1982
*Schizosaccharomyces pombe**	Smerdon *et al*. 1998
Sporotrichum (Chrysosporium) thermophile Apinis	Johri *et al*. 1991
Sterigmatomyces sp.	Majumdar *et al*. 1998
Syncephalastrum racemosum	Chopra and Chander1983
Thermomyces lanuginosus	Frich *et al*.1994
Trichosporon asteroids, Trichosporon fermentans WU-C12	Dharmsthiti and Ammaranond 1997, Chen *et al*. 1992
Trichothecium roseum	Gopinath *et al*. 1996
Ustilago maydis.	Lang *et al*. 1991
Yarrowia lipolytica	Hadeball *et al*. 1991

* indicates recombinant strain

As can be seen from the Table 1.1 and 1.2, a large number of bacteria, actinomycetes, yeasts and fungi produce lipases. The work done regarding the lipases from microbial origin as a whole is however, out of the scope of this thesis. The literature available regarding lipases from bacteria and actinomycetes is reviewed in the introduction of the thesis.

Lipase production

Bacterial lipases are mostly inducible enzymes, requiring some form of oil, fatty acid, fatty acid alcohol or fatty acid ester for induction. However, there are a few reports of constitutive lipase production by bacteria. (Elwan *et al*. 1983, Gao *et al*. 2000). Lipases are usually secreted out in the culture medium; although there are a few reports of the presence of intracellular lipases (Mourey 1981, Lee and Lee 1989) as well as cell bound lipases (Large *et al*. 1999).

The onset of lipase production is organism-specific, but, in general, it is released during late logarithmic or stationary phase (Matselis and Roussis 1992, Makhzoum *et al*. 1995).

The cultivation period from 3.5 to 168 h has been reported for the different lipase producing organisms (Table 1.5). Fast growing organisms were normally found to secrete the lipase within 12-24 h (Steur *et al*. 1986, Lee and Lee 1989, Chartrain *et al*. 1993, Lee and Rhee 1993, Lin *et al*. 1996, Imamura and Kitaura 2000).

The production of lipases from bacteria is heavily patented although the published work is rather scanty (Gawel and Chen 1977, Nakanishi and Ikeda 1986, Inoue *et al*. 1987, Holmes 1990, Ishida *et al*. 1995, Ishikawa *et al*. 1995, Lawler and Smith 2000). A few researchers have performed systematic medium optimization and fermentation studies for lipase production.

The organisms are normally grown in a complex nutrient medium containing a carbon source (usually oil), a nitrogen source (organic/inorganic), phosphorus source (sodium or potassium phosphate) and mineral salts, supplemented with micronutrients ($MgSO_4$ or $CaCl_2$). The pH of the medium is generally maintained around 7.0. A pH range between 8.0-10.0 has been used for lipase production by alkalophillic bacteria (adjusted with Na_2CO_3).

Lipase production has been done using variety of non-conventional carbon sources, like beef tallow, wool-scour effluent, whey, n-hexadecane and n-paraffins and Tweens (Gawel and Chen 1977, Gowland *et al*. 1987, Brahimi-Horn 1991, Gilbert *et al*. 1991a, Chen *et al*. 1998, Kosugi and Suzuki 1998, Fonchy *et al*. 1999).

Several researchers have studied the effects of various polysaccharides and detergents on lipase production. Winkler and Stuckmann (1979) have reported that the exolipase production by *Serratia marcescens* was enhanced by polysaccharides like glycogen, hyluronate, laminarin, pectin B and gum arabic while the exolipase production by

Pseudomonas aeruginosa was enhanced by treatment with glycogen (Schulte *et al*. 1982), hyaluronate (Jaeger and Winkler 1984) and alginate (Wingender and Winkler 1984). A "detachment hypothesis" has been proposed by Winkler and Stuckmann (1979) suggesting the interaction of polysaccharides with cell surface structures as probable mechanism of this phenomenon. They have suggested the presence of hypothetical binding sites for newly synthesized lipase. The polysaccharides were suggested to interact with these lipase molecules by either competition for these sites or by changing the conformation of exolipase. Lin *et al*. (1995) have reported that addition of Triton X-100 increased the lipase activity by 50-fold.

Most of the fermentations for lipase production have been performed in submerged, batch mode. Table 1.3 presents a brief overview of lipase production by several bacteria in batch mode, in shake flasks or in fermenters.

Table 1.3 Overview of lipase production in batch mode.

Organism	C- source	N-source	Lipase U/ml	Assay method	Reference
Acinetobacter radioresistens	Olive oil and n-hexadecane	Tryptone, yeast extract, NH_4Cl	2	Titrimetry using olive oil	Chen *et al*. 1998
Aeromonas sobria	Whey, Soybean meal	Soybean meal, yeast extract	40	Spectrophoto-metry using *p*NPP	Lotrakul and Dharmsthiti 1997a
Bacillus circulans	Sucrose	$NaNO_3$, leucine	3890 mg/ml	-	Elwan *et al*. 1985
Bacillus circulans	Soluble starch	Soybean meal, peptone	2.4	Titrimetry using tributyrin	Sztajer and Maliszewska 1988a
Bacillus thermoleovorans ID-1	Olive oil	Tryptone, yeast extract	0.7	Spectrophotometry using *p*NP-butyrate	Lee *et al*. 1999
Bacillus sp.	Sucrose	Soybean meal,	2.7	Titrimetry using	Sztajer and Maliszewska

		peptone		tributyrin	1988a
Bacillus sp.	Tween 80	NH_4NO_3	4	Titrimetry using olive oil	Gowland *et al*. 1987
Bacillus sp. THL027	Rice bran oil	Yeast extract, peptone	8.3	Titrimetry using olive oil	Dharmsthiti and Luchai 1999
Bacillus licheniformis	Maltose	Soybean meal	2.8	Titrimetry using tributyrin	Sztajer and Maliszewska 1988a
Flavobacterium	Tween 80	Yeast extract, peptone	0.2	Spectrophoto-metry using *p*NPP	Labuschagne *et al*. 1997
Pseudomonas fluorescens	Starch	Soybean meal	1.4	Titrimetry using tributyrin	Sztajer and Maliszewska 1988a
Pseudomonas aeruginosa EF2	Tween 80	KNO_3	8.3	Titrimetry using olive oil	Gilbert *et al*. 1991a
Pseudoomonas sp.	Ground soybean, Soluble starch	Corn steep liquor	87.5	Titrimetry using olive oil	Gao *et al*. 2000
Pseudomonas nitroreduscens	Soybean meal, soluble starch	$(NH_4)_2SO_4$, Urea	500	Titrimetry using olive oil	Watanabe *et al*. 1977
Pseudomonas fragi	Soybean meal, soluble starch	$(NH_4)_2SO_4$, Urea	30	Titrimetry using olive oil	Watanabe *et al*. 1977
Pseudomonas aeruginosa YS-7	Soybean oil	-	85	Spectrophotometry using *p*NP laurate	Shabtai and Daya-Mishne 1992
Pseudomonas aeruginosa MB 5001	Peptonized milk	Beef extract, peptone	100	Titrymetry using olive oil	Chartrain *et al*. 1993
Pseudomonas pseudoalcaligenes	Soymeal	Peptone, yeast extract	100	Spectrophotometry using *p*NP laurate	Lin *et al*. 1996
Pseudomonas sp.	Castor oil	Polypep-tone	105	Titrimetry using olive oil	Yamamato and Fujiwara 1988
Streptomyces sp.	Soluble starch	Peptone	1.4	Titrimetry using tributyrin	Sztajer and Maliszewska 1988a

There are a few reports of fed-batch or continuous culture studies. Suzuki *et al*. (1988) have reported mass production of lipase by *Pseudomonas fluorescens* in fed-batch culture. Lipase production as high as 2000 U/ml was achieved by feeding olive oil, in which the feed rate of oil was controlled automatically based on CO_2 evolution rate. Ishihara *et al*. (1989) have reported turbidity-dependent fed-batch culture of *Pseudomonas fluorescens*. Turbidity-dependent, automatic feeding of olive oil and Ferrous ion was performed to obtain limitation of the oily substrate in the culture liquid and the Ferrous ion content of the cells, to increase lipase production to 5600 U/ml. Wang and Chen (1998) were able to obtain increased yield of lipase by fill-and-draw culture of *Acinetobacter radioresistens*. Lechner *et al*. (1988) have used a dialysis fermenter for production of lipase from *Staphylococcus carnosus*. The bacterial culture was grown in inner 2 l compartment, separated from 10 l nutrient broth compartment by conventional dialysis membrane. The lipase production was directly coupled with increase in cell mass and reached a value of 230 mg per litre culture supernatant. Gilbert *et al*. (1991a) have optimized the lipase production by *Pseudomonas aeruginosa* EF2 by continuous culture using response surface analysis. Gerritse *et al*. (1998) have reported production of a lipase from a recombinant *Pseudomonas pseudoalcaligenes*. The organism was first grown in batch mode, using citric acid to achieve good cell growth. The feeding of soybean oil was started after the citric acid was consumed, so as to get high lipase production.

Mechanism of lipolysis

Lipases act on a variety of substrates like triacylglycerols, cholesterol esters and wax esters, which are insoluble in water. A typical reaction catalyzed by a lipase can be represented as shown in Figure 1.1

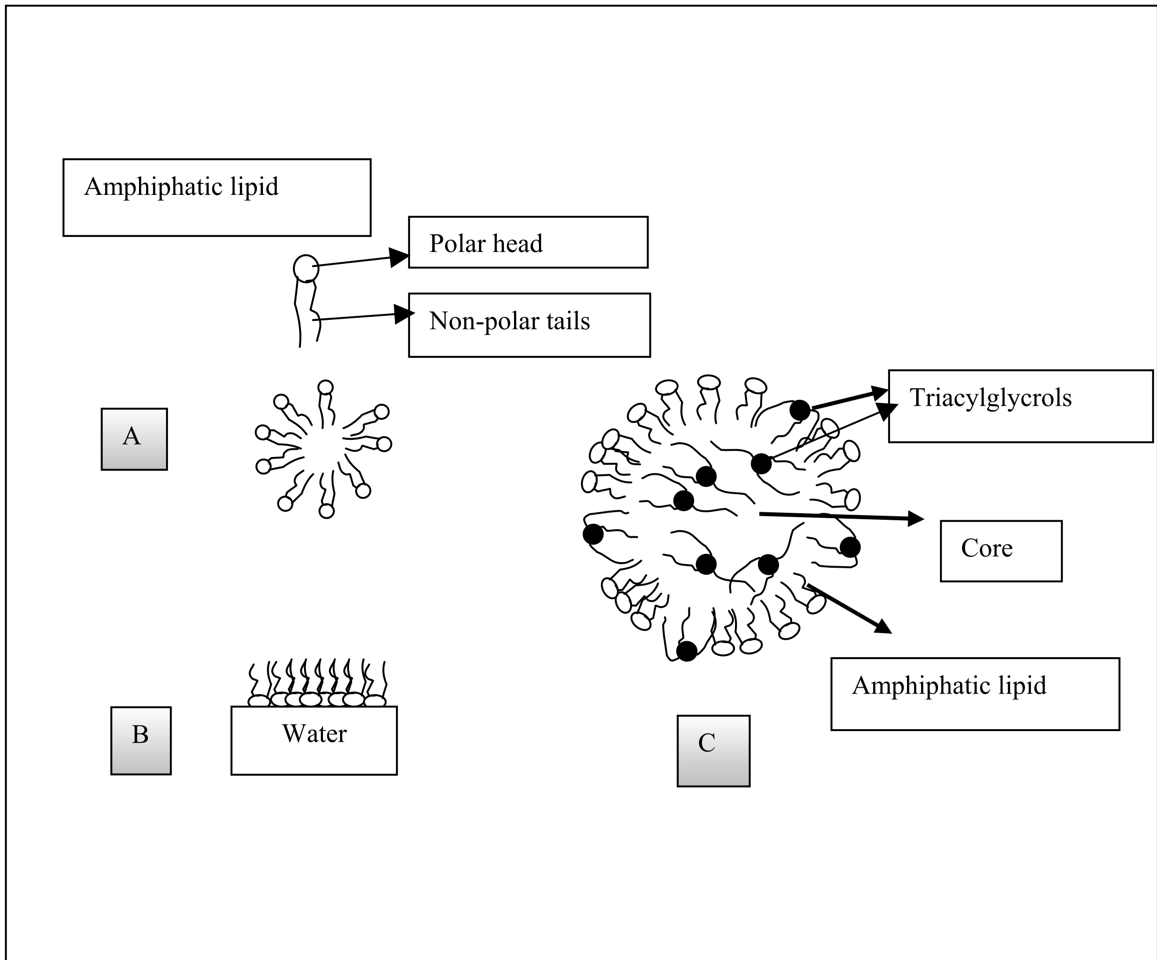
An interface

Triacylglycerols are the main substrates of lipases. They are uncharged lipids. The triacylglycerols with long chain fatty acids esterified with glycerol are insoluble in water, although those with short-chain fatty acids are sparingly soluble in water. The maximum amount of solute that can be dissolved in solvent is called its saturation value. Triacylglycerols normally form emulsion in aqueous solutions at concentrations greater than their saturation value. Phospholipids, are natural substrates for phospholipases. Phospholipids are also insoluble in water, but they form micelles when exceeding the maximum concentration of dissolved monomer at a point called critical micelle concentration. While the maximum saturation value in water for triacylglycerols can be as high as 0.330 M in the case of triacetin, it can be less than 1μM for long-chain triacylglycerols (Jaeger *et al*. 1994). Lipolysis by lipases occurs exclusively at the lipid-water interface, implying that concentration of substrate molecules at this interface (expressed in mol/ m^3) directly determines the rate of lipolysis.

Emulsions

Emulsions are characterized by a ‘core’ or a bulk lipid phase surrounded by surface monolayer of amphipathic molecules (Figure 1.2).

Figure 1.2 Different stable systems of lipid molecules in water, A) Micelle, B) Monolayer of amphipathic lipid at air water interface, C) Emulsion droplet



The formation of emulsion requires some amount of energy input such as mechanical dispersion of bulk lipid in an aqueous phase. In the absence of other system components, the dispersed lipid droplets tend to coalesce in order to minimize the apolar surface exposed to water. Amphipathic molecules must be present to form a surface monolayer on the dispersed apolar lipid and thus to stabilize the emulsion. In typical biological system, the surface components of oil emulsions can be lipids, denatured proteins or

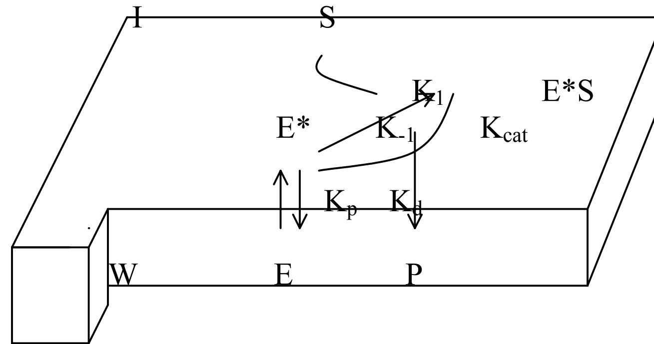
other type of amphipathic compounds. In synthetic emulsions, like emulsion of triolein in gum acacia solution, gum acacia acts as the amphipathic compound. Emulsion is the most popular lipid system used for estimation of lipase activity in laboratories.

Activation of lipolytic enzymes by interfaces

In 1958, Sarda and Deshnelle (as quoted by Verger 1980) demonstrated fundamental difference between esterase and pancreatic lipase based upon ability or inability to be activated by interfaces. They observed that in contrast to esterases, which show normal Michaelis-Menten activity dependence on substrate concentration, the lipase displayed almost no activity with the substrate when the substrate was in monomeric state. However, when the concentration of substrate *i.e.* triacetin exceeded its solubility limit, resulting in formation of an emulsion, there was a sharp increase in the observed enzyme activity with the same substrate in emulsified state.

Kinetics of lipase cannot be described with the Michaelis-Menten model because this model is valid only in the case of a single homogenous phase of soluble enzyme and substrate. A new model was proposed by Verger *et al.* (1980) to explain catalysis by lipolytic enzymes. The model consisted of two successive equilibria (Figure 1.3). It first described the penetration of a water-soluble enzyme into an interface ($E \leftrightarrows E^*$). This was followed by a second equilibrium, in which one molecule of penetrated enzyme got bound to one substrate molecule, giving the complex E*S. This was equivalent in two dimensions of classical Michaelis-Menten equilibrium. Once the complex E*S was formed, the catalytic reaction took place, regenerating the enzyme in the form E* along with the liberation of the products.

Figure 1.3 Kinetics of lipase reaction



W - Aqueous phase, I - interface

The only case considered was the one in which all products of the reaction were soluble in the water phase, they diffused away rapidly and induced no change with time in the physicochemical properties of the interface.

Hydrolysis versus synthesis

The hydrolysis of fats and oils (Figure 1.1) is a reversible reaction and it is possible to change the direction of the reaction toward synthesis by modifying the reaction conditions. The water content of the reaction mixture controls the equilibrium between forward and reverse reactions. In non-aqueous environment, lipases catalyze ester synthesis reaction. The ester synthesis reaction can be classified into simple esterification, transesterification and interesterification, depending on the nature of reaction (Figure 1.4a and 1.4b). The esterification reaction involves synthesis of glyceryl esters from glycerol and fatty acid. In transesterification, in place of fatty acid the acyl donar is an ester. The transesterification

Figure 1.4 a Transesterification reaction catalyzed by lipase enzyme

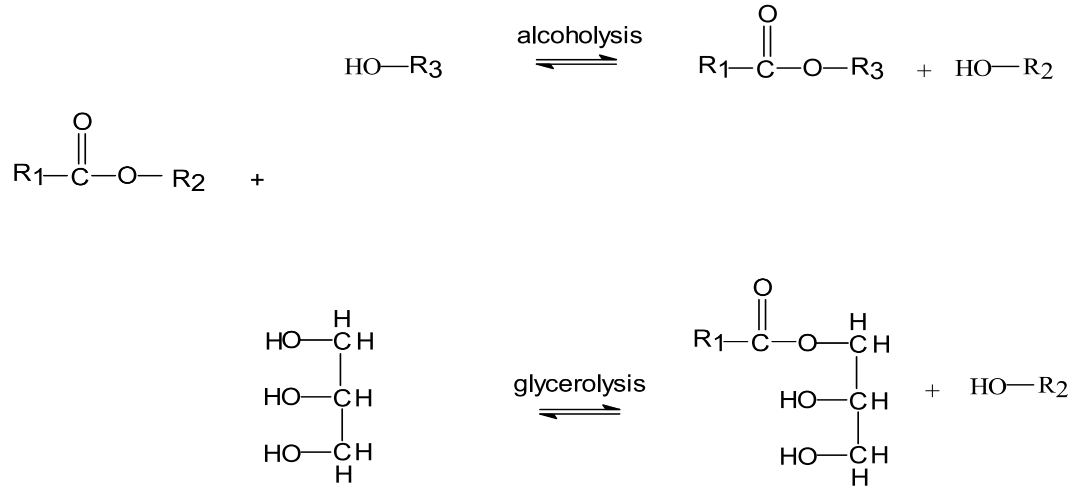
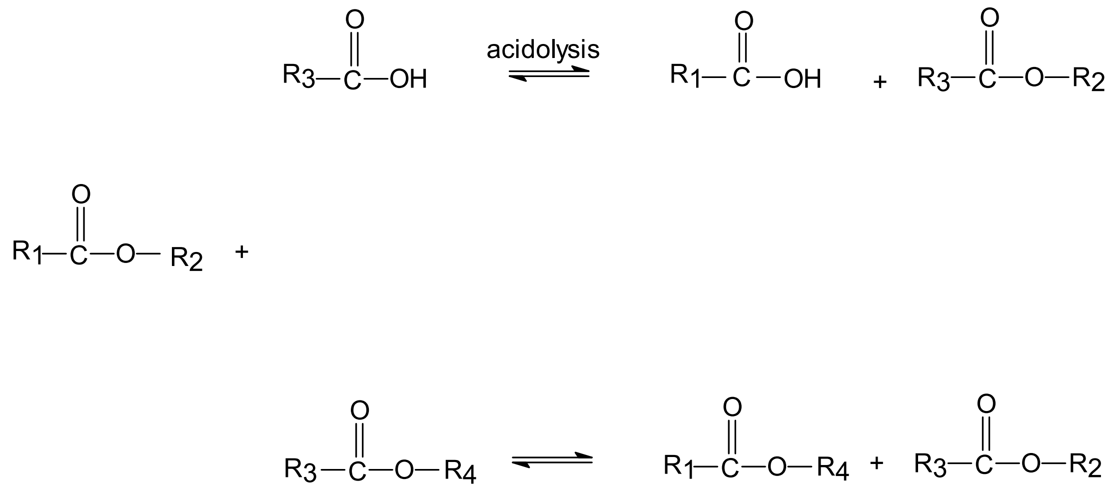


Figure 1.4 b Interesterification reaction catalysed by lipase



can be further divided into glycerolysis and alcoholysis, involving transfer of acyl group from triglyceride to either glycerol or an alcohol. In interesterification, the acyl group is exchanged between a glyceride and either a fatty acid (acidolysis) or a fatty acid ester.

Interesterification requires small amount of water, in addition to the amount needed for the enzyme to maintain active, hydrated state.

Because the lipases act on a variety of substrates, have complex kinetics and varied applications, a variety of assay methods have been developed for their qualitative and quantitative estimation as described below.

Lipase assay methods

There are numerous methods available for lipase activity estimation and they have been well reviewed in the literature (Jensen 1983, Jaeger *et al*. 1994, Beisson *et al*. 2000). Most of these methods are designed to estimate the products of hydrolytic reactions (Figure 1.1)

These assay methods can be classified as 1. Titrimetry 2, Interfacial tensiometry 3, Spectroscopy (Photometry, fluorimetry, Infrared and turbidimetry), 4. Chromatography, 5. Immunochemistry and 6. Conductimetry. The Tables 1.4a and 1.4b give an overview of different lipase assay methods.

Plate assays

Lipases secreted by microbial cultures can be detected in agar media. Plate assays have been described for screening of lipase-producing microorganisms using coloured compounds like Methyl red, Phenol red, Rhodamine B or Victoria blue B as indicators (Converse *et* al.1981, Kouker and Jaeger 1987, Samad *et al*. 1989). In these methods, emulsified oils were added to growth medium containing indicator dyes. The hydrolysis of oils caused the formation of coloured or fluorescent halos or zones around microbial colonies. The fatty acid monoesters of polyoxyethylene sobitans were covalently linked

with Remazol brilliant blue R and a plate-clearing assay was developed by Wirth (1992) using these in the growth media.

Table 1.4a Overview of Lipase assays

Plate assays			
Substrate	Reaction product	Method	Reference
Triacylglycerols	Free fatty acids	Coloured indicators (Victoria blue, methyl red, phenol red, rhodamine B)	Kouker and Jaeger 1987, Samad *et al*. 1989, Converse *et al*. 1981
Titrimetric assay			
Triacylglycerols	Free fatty acids	pH determination	Rowe and Gilmour 1981
Interfacial tensiometry			
Dicaprin	Free fatty acids	Measurement of barrier movement	Ransac *et al*. 1991
Triacylglycerols	Free fatty acids	Measurement of drop volume or decrease in surface tension	Nury *et al*. 1987, Labourdenne *et al*. 1994

Titrimetry

The lipolytic reaction liberates an acid, which can be titrimetrically assayed. Rowe and Gilmour (1981) have described a method for the estimation of serum lipase using olive oil as the substrate. The reaction was allowed to proceed for 6 h and was then stopped by addition of ethyl alcohol. The reaction mixture was then titrated with an alkali solution and the fatty acids produced were quantified.

In another method called “pH-stat”, the pH of reaction mixture was kept constant by continuous addition of NaOH solution and the volume of alkali solution was monitored as a function of time (Gargouri *et al*. 1986). The observed reaction rate was a linear function of the lipase and the substrate concentration. The substrate concentration

available in the interfacial area is important and therefore, the triacylglycerols were emulsified by methods like sonication. Goodman and Durgan (1969) have shown that sonication of olive oil emulsified with gum arabic improved the sensitivity of assay.

Controlled surface pressure and interfacial tensiometry

Lipases act at the interface between a hydrophobic substrate and the aqueous phase, and hence the surface pressure is a very important parameter, which has often been neglected. For an assay of lipolytic enzymes, this parameter is equally important as pH or temperature. The effect of the surface pressure can be studied by the monolayer technique as described by Verger (1980) and Ransac *et al*. (1991). A monomolecular substrate film was spread at the air-water interface, which was compressed with a surface barrier, changing the surface density of the substrate and thus, the interfacial tension. The lipase injected into the water sub-phase got bound to the film and hydrolyzed the substrate. The substrate (*e.g*. dicaprin, trioctanoin, didecanoin or didodecanoin) was insoluble in water, but generated soluble products. It was also possible to use substrates with longer acyl-chains under conditions where the surface pressure is above 23 milliNewtons/meter and albumin was present in the sub phase as a product-acceptor (Ransac *et al*. 1991). When the substrate got hydrolyzed, it left the interface, thereby decreasing the surface density and surface pressure, which was then compensated by compression of the film by the mobile surface barrier movement, monitored as a function of time.

Nury *et al*. (1987) developed another method to monitor the interfacial tension during lipase assays by the ‘oil-drop method’. This method consisted of forming an oil-drop in an aqueous solution, with the drop connected to a syringe containing the oil. The shape of the drop was directly correlated to the interfacial tension of oil:water. In the absence of a

detergent or fatty acid in the medium, the drop was shaped like an apple. When a lipase was added to the water phase, it bound to the oil water interface and hydrolyzed the substrate. The shape of the drop changed to a pear form and, at a certain point, it left the support. A computer-controlled device called 'oil drop tensiometer' was developed to automatically perform this type of lipase assay (Labourdenne *et al*. 1994).

Spectroscopy

Several assays for lipase activity estimation are based on spectroscopic measurements. Rollof *et al*. (1984) developed an assay, which involved direct turbidometric estimation of residual lipids, after reaction of lipase with lipid emulsion. A turbidometric assay was developed by Robinson *et al*. (1989) for estimation of lipase activity in serum.

Triacylglycerols are natural substrates of lipases and many spectrophotometric methods use them as substrates. A few spectrophotometric assays are based on methods, which render colour to fatty acids released after hydrolysis of triacylglycerols. van Autryve *et al*. (1991) used rhodamine 6G for complexation with free fatty acids liberated during lipolysis. A pink colour appeared and the absorbance was monitored at 513 nm. Medcova *et al*. (1981) assayed the monoglyceride lipase activity using Tween 20 as substrate. The lauric acid released was converted to copper laureate and measured spectrophotometrically at 435 nm. Safarik (1991) developed a method using immobilized triacylglycerols. In this method, the fatty acids released after hydrolysis were extracted with benzene and converted to their corresponding Cu (II) salts, which were measured spectrophotometrically. Rawyler and Siegenthaler (1989) described a method that used metachromatic properties of the cationic dye, safranine, to detect a change in the net negative charge at the lipid: water interface, which was monitored by the change in of

safranine. This assay is very sensitive and very low quantities of lipolytic enzyme can be detected using this method.

There are enzymatic assays based on estimation of either glycerol or fatty acids released after action of lipase on triacylglycerols. Fossati, *et al*. (1992) described a kinetic colorimetric method for assaying lipase activity in serum by using a natural long-chain fatty acid 1,2-diglyceride. In the presence of co-lipase, deoxycholate and calcium ions, pancreatic lipase hydrolyzed the clear substrate solution to produce a 2-monoglyceride, which in turn, released glycerol by the action of a 2-monoglyceride lipase. Glycerol was then assayed by a sequence of enzymic reactions (glycerol kinase, glycerol phosphate oxidase, and peroxidase) that produced a violet quinone monoimine dye with peak absorption at 550 nm. Woollett *et al.* (1984) described an enzymatic method for the determination of the amount of free fatty acids released from triglyceride by lipoprotein lipase. The quantity of free fatty acids present in the medium before and after incubation is measured spectrophotometrically by the oxidation of NADH in the final reaction of a series of coupled enzymic reactions.

Table 1.4 b **Overview of lipase assays**

Spectrophotometry					
Substrate	Product	Method	Final product	Wavelength nm	Reference
2,3-dimercaptopropan-1-ol Tributyrate	Glycerol analogue (2 over 3 positions)	Reaction with DTNB	TNB	412	Kurooka *et al*. 1977
p- Nitro phenyl esters	p-nitro-phenol	Product is colored		410	Winkler and Stuckmann 1979
Glycerides (triolein)	Free fatty acid	Enzymatic conversion	NAD	340	Woollett *et al.* 1984
Glycerides (triolein)	Free fatty acid	Negative charge	Safranine	520/560	Rawyer *et al*. 1989
Aryleethene derivatives	Hydrolysis products are colored			Variable	Richardson *et al*. 1989
Glycerides	Free fatty acid	Complex formation	Rhodamine 6G	513	van Autryve *et al*. 1991
Glycerides	Free fatty acid	Complex formation	Cu (II) salt	715	Safarik 1991
1-2- diglycerides	Glycerol	Enzymatic conversion	quinone	550	Fossati *et al*. 1992
Fluorescence					
Substrate	Reaction product	Method	Final product	Wavelength nm	Reference
Glycerides containing pyrene ring	Free acid analogues or aggregated substrate	Fluorescence shift	Free acid analogues or glyceride analogues	Ex. 340 em. 400 450	Thuren *et al*. 1987
Glycerides	Free fatty acid	Complex formation	11-(dansylamino)undecanoic acid	Ex. 350 em 500	Wilton 1990, 1991

In some of the spectroscopic assays, synthetic substrates are used. McKellar (1986) determined lipase activity in skimmed milk using β-naphthyl caprylate as substrate. The product, β-naphthol, formed was reacted with fast blue BB. The coloured product was then extracted with ethanol and measured using a spectrophotometer. Kurooka *et al*. (1977) described an assay using 2,3-dimercaptopropan-1-ol tributyrate as substrate and 5,5'dithiobis (2-nitro-benzoic acid) as chromogenic reagent. Richardson *et al*. (1989) used substituted arylethene derivatives as substrate. The hydrolysis products of these compounds are coloured and many of them are water-soluble, making them suitable precursors for chromogenic enzyme substrates. Para-nitrophenyl-esters of various chain-length fatty acids are also used as substrates (Winkler and Stuckmann, 1979). However, these compounds are not suitable for specific lipase assays because they are cleaved by esterases also (Stuer *et al*. 1986).

Some of the spectrophotometric methods can be used in the presence of organic solvents. This is useful during lipase purification with the reversed micelle method (Aires-Barros and Cabral 1991, Prazeres *et al*. 1993a and 1993b). A spectrophotometric assay that makes use of unique spectrophotometric properties of naturally occurring fatty acid, cis-parinaric (PnA) acid, was developed by Rogel *et al*. (1989). Free PnA has UV absorption at 321.2 nm. When PnA is bound to Bovine Serum Albumin (BSA), the peak absorbance shifts to 324.2 nm. The oleic acid released by lipase from triolein in the reaction mixture, displaces PnA from BSA. The reaction is monitored by measuring ratio of OD at 319 and 329 nm.

Fluorescent compounds have also been used for the lipase assay. Some assays make use of the action of lipase on substrates derived from different fatty acyl ester derivatives of

fluorophore 4- methylumbelliferon (Roy 1980, Cooper and Morgan 1981, Stead 1984). Wilton (1990, 1991) has designed a continuous assay procedure in which displacement of the fluorescent fatty acid probe 11-(dansylamino) undecanoic acid from a fatty acid binding protein was measured. The long-chain fatty acids released as a result of lipase activity, displaced the fluorescent fatty acid. It was also possible to use triacylglycerols having one of the alkyl groups substituted with a fluorescent group e.g. pyrenyl (Thuren *et al*. 1987, Negre-Salvayre *et al*. 1991). In an aggregated substrate, the pyrene groups are close to each other and fluoresce at 450 nm. When fatty acids were cleaved, the pyrene group's fluorescence shifted to 400 nm.

An infrared spectroscopy method for measuring lipase-catalyzed hydrolysis of triglycerides in reverse micelles was devised by Walde and Luisi. (1989). Using this method, lipolysis can be monitored by recording the Fourier Transformed Infra Red spectrum of entire reaction mixture. fatty acid esters and the free fatty acids can be quantified based on their molar extinction coefficient and Beer's law.

Chromatography

A lipase assay based on high-performance liquid chromatographic (HPLC) was developed by Maurich *et al.* (1991a, 1991b). This assay involved incubation of β-naphthyl laurate or *p*- nitrophenyl laurate with the enzyme, followed by the quantification of naphthol or *p*-nitro phenol in the assay solution by reversed phase HPLC. Veerraghavan (1990) also has described a HPLC-based assay method using triolein as substrate. The oleic acid formed was estimated by HPLC.

Ruiz and Roudriguez-Fernandez (1982) did quantitative analysis of released fatty acids by thin layer chromatography. The quantification was done using either densitometry or autoradiographic methods using radiolabeled triacylglycerols.

Kashyap *et al*. (1980) have described a method for the estimation of lipoprotein lipase using gas chromatography (GC). The fatty acids released by the action of enzyme on triacylglycerol-rich lipoproteins were extracted using hexane and converted to quaternary ammonium salts in the second extraction step, using trimethyl (α,α,α-trifluro-m-tolyl) ammonium hydroxide. The quaternary ammonium salts were then esterified using methyl acetate and the methyl esters formed were estimated by GC. Poluiaktova *et al*. (1995) have described a method for estimation of lipase activity in skeletal muscles. The methyl esters of oleic acid developed after hydrolysis of triolein were quantified using gas chromatography.

Other assays

It was also possible to use Nuclear magnetic resonance (NMR) for the quantification of lipase activity in biphasic macroemulsions (O'Connor *et al*. 1992). A conductometric method has been described using the short-chain substrate triacetin (Ballot *et al*. 1984).

There are a few immunological assays developed particularly to detect lipase in milk and dairy products (Birkeland *et al*. 1984, Stepaniack *et al*. 1987).

Thus, there are several types of assay methods available for analysis of lipase activity. They differ from each other in the substrates they employ as well as the techniques of end product detection and quantification. Titrimetry using pH-stat, is however, the most commonly used method for estimation of lipase. But, this method is time consuming and

requires larger quantities of substrates. Newer methods, which are more sensitive and easy to operate, therefore, continue to be developed.

Lipase purification

The purification of lipase normally involves several steps depending on the purity desired (Table 1.5). In case of extracellular lipases, normally the first step is the removal of cells by centrifugation or filtration. In case of intracellular lipases, an additional step of cell lysis is required. The crude lipase preparation can then be concentrated by ultrafiltration or can be subjected to optional solvent or salt precipitation. In most of the cases, either ion exchange chromatography or hydrophobic interaction chromatography has been effectively used for further purification of the concentrated enzyme. The final step of gel filtration normally yields a homogenous product.

In most of the lipase purification procedures, the diethylaminoethyl (DEAE) anion exchanger is used (Table 1.5). Lipases show natural affinity for hydrophobic substances, as their substrates are hydrophobic molecules. Hydrophobic interaction chromatography (HIC) is a very popular technique for purification of lipases. HIC is either used as the first or second step in the purification procedure. Bornscheuer *et al*. (1994) have reported single-step purification of lipase from a commercial preparation (*Pseudomonas cepacia* lipase) using phenyl Sepharose. Queiroz *et al*. (1999) and Diogo *et al*. (1999) have purified the *Chromobacterium* lipase using specially designed HIC matrices. In most of the cases, the enzyme was eluted from HIC matrices by decreasing salt gradient, but in a few cases, solvents were also used (Kordel *et al*. 1991). The use of HIC was generally found to result in satisfactory enzyme recovery and fold-purification (Table 1.5). In some

cases, lipases did bind very strongly to HIC matrices and could not be eluted even by use of solvents (Ihara *et al*. 1991).

Affinity matrices with fatty acids as ligands have been used for purification of microbial lipases (Horiuti and Imamura 1977, Kamimura *et al*. 1999). *Staphylococcus epidermidis* lipase has been purified using metal-affinity chromatography (Simons *et al*. 1998). A one-step purification of cloned *Bacillus licheniformis* lipase was reported by Nthangeni *et al*. (2001). The lipase was purified using Ni^{2+}- Nitriloacetic acid affinity chromatography, facilitated by 6 histidine residue tag introduced in the C-terminal region of the cloned enzyme.

Lipases have also been purified by aqueous-two-phase extraction systems. Bompensieri *et al*. (1998) have reported purification of a lipase from *Acinetobacter calcoaceticus* using an aqueous two-phase system. Aires-Barros and Cabral (1991) have reported separation of two lipases from *Chromobacterium viscosum* by aqueous two-phase extraction, using reverse micellar system formed by sodium-di-2-ethylhexylsulfosuccinate (AOT) solution in isooctane. The cultivation conditions and purification of lipases from some bacteria are summarized in Table 1.5.

Properties

Molecular weight

Lipases are reported to be monomeric proteins having molecular weights in the range of 16,000-670,000 Daltons (Table 1.6).

The most interesting feature observed with many lipases is the formation of high molecular weight aggregates.

Table 1.5 **Cultivation and lipase purification from various organisms**

Organism	Cultivation		Assay method	Purification scheme	Fold purifn
	Temp (°C)	Time (h)			
Aeromonas hydrophila MCC-2*	37		Titrimetry using tributyrin as substrate	Culture supernatant → 25-50% $(NH_4)_2SO_4$ ppt →DEAE Sepharose CL-6B → Sephadex G-100	33
Aeromonas sobria	37	48	Spectrophotometriy using *p*NPP as substrate	Culture broth → ultrafiltration → Phenyl sepharose	9.5
Acinetobacter radioresistens CMC-1	30	Till stati-onary phase	Photometry using *p*NPP OR Titrimetry using olive oil	Culture supernatant→ 30-80% $(NH_4)_2SO_4$ ppt→ Mono Q→ Phenyl sepharose CL 4B	44
Bacillus sp.	28	80	Titrimetry using olive oil as substrate	Culture supernatant → $(NH_4)_2SO_4$ ppt → Acrinol treatment→ DEAE-Sephadex A-50→ Toyopearl HW-55F→ Butyl toyopearl 650 M	7762
Bacillus sp. THL027	65	48	Titrimetry using olive oil as substrate	Crude enzyme → Ultrafiltration → Sephadex G-100	2.6
Bacillus sp. H 257	50	12	Enzymatic estimation of glycerol Monolauroylglycerol as substrate	Cell free extract →acetone ppt→ octyl sepharose CL 4B→Q sepharose→ superose 12	3028
Bacillus thermoleovorans ID-1	65	3.5	Spectrophotometry using *p*NPB as substrate	Culture supernatant→ 30-80% $(NH_4)_2SO_4$ ppt →DEAE Sephacel→ Sephacryl S-200	223
*Bacillus licheniformis**	-	-	Spectophotometry using *p*NPP	Crude extract → Ni- NTA affinity chromatography	80
Burkhordia sp. YY62	30	39	Spectrophoto-metry using *p*NP-acetate	Crude extract→ $(NH_4)_2SO_4$ ppt → DEAE-Sephacel → Sepharose CL-6B→DEAE-Sephacel→G2000 SW_{XL}→Phenyl 5PW	74.2

Table 1.5 **Cultivation and lipase purification from various organisms (Continued)**

Organism	Temp (°C)	Time (h)	Assay method	Purification scheme	Fold purifn
*Bacillus thermocatenulatus**	30	5	Photometry Using *p*NPP And pH-stat using tributyrin or triolein	Cell breakage→ Butyl sepharose→ ultrafiltration→ TSK G3000	125
Chromobacterium viscosum	26	96		Culture supernatant→ Barium aceteate treatment→ Palmitoyl cellulose →Concentration at 50 °C→ Acetone ppt→ Sephadex G-75	63.15

Flavobacterium odorum	30	24	spectrophotometry using *p*NPP and colorimetry using olive oil	Culture supernatant → ppt in Three phase partitioning using t-butanol and $(NH_4)_2SO_4$ → Super Q→ Hydroxylapatite	14
Lactobacillus casei subsp. *pseudoplantarum*	30	16	Titrimetiry using tributyrin as substrate	Cell pellet→ French press→ crude extract→ Sephadex G25 → Mono Q HR → Superose 12 → Mono Q HR	54.39
Pseudomonas aeruginosa	30	17	Titrimetry using olive oil and photometry using *p*NPP	Culture supernatant→ ultrafiltration→ CHAPS solublization→ IEF	1264.6
Pseudomonas aeruginosa MB 5001	28	24	PH stat method using olive oil	Cell-free fermentation broth → crude lipase preparation → DEAE-Sepharose CL 6B → Sephacryl S-200	298
Pseudomonas aeruginosa EF2	37	Contin uous culture	Titrimetry using olive oil	Culture supernatant→ concentrated supernatant→ Mono Q → FPLC Superose 6	31
Pseudomonas cepacia	30	72	PH-stat method using olive oil OR photometry using *p*NPP	Culture supernatant → cross flow filtration→isopropanol treatment I → isoporpanol tereatment II→ liquid/liquid extraction → phenyl sepharose	400

Table 1.5 **Cultivation and lipase purification from various organisms (Continued)**

Pseudomonas fragi			Titrimetry using lard or tributyrin	Culture supernatant→ ultrafiltration→ $(NH_4)_2SO_4$ pptn→ DEAE Sephadex	103
Pseudomonas fragi	25	96-120	Titrimetry using coconut oil	Centrifuged seudoa broth→ $(NH_4)_2SO_4$ pptn→ Dialysis → Acetone fractionation → Sephadex G-200 → DEAE cellulose	99.79
Pseudomonas putida 3SK	30	16	pH stat method using olive oil	Crude extract → ultrfiltration → DEAE Sephadex A- 50 → Sephadex G-100	20.9
Pseudomonas fluorescens 2D	10	96	PH stat method using tributyrin	Culture supernatant→ Ultrafiltration→ $(NH_4)_2SO_4$ pptn→ Phenyl sepharose CL-4B→Sephadex G 75	25
Pseudomonas fluorescens AK102.	30	72	Titrimetry using olive oil	Crude enzyme→ $(NH_4)_2SO_4$ pptn→ DEAE Toyopearl 650 M→ Phenyl Toyopearl 650 M	6.1
Pseudomonas fluorescens	25		Titrymetry using olive oil	Culture supernatant→ concentrate→ $(NH_4)_2SO_4$ pptn→ DEAE-cellulose→ Octyl sepharose CL-4B	3390

Pseudomonas fluorescens No. 33	17	168	Photometry- UHT milk as substrate and phenol red as colour reagent	Culture supernatant→ HCl precipitation (pH 4.8) → Octyl-sepharose CL-4B→ DEAE-Toyopearl 650 S→ Toyppearl HW-50S	4270
Pseudomonas glumae	-	-	Photometry using *p*NPP OR Titrimetry on olive oil	Crude enzyme preparation form Unilever→ DEAE-triacryl-M-phenyl sepharose→ alcohol-acetone precipitation→ DEAE cellulose	7.2

Pseudomonas tolassi	30	36	Photometry using β-naphthyl caprylate	Cell-free supernatant→ultrafiltration→ DEAE- cellulose DE 52→ Sephadex G-150	1000	9	1
Staphylococcus aureus	37	Stationary phase	Radioactivity of fatty acids released from triacylglycerol	Culture supernatant → Hollow fiber concentrate → $(NH_4)_2SO_4$ pptn supernatant → Octyl sepharose Cl-4B	957	30	36
Staphylococcus hyicus	-	-	Titrimetry using tributyrin	Culture supernatant → $(NH_4)_2SO_4$ ppt → Sephadex G-100/G25 → Proteolytic breakdown→ DEAE pH 8 → DEAE pH 6.5	13.1	-	50
Propionibacterium acnes	37	144-168	Titrimetry using triolein	Culture supernatant → ultrafiltration→ Sephadex G100→ CM sephadex C-50.	4800	-	18

(-) indicates no data available, * indicates product of cloned gene. For references see next page

References for Table 1.5 and 1.6

1. Baral and Fox 1997	18. Ingham *et al*. 1981	36. Rollof and Nomark 1987
2. Bozoglu *et al*. 1984	19. Jaeger *et al.* 1994	37. Rosenstein and Gotz 2000
3. Castellar *et al.* 1997	20. Kim *et al*. 1998	38. Rúa *et al*. 1997
4. Chander *et al*. 1979	21. Kojima *et al.* 1994	39. Schmidt-Dannert *et al.* 1994
5. Chartrain *et al*. 1993	22. Kotsuka *et al*. 1996	40. Schmidt-Dannert *et al.* 1996
6. Choo *et al*. 1998	23. Kumura *et al*. 1993	41. Schmidt-Dannert *et al*. 1997
7. Chuang *et al*. 1997	24. Labuschagne *et al.* 1997	42. Shabtai *and* Daya-Mishne. 1992
8. Deveer *et al*. 1991	25. Lee and Lee 1989	43. Stuer *et al*. 1986
9. Dharamsthiti and Luchai 1999	26. Lee and Rhee 1993	44. Sugihara *et al*. 1991
10. Dring and Fox 1983	27. Lee *et al*. 1999	45. Swaisgood and Bozoglu 1984
11. Dünhaupt *et al*. 1992	28. Lin *et al*. 1996	46. Sztajer *et al*. 1991b
12. Gilbert *et al*. 1991b	29. Lotrakul and Dharmasthiti 1997b	47. Taipa *et al.*1995
13. Gobbettti *et al*. 1996	30. Lu and Liska 1969	48. Talon *et al*. 1995
14. Hong and Chang 1998	31. Makhzoum *et al*. 1996	49. Tyski *et al*. 1983
15. Horiuti and Imamura 1977	32. Mencher and Alford 1967	50. van Oort *et al*. 1989
16. Iizumi *et al*. 1990	33. Nawani *et al*. 1998	51. Wang *et al.* 1995
17. Imamura and Kitaura 2000	34. Nishio *et al*. 1987	52. Yamamato and Fujiwara 1988
	35. Nthangeni *et al*. 2001	53. Yeo *et al.* 1998

Aggregate formation has been reported in lipases of both Gram-positive and Gram-negative bacteria. Among Gram-positive bacteria, aggregation has been reported with crude as well as purified *Staphylococcus aureus* lipase (Kotting *et al.* 1983). The aggregation was also observed with purified lipase produced by *Bacillus subtilis*, *Bacillus thermocatenulatus* and *Bacillus* sp. THL027 (Lesuisse *et al*. 1993, Rúa *et al*. 1997, Dharmsthiti and Luchai 1999).

In Gram-negative bacteria, it has been well documented for the members of genus *Pseudomonas*. Aggregates were reported to be formed of either pure *Pseudomonas* lipases (Mencher and Alford 1967, Finkelstein *et al.* 1970, Dring and Fox 1983, Fox and Stepaniack 1983, Gilbert *et al*. 1991b) or of the lipases associated with lipophilic molecules (Mencher and Alford 1967, Lu and Liska 1969, Steur *et al*. 1986 Kordel *et al*.

1991). The *Pseudomonas aeruginosa* PAC 1R was shown to form lipase-lipopolysaccharide aggregates (Steur *et al.* 1986). During purification of lipases, such aggregates were dissociated by treatment with detergents like Triton X-100 or CHAPS (Chartrain *et al*. 1993, Steur *et al.* 1986) or solvents like isopropanol (Dünhaupt *et al.* 1992).

Effect of metal ions, EDTA and bile salts

Most lipases are reported to be inhibited by metal ions like Hg^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+} and Cu^{2+} (Nishio *et al*. 1987, Yamamato and Fujiwara 1988, Izumi *et al*. 1990, Sugihara *et al*. 1991, Lee and Rhee 1993, Chartrain *et al*. 1993, Kumura *et al*. 1993, Kojima *et al*. 1994, Yeo *et al*. 1998). On the other hand, Ca^{2+} was reported to stimulate activity of many lipases (Gilbert *et al*. 1991b, Lee and Rhee 1993, Chartrain *et al*. 1993). EDTA (Ethylene diamine tetra-acetic acid) has been reported to inhibit activity of a few lipases (van Oort *et al*. 1989, Baral and Fox 1997, Sharon *et al*. 1998). The inactivation of some of the lipases by EDTA was overcome by treatment with divalent metal ions like Ca^{2+} (van Oort *et al*. 1989, Sharon *et al*. 1998, Baral and Fox 1997) and in some cases by Sr^{2+} (van Oort *et al*. 1989) or less efficiently by Mg^{2+} and Ba^{2+} (Baral and Fox 1997) indicating that the enzymes might have a calcium or divalent cation-binding site. Bacterial lipases have been reported to have calcium-binding site that is important for maintaining their structure (Noble *et al*. 1994, Lang *et al*. 1996, Kim *et al*. 1997). Shibata *et al*. (1998) have reported calcium-dependent reactivation of lipase by it's modular protein LipB.

Table 1.6 Properties of some lipases

Organism	Molecular weight (kDa)	Optimum		Stability		pI
		Temp (°C)	pH	Temp (% residual activity)	pH	
Aeromonas hydrophila MCC-2*	80	37	7.5-8.0	Stable below 50 °C 30 min at 55 °C: 20%	-	10.36
Aeromonas sobria LP004	97	45	6.0	Stable below 40 °C	6.5-10	-
Bacillus sp.	24[a]	75	6-8	10 min at 60 °C: 100%	7-10	4.66
Bacillus sp.	45	60	8	30 min at 70 °C: 50%	-	-
Bacillus sp.	22[a]	60	5.5-7.2	30 min at 65 °C: 100%	5-11.5	5.1
Bacillus sp. THL027	69	70	7.5	1 h at 75 °C: 80%	7.5	-
Bacilus sp. (ATCC53841)	65	60	9.5	30 min at 75 °C: 100%	5-10.5	5.15
*Bacillus licheniformis**	19.2	50-60	10-11.5	30 min at 45 °C: 90%	7-12	9.46
Bacillus stearothermophilus L1*	-	60-65	9-10	-	-	-
Bacillus thermocatenulatus	16	60-70	7.5-8	-	-	-
*Bacillus thermocatenulatus**	32	60-70	8-9	40	9-11	7.2
*Bacillus thermocatenulatus**	40	60	8	50	9-11	7.2
Bacillus thermoleovorans ID-1	34	75	7.5	30 min at 70 °C: 50%	-	-
Burkholderia sp. YY62	40	28	7	-	-	-
Chromobacterium viscosum	33[a]	50	9	t1/2 at 60 °C =0.75h	-	7.1
Flavobacterium odorum	32.5	50-65	9-10.5	1h at 60 °C: 50%	-	-
Lactobacillus plantarum 2739	65	35	7.5	D 65 °C- 18.6 min	-	-
Propionibacterium acnes	46[b]	-	6.8	-	5-6	
Pseudomonas aereuginosa YS-7	40		7	30 days at 20-55 °C: 100%	-	-
Pseudomonas aereuginosa MB 5001	29	55	8	1 h at 60 °C: 10%	7.5-8.5	-

Table 1.6 Properties of some lipases (Continued)

Pseudomonas aereuginosa EF2	29 [a]	50	9	17.5 min at 60 °C: 50 %	-	4.9
Pseudomonas fragi 22.39 B	33 [a]	65-70	9	3 d at 50 °C: 50 %	4-11	6.9
Pseudomonas fluorescens	450 [b]	55	8.0	30 min at 70 °C:60%	7-10	-
Pseudomoanas fluroscens MC50	55 [a]	30-40	8-9	513.3 s at 50 °C: 50%	6-9	-
Pseudomonas fluorescens No. 33	52 [a]	40	7.5-8.5	10 min at 30 °C: 60%	5.5-7.5	-
Pseudomonas fluorescens AFT29	16 [a]	22	7.0	1min at 50 °C: 75% 1 min at 70 °C :0%	6.2-7.6	-
Pseudomonas sp. KWI-56	33 [a]	60	5.5-7.0	24 h at 60°C:96%	4.0-10.0	5
Pseudomonas sp. f-B-24	30 [a]	60	7.0	30 min at 40 °C: 76%	9-10	-
Pseudomonas fluorescens 2D	42 [a]	40	8.5	D value (90% inactivation) at 120-140 °C = 836-207s	-	4.6-4.9
Pseudomonas fluorescens	33 [a]	55	8-10	Below 50 °C	4-10	-
Pseudomonas glumae	33	50	9.0	50 °C	At 60 °C t1/2 -0.27h	-
Pseudomonas pseudoalkaligenes F-111	32 [a]	40	6-10	Stable up to 70 °C	6-10	7.3
Pseudomonas putida 3SK	45	37	8-9	2h at 75 °C: 40%	4-11	-
Pseudomonas solanacearum SD 709	32 [a]	85	6.5-9.5	-	-	8.8
Pseudomonas tolassii	670 [b]	35	7	30 min at 50 °C: 80%	5-6.5	-
Pseudomonas sp.*	33 [a]	45	8	1h at 50 °C: 47%	6-9	-
Staphyllcoccus sp.	45 and 300	55	8	-	5-9	-
Staphyllococcus aureus	76	-	6.5	-	Up to 10	-
Staphyllococcus epidermidis	77	-	6.5	-	Up to 10	
Staphyllococcus warneri	90	25	9	-	5-9	-
Streptococcus faecalis	20.9	40	7.5	Inactivated at 90 °C	6.0-8.0	3.6

Molecular weight estimated by a: sodium dodesyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), b: gel permeation chromatography (GPC), * indicates product of cloned gene. An (-) indicates no data available, for references see page 32.

Bile salts were found to inhibit the lipase produced by *Pseudomonas fragi* 22.39 B (Watanabe *et al*. 1977). Contradictory to this report, bile salts were found to stimulate lipase activity of *Pseudomonas* sp. and *Pseudomonas aeruginosa* (Nisho *et al*. 1987, Yamamato and Fujiwara 1988, Chartrain *et al*. 1993).

Substrate specificity

a. Positional specificity

Many lipases are known to have 1-3 positional specificity (Nishio *et al*. 1987, Gilbert *et al*.1991b, Sugihara *et al*. 1991, Lesuisse *et al*. 1993, Schmidt-Dannert *et al*. 1994, Kojima *et al*. 1994, Yamamato and Fujiwara 1995, Dharmsthiti and Luchai 1999). A lipase from *Pseudomonas aeruginosa* PAC1R was found to have an absolute sterioselectivity towards sn-1 position of the triglyceride substrate trioctanoin whereas *Candida antarctica* B was found to be selective for position sn-3 (Rogalska *et al.* 1993). In the same study, Rogalska *et al*. had found that only *Candida antarctica* A lipase could preferentially cleave sn-2 ester bond.

Imamura and Kitaura (2000) have reported production of monoacylglycerol lipase from *Bacillus* sp. H257. The lipase is reported to act specifically on monoacylglycerols with highest activity with 1-monolauryl glycerol.

b. Fatty acid specificity

Many lipases are reported to be specific to esters of short chain fatty acids in the range C4 to C12 (Izumi *et al*. 1990, Choo *et al*. 1998, Dharmsthiti and Luchai. 1999). Lipases from *Pseudomoans* sp. strain ATCC 21808 and *Pseudomonas aeruginosa* MB5001 have been reported to show higher activities towards more unsaturated C18-substrates (Kordel

et al. 1991, Chartrain *et al*. 1993). Many bacterial lipases are also reported to have broad substrate specificities (Nishio *et al*. 1987, Sugiura *et al*. 1991, Jorgensen *et al*. 1991). Staphylococcal lipases were found to show higher preference towards substrate molecules that had butyric acid esterified with glycerol, *p*-nitro phenol or umbelliferon (Rosenstein and Götz 2000). Triacetyl or tripentanyl glycerol were found to be very poor substrates for these enzymes. The lipase produced by *Staphylococcus hyicus* differed from other staphylococcal lipases as it hydrolyzed esters of different fatty acids with almost equal preference. It was also different from all other bacterial lipases as it could degrade phospholipids as well as lysophospholipids along with triacylglycerols.

Biochemical Classification of the bacterial lipases

Arpigny and Jaeger (1999) have classified 47 different bacterial lipases into six families on the basis of amino acid sequence homology (Table 1.7). They have also compared the data available from three-dimensional structures of some enzymes and biochemical properties of different lipases reported by different researchers.

Family I

Family I comprised of total 22 members subgrouped into six subfamilies. *Pseudomonas* lipases were classified to subfamilies I.1 and I.2 on the basis of amino acid sequence comparison. Lipases from *Vibrio cholerae*, *Acinetobacter calcoaceticus*, *Pseudomonas wisconsinensis* and *Proteus vulgaris* had molecular masses in the range 30–32 kDa and displayed a higher sequence similarity to the *Pseudomonas aeruginosa* lipase. Ogierman *et al*. (1997) have shown that the gene coding for 33 kDa *Vibrio cholerae* O1 lipase was highly homologous to lipase gene of *Pseudomonas aeruginosa*.

Table 1.7 Classification of lipases

Subfamily		Enzyme-producing strain	Similarity (%)		Properties
			Family	Sub-family	
I	1	*Pseudomonas aeruginosa**	100		True lipases
		Pseudomonas fluorescens C9	95		
		Vibrio cholerae	57		
		Acinetobacter calcoaceticus	43		
		Pseudomonas fragi	40		
		Pseudomonas wisconsinensis	39		
		Proteus vulgaris	38		
	2	*Burkholderia glumae**	35	100	
		*Chromobacterium viscosum**	35	100	
		*Burkholderia cepacia**	33	78	
		Pseudomonas luteola	33	77	
	3	*Pseudomonas fluorescens* SIK W1	14	100	
		Serratia marcescens	15	51	
	4	*Bacillus subtilis*	16	100	
		Bacillus pumilus	13	80	
	5	*Bacillus stearothermophilus*	15	100	
		Bacillus thermocatenulatus	14	94	
		Staphylococcus hyicus	15	29	Phospholipase
		Staphylococcus aureus	14	28	
		Staphylococcus epidermidis	13	26	
	6	*Propionibacterium acnes*	14	100	
		Streptomyces cinnamoneus	14	50	
II (GDSL)		*Aeromonas hydrophila*	100		Secreted acyltransferase
		*Streptomyces scabies**	36		Secreted esterase
		Pseudomonas aeruginosa	35		Outer membrane-bound esterase
		Salmonella typhimurium	28		Outer membrane - bound esterase
		Photorhabdus luminescens	28		Secreted esterase
III		*Streptomyce exfoliatus**	100		Extracellular lipase
		Streptomyces albus	82		Extracellular lipase
		Moraxella sp.	33		Extracellular esterase

IV (HSL)	*Alicyclobacillus acidocaldarius*	100		Esterase
	Pseudomonas sp. B11-1	54		Lipase
	Archaeoglobus fulgidus	48		Carboxylesterase
	Alcaligenes eutrophus	40		Putative lipase
	Escherichia coli	36		Carboxylesterase
	Moraxella sp.	25		Extracellular esterase 2
V	*Pseudomonas oleovorans*	100		PHA-(polyhydroxyalkanoate) depolymerase
	Haemophilus influenzae	41		Putative esterase
	Psychrobacter immobilis	34		Extracellular esterase
	Moraxella sp.	34		Extracellular esterase 3
	Sulfolobus acidocaldarius	32		Esterase
	Acetobacter pasteurianus	20		Esterase
VI	*Synechocystis* sp.	100		Carboxylesterases
	Spirulina platensis	50		
	*Pseudomonas fluorescens**	24		
	Rickettsia prowazekii	20		
	Chlamydia trachomatis	16		
VII	*Arthrobacter oxydans*	100		Carbamate hydrolase
	Bacillus subtilis	48		p-Nitrobenzyl esterase
	Streptomyces coelicolor	45		Putative carboxylesterase
VIII	*Arthrobacter globiformis*			Stereoselective esterase
	Streptomyces chrysomallus	43		Cell-bound esterase
	Pseudomonas fluorescens SIK W1	40		Esterase III

* Lipolytic enzyme with known 3D structure.

Enzymes from subfamily I.2 were characterized by a slightly larger molecular mass (33 kDa) owing to an insertion in the amino acid sequence forming an anti-parallel double-strand at the surface of the molecule. The *Pseudomonas luteola* lipase was found to possess this insertion (residues 254–272 in the preprotein) and showed a high similarity to the *Burkholderia* enzymes, notably in this region. The expression in an active form of lipases belonging to subfamilies I.1 and I.2 was found to depend on a chaperone protein named lipase-specific foldase ('Lif'). However, such specific helper proteins have not

been described for *Pseudomonas fluorescens* C9, *Pseudomonas fragi, Pseudomonas vulgaris* and *Pseudomonas luteola*. Both subfamilies also shared important structural features. Apart from the residues forming the catalytic triad, two aspartic residues involved in the Ca2+-binding site described in the crystal structures were found at homologous positions in all sequences. Two cysteine residues forming a disulphide bridge were conserved in a majority of sequences. Because the residues involved in the formation of both the Ca2+-binding site and the disulphide bridge are located in the vicinity of the catalytic His and Asp residues, they were believed to be important in the stabilization of the active center of these enzymes. The two Cys residues of the *Pseudomonas fluorescens* C9 lipase were found not to lie at equivalent positions and no information was available on the possible existence of a disulphide bridge in this molecule. *Pseudomonas fragi* and *Pseudomonas vulgaris* lipases contained only one Cys residue.

Subfamily I.3 contained enzymes from at least two distinct species: *Pseudomonas fluorescens* and *Serratia marcescens*. These lipases had in common a higher molecular mass than lipases from subfamilies I.1 and I.2 (*Pseudomonas fluorescens*, 50 kDa; *S. marcescens*, 65 kDa) and the absence of an N-terminal signal peptide and of Cys residues. The secretion of these enzymes was found to occur in one step through a three-component ATP-binding-cassette transporter system. They showed regioselectivity towards 1-3 positions of triglycerides. Akatsuka *et al.* (1994, 1995) showed that *Serratia marcescens* lipase is 62 kDa protein lacking the typical N-terminal signal sequence. The secretion of this lipase required three other proteins. The proteins were identified to be products of genes LipB, LipC and LipD. The LipB and LipC were identified to be inner

membrane proteins and LipD was an outer membrane protein involved in transport of lipase across the membrane.

Lipases from Gram-positive organisms

The various lipases produced by Bacillus sp. were found to have an alanine residue replacing the first glycine in the conserved pentapeptide: Ala-Xaa-Ser-Xaa-Gly. The lipases from the two mesophilic strains B. subtilis and B. pumilus were different from other Bacillus lipases. They were the smallest true lipases known (approximate molecular mass 20 kDa) and shared very little sequence similarity (approximately 15%) with the other lipases. They were included in family I.4.

The high molecular weight Bacillus lipases and Staphylococcus lipases were included in subfamily I.5.B. thermocatenulatus and B. stearothermophilus produced lipases with similar properties. Their molecular mass was approx. 45 kDa and they displayed maximal activity around pH 9.0 and 65 °C. Staphylococcal lipases were enzymes with larger molecular mass (approx. 75 kDa) that were secreted as precursors and cleaved in the extracellular medium by a specific protease, yielding a mature protein of approximately 400 residues. The propeptide (207–267 residues) presumably acted as an intramolecular chaperone and facilitated the translocation of the lipase across the cell membrane. Rollof and Nomark (1992) have shown that Staphylococcus aureus lipase was secreted as 82 kDa propeptide, which was processed by a metallocysteine protease to yield mature 46 kDa lipase. The Staphylococcus epidermidis lipase was preproprotein with molecular mass 77 kDa, and was processed to yield a 43kDa mature protein (Farrell et al. 1993). Interestingly, the lipase from Staphylococcus hyicus also displayed a remarkable phospholipase activity, which was unique, among true lipases.

Nthangeni *et al*. (2001) recently suggested modification in classification of Arpigny and Jaeger. They classified *Bacillus* lipases in two subfamilies, based on amino acid analysis and biochemical characteristics. The subfamily I.4 included lipases with low molecular mass in the range 19-20 kDa from *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus*. The lipases from *Bacillus themocatenulatus, Bacillus thermoleovorans*, and *Bacillus stearothermophilus* were included in the subfamily I.5 and they had molecular mass around 43 kDa. They suggested that the Staphylococcal lipases should be included in separate family of their own.

Other lipases

The lipases from *Propionibacterium acnes* (339 residues) and *from Streptomyces cinnamoneus* (275 residues) were found to have a molecular mass of 50 kDa (Sommer *et al.* 1997) and showed significant similarity to each other (39% identity, 50% similarity). The central region of these proteins (residues 50–150) was approximately 50% similar to the lipases from *Bacillus subtilis* and from subfamily I.4. No similarity was found between the *Streptomyces cinnamoneus* lipase and other *Streptomyces* lipases known so far.

Family II (The GDSL family)

The enzymes grouped in family II did not exhibit the conventional penta-peptide Gly-Xaa-Ser-Xaa-Gly but rather displayed a Gly-Asp-Ser-(Leu) [GDS(L)] motif containing the active-site serine residue. In these proteins, this important residue was found to lie much closer to the N-terminus than in other lipolytic enzymes.

Family III

This family of lipases was identified primarily by Cruz *et al*. (1994) and mentioned by Wei *et al*. (1998) who solved the 3D structure of the *Streptomyces exfoliatus* (M11) lipase. This enzyme displays the canonical fold of α/β -hydrolases and contains a typical catalytic triad.

Family IV-The hormone-sensitive lipase (HSL) family

A number of bacterial enzymes (family IV) displayed a striking amino acid sequence similarity to the mammalian HSL. They were included in family IV.

Family V

Like proteins in the HSL family, enzymes grouped in family V originated from mesophilic bacteria (*Pseudomonas oleovorans*, *Haemophilus influenzae, Acetobacter pasteurianus*) as well as from cold-adapted (*Moraxella* sp., *Psychrobacter immobilis*) or heat-adapted (*Sulfolobus acidocaldarius*) organisms.

Family VI

With a molecular mass in the range 23–26 kDa, the enzymes in this family were among the smallest esterases known.

Family VII

A number of rather large bacterial esterases (55 kDa) share significant amino acid sequence homology (30% identity, 40% similarity) with eukaryotic acetylcholine esterases and intestine/liver carboxylesterases. They were classified in family VII.

Applications of lipases

Lipases possess the unique feature of acting at an interface between the aqueous and non-aqueous (*i.e.* organic) phase; this feature distinguishes them from esterases. Lipase activity generally depends on the available surface area. Lipases are the most versatile biocatalysts and they bring about a range of bioconversion reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. Lipases act under extremely mild conditions (Sharon *et al*. 1999). They can be used in a variety of organic solvents and often show selectivity for a specific reaction type.

Lipases are an excellent alternative to many classical organic techniques in the selective transformation of complex molecules. They possess many features that favor their use as an excellent biocatalyst. They impart specificity to a reaction, in which a chemical process is typically more non-specific. In addition, the use of enzyme can decrease side reactions and simplify post-reaction separation problems (Pandey *et al*. 1999). Lipase-catalyzed processes are reported to offer cost-effectiveness too, in comparison with traditional downstream processing in which energy consumption and toxic by-products might often pose problem (Jansen, *et al.* 1996). The alkaline thermophilc lipases find application in detergent industry Many fatty food stains and human sebum contain triglycerides which are hydrolyzed by lipases to produce fatty acids, monoglycerides and diglyceries, which are easier to remove than unhydrolyzed Triglycerides (Fuji *et al*. 1986). Table 1.8 enumerates a few of the most significant industrial applications of microbial lipases.

Table 1.8 Applications of lipases

Source	Application
Acinetobacter sp. *A. calcoaceticus*	Waste management -Heating oil/furnace oil, removal of fats, oils and greases (Mrin *et al*. 1995, wakelin and Forster 1997)
Arthrobacter sp.	Pesticide Pyrethroids (Danda *et al.* 1991) and insecticide synthesis (Mitsuda *et al.* 1988)
Bacillus subtilis	Biomedical applications- Cephalosporin derivative (Usher *et al.* 1995)
Chromobacterium viscosum	Biomedical applications- precursors of Vitamin D (Fernandez *et al.* 1995), Verlukast - synthesis of an LTD4 antagonist (Hughes *et al.* 1993)
Pseudomonas sp.	Biomedical applications- Synthesis of (-)-Indolmycin (Akita *et al.* 1997), Pesticide syntheisis- Triazole/morpholine (Bianchi *et al.* 1992a) Lipase as detergent additive (Yokoe and Mase 1988, Mukoyama and Umehara 1989)
Pseudomonas cepacia	Biomedical applications- Syntheisis of Rapamycin-42 (Adamezyk *et al*. 1994) Pesticide synthesis- Nikkomycin-B (Akita *et al*. 1995), Pyrenophorin (Sugai *et al*. 1995), fenpropimorph (Avdagic *et al.* 1994), racemic morpholine (Bianchi *et al.* 1992b), cyanohydrin acetate (Inagaki *et al.* 1992), pyrethroids (Inagaki *et al*. 1992)
Pseudomonas fluorescens	Biomedical applications- Hydantoins (Yokomatsu *et al.* 1995), Lamivudine (3TC) (Milton *et al*. 1995), racemic 2-tetradecyloxirane-carboxylate (Jimenez *et al*. 1997) Pesticide synthesis- Tetraconazole (Bianchi *et al*. 1990)
Staphylococcus warneri Staphylococcus xylosus	Food industry- production of flavour esters (Talon *et al*. 1996)
Streptomyces sp.	Pancreatic lipase inhibitor- Panclicins production (Yoshinari *et al.* 1994)

Lipases in food industry

Lipases have become an integral part of the modern food industry. The use of enzymes to improve the traditional chemical processes of food manufacture has been developed in the past few years.

Yoneda *et al*. (1996) have patented a process on *Pseudomonas* lipase, which was claimed to be useful in, for example, food processing and oil manufacture. Alcoholysis of cod

liver oil for the production of omega- 3 polyunsaturated fatty acids was investigated by using *Pseudomonas* lipase (Zuyi and Ward 1993). A few bacteria produce flavour esters and find use in cheese industry. The production of flavour esters by lipases of *Staphylococcus warneri* and *Staphylococcus xylosus* has also been described by Talon *et al.* (1996). Synthesis of fatty acid esters by a recombinant *Staphylococcus epidermidis* lipase has been described by Chang *et al.* (2001). *Chromobacterium viscosum* lipase was shown to have good potential for the instant generation of aroma and flavour compounds and could be stored at least for one month. In this case, the lipase activity was immediately regenerated on dehydration (Carlile *et al*. 1996).

Lipases in biomedical application

Because of their excellent capability for specific regioselective reactions in a variety of organic solvents with broad substrate recognition, lipases have emerged as an important biocatalyst in biomedical applications. Recently, Parmar *et al*. (1996) have reviewed a variety of substrates accepted by hydrolytic enzymes, including lipases, to produce compounds in high enantiomeric excess, which can be used as chiral building blocks for the synthesis of compounds of pharmaceutical interest.

Conventional chemical synthesis of drugs containing a chiral center generally yields equal mixtures of enantiomers. During the past decade, many studies have shown that racemic drugs usually have the desired therapeutic activity residing mainly in one of the enantiomers and the other enantiomer might interact with different receptor sites, which can cause unwanted side effects (Pandey *et al*. 1999).

Akita *et al*. (1997) have performed enzymic hydrolysis in organic solvents for the kinetic resolution of water-insoluble acetyloxy esters using immobilized *Pseudomonas* sp. lipase

to produce chiral intermediates for the synthesis the antibiotic (-)-indolmycin. A method was developed by Jimenez *et al*. (1997) to synthesize methyl (R)- and (S)-2-tetradecyloxiranecarboxylate through sequential kinetic resolution catalysed by *Pseudomopnas* sp. lipase. Both the enantiomers are a potent anti-diabetic and antioxidant agent.

Lipases in pesticides

A variety of pesticides (insecticides, herbicides, fungicides or their precursors) made with the applications of lipases are currently in the use (Pandey *et al.* 1999). The most important application of lipases has been in the organic synthesis of pesticides for the production of optically active compound (Reddy 1992). Generally, these compounds were produced through the resolution of racemic mixtures of alcohol or carboxylic esters; stereospecific synthesis reactions were also employed. Akita *et al*. (1995) described a highly stereospecific synthesis of the versatile chiral synthon possessing two stereogenic centres, which was subsequently converted into a homochiral intermediate for the synthesis of the biologically active potent pesticide nikkomycin-B. Mitsuda *et al*. (1990) have reported use of *Achromobacter* lipase for enantioselective hydrolysis of the acetic acid ester of racemic a-cyano-3-phenoxybenzyl alcohol (CPBA) for the production of (S)-CPBA, an active insecticidal stereoisomer.

Lipases in detergents

Lipases were generally added to the detergents primarily in combination with proteases and cellulases. However, other enzymes such as amylases, peroxidases and oxidases are also reported to be added in detergent preparations (Kottwitz *et al.* 1994).

Removal of oil/fatty deposits by lipase is attractive owing to its suitability under milder washing conditions. To be a suitable additive in detergents, lipases should be both thermostable as well as alkalophilic and capable of functioning in the presence of the various components of washing powder formulations (Jaeger *et al.* 1994).

Pseudomonas lipase preparations have been used for preparation of washing powder formulations. *Pseudomonas medocina* (Lumafast®) and *Pseudomonas alcaligenes* (Lipomax®) lipases have been manufactured by Genencor international USA, as detergent additive (Jaeger *et al*. 1994, Reetz and Jaeger 1998). The Novo group has reported a highly alkaline, positionally non-specific lipase, from a strain of *Streptomyces* sp. that was useful in laundry and dish-washing detergents as well as industrial cleaners (Pandey *et al*. 1999). Several lipase-producing organisms and their manufacturing processes are patented for preparation of detergent lipases (Yokoe and Mase 1988, Mukoyama and Umehara 1989, Holmes 1993, Ishida *et* al. 1995, Lawler and Smith 2000).

Lipases in the leather industry

Leather processing involves the removal of subcutaneous fat, de-hairing and stuffing. Tanning processes are usually performed in an alkaline environment, so alkalophilic microbes ought to be better for exploration. Many *Bacillus* sp. strains, which grew successfully under highly alkaline conditions, were found to be useful in leather processing (Haalck *et al*. 1992).

Lipases in environmental management

Lipases have been used for the degradation of wastewater contaminants such as olive oil from oil mills (Vitolo *et al*. 1998). The treatment process involved the cultivation of

lipase-producing microbial strains in the effluents. Wakelin and Forster (1997) investigated the microbial treatment of waste from fast-food restaurants for the removal of fats, oils and greases. They cultivated pure and mixed microbial flora known to produce lipases and other enzymes. *Acinetobacter* sp. was the most effective of the pure cultures, typically degrading 60–65% of the fatty material.

Lipases in the cosmetics and perfume industry

Monoacylglycerols and diacylglycerols, prepared by the lipase-catalysed esterification of glycerol, are useful as surfactants in the cosmetics (Pandey *et al*. 1999). The monoacylglycerol synthesis has been reported using *Pseudomonas* sp. LP7315 moacylglycerol lipase. Izumi *et al*. (1997) performed the transesterification of 3,7-dimethyl-4,7-octadien-1-ol with lipases from various microbial sources to prepare rose oxide, which is an important fragrance ingredient in the perfume industry.

Scope of this thesis

Alkaline thermophilic lipases find application in laundry detergents for removal triglyceride stains of human sebum and fatty food, which are difficult to remove under normal washing conditions. Most of the lipase preparations included in laundry detergents sold in India are imported and are responsible for high market price of such detergents. Therefore development of indigenous technology for alkaline lipase production is necessary.

India is the largest producer of castor seed in the world, contributing 750,000 tonnes annually, and accounting for over 60% of the entire global production. Castor Oil is natural oil obtained from the seed of the castor plant. It is unique among all fats and oils in that it is the only commercially important oil composed of approximately 90% of a hydroxy, unsaturated C18 fatty acid-ricinoleic acid. Owing to the presence of two reactive functional groups, ricinoleic acid finds application in a number of cosmetic and food industries. It is also used to synthesize water-soluble lubricants, gelatinizer and poly vinyl chloride (PVC) stabilizers. At present, the industrial hydrolysis of fat and oil is usually done at high temperature and high pressure, using a process called Colgate-Emery process. Although the process is simple, it has several drawbacks like large energy consumption, induction of polymerization, colour development and requirement of subsequent purification by distillation. On the other hand, hydrolysis of oils and fats using lipases is advantageous due to low energy consumption, the colourless product and decreased quantities of wastes as compared with Colgate-Emery process. Therefore lipase that can cleave castor oil efficiently has great industrial potential.

In view of these needs, the work was carried out on alkaline thermophilic lipase of microbial origin. The objectives for this work were as follows-

Objectives

1. Isolation of an organism that can produce an alkaline thermophilic lipase, able to hydrolyze castor oil.
2. Optimization of media for lipase production
3. Purification to homogeneity and characterization of this lipase
4. Optimization of process parameters for downstream processing of lipase using novel technique – foam separation.
5. Study of cell growth and lipase production in fermenters.
6. Formulation of product, which can be used commercially as detergent additive.
7. Immobilization of lipase and optimization of conditions for use of this lipase for hydrolysis of castor oil to produce ricinoleic acid.

The work is presented in following chapters

Chapter 2 describes design of new method for estimation of lipase activity using gas chromatography. The assay used tributyrin as substrate and volatile end product butyric acid was estimated on GC by internal standard method. The assay conditions were optimized with respect to emulsifier, substrate concentration, sonication time, and incubation time. The effect of pH and temperature on substrate stability was studied. This assay was routinely used in the work.

Chapter 3 describes isolation of an alkaline lipase-producing *Pseudomonas* sp. isolated from soil and its taxonomical identification. The chapter further describes optimization of production of lipase from this organism in shake flasks using a statistical experimental design. The effect of various components in the basal medium like carbon, nitrogen and mineral source was studied and the optimum concentration of selected media components was determined using factorial design. Two-level factorial design was constructed in four variables *viz*. ammonium di-hydrogen orthophosphate, groundnut oil, calcium chloride and magnesium sulfate concentrations. The cell growth and lipase production was studied in shake flask in the optimized medium. The effect of inducers like fatty acids, glycerol and alcohols on growth and lipase production was studied in shake flask. The effect of feeding oil and oleic acid was also studied in shake flask.

In **Chapter 4**, the lipase enzyme from this strain was purified to homogeneity. The purified enzyme was characterized in terms of its molecular weight, isoelectric point, pH optimum and stability, temperature optimum and stability, effect of metal ions, substrate specificity and regioselectivity.

Chapter 5 describes the optimization of process parameters for selective enrichment of the lipase from fermentation broth of *Pseudomonas fluorescens* using foam separation. The process parameters were first optimized for batch process and were further applied to continuous foam separation. The parameters studied using factorial design were foam bed height, airflow rate and liquid pool height. The optimum pH, temperature and salt concentration for maximum enrichment of lipase were also studied in batch mode of operation

Chapter 6 describes the production of lipase was in 1 l and 10 l fermenters using optimized medium. The effect of temperature, pH, and agitation speed and on enzyme production was investigated.

Chapter 7 describes formulation of detergent additive by spray drying and immobilization of lipase on Celite. The suitability of spray dried preparation as detergent additive was studied. The immobilized preparation was used to study castor oil hydrolysis.

Chapter 8 summarizes the conclusions of our work and offers suggestions for future studies in this area.

References

References are listed in Chapter 9

CHAPTER 2

Simple gas chromatography method for lipase assay : Optimization of assay conditions

Summary

A gas chromatography method for lipase assay was developed using tributyrin as substrate. Tributyrin was hydrolyzed by lipase to produce free butyric acid that was directly quantified by gas chromatography. The estimation of butyric acid took only a few minutes after enzyme reaction. The technique needed a small enzyme sample and was useful for analysis of large number of lipase samples. The method was able to detect lipase activity as low as 0.04 IU.

Introduction

Lipases (EC 3.1.1.3) are usually estimated using triolein or olive oil where fatty acids released from triacylglycerols are titrimetrically quantified. The spectroscopic lipase assay methods, presently practiced, use either natural substrates or chromogenic synthetic substrates. The fatty acids released from natural substrates react with compounds like Rhodamine 6G or copper salts to form coloured products (Jaeger *et al.* 1994). The chromogenic synthetic substrates, like *p*-nitrophenyl esters, (Winkler and Stuckmann 1979) allow spectrophotometric estimation of the enzyme activity, directly.

Lipases act on oil:water interface. As the natural substrate of lipases, triacylglycerols, are insoluble in water, it is necessary to make their emulsions so as to increase the available reaction surface. Triacylglycerols can be effectively emulsified by methods like sonication. Goodman and Durgan (1969) have shown that sonication of olive oil emulsified with gum Arabic, improved the sensitivity of assay.

Although there are several methods reported for estimation of lipase activity, (Beisson *et al*. 2000) simple and sensitive method needs to be developed for estimating lipase activity from batch cultures and column fractions.

GC and HPLC are common laboratory equipments. The quantification of organic compounds based on these instruments is more reliable and reproducible as compared to other analytical techniques. These instruments can be automated, require small quantities of samples and have very high sensitivity of estimation. The lipase assay method based on use of HPLC could detect enzyme activity as low as 0.02 units (Veeraraghavan 1990). Although it is possible to quantify the hydrolysis products released from substrates like triacylglycerols directly by HPLC, it involves rather high recurring expenditure as compared to GC.

Kashyap *et al*. (1980) have described method for estimation of lipoprotein-lipase using gas chromatography. The fatty acids released by the action of enzyme on triacylglycerol-rich lipoproteins were extracted using hexane and converted to quaternary ammonium salts in the next extraction step using trimethyl (α,α,α-trifluro-m-tolyl) ammonium hydroxide. The quaternary ammonium salts were then esterified using methyl acetate. The methyl esters formed were estimated by GC. Poluiaktova *et al*. (1995) have described a method for estimation of lipase activity in skeletal muscles. The methyl esters of oleic acid developed after hydrolysis of triolein were quantified using GC. Omar *et al.* (1987) have described estimation of hydrolysis products of triolein by gas chromatography after converting them to trimethylsilyl derivatives. Lipases hydrolyze oils to form non-volatile higher fatty acids, which are converted to volatile esters for

estimation by GC. The lower fatty acids (C1-C7) are volatile and need not be converted to their methyl esters before analysis by GC.

The gas chromatographic fatty acid analysis methods published earlier require cumbersome and expensive derivatization techniques that make them unsuitable for quantitative, routine analysis of lipase activity.

This chapter describes a new assay method for lipase activity, using gas chromatography. The synthetic substrate, tributyrin, was hydrolyzed by lipase to produce butyric acid that was directly estimated by gas chromatography. Two commercially available enzymes, studied widely for different applications, AmanoPS (*Pseudomonas florescence*) and Lipase type II (from porcine pancreas) were used for the method development of lipase assay.

Materials and methods

AmanoPS (*Pseudomonas florescence*) lipase was a kind gift from Amano Pharmaceutical Co. (Nagoya, Japan). The Lipase type II (from porcine pancreas) and tributyrin were from Aldrich. Triolein, polyvinyl alcohol, n-butyric and n-caproic acids were from Sigma. All other chemicals of analytical grade were from S. D. Fine Chemical Co. (Boisar, India). The Free fatty acid phase (FFAP) GC column was from Chromatopak, Mumbai, India.

Butyric acid estimation by GC

A gas chromatograph with following conditions was used in the present method. Column- 10% free fatty acid phase on Chromosorb W (AW), SS; 3 m, 2.2 mm ID, packed column, Carrier - nitrogen at 40 ml/min, Fuel- hydrogen at 30 ml/min, Air 300

ml/min, Column temperature 180 °C, Injector temperature 200 °C, Flame ionization detector (FID) 220 °C. The samples were to be injected without any clean-up and therefore, a pre-column of 4 cm length was fitted before the analytical column to protect the analytical column. The pre-column was packed with acid washed glass wool. Linearity of the FID was determined by injecting different amounts of butyric acid dissolved in 147 mM orthophosphoric acid, as standard solutions for GC analysis.

Substrate preparation

Tributyrin, the synthetic triacylglycerol, was used in the form of emulsion as substrate for lipase assay. Ten ml of tributyrin were emulsified in 90 ml of emulsifier solution (20 g/l PVA) by sonication using a Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT, USA). The sonication was done in an ice bath, in cycles of 1min followed by 1min gap using a microprobe, in pulser mode. The mixture of emulsifier and tributyrin was cooled to 4 °C prior to sonication, so as to minimize the hydrolysis of substrate. The output control of sonifier was kept on 5 and sonication was done at 50% duty cycle. The sonifier was operated at 20 KHz.

Assay by gas chromatography

The assay was carried out in 1.5 ml microcentrifuge tubes. The reaction mixture composed of 250 µl tributyrin emulsion, 250 µl Tris- HCl buffer (0.1 M, pH 7.5) and 400 µl lipase solution (Amano PS 0.5 mg/ml or Lipase type II 5 mg/ml) was incubated at 30 °C in shaking water bath at 180 rpm for 30 min. The reaction was then stopped by adding 100 µl orthophosphoric acid (14.5 M) and tubes were centrifuged at 10,000 *g* for 10 min. After centrifugation, 500 µl of the aqueous phase was withdrawn, mixed with 125 µl

caproic acid (4 mg/ml) as the internal standard and 5 μl of this mixture was injected. The enzyme activity was measured in terms of free butyric acid produced. One unit of enzyme was defined as the amount, which produces 1μmol of butyric acid per minute under above conditions. A blank was prepared using distilled water instead of enzyme.

Optimization of assay conditions

Effect of emulsifiers and sonication time

Ten ml of tributyrin were emulsified in 90 ml of emulsifier solutions (cold water soluble polyvinyl alcohol, hot water soluble polyvinyl alcohol or gum *Acacia*, 20 g/l) by sonication. The sonication was done using Branson sonifier as described above. Because the cold water-soluble PVA was found to be a suitable emulsifying agent, it was further studied. The sonication time with PVA was varied from 5 seconds to 10 minutes. The emulsion stability was studied over 72 h by microscopy, using Leitz Labolux 12 POL S microscope.

Effect of substrate concentration on lipase assay

The tributyrin substrate was prepared in concentration ranging from 1 to 20% (v/v) tributyrin in 20 g/l cold water soluble PVA solution. Amano PS was used as the enzyme (1mg/ml) for activity estimation using GC method, as described above.

Effect of pH and temperature on substrate

The stability of 10% tributyrin emulsion was tested at different pH values from 3-11 for 2 h. The buffers used were, citrate- phosphate, pH 3 to 7; sodium phosphate, pH 8; glycine-

NaOH, pH 9 and 10; sodium phosphate-NaOH, pH 11. The effect of temperature on the substrate emulsion was studied at pH 9.4 using glycine-NaOH buffer, at 20-80 °C for 2 h. The stability of the substrate emulsion was studied by incubating 250 µl of substrate and 650 µl of buffer at different pH and temperatures. After incubation, 100 µl of phosphoric acid was added and the tributyrin emulsion was disrupted by centrifugation at 10,000 *g* for 10 min. The butyric acid produced was estimated as described before using Gas Chromatograph.

Assay by titrimetry

Twenty g polyvinyl alcohol (PVA) was dissolved in one liter distilled water. Substrate was prepared by emulsifying ten ml of tributyrin with 90 ml of PVA solution by sonication. The reaction mixture composed of 5 ml substrate emulsion; 4 ml 0.1 M Tris HCl (pH 7.5) and 1 ml lipase solution (Amano PS 0.5 mg/ml or Lipase type II 5 mg/ml) was incubated at 30 °C for 30 min. The reaction was stopped by adding 20 ml acetone:ethanol (1:1 v/v). The liberated fatty acids were titrated with 0.01 M NaOH. One unit of activity was defined as the amount of enzyme, which liberated 1 µmol of butyric acid, per min, under assay conditions.

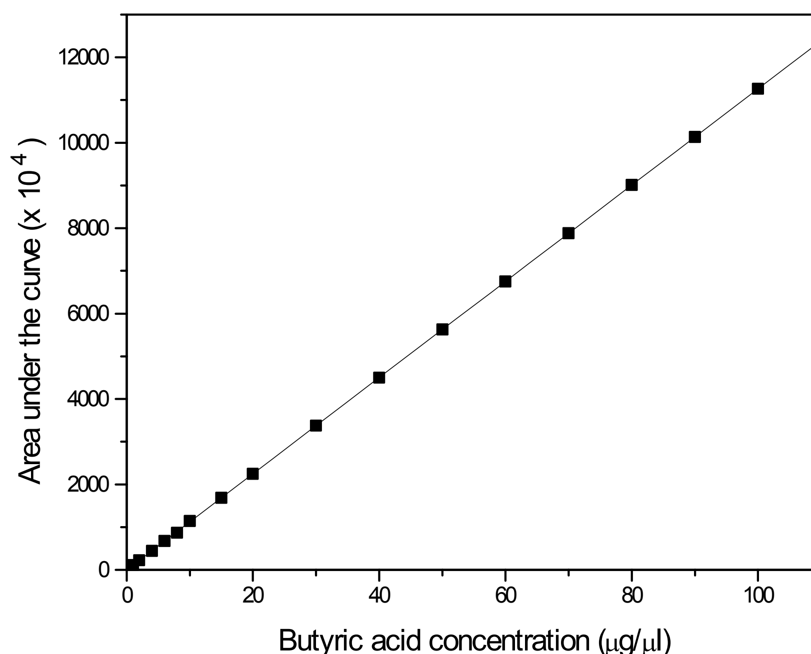
Results and Discussion

Butyric and caproic acid peaks were well resolved during GC analysis. A chromatogram showing the separation of butyric and caproic acid is presented in Figure 2.1. Caproic acid was found to be a suitable internal standard as it eluted close to butyric acid and had similar response factor.

Figure 2.1 Estimation of butyric acid using internal standard method a) Butyric acid standard (Retention time (R.T.) 2.40 min- Butyric acid, R.T. 4.83 min- caproic acid) b) Tributyrin substrate hydrolyzed by lipase.

Figure 2.2 shows the standard graph of butyric acid. The butyric acid estimation had a linear response up to 100 μg (regression coefficient 0.999) and the internal standard method had a high precision (standard deviation 0.057 μg). The method could estimate as low as 0.04 μmole of butyric acid, which was equal to 0.04 IU of lipase.

Figure 2.2 Standard graph of butyric acid



Effect of emulsifiers and sonication time

The lipase activity is closely related to the quality of emulsion formed. Among the emulsifiers tested, cold water soluble PVA was found to be the best, followed by gum *Acacia* and hot water soluble PVA giving 11.3, 10.8 and 4.0 U/ml lipase activity, respectively, with 0.5 mg/ml Amano PS lipase. It was observed that the substrate emulsion using cold water soluble PVA was stable for at least 24 h (data not shown) when sonication time was 4 min, and therefore was used for subsequent emulsion

preparation. When the substrate emulsion stability was examined over a period of 72 h by microscopy, it was found that the emulsion was not stable for more than 36 h. The substrate droplets tend to coalesce resulting in phase separation and sedimentation of tributyrin after 48 h. Therefore, it is ideal to use freshly prepared emulsion for lipase assay. Koseki *et al.* (1989) had studied the effect of gum arabic on lipase activity and found that it stabilized substrate emulsion and enhanced lipase activity. Yasuhiro (1987) and Yasuhiro and Satoru (1988) have also studied the effect of PVA as emulsifier for lipase substrate. They have reported that the use of a mixture of PVA 117 and PVA 205 gave better stability to emulsion. In present study cold water soluble PVA was found to be the best emulsifier and was therefore used as an emulsifying agent in all the assays subsequently.

Effect of substrate concentration on lipase assay

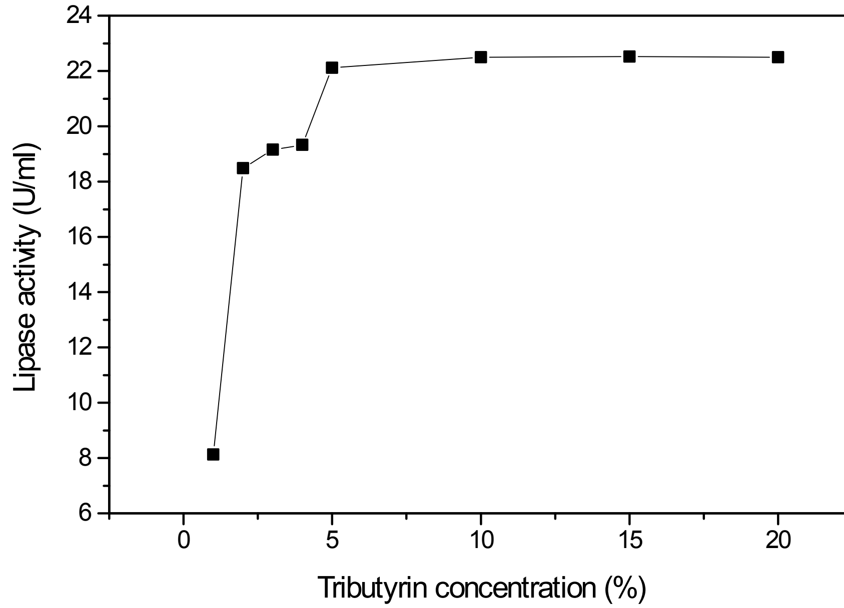
It was observed that the lipase activity measured, increased when the substrate concentration was increased from 1 to 5% reaching a plateau at 5% concentration. Further increase in substrate concentration did not have any effect on lipase activity (Figure 2.3).

Effect of pH and temperature on substrate

The tributyrin emulsion was stable from pH 3 to 11. The substrate produced a maximum of 0.1702 μmole of butyric acid at pH values higher than 10, in the assay mixture, when incubated in the absence of enzyme for two hours.

The substrate was stable in the temperature range 20-80 °C producing only 0.3404 μmole of butyric acid in 2 h, at higher temperatures.

Figure 2.3 Effect of substrate concentration on lipase assay



Results of the titrimetric and gas chromatographic assay with two commercial enzymes were comparable (Table 2.1).

Table 2.1. Comparison of lipase activity by different assay methods.

Enzyme	Substrate	Method	Estimated lipase activity (U/mg protein)
AmanoPS[a]	Tributyrin	GC	10.92 ± 0.45
		Titrimetry	9.60 ± 0.08
Lipase type II[b]	Tributyrin	GC	6.72 ± 0.31
		Titrimetry	5.88 ± 0.06

The conventional olive oil based assays gave true lipase activities but required large substrate and sample volumes, longer processing time for titration and are therefore

unsuitable for large number of samples. The spectrophotometric assay using *p*NPP or β-naphthyl caprylate (McKellar 1986) are quick, require small sample volume but are not specific for true lipases because these substrates can be cleaved even by esterases (Jaeger *et al.* 1994). It was found that the enzymes that hydrolyzed triolein always hydrolyzed pNPP and pNPB but hydrolysis of pNPP and pNPB did not predict lipolytic activity with triolein (Vorderwülbecke *et al.* 1992). In addition, some of these substrates are useful in a rather narrow pH range.

Guillou and Chevrier (1977) have previously described lipase detection using GC for estimation of butyric acid produced by anaerobic cultures grown on tributyrin. In their assay, tributyrin was used as medium ingredient, which acted as substrate for lipase as well as carbon source for the growth of the bacteria. The butyric acid produced, thus, could be in fact, a product of cell bound or extracellular lipase. The method also involved extraction with of the butyric acid, in ether, before estimation on GC.

The present assay method requires a small sample, uses a substrate that is chemically closer to a true lipid as compared to chromogenic substrates, can be used in a wide range of pH and temperature as studied here and takes only six minutes for accurate estimation of the liberated fatty acids. The assay is very sensitive as it can estimate lipase activity as low as 0.04 IU. The assay has an excellent accuracy and precision. An added advantage of present method is that it can be automated by use of modern auto-samplers and chromatography software.

CHAPTER 3

Application of factorial designs for optimization of lipase production from *Pseudomonas fluorescens* NS2W

Summary

An alkaline lipase producing bacterium was isolated from soil and taxonomically identified to be *Pseudomonas fluorescens* NS2W. Production of the lipase from *Pseudomonas fluorescens* NS2W was optimized in shake flasks, using a statistical experimental design. Two-level factorial design was constructed with four variables *viz*. ammonium di-hydrogen orthophosphate, groundnut oil, calcium chloride and magnesium sulfate. The optimum medium composition, thus found, consisted (g/l) groundnut oil 7.5, ammonium di-hydrogen orthophosphate 7.7, yeast extract 2.5, calcium chloride 0.3 and magnesium sulfate 0.3, at pH 7.4. The maximum lipase activity obtained was 69.7 U /ml. The optimized medium resulted in about 5-fold increase in the enzyme production, as compared to that obtained in the basal medium. Among different inducers tested, oleic acid was found to be the best inducer. Feeding oleic acid to shake flask cultures at 8, 16 and 24 h increased cell mass by 47% and lipase production by 12%. Feeding groundnut oil increased the cell mass by 50% but did not increase lipase production.

Introduction

Lipases show different biotechnologically important properties and as a consequence, they find use in various industries like food, chemical, pharmaceutical, cosmetic and leather processing. Lipases also find use in detergent industry (Starace 1983, Tatara *et al*. 1985). Triglycerides present in stains on fabric are difficult to remove because they are hardly saponified as compared to fatty acids. Therefore, lipases functioning at alkaline pH values are required.

In the conventional method of medium optimization for the production of biochemicals by microbial cultures, the concentration of one component is varied at a time while

keeping the concentrations of other components constant. This method is time consuming and is also found to be inaccurate when interactions between different components are present. In contrast, the factorial approach for process optimization is convenient and can yield several-fold improvement in the process as demonstrated in many cases. (Harris *et al*. 1990, Gilbert *et al*. 1991a, Gawande and Patkar 1999, Gao *et al*. 2000). The present chapter deals with the isolation of a lipase-degrading organism, its identification and medium optimization for lipase production, using a two-level full factorial design with four variables.

Material and Methods

Materials

Tributyrin and olive oil were from Aldrich. Poly vinyl alcohol (PVA, average molecular wt. 30,000-70,000), butyric acid and caproic acid were from Sigma. All other chemicals, of analytical grade, were from S. D. Fine Chemicals, Boisar, India. Media components were from HiMedia, Mumbai, India, while Proflo and Pharmamedia were gifts from Traders Protein, Memphis, USA. The refined vegetable oils were purchased locally.

Methods

Isolation and screening of lipolytic microorganisms

Samples were collected from three different sites- 1. Soils exposed to vegetable oils for long periods, 2. Spoiled coconut, 3. Soil from a vegetable oil mill unit in Pune.

The isolation was carried out in two steps, followed by screening for lipase activity.

1. Enrichment in liquid medium
2. Isolation and selection on solid medium
3. Screening for lipase activity on oil containing solid media

4. Screening for lipase activity in liquid medium

Table 3.1 Media used for enrichment, isolation and screening of bacteria producing extracellular lipases.

Medium no.	Composition
1	(g/l) Olive oil or castor oil 20, K_2HPO_4 2.5, $(NH_4)_2SO_4$ 1.3, $MgSO_4.7H_2O$ 0.5, yeast extract 0.5. Filter sterilized urea 6.5 ml (200 g/l stock), added to the medium after autoclaving. pH 7.0 or adjusted by sterile sodium carbonate: bicarbonate mixture (75:25 g/l) to 8.5, after autoclaving.
2	(g/l) Castor oil 2.0 or olive oil 1.0, $(NH_4)_2SO_4$ 5.0, $MgSO_4.7H_2O$ 1.0, NaCl 1.0, yeast extract 0.5, filter-sterilized urea solution 1 ml (200 g /l stock), agar 20, at pH 7 or pH 8.5 by sterile 10% Na_2CO_3 solution. The above mixture was sonicated and then autoclaved.
3	Tributyrin agar composed of [A] (g/l) gum acacia, 1; Na_2HPO_4, 8.63; NaH_2PO_4, 6.08; tributyrin, 10; agar, 20; distilled water 90 ml, [B] Ammonium nitrate 120 g/l, [C] Magnesium sulfate 100 g/l, [D] Sodium chloride 100 g/l, [E] Calcium chloride 50 g/l. All the components were autoclaved separately at 15 lbs for 20 min. 10 ml of each stock solutions [B], [C], [D] and [E] were added to 960 ml of component [A], after sterilization and cooling.
4	(g/l) Peptone 10, castor oil 10, yeast extract 5, NaCl 1, NaH_2PO_4 6.08, Na_2HPO_4 8.63, at pH 7.4. One ml of sterile $MgSO_4.7H_2O$ stock solution (500 g/l) was added after autoclaving.
5	(g/l) Peptone 5, yeast extract 2.5, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.2 and castor oil 5 (as an emulsion with gum Acacia), at initial pH 7.4.

1. Enrichment in liquid medium

The enrichment was carried out using two different methods.

Method 1

The samples were suspended in sterile distilled water to form 10% (W/V) suspension. One ml of the suspension was used to inoculate 250 ml Erlenmeyer flasks containing 25 ml of Medium 1 (Table 3.1). The flasks were incubated at 30 °C for 24 h, at 220 rpm, on a rotary shaker.

Method 2

All samples were heat treated to select spore-producing cultures. Ten ml of the sample suspension was taken in 100 ml conical flask, and incubated in shaking water bath at 80^0C, for 10 minutes at 120 rpm. The flasks containing Medium 1 were inoculated with these heat-treated samples and incubated at 30 °C on rotary shaker for 24 hours.

After the incubation, the culture fluid was used to inoculate another set of flasks or Medium 2 plates (Table 3.1). The enrichment was repeated 2-3 times in Medium 1.

2. Isolation and screening of lipase producing microorganism

The isolates were screened and maintained on Medium 2. Appropriate dilution of enriched culture was spread on plates and the plates were incubated up to 5 days, at 30 °C. Colonies having clearance zone around them due to oil hydrolysis were selected and transferred to agar slants.

3. Screening for lipase activity on solid medium

The extracellular lipase production by all the isolates was compared on five different agar media. The lipase production of all the isolates was screened on Medium 2 plates with Olive oil or Castor oil as the sole carbon source at pH 7.0 and pH 8.5, and Medium 3 plates having tributyrin. The agar plates of different media described above were spot

inoculated with the isolates and the plates were incubated at 30 °C for 2 days. The diameters of the colonies and clearance zones were measured after 24 and 48 hours.

4. Screening for lipase activity in liquid medium

All the isolates were tested for the production of lipase activity in Medium 4. The pH used for cultivation of isolates was similar to their respective isolation media. Inoculum was prepared in the Medium 4, devoid of oil. The inoculum flasks were inoculated from a slant culture and incubated at 30 °C for 24 h. The Medium 4 flasks, were inoculated with 10% inoculum (v/v) and incubated on rotary shaker at 220 rpm, for 48 h. Samples were withdrawn at 24 and 48 h and cells were removed by centrifugation at 20,000 *g* for 10 min. The lipase activity in the supernatant was estimated by titrimetry as well as gas chromatography assay (Kulkarni and Gadre 1998). The bacterial isolate that produced maximum lipase was selected for further work.

Lipase assay

Titrimetry assay

Twenty ml olive oil was added to 80 ml of 20 g/l polyvinyl alcohol solution and sonicated using Branson sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT, USA) as described in chapter 2. The reaction mixture composed of 5 ml olive oil emulsion, 4 ml glycine-NaOH buffer (0.1 M, pH 9.0), and 1 ml of enzyme sample was incubated at 30 °C in shaking water bath at 180 rpm for 1 h. At the end of the incubation, the emulsion was broken by addition of 20 ml acetone: ethanol mixture (1:1) and the liberated fatty acids were titrated with 0.05 N NaOH.

Gas chromatography assay

Tributyrin was used as the enzyme substrate for the gas chromatography assay as described earlier in Chapter 2.

Taxonomical studies

The strain was identified according to Palleroni (1992) and "Bergey's Manual of Systematic Bacteriology" (Palleroni 1984).

Medium optimization

A 5% inoculum (v/v) was added to a 500 ml Erlenmeyer flask containing 50 ml of Medium 5. The flasks were incubated on rotary shaker at 220 rpm, at 30 °C. Samples were removed at regular intervals for growth and lipase activity measurement. One ml aliquot of the sample was centrifuged at 20,000 *g* for 10 min and lipase activity in the supernatant was quantified using GC method. For estimation of cell growth, the pellet was suspended in one ml saline (8.5 g/l NaCl solution), it was appropriately diluted further, if required and the absorbance was measured at 600 nm.

For all the experiments, the inoculum was grown in Medium 5, devoid of oil, for 10 h at 30 °C. For testing the effect of different carbon sources, castor oil was replaced by the respective carbon source, at equal carbon content. Replacing peptone with various N sources, on equal nitrogen content basis, assessed the effects of nitrogen sources.

Factorial design

Factorial experiment was designed using the approach given in standard texts on design of experiments (Box *et al*. 1978, Davies 1993). The variability in lipase production was

determined using five flasks of identical medium, initially (at the center of first factorial design). Effects of concentrations of the four variables, namely ammonium di-hydrogen phosphate, groundnut oil, calcium chloride and magnesium sulfate on lipase production were studied using two-level factorial design. The data obtained from the factorial experiments were fitted to the following polynomial.

$$\text{Activity} = \alpha_o + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_4 x_4 + \alpha_{12} x_1 x_2 + \alpha_{13} x_1 x_3 + \alpha_{14} x_1 x_4 + \alpha_{23} x_2 x_3 + \alpha_{24} x_2 x_4 + \alpha_{34} x_3 x_4$$

Where the 'α' are fitted constants, x_1, x_2, x_3 and x_4 are coded variables for ammonium di-hydrogen phosphate, groundnut oil, calcium chloride and magnesium sulfate, respectively. The 'α' were calculated from the main effects and interactions as described by Box *et al*. (1978). Alternatively, these coefficients can also be obtained by simple linear regression.

Coefficients smaller than "two times the standard error" were presumed to be due to experimental error and were therefore neglected (Montgomery 1991). The direction of maximum increase in lipase production is given by the gradient of the above polynomial and the next set of experiments was conducted along this direction. This amounted to one-dimensional optimization along the line of steepest increase.

Time course of lipase production

Time course of the lipase production was studied with the optimized medium in shake flasks, for 60 h. A 5% (v/v) inoculum was added to 50 ml medium, in 500 ml Erlenmeyer flask and incubated at 220 rpm on a rotary shaker, at 30 °C, for 60 h. Samples were removed periodically and growth as well as lipase activity in the culture supernatant was determined.

Effect of inducers on lipase production

The effect of various inducers on lipase production was studied in shake flaks using Medium 5. The castor oil in the medium was replaced by different inducers. Different fatty acids, glycerol and alcohol were used as inducers at concentration equivalent to carbon content of 5 g/l of triolein. The volatile fatty acids (butyric, caproic, valeric and heptanoic) were tested as free fatty acids as well as their sodium salts. Samples were removed at regular intervals for growth and lipase activity estimation. One ml aliquots of the samples were centrifuged at 20,000 *g* for 10 min and lipase activity in the supernatant was determined. The lipase activity was estimated using GC method. For estimation of cell growth, the pellet was suspended in one ml saline (8.5 g/l NaCl solution), appropriately diluted and absorbance measured at 600 nm.

Fed-batch studies

Fed-batch experiments were done in shake flasks by growing NS2W in the medium optimized by factorial design. Oleic acid or Groundnut oil was used for feeding. Oleic acid was fed at concentration 1.92 ml/l from zero to 32 h. Feeding was done such that a set of five flasks received one shot each, at 8 h interval. Another set of 3 flasks received 2 shots each (at16 and 24 h, 24 and 32 h, 16 and 32 h). Samples were removed at intervals and cell growth as well as lipase activity in the culture supernatant were estimated.

In experiment with groundnut oil sterile groundnut oil was fed at 7.5 g/l concentration as an emulsion in 20 g/l gum *Acacia*. The feeding was done from 0 h to 32 h, at 8 h interval, in a set of 5 flasks. The extracellular, intracellular and cell bound lipase activities were estimated as described below. Ten ml of the culture broth was centrifuged at 20,000 *g* for 20 min. The supernatant was used for estimation of extracellular lipase activity as

described before. For estimation of cell-bound activity, the cell pellet was washed twice by suspension and centrifugation in physiological saline and was then suspended in 5 ml distilled water and sonicated using Branson Sonifier. The sonication was done in ice bath, for 4 cycles of 2 min each, with 2 minutes cooling between the cycles using a microprobe, in pulser mode. The output control of sonifier was kept on 5 and sonication was done at 50% duty cycle. The sonifier was operated at 20 KHz. The sonicated cell lysate was centrifuged at 20,000 *g* for 10 min. The supernatant was used for estimation of intracellular activity and the pellet was washed twice as described before, with distilled water and used for estimation of cell-bound lipase activity.

Results and discussion

Screening of lipase producing microorganism

The lipase production by 20 bacterial, one yeast and four fungal strains isolated from 6 soil samples and one spoiled coconut sample were examined. The results obtained from screening on solid medium gave good hydrolysis zones for 3 bacterial isolates (Colony diameter 7, 5, and 3 mm and zone diameter 12, 7, and 5 mm respectively on Medium 2 with castor oil, pH 7). All the isolates were screened for lipase activity in liquid medium. The lipase produced by fungal cultures was more active at acidic pH as compared to alkaline pH. The 3 bacterial strains showed more than 2 U/ml lipase activity at pH 9, as determined by GC method. Among them, NS2W was selected as the highest lipase producer. It produced 10 U /ml lipase in the Medium 5, at pH 7.

Identification of NS2W

From growth, morphology and nutritional characteristics, the organism was identified to be *Pseudomonas fluorescens* and named as *Pseudomonas fluorescens* NS2W. The isolate is deposited in National Collection of Industrial Microorganisms, Pune, India, as NCIM 5145. The results of identification are summarized below.

Growth characteristics

Gram character	Gram negative, short rods
Motility	Motile, showing tumbling motility
Spore	Not formed
Capsule	Not formed
Growth on nutrient agar plate	Good growth, colonies with irregular margin and wrinkled surface, 2-3 mm dia. (48 h). Produced yellow diffusible pigment.
Nutrient agar slant	Good growth along streak line
King's B agar	Produced yellow, diffusible pigment that fluoresced at 274nm.
Response to oxygen	Strict aerobe
pH range of growth	4-8
Growth at 4 °C	Positive
Growth at 40 °C	Positive

Nutritional characteristics

Levan formation from sucrose	Negative
Arginine dihydrolase	Negative
Oxidase reaction	Positive
PHB accumulation	Positive
Denitrification	Positive
Gelatin hydrolysis	Positive
Starch hydrolysis	Negative
Lecithinase	Positive
Lipase	Positive
Catalase	Positive
Urease	Positive
Nitrate reduction	Positive

Catechol *ortho* cleavage	Positive
Citrate utilization (Simmon's)	Positive
H_2S production (TSI)	Negative
Voges-Proskauer test	Negative
Methyl red test	Negative
Indole production	Negative
Phenylalanine deaminase	Negative

Utilization of different carbon sources

D-Arabinose, xylose, sorbose, glucose, fructose, mannose, cellobiose, trehalose, sucrose, lactose, xylitol, sorbitol, mannitol, glycerol, L-tryptophan, L-cysteine, L-threonine, L-serine, L-tyrosine, L-proline, L-arginine, L-aspartic acid, L-leucine, L-histidine, L-ornithine	Positive
Starch, dextrin, maltose, rhamnose, raffinose, erythritol	Negative
p- hydroxybenzoate, benzoate	Positive
m-hydroxybenzoate	Negative
Gluconate	Negative
Malonate	Negative
Tryptamine	Positive
Benzylamine	Negative
Glycine	Positive
Creatine	Negative
Hippurate	Positive

The results confirmed that the isolate NS2W belonged to species *fluorescens* of genus *Pseudomonas*. The organism varied in two tests from the reported characteristics. It failed to give positive test for arginine dihydrolase and was able to grow at 4 °C as well as 40 °C.

Effect of carbon source on lipase production

The study of lipase production with various carbon sources in the medium showed that the enzyme production was maximal, when groundnut oil was used as C-source (Table

3.2). *Pseudomonas fluorescens* NS2W could utilize several vegetable oils as well as hexoses and pentoses but showed poor growth with disaccharides and polysaccharides. The isolate secreted more than 12 U/ml lipase with all examined vegetable oils as carbon source. With tributyrin as sole carbon source for growth, the secreted enzyme activity was only 2.6 U/ml. Among sugars used as carbon source, it secreted maximum lipase with glucose. Sztajer and Maliszewska (1988a) have reported that starch was the best C-source for lipase production from *Pseudomonas fluorescens*. Makhzoum *et al*. (1995) have reported that glucose inhibited lipase production by *Pseudomonas fluorescens* 2D. The present culture was able to synthesize lipase in presence of glucose. The lipase production was enhanced in the presence of oil.

Table 3.2 Effect of C-source on lipase production

C – Source	Cell growth (OD 600 nm)	Lipase yield (U/ml)
Arabinose	8.8	2.6
Coconut oil	13.5	17.2
Fructose	9.1	4.7
Glucose	11.7	8.0
Groundnut oil	15.0	21.3
Lactose	3.7	4.5
Maltose	4.2	6.5
Mustard oil	17.1	15.2
Olive oil	14.8	14. 7
Sesame oil	15.7	13.9
Soluble starch	4.4	1.9
Sucrose	3.4	4.0
Sunflower oil	16.8	18.8
Tributyrin	7.7	2.6
Xylose	12.7	3.0

Castor oil	16.7	14.7

The medium contained (g/l) peptone 5, yeast extract 2.5, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.2, to which different carbon sources with carbon content equivalent to 5 g/l of triolein, at 30 ºC, were added. Samples were diluted suitably for OD and enzyme activity measurement.

Effect of nitrogen source on lipase production

The *Pseudomonas fluorescence* NS2W grew well with all nitrogen sources tested and the optical density reached above 10. Ammonium di-hydrogen phosphate was the best among all the nitrogen sources tested, giving 56.9 U/ml lipase, which was almost three-fold more as compared to the basal medium (Table 3.3). The lipase production was very low with soybean meal and corn steep liquor; whereas other organic and inorganic nitrogen sources produced more than 10-27 U/ml of enzyme.

Makhzoum *et al*. (1995) have reported that arginine, threonine and lysine as well as some ammonium salts of mineral acids supported good growth and lipase production by *Pseudomonas fluorescens* 2D. Tryptone and casamino-acids in combination with ammonium were reported to be the best nitrogen sources for lipase production by *Acinetobacter calcoaceticus* strains (Cordenons *et al*. 1996).

Table 3.3 Effect of N-source

N-Source	Cell growth (OD 600 nm)	Lipase yield (U /ml)
Ammonium nitrate	10. 8	17.7
Ammonium di hydrogen phosphate	15.6	56.9
Ammonium sulphate	12.5	21.9
Beef extract	15.9	23.4
Casein Acid hydrolysate	13.6	17.4
Corn steep liquor	15.3	2.9
Peptone (Basal medium)	19.3	19.5
Pharma media	NA	11.8
Potassium nitrate	12.7	17.7
Proflo	NA	12.8

Sodium nitrate	14.8	18.6
Soyapeptone	15.8	18.5
Soybean Meal	NA	3.2
Tryptone	15.5	24.5
Urea	11.6	27.4
Yeast extract	21.6	19.8

The medium contained (g/l) yeast extract 2.5, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.2, 5 g/l groundnut oil as gum acacia emulsion. Different nitrogen sources with nitrogen content equivalent to 5 g/l of peptone were added. NA – Not attempted due to particulate N- source. Samples were diluted suitably for OD and lipase activity measurement.

Effect of minerals on lipase production

The investigations on the effects of $MgSO_4.7H_2O$, NaCl, and $CaCl_2.2H_2O$ on the lipase production by *P. fluorescence* NS2W done individually as well in combination showed that addition of $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ increased the lipase production by 26% while NaCl decreased the lipase production (Table 3.4).

Table 3.4 Effect of mineral salts on the lipase production

Salt (200 mg/l individually)	Cell Growth (OD 600 nm)	Lipase yield (U/ml)	Change %
None	11.6	47.2	0
$MgSO_4.7H_2O$	11.4	57.8	22
$CaCl_2.2H_2O$	10.9	50.4	7
NaCl	13.8	32.2	-32
$MgSO_4.7H_2O$ + $CaCl_2.2H_2O$	13.1	59.3	26
$MgSO_4.7H_2O$ + NaCl	11.9	54.6	16
$CaCl_2.2H_2O$ + NaCl	11.3	48.8	3
$MgSO_4.7H_2O$+ $CaCl_2.2H_2O$ + NaCl	12.0	52.3	11

Cultivated in medium containing (g/l) Groundnut oil 5, $NH_4H_2PO_4$ 5.134, K_2HPO_4 1, yeast extract 2.5, each mineral salt 0.2 g/l. Samples diluted, if necessary.

Several workers have reported stimulatory effect of sodium, magnesium and calcium ions on lipase production by different organisms. El-Gammal and Rizk (1989) reported that

addition of 0.01% NaCl to fermentation medium stimulated lipase production in *Candida utilis* and *Debaryomyces hansenii*. In present investigation, addition of NaCl at 0.02% (w/v) caused decrease in lipase activity. Among several metal ions, calcium was shown to have stimulatory effect on lipase production by *Pseudomonas fluorescens* 2D while magnesium did not have any stimulatory effect (Makhzoum *et al* 1995). In case of *Pseudomonas fluorescens* B52, lower yield of lipase was observed in the absence of calcium when the culture was grown in a minimal medium (McKellar and Cholete. 1986). Secretion of extracellular lipase by *Pseudomonas aeruginosa* and *Aspergillus terreus* were also found to increase by addition of Ca^{2+} and Mg^{2+} ions to growth medium (Abdelraham and Yousef 1997, Gulati *et al*. 2000). In the present study, Ca^{2+} and Mg^{2+} when added alone or in combination proved to be useful for increasing lipase yield.

Factorial design

The effect of concentrations of the four variables, namely ammonium di-hydrogen phosphate, groundnut oil, calcium chloride and magnesium sulfate on lipase production was studied using two-level full factorial design. Using five flasks containing identical medium, the standard error in enzyme production was calculated to be 2.57.

The experimental results obtained from the factorial design showed that all the variables, except $MgSO_4.7H_2O$, had significant effect on lipase production. $MgSO_4.7H_2O$ had negligible effect at the concentration selected for factorial design experiment. $NH_4H_2PO_4$ had positive effect whereas groundnut oil and $CaCl_2.2H_2O$ had negative effects (Table 3.5). The response surfaces obtained from predicted lipase yields are shown in Figure 3.1a and Figure 3.1b.

An experiment was then conducted in the linear direction of steepest increase keeping $MgSO_4.7H_2O$ concentration at optimum level. However, further increase was not observed in the lipase production (Table 3.6).

Table 3.5 Results of factorial design.[a, b]

Flask No.	$NH_4H_2PO_4$ (X_1)	Groundnut oil (X_2)	$CaCl_2.2H_2O$ (X_3)	$MgSO_4.7H_2O$ (X_4)	Lipase (U/ml)
1	+	+	+	+	61.7
2	+	+	+	–	60.8
3	+	+	–	+	60.6
4	+	+	–	–	64.2
5	+	–	+	+	67.4
6	+	–	+	–	66.7
7	+	–	–	+	58.1
8	+	–	–	–	54.3
9	–	+	+	+	12.4
10	–	+	+	–	17.6
11	–	+	–	+	26.4
12	–	+	–	–	27.3
13	–	–	+	+	16.0
14	–	–	+	–	12.9
15	–	–	–	+	53. 7
16	–	–	–	–	73.7

[a]Levels (g/l)– $NH_4H_2PO_4$, (+) 10.26 and (–) 5.134; Groundnut oil, (+) 10 and (–) 5; $CaCl_2.2H_2O$, (+) 0.4 and (–) 0.2; $MgSO_4.7H_2O$, (+) 0.4 and (–) 0.2.

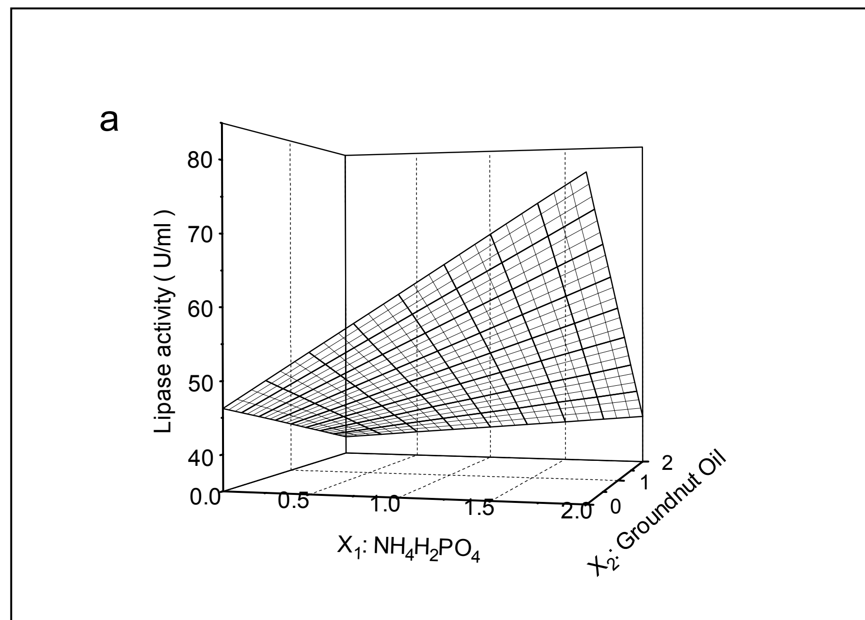
[b]Effects α_1: 30.94, α_2 : -8.52, α_3 : -13.47, α_4 : -1.86, α_{12}: 8.74, α_{13}: 18.13, α_{14}: -2.32, α_{23}:5.57 α_{24}:0.8775, α_{34}: 2.11.

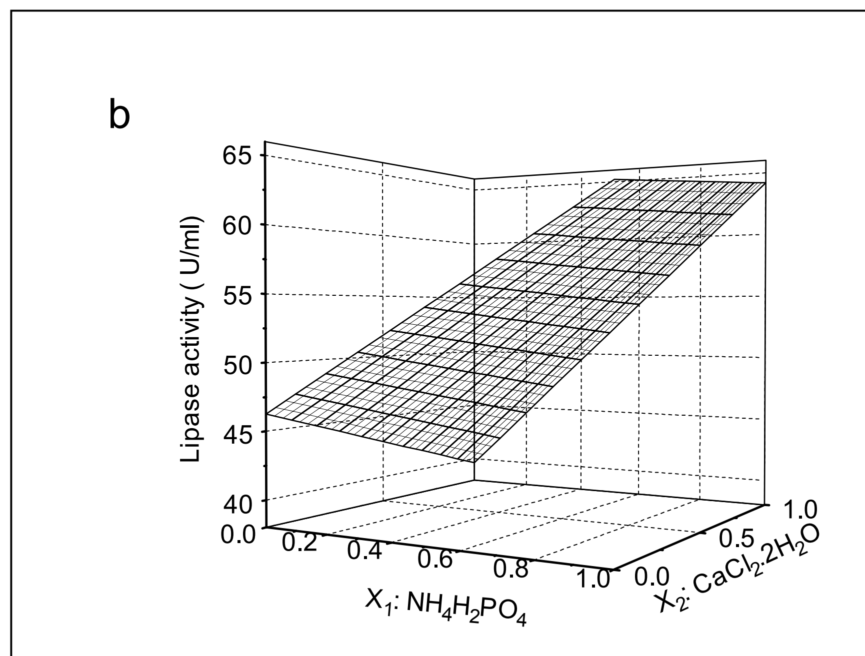
Table 3.6 Experiments conducted in the direction of steepest ascent

Flask No.	$NH_4H_2PO_4$ (g/l)	Groundnut oil (g /l)	$CaCl_2.2H_2O$ (g /l)	$MgSO_4.7H_2O$ (g/l)	Lipase (U /ml)
1	7.7	7.5	0.3	0.3	69.7
2	10.6	6.8	0.3	0.3	63.1
3	17.9	5.1	0.2	0.3	57.2
4	29.5	2.9	0.1	0.3	35.6
5	45.2	0.6	0.00	0.3	16.8

Flask no. 1 denotes the centroid of the factorial experiment

Figure 3.1 Response of the lipase production to variation in a) $NH_4H_2PO_4$ and groundnut oil concentrations when $MgSO_4.7H_2O$ and $CaCl_2.2\ H_2O$ were set at zero. b) $CaCl_2.2H_2O$ and $NH_4H_2PO_4$ concentrations when $MgSO_4.7H_2O$ and groundnut oil were set at zero. Zero value refers to center of first factorial design.





It was observed that the pH of the medium dropped down to 5.8 or even lower when the culture was grown in shake flask. As the concentration of $NH_4H_2PO_4$ was increased, the pH dropped down very fast and this might have inhibited growth and lipase production. Thus, the optimum medium contained (g/l) groundnut oil 7.5, ammonium di-hydrogen orthophosphate 7.7, yeast extract 2.5, calcium chloride 0.3 and magnesium sulfate 0.3.The medium optimization thus resulted in 5-fold increase in lipase production as compared to the basal medium.

Growth and lipase production

Lipase production from *Pseudomonas fluorescens* NS2W was studied in shake flask (Figure 3.2). The lipase production in shake flasks reached maximum in the late logarithmic phase. The lipase activity obtained with optimized medium in shake flask was 69.7 U/ml. There was a lag of 12 h before the extracellular lipase activity was detected. Tan and Gill (1985) have described occurrence of initial lag phase with *Pseudomonas fluorescens* culture, when olive oil containing medium was inoculated with cells from nutrient broth.

Effect of inducers on lipase production

Among different fatty acids tested, oleic acid was found to be the best inducer and gave 17-fold increase in the activity as compared to the basal medium (Table 3.7). Volatile fatty acids (C2-C6), their salts and ethanol inhibited the growth of the organism. Glycerol supported good growth but produced only 1.84 U/ml lipase.

Figure 3.2 Time course of lipase production with optimized medium in shake flask

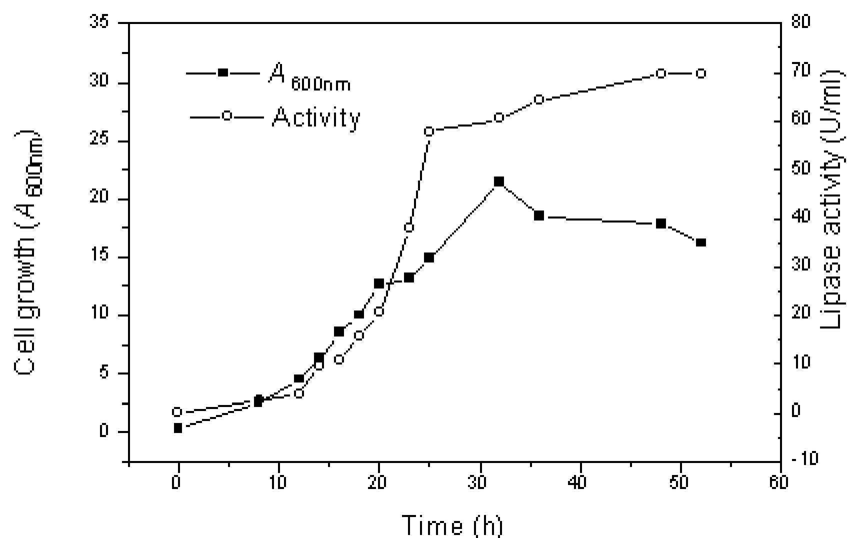


Table 3.7 Effect of various inducers on growth and lipase production

Inducer	O. D. 600	Lipase yield (U/ml)
Control	2.78	3.04
Glycerol	11.96	1.84
Groundnut oil	18.54	29.55
Oleic acid	10.10	51.97
Palmitic acid	6.50	31.16
Stearic acid	10.54	30.87

Several workers have reported that triglycerides were necessary for lipase production by microorganisms and very low quantities of lipases were detected in the absence of such inducers (Suzuki *et al*. 1988, Iizumi *et al*. 1990, Sugihara *et al*. 1991). The present culture was also found to secrete more lipase in the presence of vegetable oils (Table 3.2). A

variety of inducers, including fatty acids and fatty acid derivatives, have been used for lipase production (Chander *et al*. 1979, Shimada *et al*. 1992). The long chain fatty acids like oleic acid, linoleic acid and their esters were found to induce lipase production by *Geotrichum candium* effectively as compared to short chain fatty acids (Shimada *et al*. 1992) as found with the present culture. Gilbert *et al*. (1991a) have reported that the lipase production by *Pseudomonas aeruginosa* EF2 was repressed by oleic acid. The fatty acid esters like triglycerides, spans and tweens (Chakrabarti *et al*. 1987, Gilbert *et al*. 1991a), fatty acid alcohols (Ushio *et al*. 1996) are reported to be good lipase inducers. The lipase production by *Bacillus stearothermophilus* was found to be stimulated by glycerol (Bradoo *et al*. 1999).

Fed-batch studies

The feeding of oleic acid to the shake flask cultures was found to increase the lipase production by 12 % and the cell mass by 45% (Table 3.8). The feeding of oleic acid once, between 8 to 24 hours and two times at 16 and 24 h improved the lipase production. Feeding oleic acid increased the cell mass in general, with maximum increase in flasks, which were fed twice, at 16-24 h and 24-32 h.

The feeding of groundnut oil to shake flask cultures resulted in 2-fold increase in the cell mass by but failed to improve the lipase secretion. The intracellular and cell-bound lipase activities of cultures in the flasks were always lesser than the extracellular activities (Table 3.9). The proportion of intracellular and cell-bound lipase increased with increase in the culture age. The percentage of cell-bound enzyme at particular time, was almost similar in samples of all the flasks.

Table 3.8 Effect of feeding of oleic acid on growth and lipase production

Flask no.	Oleic acid fed at (h)	O. D. 600 nm	Lipase production (U/ml)
1	Control	17.04	65
2	0	20.8	23
3	8	25.72	71
4	16	24.08	70
5	24	24.68	71
6	32	23.12	60
78	16, 24	29.96	70
9	24, 32	30.04	45
10	16, 32	21.72	9

Table 3.9a Effect of feeding of groundnut oil on lipase production and cell growth

Addition of oil	Time	Lipase			DCW (g/100 ml)
		Extracellular u/ 100 ml	Intracellular (U/g)	Cell bound (U/g)	
Control	32h	2456	0	154.2752	0.37
	48h	3815	1076.496	609.888	0.6
	168h	13415	4448.2	1772.791	0.34
oil fed at 0 h	32h	2726	439.9412	150.7308	0.76
	48h	3414	1155.886	842.184	1.26
	168h	5499	3459.688	1101.84	0.4
oil fed at 8 h	32h	2508	118.4131	67.6635	0.79
	48h	3669	1465.555	750.7097	1.27
	168h	5282	3379.962	1080.354	0.41
oil fed at 16 h	32h	2442	213.2064	98.6184	0.72
	48h	2950	1646.039	1473.471	1.63
	168h	5447	2242.54	1160.513	0.41

Table 3.9b % distribution of lipases on feeding of groundnut oil at different time intervals

Addition of oil	Time	Lipase			DCW (g/100 ml)
		% Extracellular	% Intracellular	% Cell bound	
Control	32h	94.08	0	5.91	0.37
	48h	69.34	19.56	11.08	0.6
	168h	68.31	22.65	9.02	0.34
oil fed at 0 h	32h	82.19	13.26	4.54	0.76
	48h	63.08	21.35	15.56	1.26
	168h	54.65	34.38	10.95	0.4
oil fed at 8 h	32h	93.09	4.39	2.51	0.79
	48h	62.34	24.90	12.75	1.27
	168h	54.21	34.69	11.08	0.41
oil fed at 16 h	32h	88.67	7.74	3.58	0.72
	48h	48.60	27.11	24.27	1.63
	168h	61.54	25.33	13.11	0.41

The intracellular and cell bound activities were determined by converting the U/g of culture broth to total activity obtained from 100 ml of broth.

Conclusions

A strain of *Pseudomonas fluorescens* was isolated and identified using biochemical tests. It was selected for further studies as it produced considerable quantities of an alkaline, thermostable lipase. Two-level full factorial design, in four variables was used for optimization of medium composition for production of the alkaline lipase from *Pseudomonas fluorescens* NS2W. The optimized medium resulted in 5-fold increase in the production of lipase, as compared to that in the basal medium.

Oleic acid was the best inducer and feeding of oleic acid to shake flask cultures enhanced the lipase production by 12%.

CHAPTER 4

Purification and characterization of alkaline lipase from *Pseudomonas fluorescens* NS2W

Summary

An alkaline lipase-producing organism was isolated from a soil sample and taxonomically identified as *Pseudomonas fluorescens*. The extracellular lipase produced by this organism was purified to homogeneity by foam separation, acetone precipitation and gel filtration chromatography. The molecular weight of the pure protein was estimated to be 33 kDa by SDS-PAGE. The lipase formed high molecular weight aggregates with molecular weight more than 100,000 kDa. The isoelectric point of the pure protein was in the range 4.8 to 5. The lipase had optimal activity at pH 9 and it retained more than 70% activity over the pH range of 3 to 11. The lipase had optimal activity at 55 °C and was stable up to 60 °C with more than 70% activity retention for at least 2 h. Metal ions Cu^{2+}, Hg^{2+}, Zn^{2+} and EDTA inhibited the activity. The enzyme showed marked regioselectivity for the 1,3-oleyl residues of triolein as analyzed by HPLC.

Introduction

Lipases are enzymes that catalyze the hydrolysis of triglycerides at oil water interface. They occur widely in bacteria, fungi and yeasts (Sztajer *et al*. 1988). Lipases have a wide range of properties with respect to positional specificity, fatty acid specificity, thermostability and pH optimum, depending on their source (Table 1.6). Because of these properties, lipases find use in various chemical, pharmaceutical, food, leather and detergent industries. Several *Pseudomonas* sp. are known to produce lipases (Table 1.5) and many of them produce alkaline and thermostable lipases. *Pseudomonas* lipases are used industrially as detergent additives, to catalyze hydrolysis of oils and fats, and in organic synthesis (Reetz and Jaeger 1998). We have isolated a strain of *Pseudomonas fluorescens*, producing an alkaline, thermostable lipase. This chapter deals with the purification and characterization of this lipase.

Materials and Methods

Materials

Sephadex G-100, molecular weight calibration kits for electrophoresis and the HPLC standards (1-monoolein, 2- monoolein, 1-3 diolein, 2-3 diolein, triolein and oleic acid) were purchased from Sigma (USA). Sephacryl S-200 was procured from Pharmacia Fine Chemicals (Uppsala, Sweden). The refined vegetable oils used were purchased locally. Metal ion salts and buffer ingredients and all other chemicals were AR grade, from S. D. Fine chemicals, Boisar, India.

Methods

Protein estimation

Protein content was estimated according to Lowry *et al*. (1951) using bovine serum albumin (BSA) as a standard.

Lipase assay

The lipase activity was estimated by gas chromatography method, using tributyrin as substrate, as described in Chapter 2 unless stated otherwise. One unit of enzyme was defined as the amount of enzyme that liberates 1μmol of butyric acid, per minute.

Lipase purification

Crude enzyme preparation

The culture was grown in the optimized medium described in Chapter 3 for a period of 52 h. At the end of incubation, the bacterial cells in the fermentation broth were removed by centrifugation at 20,000 *g* for 20 min at 10 °C. The cell-free supernatant was used as a crude enzyme preparation and purified further as described below.

Foam separation

The cell-free supernatant was subjected to foam separation in a specially designed column (Figure 5.1). The column had jacketed reservoir for holding liquid. At the base of the reservoir, there was sintered glass filter through which air could be bubbled in to the liquid. The upper part of column had outlets, at different heights, designed to collect foam. The conditions for foam separation were as follows. Temperature- 30 °C, pH of cell-free broth - 5.8, volume of feed- 162 ml, air flow rate- 75 ml/min, pool height- 19.5 cm, foam collected at - 22.5 cm. The foam generated was collapsed using vacuum and

collected in a flask through sintered glass funnel. The lipase activity as well as protein in feed and foamate was quantified. Foam separation of the present lipase was studied in details as presented in Chapter 5. The parameters for maximum enrichment of lipase by foam separation were optimized to give best enrichment of lipase (highest enzyme, per unit protein) in the foamate.

Acetone precipitation

The enzyme concentrate obtained after foam separation, was precipitated by 1:2 volumes of chilled acetone (-10 °C). The precipitate was stored overnight, at -20 °C and recovered by centrifugation at 20,000 *g* for 20 min. The precipitate was dried in vacuum dessicator at room temperature, so as to remove traces of acetone. The precipitate was then re-suspended in 10 mM phosphate buffer, pH 6.4.

Ultrafiltration

The resuspended acetone precipitate was diafiltered using stirred-cell filtration assembly (Amicon, Beverly, USA) with YM50 membrane (molecular weight cut off of 50 kDa), at 4 °C.

Gel filtration chromatography

The ultrafiltered enzyme preparation was loaded on a Sephadex G-100 column (2 cm dia × 150 cm length) pre-equilibrated with 10 mM sodium phosphate buffer, pH 6.4. The elution was carried out in the same buffer at a flow rate of 0.23 ml/min at room temperature and 4.6 ml fractions were collected. The protein content of fractions was

determined by measuring optical density at 280 nm. The protein-containing fractions were assayed for lipase activity.

Properties of lipase

Molecular weight determination

Molecular weight of the protein was estimated by SDS-PAGE, performed according to Laemmli (1970) on a vertical slab 8% (w/v) polyacrylamide gel, at a constant voltage of 200 V, for 5 h, at 25 °C. α- Lactalbumin (14.2 kDa), β-lactaglobulin (18.4 kDa), Carbonic anhydrase (29 kDa), Fumarase (48.5 kDa), bovine serum albumin (66 kDa) were used as standard protein molecular weight markers. The gel was stained with the silver-staining method of Blum *et al*. (1987).

Effect of propan-2-ol on enzyme activity

The effect of propan-2-ol on pure enzyme was studied by incubating the pure enzyme in presence of 0 to 40% (v/v) propan-2-ol for 10 min. The residual activity was then estimated by using *p*-nitro phenyl palmitate (*p*NPP) as substrate as described by Winkler and Stuckmann (1979). The *p*NPP substrate was prepared as follows. Thirty mg of *p*NPP dissolved in 10 ml of propan-2-ol were emulsified in 90 ml of 0.05 M Tris-HCl pH 8 buffer containing 200 mg Triton X-100 and 0-40 % propan-2-ol. The reaction mixture containing 0.9 ml of substrate and 0.1 ml of enzyme was incubated at 30 °C for 20 min. The reaction was terminated by addition of 150 μl of 0.1 M Na_2CO_3 and the yellow colour developed was read at 410 nm. A blank was prepared using distilled water instead of the enzyme. One unit of enzyme was defined as the amount of enzyme that liberates one μmol of *p*-nitro phenol per minute.

Isoelctric focusing

Isoelectric focusing was carried out in a vertical slab gel apparatus using 5% (w/v) polyacrylamide gel and carrier ampholyte (Ampholine, Sigma Chemical Company) with a pH range 3.5 to 10, as described by Robertson *et al*. (1987) at a constant voltage of 250 V for 2 h. The gel was stained with silver nitrate staining procedure according to of Blum *et al*. (1987). The gel was cut vertically in two halves. One half was used for staining. The other half was cut longitudinally in pieces of 5 mm breadth. Each piece was suspended in 250 µl distilled water, in 1.5 ml Eppendorf tubes and crushed with the help of glass rod. The mixture was centrifuged at 10,000 *g* for 10 min and pH of the supernatant was measured. Lipase activity of the eluted enzyme was estimated as follows. The activity was estimated qualitatively using *p*NPP as substrate in a micro-titer plate using modification of the method described by Winkler and Stuckmann (1979). The development of yellow colour indicated presence of lipase. A blank was run, by adding distilled water, instead of the eluted supernatant.

Optimum pH and stability

The effect of pH on enzyme activity was studied by incubating the enzyme with tributyrin substrate, prepared in different buffers in the pH range 4 to 10.The buffers used were, citrate-phosphate (pH 4 – 7), sodium phosphate (pH 7 – 8), glycine-NaOH (pH 9 – 10) and sodium phosphate- NaOH (pH 11). The reaction mixture containing 0.5 ml of 5% tributyrin substrate (prepared in 0.1 M buffers having different pH values), 0.3 ml buffer and 0.1 ml enzyme was incubated at 30 °C, for 30 min, in shaking water bath at 180 strokes, per min. The butyric acid liberated was estimated by gas chromatography method. The enzyme stability was determined by incubating the enzyme preparation in

10 mM buffers of different pH, in the range of 4 to 11 for 2 h, at 30 °C and quantification of the residual activity, by gas chromatography method at pH 9 (0.1 M glycine-NaOH buffer), as above.

Optimum temperature and stability

The temperature optimum of the enzyme was determined in the range 30 to 80 °C, at pH 9, as above. The enzyme stability at different temperatures was studied by incubating the enzyme in 10 mM sodium phosphate buffer pH 6.4 at different temperatures for 2 h, followed by the activity estimation at 30 °C at pH 9.0.

Effect of metal ions, EDTA and bile salt on lipase activity

The effect of metal ions was studied by estimation of the activity in presence of 1mM solution of metal salts. Sulfate or chloride salts of metal ion were used. The effect of EDTA was studied by estimating activity in the presence of 1mM EDTA. The effect of bile salt was studied at 2 mg/ml concentration, as above. The enzyme was incubated in presence of metal ions, EDTA and bile salt for 30 min followed by estimation of activity by gas chromatography method.

Positional specificity of lipase

The positional specificity of lipase was determined by analyzing the products formed by hydrolysis of triolein using HPLC (Thermo Separation Products, USA). The chromatographic conditions were as follows. Column, CLC-ODS 150 x 4 (Shimadzu); mobile phase, acetonitrile:acetone (60:40); mobile phase flow rate, 0.5 ml/min; column

temperature 45 °C (CTO 10 column oven, Shimadzu); refractive index detector (Shodex RI-71, Japan).

The reaction mixture contained 0.5 ml triolein emulsion (40 g/l in 20 g/l PVA), 0.4 ml of 0.1 M glycine -NaOH buffer pH 9.0, and 0.1 ml of enzyme (3.2 units). It was incubated at 30 °C, 220 rpm for 16 h. After incubation, 3 ml of di-ethyl ether was added to the reaction mixture to stop the reaction and to extract the reaction products. The di-ethyl ether phase was separated and the solvent was then removed under vacuum using rotary vacuum evaporator (BÜCHI R-124) at 40 °C. The extracted mixtures of reaction products were suspended in mobile phase, filtered through 0.45µ membrane filter (Millipore, USA) and injected through a Rheodyne 7725 (USA) injector with 20 µl loop.

Substrate specificity of the enzyme

The substrate specificity of the enzyme was studied by titrimetry. The reaction was done in 20 ml baffled Erlenmeyer flask. The substrates used were, castor oil, coconut oil, cottonseed oil, groundnut oil, olive oil, sunflower oil, Span 20, triacetin, tributyrin, triolein and Tween-20. The reaction mixture was composed of 1 g of the above substrate, 23.9 ml glycine-NaOH buffer (0.1 M, pH 9.0) and 0.1 ml enzyme. The mixture was incubated at 30 °C on rotary shaker at 160 rpm for 1 h. The reaction was terminated by addition of 50 ml of acetone-ethanol mixture (1:1). The fatty acids liberated were estimated by titration with 0.05 M NaOH. One unit of enzyme was defined as the amount of enzyme, which liberated one µmol of fatty acid, per minute.

Results and discussion

Purification

The crude lipase was purified to homogeneity in three steps (Table 4.1). In the first step of foam flotation, the enzyme was concentrated two fold. In the next step of acetone precipitation, it got further concentrated by 8.83 fold, with 60% recovery of activity. The yellow pigment produced by the organism was removed during acetone precipitation. The diafiltration step removed traces of acetone as well as the low molecular weight proteins. The protein formed high molecular weight aggregates and hence it was possible to concentrate the 33 kDa protein-aggregates using 50 kDa molecular weight cut off membrane. In the gel filtration step, almost all the lipase activity was found to coincide with one major protein peak (Figure 4.1). The purification protocol resulted in a pure protein, giving a single band on a silver-stained SDS-PAGE gel (Figure 4.3), with overall 290-fold purification. The results of the purification scheme are summarized in Table 4.1.

Table 4.1 Summary of purification results

Step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold purification	Percent yield
Cell-free supernatant	162	623.7	11291	18.1	-	100
Foam flotation	75	298.8	10923	36.6	2.01	96.7
Acetone precipitation. and diafiltration	15	40.9	6554	160	8.83	60
Gel filtration on Sephadex G-100	396	19.8	5753	290.6	16.05	50.9

Figure 4.1 Elution profile of lipase on Sephadex G-100 in 10 mM phosphate buffer, pH 6.4. □, absorbance at 280 nm; ■, Lipase activity.

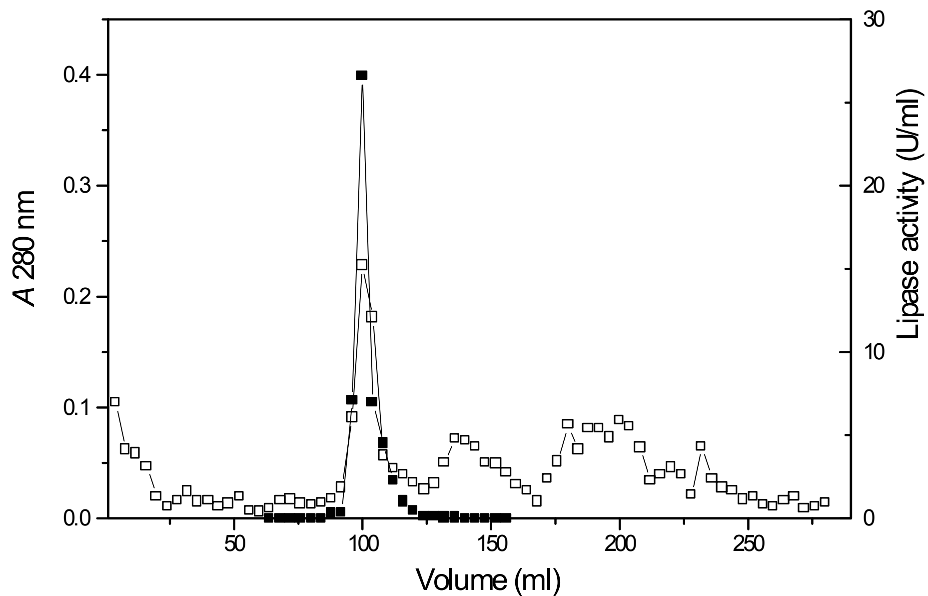


Figure 4.2 Elution profile of the lipase on Sephacryl S-200 in 10 mM phosphate buffer pH 6.4. □, absorbance at 280 nm; ■, Lipase activity.

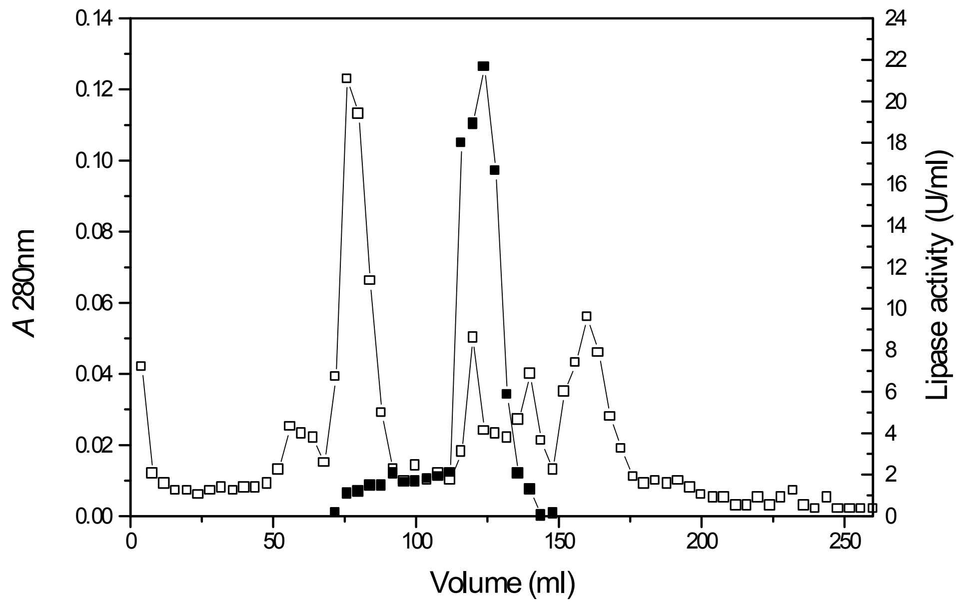
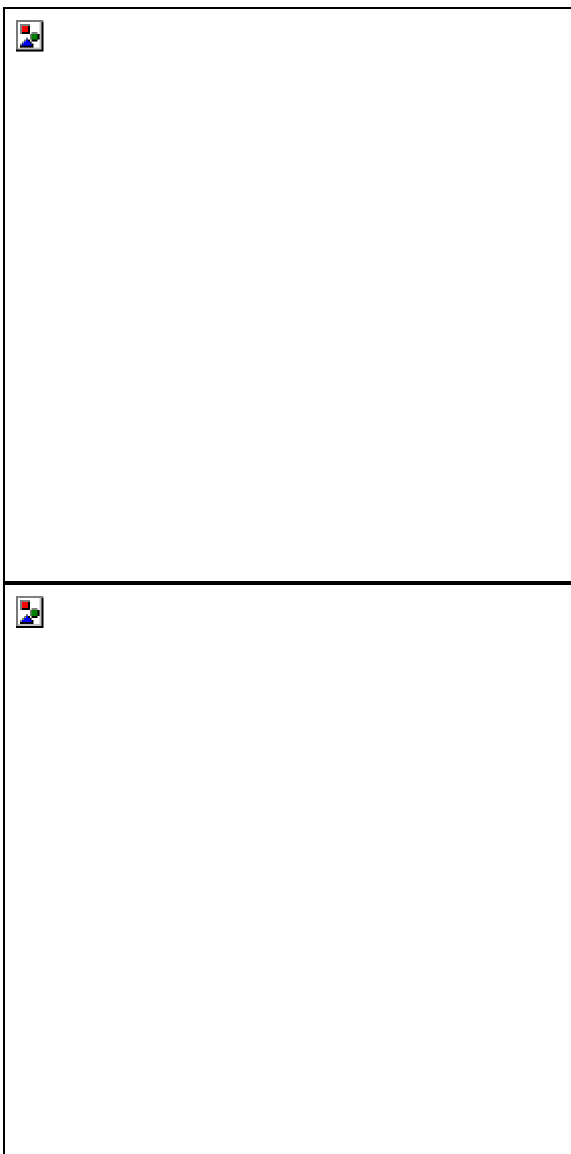


Figure 4.3 Determination of molecular weight on SDS-PAGE. Lane 1, 5 molecular weight markers; Lane 2, crude enzyme (27.3 μg); Lane 3, Foamate (21 μg); Lane 4 gel filterd enzyme (2.4 μg).

Molecular weight



Properties

Molecular weight

The molecular weight of the enzyme was estimated to be 33 kDa by SDS-PAGE (Figure 4.3). On gel filtration column (Sephadex G-100), the protein exhibited apparent molecular mass more than 100,000 kDa and was eluted in fractions immediately after the void volume. On Sephacryl S-200, the lipase eluted in fraction 17 to fraction 37, in a total volume of 60 ml, indicating presence of high molecular weight aggregates with variable association (Figure 4.2). The major activity, however, was found in the fractions between 28 and 35.

The present lipase failed to bind to ion exchange and hydrophobic interaction chromatography matrices tested. The lipase eluted as a high molecular weight protein from the gel filtration column. The pure protein smeared badly on native PAGE but showed single band with molecular weight of 33 kDa on SDS-PAGE. This indicated that the lipase might be forming high molecular weight aggregates with other lipophilic materials. Such behavior of lipases has been previously reported by several workers (Stuer *et al*. 1986, Gilbert *et al*. 1991b, Dühaupt *et al*. 1992, Chartrain *et al.* 1993). *Pseudomonas fluorescens* lipases are reported to have different molecular weights like 20.9 kDa, 33kDa, 48kDa and 45 kDa (Chung *et al*. 1991, Tan and Miller 1992, Kojima *et al*. 1994, Costa *et al*. 1997).

Lipases are reported to be monomeric proteins having molecular weights in the range of 16 kDa-670 kDa (Table 1.6). Among Gram-positive bacteria, the Bacillus lipases were classified in to two subfamilies by Nthangeni *et al*. (2001). The first subfamily included lipases with low molecular mass in the range 19-20 kDa from *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus*. The lipases from *Bacillus themocatenulatus, Bacillus thermoleovorans*, and *Bacillus stearothermophilus* were included in the second

subfamily and they had molecular mass around 43 kDa. In Gram-negative bacteria, lipases from *Vibrio cholerae*, *Acinetobacter calcoaceticus*, *Pseudomonas wisconsinensis* and *Proteus vulgaris* were reported to have molecular masses in the range 30–32 kDa. The lipases from *Burkholderia glumae*, *Chromobacterium viscosum*, *Burkholderia cepacia*, *Pseudomonas luteola* were reported to have molecular weight around 33 kDa. The lipases from *Pseudomonas fluorescens* and *Serratia marcescens* were reported to have molecular weight 50 kDa and 65 kDa respectively (Arpigny and Jaeger 1999).

Effect of propan-2-ol on enzyme activity

Incubating the enzyme with propan-2-ol increased the enzyme activity distinctly. The increase in lipase activity was in proportion with increase in the concentration of propan-2-ol, till 25% (Table 4.2). Further increase in the concentration, however, decreased the lipase activity (data not shown). The enzyme activity increased 37 fold when the enzyme was incubated with 25% propan-2-ol. Addition of propan-2-ol to the lipase gave substantially higher activity values presumably due to dissociation of enzyme aggregates. The lipase was found to elute as a high molecular weight species from gel filtration column in present work. Dünhaupt *et al*. (1992) have reported successful use of propan-2-ol to disintegrate aggregates of *Pseudomonas cepacia* lipase. Several authors have used surfactants like Triton X-100 or 3-[(3-Cholamidopropyl)dimethylaamonio]-1-propanesulfonate (CHAPS) for disintegration of aggregates formed by *Pseudomonas* lipases during purification procedures (Stuer *et al*. 1986, Chartrain *et al*. 1993).

Table 4. 2 Effect of propan-2-ol on lipase activity.

propan-2-ol	Fold increase in lipase

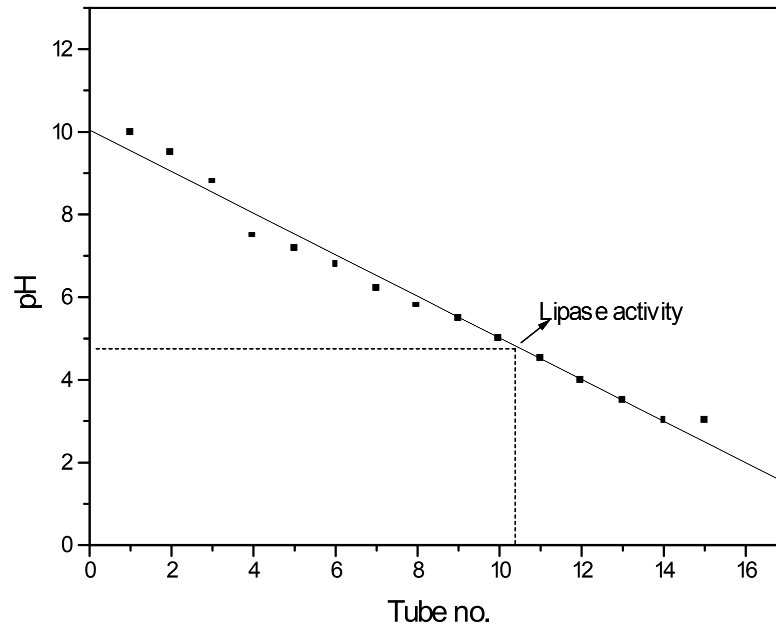
concentration (% v/v)	activity
0	0
5	1.47
10	2.97
15	11.83
20	31.79
25	37.03

Isoelectric point

The isoelectric point of the lipase was found to be between 4.6 and 5.0 (Figure 4.4). The isoelectric point in the range 4.6 to 4.9 has been reported previously by Makhzoum *et al*. (1996) for *Pseudomonas fluorescens* lipase.

A wide range of isoelectric points has been reported for the lipases. Among Gram-positive organisms, *Bacillus* lipases are well studied. Nthangeni *et al*. (2001) have classified *Bacillus* lipases in to two subfamilies, based on biochemical properties and amino acid sequence analysis (Chapter1). Subfamily I.4 *Bacillus* lipases were described to contain *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis*, having pI in alkaline range, around 9. The subfamily I.5 lipases included lipases produced by *Bacillus stearothermophilus*, *Bacillus thermoleovorans* and *Bacillus thermocatenulatus* with acidic pI around 6.

Figure 4.4 Determination of isoelectric point by isoelectric focusing of lipase.



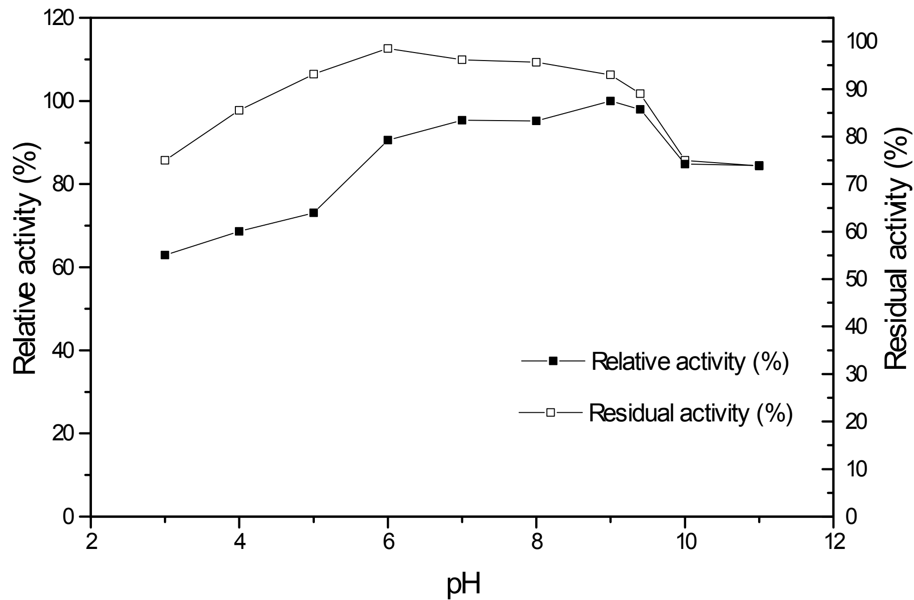
Among Gram-negative bacteria, lipases produced by *Pseudomonas* sp. are well characterized. They have been reported to have acidic pI around 4.8 (Gilbert *et al*. 1991b, Makhzoum *et al*. 1996), a near-neutral pI (Nishio *et al*. 1987, Lin *et al*. 1996) or an alkaline pI near 8.8 (Kotsuka *et al*. 1996).

Effect of pH on the enzyme activity and stability

The enzyme was most active in the pH range of 5.5 to 9.5 (Figure 4.5) with maximum activity at pH 9, whereas it was most stable in the pH range 4 to 9, retaining more than 80% activity. The enzyme retained more than 70% activity in pH range 3-11. It was most stable at pH 6. There was a decrease in activity below pH 4 and above pH 10.

Most of the bacterial lipases are reported to have pH optimum on alkaline side (Table 1.6). Among Gram positive bacteria, a *Bacillus subtilis* lipase was shown to have a very alkaline pH optimum between 10 and 11.5 (Nthangeni *et al*. 2001). The lipases from *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Propionibacterium acnes* were found to be exceptions to other bacterial lipases (Ingham *et al*. 1981, Simons *et al*. 1996 and 1998,) with pH optimum around 6.

Figure 4.5 Effect of pH on lipase activity and stability



Talon *et al*. (1995) have reported pH optimum of 9 for *Staphylococcus warneri*. *Staphylococus haemolyticus* and *Staphylococus hyicus* also have alkaline pH optimum Rosenstein and Götz 2000). *Pseudomonas* lipases are reported to have optimum pH in acidic (Iizumi *et al*. 1990) as well as alkaline environment (Sztajer *et al*. 1991b, Kojima *et al.* 1994, Castellar *et al.* 1997). The present lipase also had optimum activity on alkaline side, as reported for many other *Pseudomonas* lipases.

The lipases from *Pseudomonas* and *Bacillus* lipases were reported to be more stable in alkaline pH range (Table 1.6). The *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Propionibacterium acnes* (Ingham *et al*. 1981) lipases were again exception to this, being more stable in the acidic range.

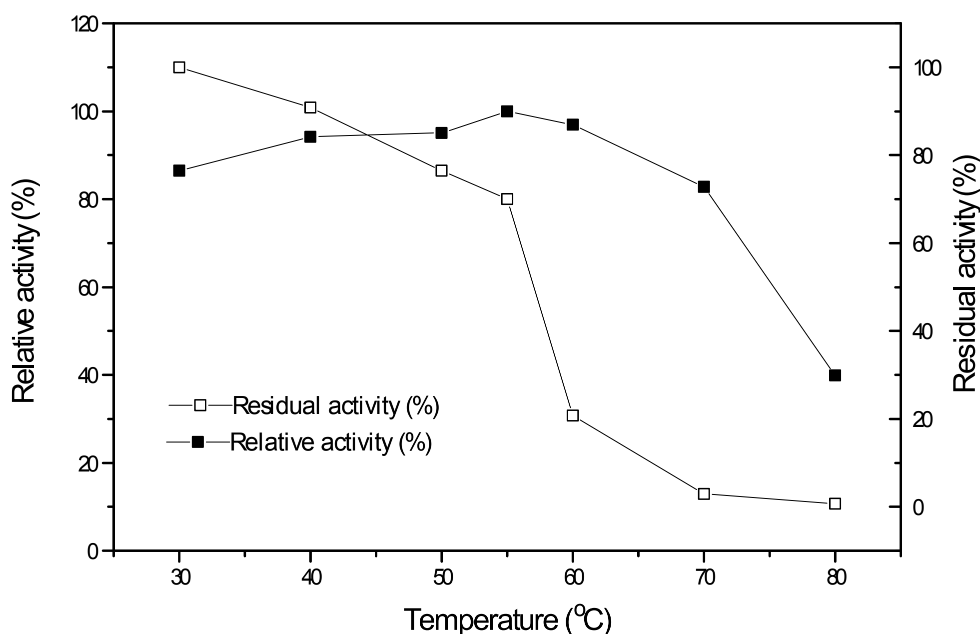
Sztajer *et al*. (1991b) have reported that a lipase from *Pseudomonas fluorescens* retained more than 80% of its activity following treatment at a pH range of 7-11.5 for 1h. Kojima *et al*. (1994) have reported a lipase from *Pseudomonas fluorescens* AK102 to be stable over pH range 4-11. A lipase from *Pseudomonas* sp. was previously reported by us to be stable over pH range 5-9 with more than 70% activity retention for 2 h (Kulkarni and Gadre 1999). The present lipase was found to be stable over wider pH range than the *Pseudomonas* lipases studied before with more than 70% activity retention in the pH range 3-11.

Effect of temperature om lipase activity and stability

The present lipase was most active in temperature between 50 and 60 °C. It retained more than 80% activity till 55 °C (Figure 4.6). The activity dropped rapidly above 60 °C. Sztajer *et al*. (1991b) have reported a temperature optimum for oil hydrolysis between 50

and 55 °C, for a lipase from *Pseudomonas fluorescence*. The temperature optima around 55-65 °C have been reported for lipases of *Pseudomonas* spp. (Yamamato and Fujiwara 1988, Iizumi *et al*. 1990, Kojima *et al*. 1994, Castellar *et al*. 1997, Kulkarni and Gadre 1999).

Figure 4.6 Effect of temperature on lipase activity and stability



The temperature optima of lipases from mesophilic microorganisms are found to be normally in the range of 45-60 °C. A lipase from *Pseudomonas fragi* was reported to have temperature optimum between 65 and 70 °C (Nishio *et al*. 1987). The highest temperature optimum of 85 °C has been reported for the lipase from *Pseudomonas solanacearum* SD 709 (Kotsuka *et al.* 1996). Many thermophilic *Bacillus* strains have been reported to produce lipases that are active at temperatures between 60 and 75 °C. The lipases from thermophilic bacilli are relatively more stable at higher temperatures

(above 60 °C), than those from mesophilic organisms. The lipases from mesophilic *Pseudomonas* sp. are stable till 50 °C, although there are a few repots of thermostable lipases from *Pseudomonas* sp. (Iizumi *et al*. 1990, Lin *et al*. 1996).

Many workers have observed an interesting phenomenon of low temperature partial inactivation (LTI) for lipases from psychrotrophic *Pseudomonas* sp. (Swaisgood and Bozoglu 1984, Kumura *et al*. 1993, Baral and Fox 1997, Konstantinou and Roussis 1998). These lipases were found to loose 80-90 % of their activity at temperatures between 40-90 °C. The thermal denaturation curves were, however, biphasic with very low activities still retained at temperatures as high as 140 °C. Due to this property, many lipases presumably survived the high temperature short time (HTST) and ultra high temperature (UHT) treatments used for pasteurization of milk and were presumably responsible for the spoilage of milk products.

Effect of metal ions, EDTA and bile salt on the lipase activity

As presented in Table 4.3, the enzyme was inhibited by metal ions like Hg^{2+}, Zn^{2+} and Cu^{2+}. Barium and manganese were found to stimulate lipase activity. Mg^{2+} and Ca^{2+} did not have any significant effect on the lipase activity. EDTA was found to inhibit lipase activity by 20 % whereas bile salt did not have significant effect.

Metal ions like Hg^{2+}, Zn^{2+} and Cu^{2+} are reported to have inhibitory effect on *Pseudomonas* lipases by several workers (Yamamato and Fujiwara 1988, Iizumi *et al*. 1990, Kumura *et al*. 1993, Chartrain *et al*. 1993). Barium was shown to have marginal stimulatory effect in case of *Pseudomonas sp*. lipase (Yamamato and Fujiwara 1988). Sr^{2+} have been reported to have stimulatory effect in the case of *Staphylococcus hyicus*

lipase (van Oort *et al*. 1989). Several workers have found that calcium ions are able to stimulate lipase activity (Chartrain *et al*. 1993, Lee and Rhee 1993). As with the present lipase EDTA has been reported to inhibit activity of a few lipases (van Oort *et al*. 1989, Baral and Fox 1997, Sharon *et al*. 1998). Bile salts were found to stimulate lipase activity of *Pseudomonas* sp. and *Pseudomonas aeruginosa* (Nisho *et al*. 1987, Yamamato and Fujiwara 1988, Chartrain *et al*. 1993). Contradictory to these reports, bile salts were found to inhibit the lipase produced by *Pseudomonas fragi* 22.39 B (Watanabe *et al*. 1977). The present lipase was not inhibited by bile salts.

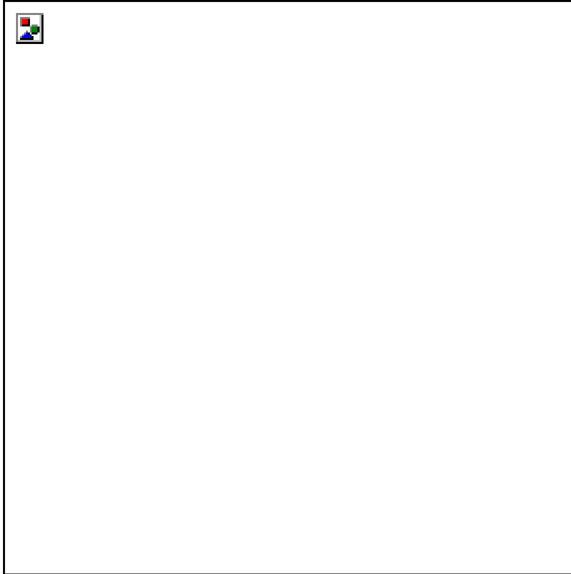
Table 4.3 Effect of metal ions on lipase activity.

Metal ion	Residual activity (%)
Cu^{2+}	90
Ca^{2+}	99.6
Mg^{2+}	99.1
Ba^{2+}	118.7
Zn^{2+}	65.6
Mn^{2+}	122.2
Hg^{2+}	54.8
Control	100

Positional specificity of the lipase

The present lipase was found to have 1-3 positional specificity. The results are presented in Figure 4.5a-4.5f and Figure 4.6. During the studies on hydrolysis, 1-2 (2-3)-diolein was found to accumulate as a product in all the samples taken from 15 min onwards.

Figure 4.5 Standards a) 1- monoolein (R. T.- 4.86 min), b) 2-monoolein (R. T.- 4.84 min)



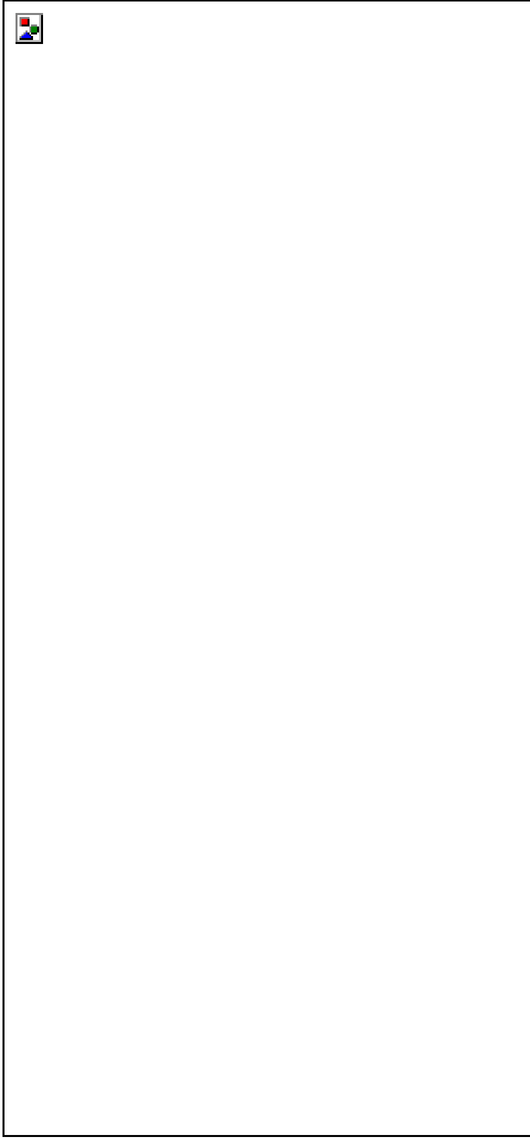
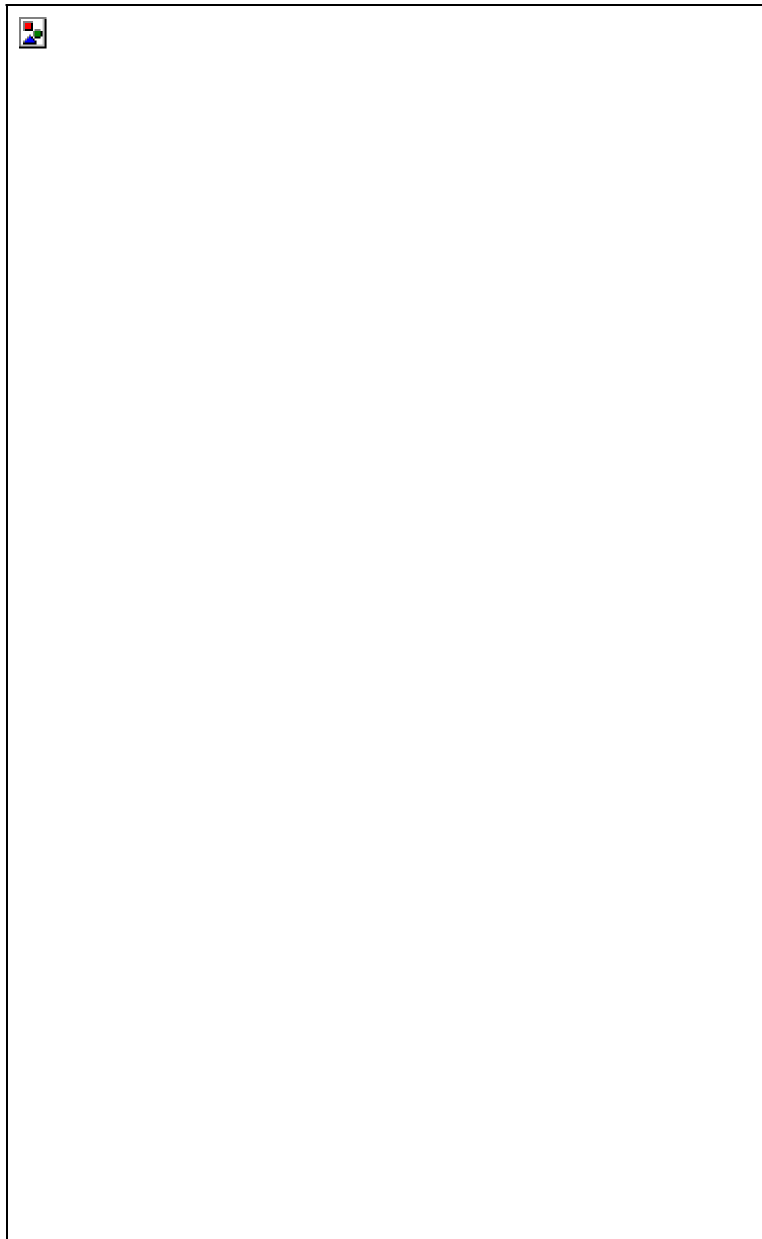


Figure 4.5 Standards c) 1-2(2-3) Diolein (R. T.- 12.21 min), d) 1-3Diolein (R. T. 11.84 min)



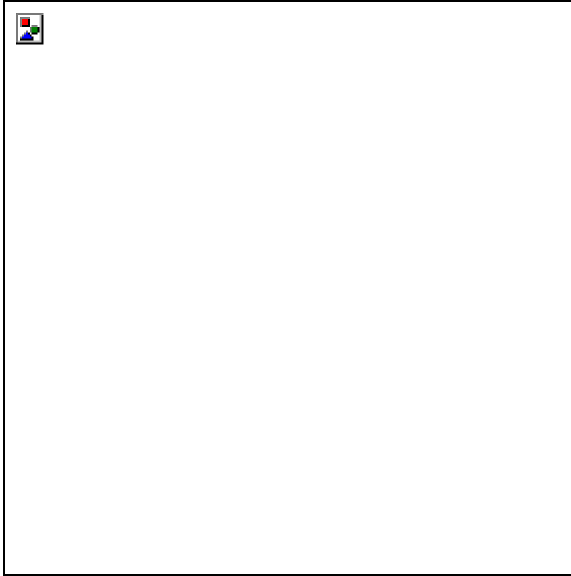
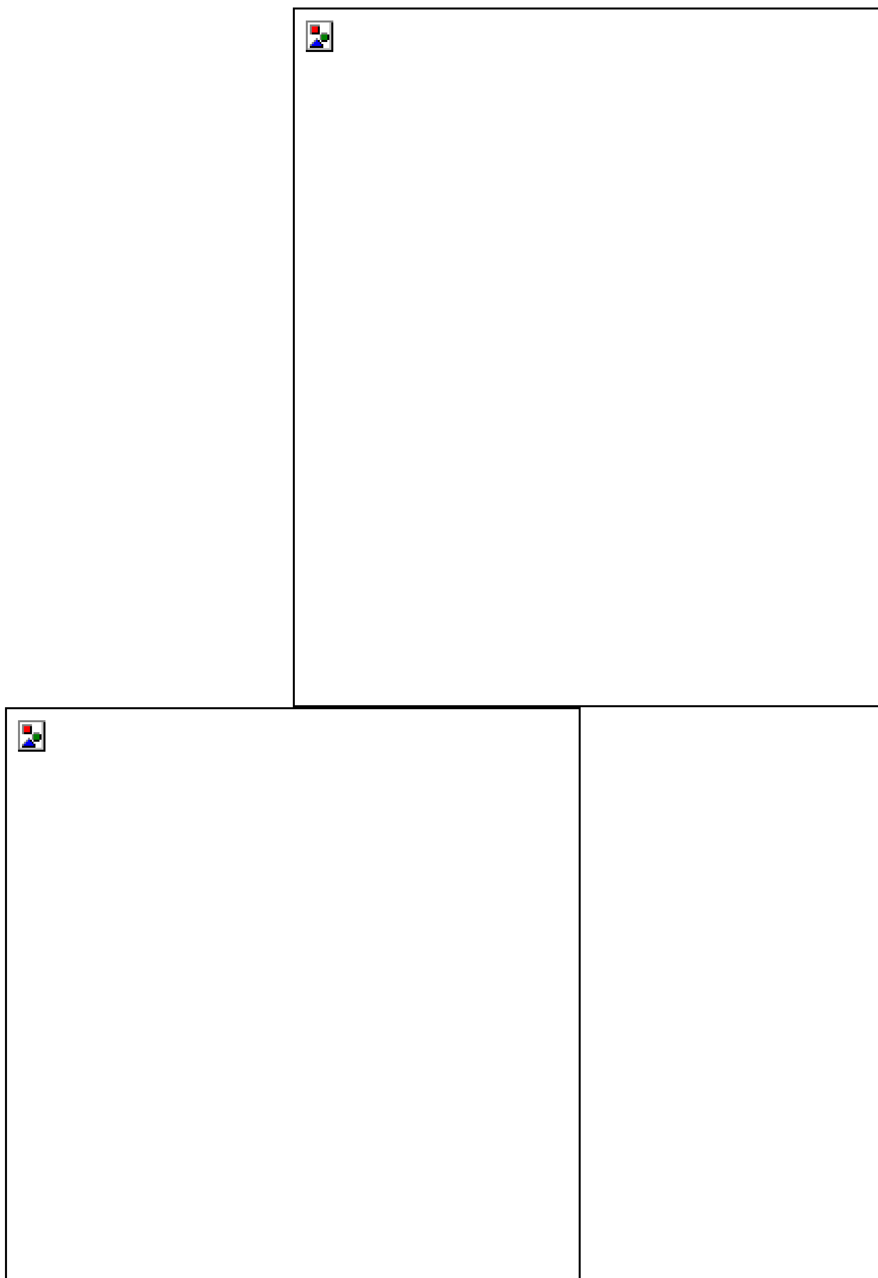
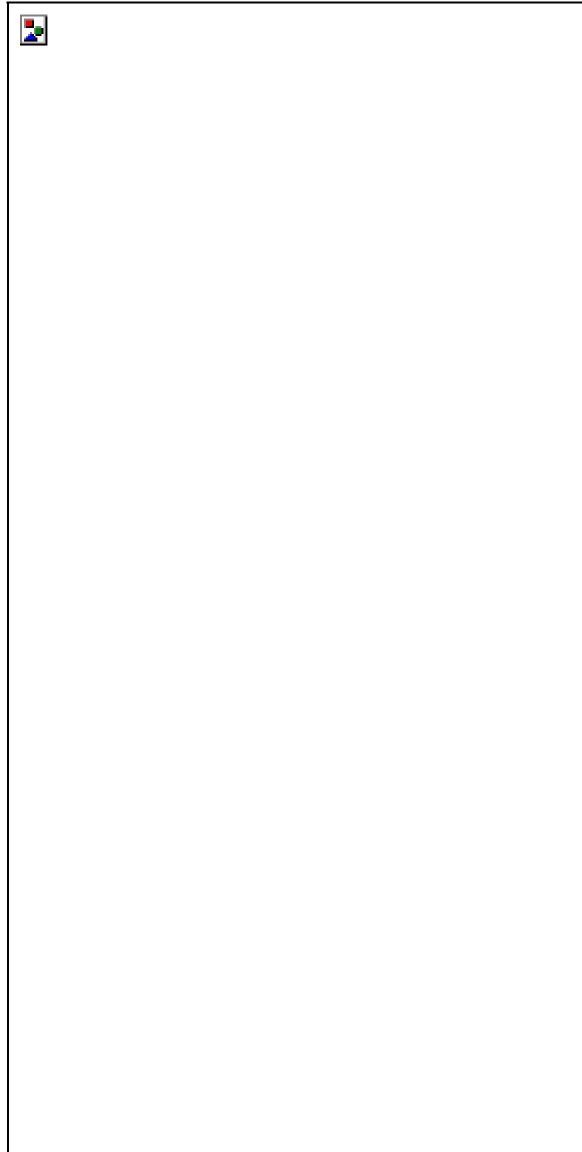


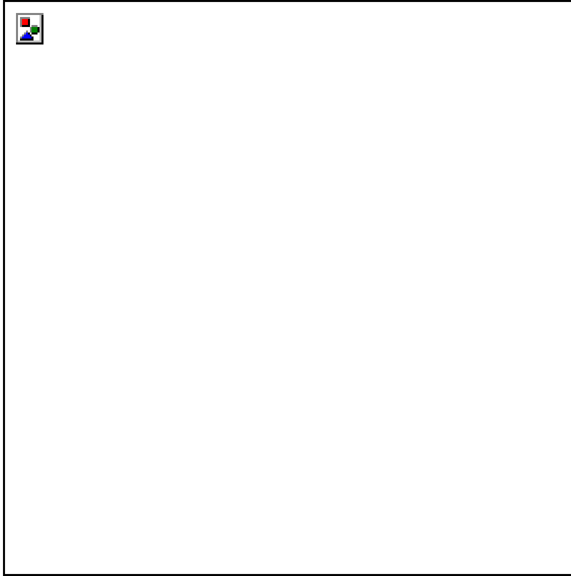
Figure 4.5 e) Oleic acid (R. T. 5.37 min)



The concentration of 1-2 (2-3)- diolein went on increasing as the reaction proceeded. 1-3 monooloein was detected only after 6 h of reaction (Figure 4.6). This indicated that the lipase was not acting on ester linkage at 2-position of triolein.

Figure 4.6 Products of hydrolysis of triolein using 3.2 U of lipase, a) Triolein blank, b) products after 2h of hydrolysis, c) products after 6h of hydolysis (R. T. - 4.85 min, 1-monoolein; 5.37 min, oleic acid; 11.84 min, 1-3 diolein; 12.21 min, 1-2(2-3) diolein)





Some *Pseudomonas* lipases have been reported to have 1-3 positional specificity (Chung *et al*. 1991, Gilbert *et al*. 1991b). A *Bacillus* sp. was reported to have 1-3 positional specificity by Sugihara *et al*. (1991). A lipase from *Pseudomonas aeruginosa* PAC1R was found to have absolute sterioselectivity towards sn-1 position of the triglyceride substrate trioctanoin, whereas, *Candida antarctica* B was found to be selective for position sn-3 (Rogalska *et al.* 1993). In the same study, Rogalska *et al*. had found that only *Candida antarctica* A lipase could preferentially cleave sn-2 ester bond.

Substrate specificity of enzyme

The lipase caused rapid hydrolysis of the vegetable oils containing C-16 and C18 fatty acid esters (Table 4.4). This indicated that the present lipase is a “true” lipase. It acted preferentially on oils with lower chain fatty acid esters. It showed more than three-fold activity with tributyrin as compared to triolein. Triacetin was a poor substrate and was hardly hydrolyzed (Table 4.4). It was also observed that the lipolytic activity on vegetable oils with C-18 unsaturated fatty acids increased with increase in the degree of unsaturation and the percentage of unsaturated fatty acids. (Olive oil< cottonseed oil < Sunflower oil).

Schmidt-Dannert *et al*. (1997) have reported that the lipases from *Bacillus thermocatenulatus*, *Staphylococcus hyicus* and *Staphylococcus epidermidis* hydrolyzed tributyrin preferentially. They have also reported that a lipase from *Bacillus thermocatenulatus* failed to hydrolyze triacetin as observed with the present lipase. Chartrain *et al*. (1993) have reported that *Pseudomonas aeruginosa* MB 5001 lipase was more active towards lipids containing C-18 unsaturated fatty acid and showed similar

increase in activity as the percentage of unsaturated fatty acid in the oils increased, as found with the present lipase.

Table 4.4 Substrate specificity of the lipase

Substrate	Relative activity (%)
Castor oil	35.7
Coconut oil	142
Cottonseed oil	112
Groundnut oil	34.5
Olive oil	105
Sunflower oil	121
Span 20	23
Triacetin	0
Tributyrin	357
Triolein	100
Tween 20	23

Conclusions

An alkaline lipase produced by *Pseudomonas fluorescens* was purified to homogeneity. The purification involved use of a novel enrichment and concentration technique of foam separation. The lipase was purified in three steps, involving use of only one column chromatography step. The present lipase formed high molecular weight aggregates. The enzyme was active at alkaline pH and was stable over range 3-11. It had temperature optimum at 55°C and retained more than 80% activity at 55 °C for 2h. Thus, this lipase can find application in the detergent industry. The lipase showed marked regioselectivity for 1-3 positions of triolein and hence, can also be useful in chiral synthesis.

CHAPTER 5

Optimization of physicochemical parameters for downstream processing of alkaline lipase from *Pseudomonas fluorescens* NS2W using foam separation

Summary

The parameters for optimum operating conditions for foam separation of the lipase from culture supernatant of *Pseudomonas fluorescens* NS2W were studied using factorial design. In batch process, the maximum enrichment was obtained with more foam column height and lower airflow rate. Liquid pool height did not have any effect on enrichment of lipase in the foamate. The optimum pH, temperature and salt concentration for enrichment of lipase in foamate in batch process were, 4.8, 30 °C, and 0.05 M NaCl, respectively. Dilution of fermented broth resulted in better enrichment of the lipase in foamate. The continuous foam separation was performed under conditions optimized by batch process. The highest enrichment ratio values obtained for batch process and continuous process were 22.7 and 22.9, with simultaneous recovery values of 86% and 71%, respectively.

Introduction

With advances in enzyme based biotechnological processes, it has become important to develop cheaper methods for isolation and purification of enzymes from microbial culture filtrates. A biotechnological process for production of microbial proteins can be divided in two parts, the fermentation as synthesizing step and downstream processing for isolation and purification of the desired product. The conventional separation techniques used in laboratories like precipitation, membrane separation and chromatographic procedures have limitations in large scale continuous or batch processes as well as economics of the process.

Foam separation

Foam concentration or foam fractionation is a separation technique in which surface-active solutes are concentrated from very dilute solutions by preferential adsorption at a gas-liquid interface, created by sparging an inert gas through a surfactant solution (Uraizee and Narsimhan 1995a). These gas bubbles entrain the surfactant to form stable foam with large gas-liquid interfacial area. As the foam moves upwards through the column, the surfactant solution tends to drain due to gravity and capillary forces. Because of this, the liquid holdup in the foam decreases, resulting in a larger interfacial area per unit volume of the liquid. Collapse of the foam, therefore, results in the enrichment of surfactant and recovery of adsorbed surfactant in the bulk. The extent of enrichment depends upon the relative amount of adsorbed surfactant compared to that in the bulk. A very large gas-liquid interfacial area would ensure larger amounts of adsorbed surfactant. In addition, the nature of adsorption isotherm of surface-active materials leads to much higher proportion of adsorbed surfactant at very low bulk concentrations. Consequently, foam concentration is found to be very efficient for recovery of substances like proteins from extremely dilute solutions, leading to the separation of mixtures.

Design and operation of foam separation column

A schematic diagram of foam separation column is shown in Figure 5.1. An inert gas is sparged in to the liquid pool maintained at the bottom of the column. The surface-active compounds adsorb on to the gas bubbles during their movement through the liquid pool. Upon reaching the top of the liquid pool, the bubbles form foam, which moves upward in the column. As the foam moves upward in the column, the liquid entrained by the foam gets drained as a result of gravitational and capillary force. Consequently, the liquid

holdup of foam decreases as it moves up the column. The foam from the top of the column is transferred to a foam breaker where the foam is broken by the application of mechanical shear. Because of very small liquid holdup, the foam at the top of the column has an extremely large gas-liquid interfacial area per unit volume of the liquid. Collapse of the foam recovers adsorbed surface-active material from this large interfacial area in to the bulk resulting in several-fold concentration of the material.

In case of a mixture of several compounds, a material that adsorbs preferentially at the gas-liquid interface will be enriched in the top product, leading to its separation. The foam column can be operated in a batch or continuous mode. In batch mode, the surface-active material is continuously depleted from the liquid pool and recovered in the top product. As a result, its concentration in the liquid pool decreases with time. In continuous mode, the column is continuously fed with inlet stream and the bottom product is continuously withdrawn. In some cases, the top product is refluxed back in to the column in order to increase separation efficiency (Uraizee and Narsimhan 1995a).

The idea that foam separation can be used for protein recovery is not new (Uraizee and Narsimahan 1990 a, b). Several workers have investigated the foam separation technique for the separation of enzymes from crude preparations and culture filtrates (London and Hudson 1953, Sugiura and Isobe 1975, Holmström 1968, Sarkar *et al*. 1987, Bhattacharya *et al*. 1991, Zeh *et al*. 1992, Banerjee *et al*. 1993, Montero *et al*. 1993).

During foam separation, a selective separation of proteins takes place because of differences in the surface activities of different proteins. The surface activity of the solutes, like proteins, depends on physicochemical characteristics, *e.g.* size, charge and

hydrophobicity, as well as the environmental conditions like ionic strength, pH and presence of other additives (Brown *et al*. 1999a).

The foam separation process can be carried out in a batch mode or continuous mode. The effect of process variables on separation efficiency of protease from placental extracts was studied by Sarkar *et al*. (1987) in batch mode and by Bhattacharya *et al*. (1991) in continuous mode.

There have been very few comprehensive studies on the effect of process conditions on process performance and Bovine serum albumin (BSA) has been studied, as the model protein, almost exclusively (Brown *et al*. 1990, Uraizee and Narsimhan 1995b, 1996). Wenzig *et al*. (1993) have studied different methods of downstream processing for *Staphylococcus hyicus* lipase fermentation broth. They studied effect of various process variables like superficial gas velocity, foam height, liquid height, temperature, pH-value, protein concentration and feed rate for foam fractionation of the lipase. Brown *et al*. (1999a, 1999b) have studied the interactive effect of process variables and optimized the operating conditions for continuous foam separation, using β-casein, by factorial design. They have further studied selective recovery of proteins using binary mixtures, under these optimized conditions.

Lipases are industrially very important enzymes and find applications in many fields including detergent, chemical and pharmaceutical industry. Sugiura and Isobe (1975) have reported that, the hydrophobic amino acid content of various lipases was higher than that of other enzymes making lipases more hydrophobic. Aqueous solutions of lipases and denatured proteins were shown to have surface activity as strong as that of synthetic detergents. Sugiura (1984) compared the ability of several proteins to bind to the air-

water interface using foam fractionation. It was observed by him that lipases strongly adsorbed to the bubbles, whereas other enzymes and proteins including, esterase, amylase, protease *etc.* were not significantly concentrated in foamate.

In this chapter, optimization of process parameters for selective enrichment of a lipase from fermentation broth of *Pseudomonas fluorescens* NS2W using foam separation technique is presented. The process parameters were first optimized for batch process and were further applied to the continuous foam separation of the lipase.

Materials and Methods

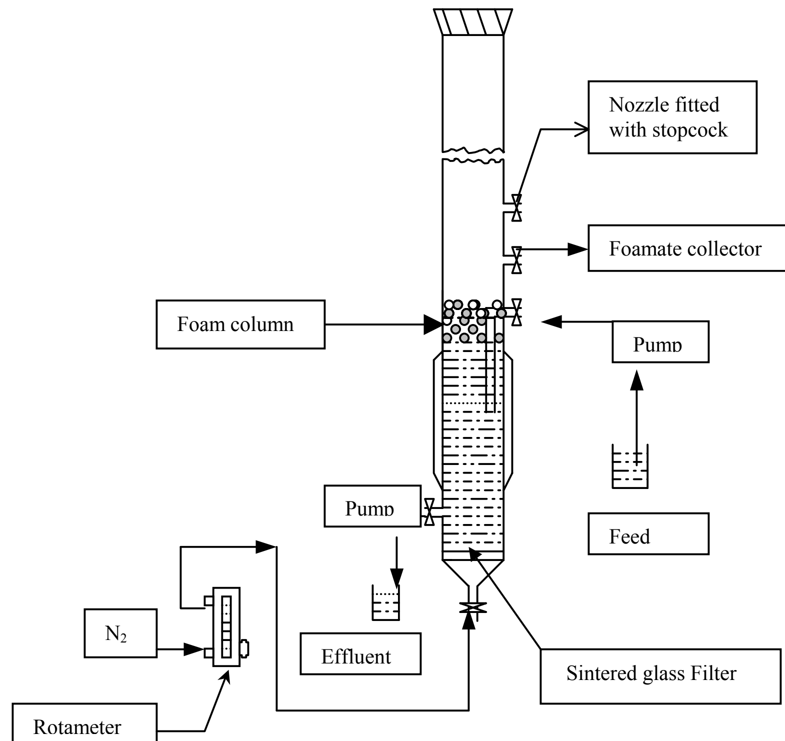
Foam fractionation column

The foaming system was constructed as presented in the schematic diagram in Figure 5.1. The basic components were foaming column, an air compressor, gas-flow meter, foam collecting vessel, vacuum pump and a controlled temperature water bath. The foaming column had an internal diameter of 32 mm and had a glass jacket. The column was fitted with sintered glass filter with pore size 15 to 40 micrometers. The jacketed part held 160 ml of liquid. The column was fitted with a feed inlet and a feed outlet. The foam outlet nozzles were at every 6 cm interval, the first starting from 6 cm above the jacketed part. The foam column height was varied with the help of these nozzles during the time of experiment, whenever necessary.

Flow rate of the air being sparged in to the column was controlled using a rotameter. Two peristaltic pumps (Watson and Marlow, UK) were used to pump the liquid, in and out, at defined flow rates. The foam was allowed to flow into a sintered glass funnel and was made to collapse into a collecting flask, using vacuum. The volumes of retentate and

foamate were measured for all the experiments. The system was operated under batch and continuous mode.

Figure 5.1 Schematic diagram of a foam separation column



Performance criteria

The following performance parameters were determined for each foaming experiment.

1) Enrichment ratio = Lipase units per ml of foamate /Units per ml of lipase in the initial feed

2) Recovery (%) = (Total units of lipase in the foamate /Total units of lipase in the initial feed) X 100

Lipase production

The enzyme was prepared by growing *Pseudomonas fluorescens* NS2W for 52 h, in the medium optimized by factorial design, as described in Chapter 3. The cells were removed from the broth by centrifugation at 20,000 *g* for 10 min. The supernatant was used for foam separation studies.

Enzyme activity measurement

The enzyme activity was measured using gas chromatography method as described in Chapter 2.

Protein estimation

The protein measurement was done by colorimetric method by Lowry *et al*. (1951), using Bovine serum albumin as the standard protein.

Factorial design

Factorial design was used to study the effect of three physical parameters on foam separation of the lipase from fermentation broth. The experiment was done in batch mode of operation. A two-level, three-variable factorial was designed using the approach given in standard texts on design of experiments to study the effect of variation of, liquid pool height, foam bed height and gas flow rate on foam separation of the lipase (Box *et al*. 1978, Davies *et al*. 1993). The experimental standard error was determined by doing five identical batch experiments.

The next set of experiments was performed in the direction of steepest increase, keeping the liquid pool height constant, to obtain the optimized foam separation conditions.

Effect of temperature , protein concentration, salt concentration and pH on foam separation of lipase

The effect of varying temperature, pH and protein concentration in the initial feed and the effect of addition of salt to the feed were studied independently, in batch mode. All these experiments were carried out at constant foam bed height, pool height and gas flow rate values of 22.5 cm, 19.5 cm, and 75 ml/min, respectively. The effect of liquid pool temperature on lipase separation was studied by maintaining the temperature of the liquid by circulation of water through the water jacket, from a controlled temperature water bath. The effect of temperature was studied at 25, 30 and 35°C. The effect of protein concentration in the feed solution was studied by diluting the fermentation broth with distilled water. The effect of salt concentration was studied by addition of solid NaCl to the fermentation broth to make final salt concentration in the range 0.05 to 1 M. The effect of pH was studied by changing the pH of fermentation broth, by addition of solid citric acid or sodium carbonate to get desired pH in the range 3.7 to 6.8.

Enrichment of lipase in the foam as a function of time

A foam separation experiment was done under conditions optimized by batch studies. The foam bed height was 28.5 cm; the pool height was 19.5 cm and gas flow rate used was 45 ml/min. The temperature was maintained at 30 °C and pH was adjusted to 4.8 using citric acid. Foamate samples were collected at regular intervals of 30 min, for 2.5 h and analyzed for the lipase and protein content, as described before. The pattern of proteins present in the foamate as the experiment progressed was studied by SDS-polyacrylamide gel electrophoresis of the foamate samples.

Continuous foam separation

Continuous foam separation was investigated under conditions optimized by the factorial design for batch process. The foam bed height was maintained at 28.5 cm; the pool height at 19.5 cm and the gas flow rate used was 45 ml/min. The fermentation broth without any additives was used for the study. The pH of broth was 5.8 and the temperature of liquid pool and feed reservoir was maintained at 30 ° C. The effect of change in the feed flow rate on the enrichment of lipase was studied under continuous mode of operation.

Results and Discussion

Factorial design

The effect of three variables *viz*. foam bed height, liquid pool height and gas flow rate, on the separation of lipase by foam separation was studied using two-level three-variable factorial design. The standard error calculated from 5 identical experiments was found to be 0.05. All the calculations were done using enrichment ratio as the criterion. The effects having values "less than two times standard error" were presumed to be due to experimental error and were neglected.

The values obtained for the main effects showed that, the enrichment of lipase from culture supernatant was significantly influenced by foam bed height and gas velocity (Table 5.1). Liquid pool height did not have any significant effect on the enrichment as well as the recovery of the lipase.

Effect of foam bed height

Foam bed height was found to have a positive effect on enrichment, indicating that further increase in foam bed height will yield better enrichment values. Increase in the foam column height tends to enhance the enrichment of lipase in the foamate. In general, the conditions that give “drier” foam were found to enhance enrichment of lipase in the foamate. The lower liquid holdup in dry foam promoted greater protein concentration in the foam phase, resulting in higher enrichment ratios.

Table 5.1 Results of factorial design

Batch	Foam height (X_1)	Pool height (X_2)	Air flow (X_3)	Enrichment ratio
1	+	+	+	0.90
2	+	+	-	2.33
3	+	-	+	1.61
4	+	-	-	2.31
5	-	+	+	1.42
6	-	+	-	1.78
7	-	-	+	1.33
8	-	-	-	1.53

Levels – Foam bed height (+) 28.5 cm, (-) 16.5 cm; pool height (+) 25.5 cm, (-) 13.5 cm; gas flow rate (+) 100 ml/min, (-) 50 ml/min. Effects- α_1 0.2763, α_2 -0.0888, α_3 -0.6702

This is in agreement with previous research findings. Several workers have reported that enrichment of protein in foamate increased with increase in foam height (Uraizee and Narsimhan 1996, Gehle and Schügerl 1984, Brown et al. 1999a).

Effect of gas flow rate

The gas velocity was found to have a negative effect on enrichment indicating that the decrease in gas flow rate will give better enrichment values. Enrichment ratio was found to decrease, as the gas flow rate was increased. Higher gas flow rate was found to decrease the residence time of foam in the column and caused more liquid entrapment in the foam. This resulted in lesser protein concentration in the foamate and hence lower enrichment ratio. Lower gas flow rates, in contrast, allowed more drainage of liquid from the foam, increased the protein concentration in the foamate and resulted in higher enrichment ratios. Similar results were observed in case of BSA (Uraizee and Narsimhan 1996) and β-casein (Brown *et al*. 1999a). In contrast to enrichment values, the recovery increased as gas flow rate was increased due to increased entrainment. The gas flow rate was found to have very strong positive effect value (+ 26.01) for recovery of the lipase in foamate, when the results of factorial design were analyzed in terms of "% recovery" value (data not shown). Brown *et al*. (1990) have shown that recovery of BSA increased with increase in gas flow rate. Sarkar *et al*. (1987) have reported that the decrease in gas flow rate increased the concentration of human placental protease in the foamate. Bhattacharya *et al*. (1991) studied effect of several physicochemical parameters on enrichment of proteins from placental extract using continuous foam fractionation. They observed that at particular feed flow rate, the enrichment ratio decreased with increase in the nitrogen flow rate.

In the present work, air was used in place of inert gas to generate foam. It was observed that the use of air, instead of nitrogen, did not result in any loss of activity of the lipase. This is understandable considering that the enzyme was secreted by the organism during

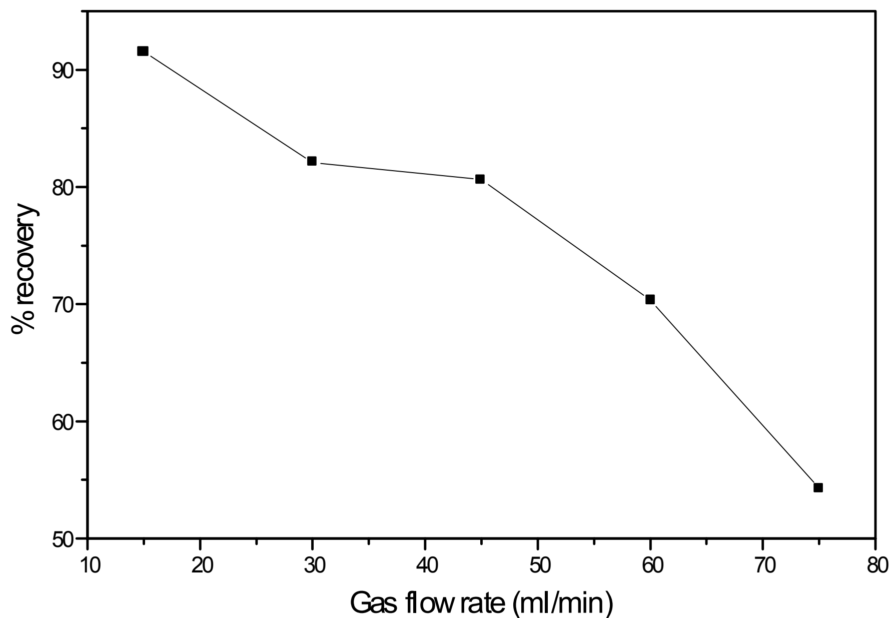
an aerobic fermentation and was exposed to oxygen for several hours before the fermenter was harvested. It has been reported previously that frothing can promote air-oxidation of proteins (e.g. its sulfhydryl group) and surface denaturation (Englard and Siefter 1990). Many workers have used inert gas, like nitrogen, for foam flotation of proteins to avoid such inactivation (Bhattacharya *et al*. 1991, Uraizee and Narsimhan 1996, Lockwood *et al*. 2000). The use of air, in place of N_2, can decrease the operational cost of foam separation, as seen in the present investigation.

The experiment conduced in the direction of steepest ascent further confirmed that the increase in foam bed height and decrease in gas flow rate increased the enrichment of lipase in the foamate (Table 5.2). The increase in gas flow rate was also shown to increase the recovery of lipase in the foam (Figure 5.2).

Table 5.2 Experiments conducted in the direction of steepest ascent

Parameter	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Foam bed height (cm)	22.5	28.5	28.5	34.5	34.5
Pool height (cm)	19.5	19.5	19.5	19.5	19.5
Gas flow rate (ml/min)	75	60	45	30	15
Run time (h)	1.5	2.5	3.5	6	11
Enrichment ratio	2.42	3.11	9.48	15.86	22.66

Figure 5.2 Effect of gas flow rate on recovery of lipase in the foamate



Effect of temperature

The effect of temperature on enrichment of the lipase in foamate was studied at temperatures 25°C, 30°C and 35°C. The results show that maximum enrichment and recovery was obtained at 30°C (Table 5.3).

Table 5.3 Effect of temperature on enrichment and recovery of lipase

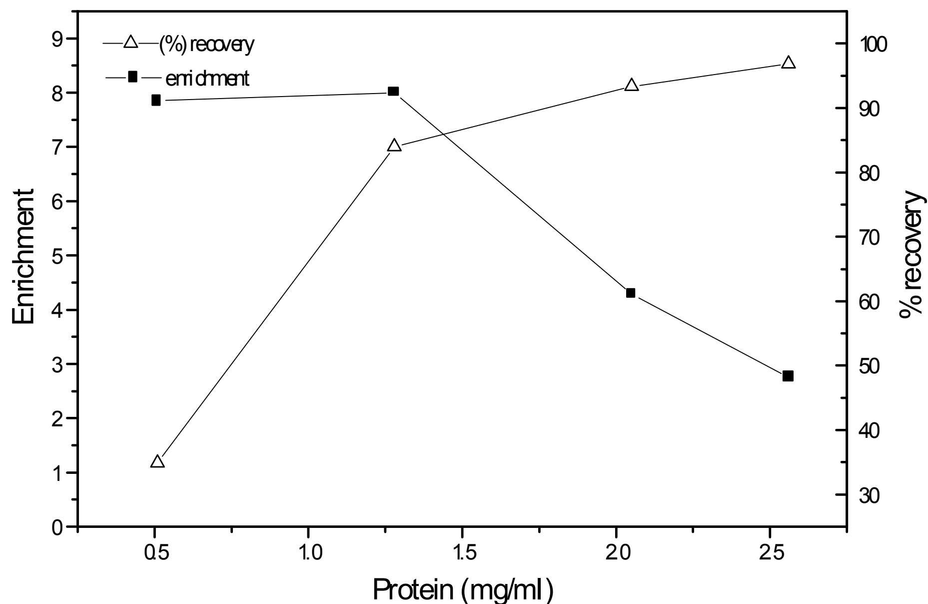
Temperature (°C)	Enrichment	% Recovery
25	2.01	69.48
30	2.91	89.90
35	2.15	78.53

Wenzig *et al*. (1993) have reported that temperature exerted large influence on enrichment of *Staphylococcus hyicus* lipase, and 30 °C was found to be the optimum temperature.

Effect of the protein concentration

The effect of protein concentration on foam separation was studied by diluting the culture supernatant with distilled water. Enrichment ratio of the lipase was found to increase as the protein concentration in liquid decreased (Figure 5.3). In contrast, the recovery was found to decrease with decrease in the protein concentration. Sarkar *et al*. (1987) have shown that the purification and recovery of a protease from human placental extract varied significantly with total protein concentration, attaining a distinct optimum at a concentration of 0.25 mg/ml. They observed that protein did not concentrate enough in the dilute solutions to form stable bubbles, which can climb the column, whereas excess protein made bubbles too stable to allow for effective drainage.

Figure 5.3: Effect of protein concentration on enrichment and recovery of the lipase



Effect of salt concentration

The effect of salt concentration was studied by addition of NaCl to have final salt concentrations in the range 0.05 M to 1 M and it was observed that the addition of 0.05 M NaCl increased enrichment ratio from 1.48 to 4.83. Further increase in ionic strength did not have any effect on enrichment of the lipase in the foamate. At lower ionic strength the larger foam bubbles were formed resulting in higher enrichment values.

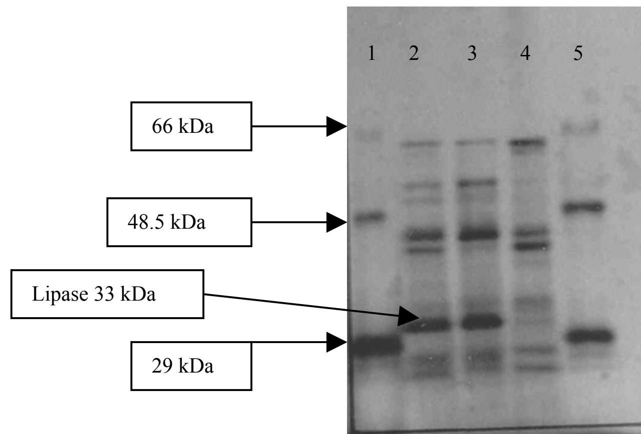
Brown *et al*. (1990) have studied the effect of addition of NaCl in the range 10 mM to 1 M and found that the enrichment of BSA decreased with increase in ionic strength at lower values of ionic strength. Beyond a concentration of approximately 0.1 M NaCl, the addition of more salt was found to have very little effect on the enrichment. The bubble size in the foam was found to increase as the ionic strength decreased, resulting in more enrichment.

Effect of pH

The effect of pH on separation of the lipase in the foamate was studied in the range 3.7 to 6.8 and the maximum enrichment was found at pH 4.8 (Figure 5.5). The enrichment ratio decreased above and below this pH. The isoelectric point of the present lipase was found to be in the range 4.8 to 5 (Chapter 4). The lipase was thus enriched maximally at its isoelectric point during foam separation. It was also observed that the pH of liquid affected the bubble size. At alkaline pH, the bubbles became very fine, resulting in entrainment of more liquid in foam. This, in turn, resulted in lower enrichment values. The bubble size increased with decrease in pH, resulting in more enrichment of lipase at acidic pH. At pH 3.72, the foam was not able to form a stable column and hence it was

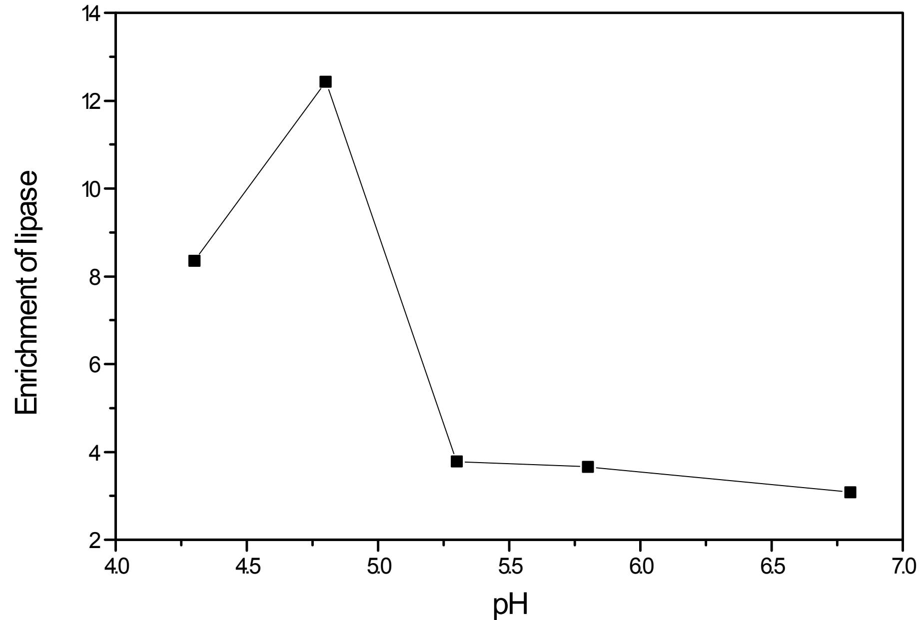
not possible to collect any foam from the port at 22.5 cm. The foam could, however, be collected from the port at lower height (16.5 cm) and it gave enrichment value of 11.22. The protein pattern of foamate and retentate of run done at pH 4.8 was studied on SDS-polyacrylamide gel (Figure 5.4). The lipase (33 kDa) was preferentially concentrated in foamate. The specific activity of lipase increased 5 fold after the foam flotation run with 12.43 fold enrichment. It can be seen that the retentate had very low lipase content.

Figure 5.4 SDS- PAGE of foam separation run at pH 4.8; lane 1 and 5, standard molecular weight markers; lane 2, crude broth (27.3 μg); lane 3, foamate (21 μg); lane 4, retentate (24.4 μg).



Proteins are reported to be least soluble and have minimum surface tension at their isoelectric point. Charm *et al*. (1966) have shown that when dilute solutions of partially purified amylase were foamed at its isoelectric point, the specific activity was 4.7 times higher in the foamate than that in initial feed.

Figure 5.5 Effect of pH on enrichment of the lipase in the foam



Sarkar *et al*. (1987) have studied the effect of pH on enrichment and purification of a protease from human placental extracts. The fold purification of protease was maximal at pH 8, close to the isoelectric pH, at which the surface adsorption of the protein on the foam bubbles was maximum. The present lipase was also enriched maximally in foamate near its isoelectric point, with 5.4 fold increase in the specific activity. Holmström *et al*. (1968) have studied the effect of pH of the liquid on foam separation of a streptokinase from crude culture filtrates of a streptococcal culture. At a pH close to the isoelectric point, 40 –50% of the enzyme was inactivated, but at a pH close to 7, about 80% of the enzyme was recovered. In contrast, the present lipase was not inactivated at its isoelectric point and could be enriched maximally in the foam.

Brown *et al*. (1990) reported that the enrichment of BSA was higher at pH 7 as compared to its isoelectric point (4.8). The enrichment increased as the protein concentration in the feed solution was decreased. They studied the change in the bubble size with change in pH and found that the bubble size was minimal at pH 4.8. This resulted in higher liquid hold up and hence lowered the enrichment. The bubble size was increased again at pH 3.5. This change in the bubble size may mask any other effect caused by pH change.
Wenzig *et al*. (1993) have studied effect of pH on enrichment of a lipase in foam. They reported that pH had weak effect on enrichment as compared to other process variables tested and slightly acidic medium was found to have favorable effect on enrichment. In the present work it was found that pH had significant effect on enrichment. The present lipase was also enriched more at acidic pH.

Continuous foam separation

Continuous foam separation batches were performed at different feed flow rates and it was observed that the enzyme enrichment increased as the feed flow rate was decreased. This was presumably because of the higher residence time of the liquid in the foam separation column. The maximum enrichment ratio obtained was 9.4 at pH 5.8 at a feed flow rate of 0.5 ml/min. A continuous run was also done at pH 4.8 to give maximum enrichment of the present lipase resulting in an enrichment ratio of 22.9 and specific activity of the lipase was found to increase by 4.2 fold.
Gehle and Schügerl (1984) have reported that low feed flow rates increased enrichment of BSA in foam in continuous foam flotation runs. Brown *et al*. (1999) have observed that the low feed flow rates promoted higher enrichment of β-casein in foam. In the present work, lower feed flow rates were found to promote higher enrichment of lipase.

Effect of time on enrichment of lipase in foam

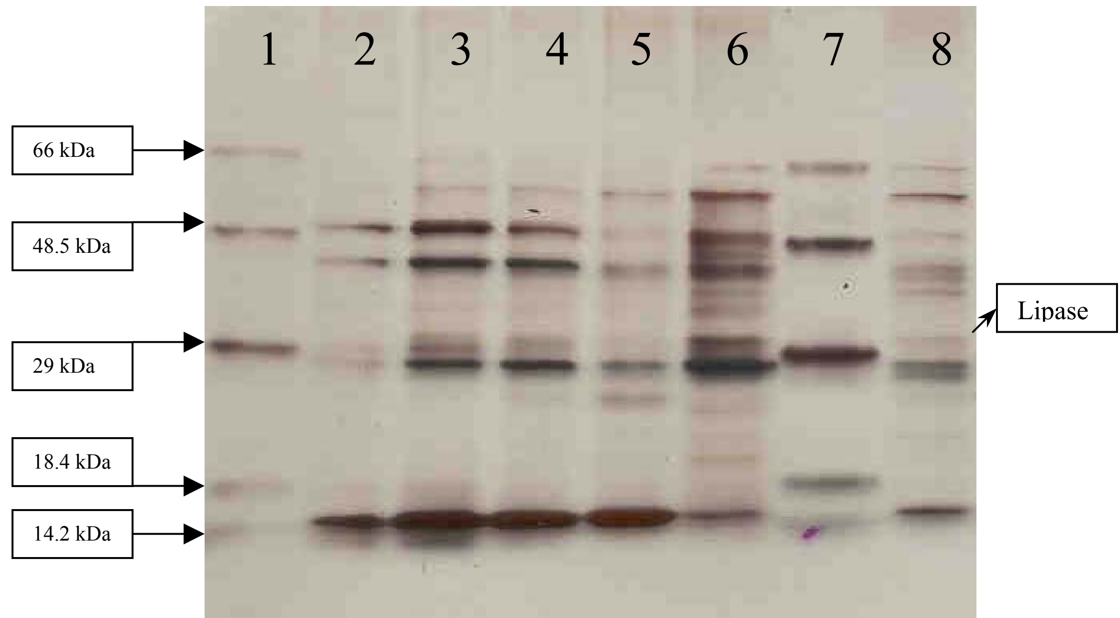
The lipase was found to be enriched maximally in foamate in fraction- 4 taken at 2 h, whereas the specific activity was maximal in fraction-1, taken at 30 min run time (Table 5.4). It was observed that the bubble size of the foam changed as the run time increased.

Table 5.4 Effect of time on enrichment of lipase in the foam

Fraction no.	Time (min)	Lipase (U/ml)	Protein (mg/ml)	Enrichment	Specific activity (U/mg)
1	30	203.1	4.66	19.9	43.5
2	60	153.9	11.91	15.0	12.9
3	90	331.9	16.21	32.5	20.5
4	120	425.6	17.95	41.7	23.7
5	150	333.3	18.96	32.6	17.6
Crude broth	-	10.2	2.24	-	4.55

The bubble size was very fine initially and this resulted in higher entrapment of liquid in earlier fractions. This might be the reason for lower enrichment value observed for fraction-1, as compared to fractions 3, 4 and 5. The electrophoresis of the fractions also showed prominent band of the lipase (33 kDa) in the first fraction, supporting the higher specific activity observed (Figure 5.6). It was also observed that there were more proteins with higher molecular weight in first fraction.

Figure 5.5 SDS- PAGE of foam fractions; lane 1 and 7, standard molecular weight markers; lane 2, fraction 5 (21 μg); lane 3, fraction 4 (21 μg); lane 4, fraction 3 (20.0 μg); lane 5, fraction 2 (21.6 μg); lane 6, fraction 1 (23.2 μg); lane 8, crude broth (22.4 μg).



The low molecular weight protein (~ 15 kDa) was enriched more with time, showing maximum enrichment in the fraction 4.

Conclusions

The process variables were optimized to obtain best enrichment of *Pseudomonas fluorescens* NS2W lipase using foam separation. The process was optimized for batch mode of operation and successfully applied to continuous mode of operation. The technique is very easy and cost effective and can be used for downstream processing of lipase on large scale.

CHAPTER 6

Fermentation studies on alkaline lipase from *Pseudomonas fluorescens* NS2W

Summary

Lipase production from *Pseudomonas fluorescens* NS2W was studied in batch mode, in automated bioreactors. Effect of various fermentation parameters on the cell growth and enzyme production was studied. The specific growth rate of the organism was highest at 25 °C, whereas the maximum lipase production was observed at 30 °C. In the fermentation batch with pH control at 7.2, the enzyme production was more than that in the fermentation batches in which pH was controlled either at 6.2 or 8.2. The cell growth as well as lipase production was more at agitation speed 300 and 400 rpm, as compared to agitation speed 500 and 600 rpm.

Introduction

Lipases are the extracellular enzymes that are secreted in the culture medium, although a few intracellular lipases have been reported (Mourey 1981, Lee and Lee 1989). In most of the microorganisms, lipase production is growth associated and the concentration of the enzyme is found to reach its maximum in the late exponential phase or stationary phase of growth curve.

Lipases are mostly inducible enzymes although there are a few reports of constitutive lipase production (Elwan *et al*. 1983, Gao *et al*. 2000). Production of lipases is reported to be enhanced in media containing oils or oil-related compounds (Suzuki *et al*. 1988, Iizumi *et al*. 1990, Sugihara *et al*. 1991). However, production of lipase using sugars or other carbohydrates as carbon source has also been reported (Sztajer and Maliszewska 1988a, Gao *et al*. 2000).

Lipase production by microorganisms can be increased by optimization of various fermentation parameters. There are relatively few published reports of detailed fermentation studies for lipase production in batch mode of operation. Harris *et al*. (1990) have optimized the pH, temperature and degree of aeration, for lipase production by *Pseudomonas fluorescens* in shake flasks, using response surface methodology. Watanabe *et al*. (1977) have reported production of lipase by *Pseudomonas nitroreduscens* and *Pseudomonas fragi* in 20 l fermenters. The culture conditions were optimized with respect to medium pH, incubation temperature, agitation speed and the type of antifoam used for the production of lipase in batch mode. Gilbert *et al*. (1991a) have studied effect of temperature and medium pH on the lipase production by *Pseudomonas aeruginosa* EF2 in Tween 80-limited, continuous culture.

In this chapter, we report optimization of the various fermentation parameters like temperature, pH and agitation for lipase production by *Pseudomonas fluorescens* NS2W.

Materials and methods

Chemicals and media components

Analytical grade $NH_4H_2PO_4$, $CaCl_2.2H_2O$, $MgSO_4$ $7H_2O$ and gum *Acacia* were from S. D. Fine Chemicals, Boisar, India. Peptone and yeast extract were from HiMedia, Mumbai, India. The refined groundnut oil was purchased locally.

Bacterial strain

The alkaline lipase producing strain of *Pseudomonas fluorescens* NS2W (NCIM 5145), isolated from a soil sample as described in Chapter 3 was used in this study. Experiments

to assess the effect of temperature, pH and agitation were carried out in 1 l and 14 l fermenters, in the medium previously optimized by factorial design at flask level (Chapter 3).

Lipase assay

Lipase activity was determined using gas chromatography method as described in Chapter 2.

Cell growth measurement

Cell growth was monitored by measurement of turbidity at 600 nm using a UV-Visible spectrophotometer (Shimadzu UV-240, Japan). Each sample was diluted with physiological saline (8.5 g/l NaCl) and the absorbance was measured immediately at 600 nm. For dry cell weight (DCW) estimation, the sample (usually 10 ml) was centrifuged at 20,000 *g* for 20 min, supernatant was used for estimation of lipase activity and cell pellet was taken to dryness in a vaccum oven, at 60 °C, to a constant weight.

Lipase production in the fermenters

The lipase production was studied in a 1 l bioreactor (working volume 400 ml, magnetic bottom drive, Gallenkamp, UK) as well as in a 14 l bioreactor (working volume 5 l, New Brunswick Scientific, USA). The New Brunswick bioreactor was equipped with three six-bladed Rushton-disk impellers, fitted on a top-driven shaft. The inoculum was grown in the medium composed of (g/l) peptone 5, yeast extract 2.5, K_2HPO_4 1 and $MgSO_4.7H_2O$ 0.2; for 10 h. A 5% (v/v) inoculum was transferred to the fermenter. Antifoam (Sigma 289) was used at a concentration of 0.05 ml/l in the fermentation

medium. A 10% (v/v) suspension of the antifoam in water was sterilized and added drop wise, to control the foaming. Samples were withdrawn from the fermenters using a peristaltic pump (Watson and Marlow, UK). The sample sizes for 1 l and 14 l fermenters were 1 and 10 ml, respectively.

Time-course of the lipase production

The profile of lipase production was studied in the 1 l fermenter (Gallenkamp, UK). A 40 ml inoculum was added to 400 ml of optimized, sterile medium in the fermenter. The fermenter was operated at 30 °C with 0.7 l/min airflow rate and 400 rpm agitator speed. Samples were removed periodically and analyzed for growth and lipase activity.

Effect of temperature and pH on growth and lipase production

The effect of temperature on the growth of the strain and the lipase production was studied by growing it in a medium having 7.4 pH, at temperatures ranging from 20 to 35 °C, in the 1 l fermenter. The enzyme production was investigated at pH 6.2, 7.2 and 8.2. The pH was controlled at desired set points by automatic addition of 5 N NaOH or HCl.

Effect of agitation on growth and lipase production

The effect of agitation was studied in 14 l New Brunswick bioreactor. The speed of agitation was varied between 300 and 600 rpm. The cell growth and lipase activity were determined for all the experiments, as described before.

The consumption of groundnut oil under optimized conditions of pH, temperature and medium composition was investigated in the 14 l bioreactor. Samples of the culture broth were removed at regular interval. A 1 ml portion of each sample was centrifuged at

20,000 *g* for 20 min. The supernatant was used for estimation of lipase activity. The pellet was suspended in physiological saline and used for determination of the cell mass by measurement of O. D. at 600 nm, as described before. Another 10 ml portion of the sample was centrifuged as above, the pellet was washed by suspension and centrifugation in physiological saline three times and used for the determination of dry cell weight, as described before. The oil in the supernatant of 10 ml sample was extracted with equal volume of hexane, twice. The hexane was removed at 60 °C and the residual oil was weighed.

Results and discussion

Time- course of lipase production

The lipase was found to be secreted mainly in the late logarithmic growth phase as could be seen from Figure 6.1. The lipase concentration in the culture supernatant reached maximum at 48 h, when the culture was in the stationary phase. The lipase activity obtained in the optimized medium in 1 l fermenter was 68.7 U/ml, at an initial medium pH of 7.2. The Figure 6.2 shows the setup of 1 l fermenter.

Fox and Stepaniak (1983) have reported that secretion of a lipase by *Pseudomonas fluorescens* AFT 36 occurred mainly at the end of logarithmic phase and during early stationary phase. Al-Saleh and Zahran (1999) have reported that *Pseudomonas fluorescens* RM_4 secreted lipase maximally during late logarithmic phase, followed by a decrease in the lipolytic activity with the onset of stationary phase. Makhzoum *et al*. (1995) have reported that the lipase production by *Pseudomonas fluorescens* 2D

paralleled the growth of the organism. The entire enzyme was found to appear during active growth and its production stopped before stationary phase was reached.

Figure 6.1 Lipase production in 1 l fermenter

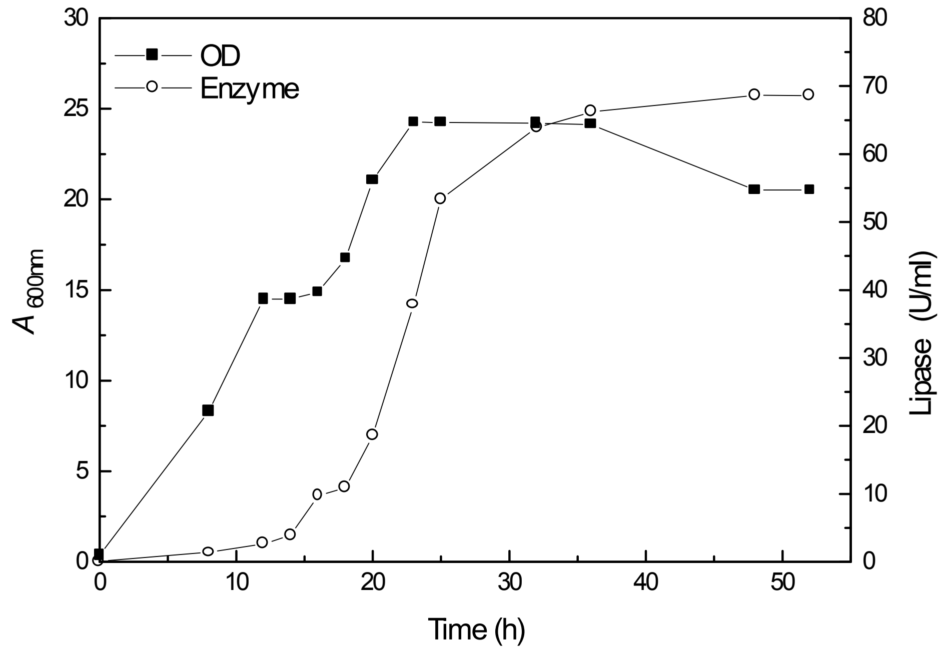
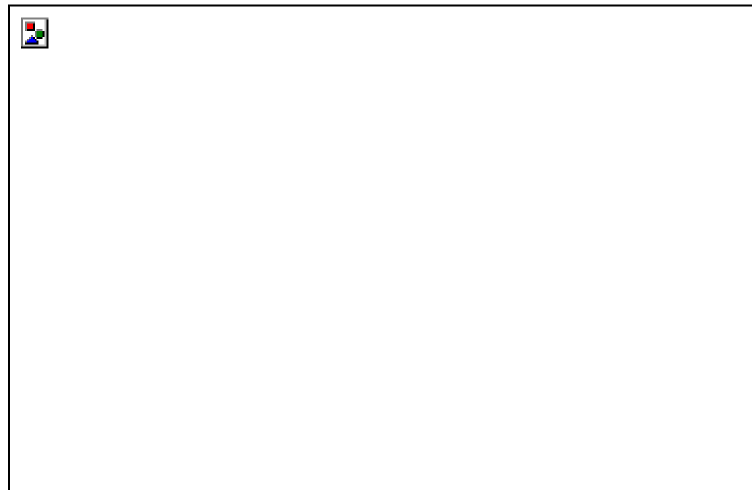
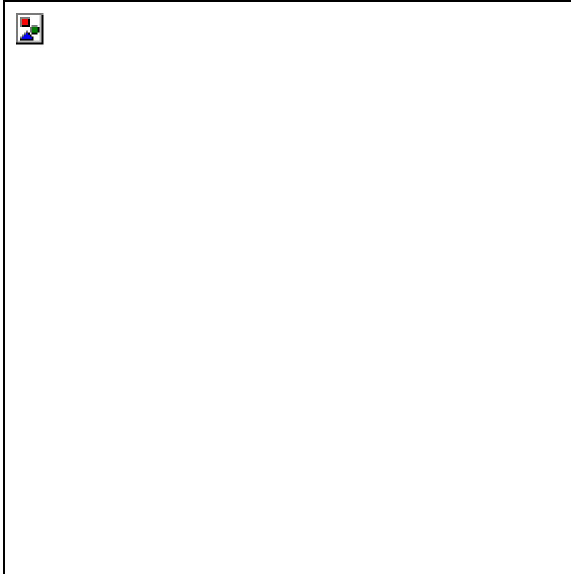


Figure 6.2 Setup for production of lipase in 1 l fermenter





The secretion of present lipase started after 8 h of growth when OD (600nm) 8.3 was reached. The concentration of the lipase reached its maximum (68.6 U/ml) when the culture reached the stationary phase. The enzyme was found to be stable and the lipolytic activity did not decrease till 72 h, even when the culture entered death phase.

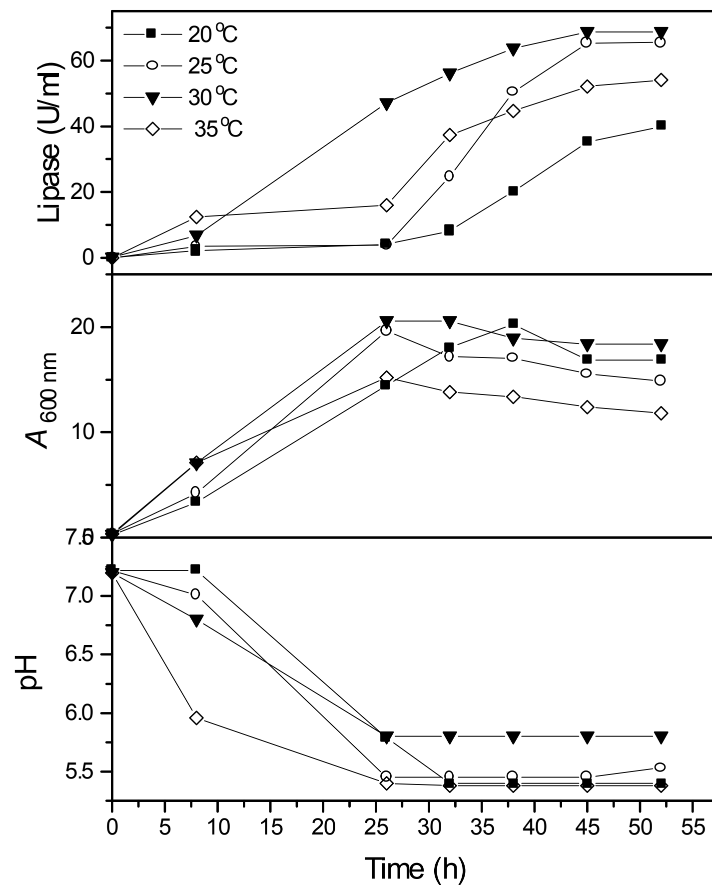
Effect of temperature

The organism was cultivated in the optimized medium in a 1 l bioreactor at various temperatures ranging from 20 to 35 °C at 5 °C intervals (Figure 6.3). The specific growth rate at 20 °C (0.18 h^{-1}) and 25 °C (0.20 h^{-1}) were comparable. *Pseudomonas fluorescens* NS2W is a mesophillic organism and produced maximum lipase at 30 °C. Although the specific growth rate at 30 °C (0.10 h^{-1}) was lesser than that at 20 or 25 °C, the organism produced maximum lipase (68.6 U/ml) at 30 °C. It was observed that at 35 °C, specific growth rate decreased to 0.063 h^{-1} and the enzyme production was 78% of that produced at 30°C.

The final pH of the medium was recorded for the fermentation batches performed at all the temperatures. The end pH values were found to be 5.45, 5.53, 5.8 and 5.38 at 20, 25, 30 and 35°C, respectively. The drop in the pH was relatively slow at 30 °C as compared to other temperatures. Further experiments were carried out at 30 °C.

The optimum temperature for lipase production varies greatly for different microorganisms. For mesophilic microorganisms, the optimum lies between 30 and 40 °C, whereas for thermophilic microorganisms, it is often between 50 and 60 °C.

Figure 6.3 Effect of temperature on growth and lipase production by *Pseudomonas fluorescens* NS2W

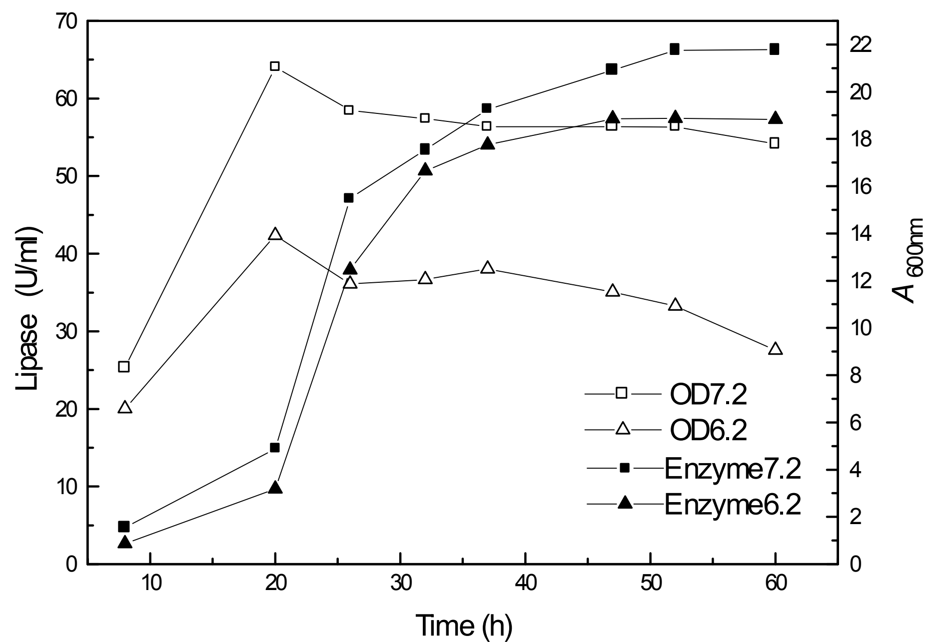


Bacillus thermoleovorans ID-1 was found to grow at a maximum specific growth rate of 2.50 h^{-1} in a medium with olive oil as carbon source at 65 °C, which is the highest growth rate on lipid substrates among thermophilic bacilli reported so far (Lee *et al*. 1999). Sztajer and Maliszewska (1988a) studied the effect of temperature on growth and lipase production by several bacterial cultures in the range 22 to 42 °C. The lipase production by different *Bacillus* sp. was found to be maximal at 42 °C. *Pseudomonas fluorescens* and *Streptomyces* were found to produce maximum lipase at 22 and 28 °C, respectively. *Pseudomonas fluorescens,* a psychrotrophic microorganism has been well studied for its lipase production. Different workers have reported different optimal temperatures for the production of lipase from this organism. Harris *et al*. (1990), have studied lipase production from *Pseudomonas fluorescens* by response surface methodology and found that 25 °C was the optimal temperature for lipase production. Guillou *et al*. (1995) have studied lipase production by *Pseudomonas fluorescens* in chemostat culture and reported that at lower dilution rates, the lipase production was maximal at 17 °C. Makhzoum *et al*. (1995) have reported an optimum temperature for lipase production to be 20 °C, for a psychrotrophic strain of *Pseudomonas fluorescens*. Al-Saleh and Zahran (1999) have studied the effect of temperature on growth and lipase production by the psychrotrophic strain, *Pseudomonas fluorescens* $RM_{4,}$ in the range 7 to 37 °C and observed that optimum temperature for lipase production and growth were 25 and 37 °C, respectively. The present isolate produced maximum lipase at 30 °C.

Effect of pH

Cell growth and lipase production were studied in optimized medium, at different pH values from 6.2 to 8.2 controlled on-line (Figure 6.4). Specific growth rate and lipase production at pH 7.2 were 25% and 20% more than that observed at pH 6.2. The lipase production was maximum at pH 7.2. At pH 8.2, excess foaming occurred between 12 and 20 h during the fermentation run. Also the organism did not grow well at this pH and gave a final optical density (600 nm) of 3.94 and the maximum lipase produced was only 4.87 U/ml at the end of fermentation (24 h).

Figure 6.4 Effect of pH on growth and lipase production by *Pseudomonas fluorescens* NS2W



Harris *et al.* (1990) have optimized lipase production by *Pseudomonas fluorescens* using response surface methodology. They found that optimum pH for lipase production was

8.07. Al-Saleh and Zahran (1999) have reported that *Pseudomonas fluorescens* RM_4 was able to secrete lipase over a wide pH range between 5.5 and 8.5, and the maximum lipase production was between pH 7.5 and 8.0. Gilbert *et al*. (1991a) studied effect of pH on the lipase production in Tween 80-limited continuous culture of *Pseudomonas aeruginosa* EF2 and found that maximum lipase was produced at pH 6.5. The present organism was able to synthesize lipase at 6.2 as well as 7.2. The lipase yield obtained by controlling pH at 7.2 was comparable to that obtained by growing culture without on-line pH control, but when the initial pH of the medium was 7.2. Hence in further experiments the culture was grown at 30 °C in medium with initial pH 7.2.

Effect of agitation

Pseudomonas fluorescens NS2W was cultivated in optimized medium at agitator speeds from 300-600 rpm in 14 l fermenters (Figures 6.5-6.8). At 400 rpm, the dissolved oxygen (DO) concentration decreased below 20% of saturation at about 5 h, remained between 4 and 6% for next seven hours and then started rising after 12 h incubation period (Figure 6.6). The maximum concentration of lipase was observed between 48 and 52 h of cultivation.

At 300 rpm, the DO decreased rapidly in first 12 h, remained at 3-8% of saturation till about 20 h and then started rising (Figure 6.5). The growth as well as lipase production was delayed at 300 rpm. The maximum yield of lipase was obtained at around 60-72 h of incubation. The lipase yield obtained at 300 and 400 rpm was comparable as were the yields of cell mass (Table 6.1).

The dissolved oxygen concentration reached a minimum of 28% saturation, at 10 h, in the 500 rpm run and to 58% saturation, at 10 h, in the 600 rpm run. The enzyme production in the batches performed at 500 rpm and 600 rpm was 64% and 29% of that obtained at 400 run. At 500 and 600 rpm, low cell yield was obtained as compared to that at 400 and 300 rpm fermentation runs (Table 6.1).

Table 6.1 Cell mass and lipase production yields at various agitation speeds in batch culture

RPM	Cell mass X_{max} (g/l)	Specific growth rate μ_{max} (h^{-1})	Cell yield X_{max} / Total substrate (g/g)	Lipase P_{max} (U/ml)	Specific enzyme productivity P_{max} / X_{max} (U/g)	Lipase yield (U/l)
300	10.5	0.03	1.4	54	38.57	5400
400	11.32	0.11	1.51	52.6	34.85	5260
500	4.98	0.05	0.66	34	51.20	3400
600	3.91	0.06	0.52	15.2	29.16	1520

Figure 6.5 Cell growth and enzyme production at 300 rpm agitator speed

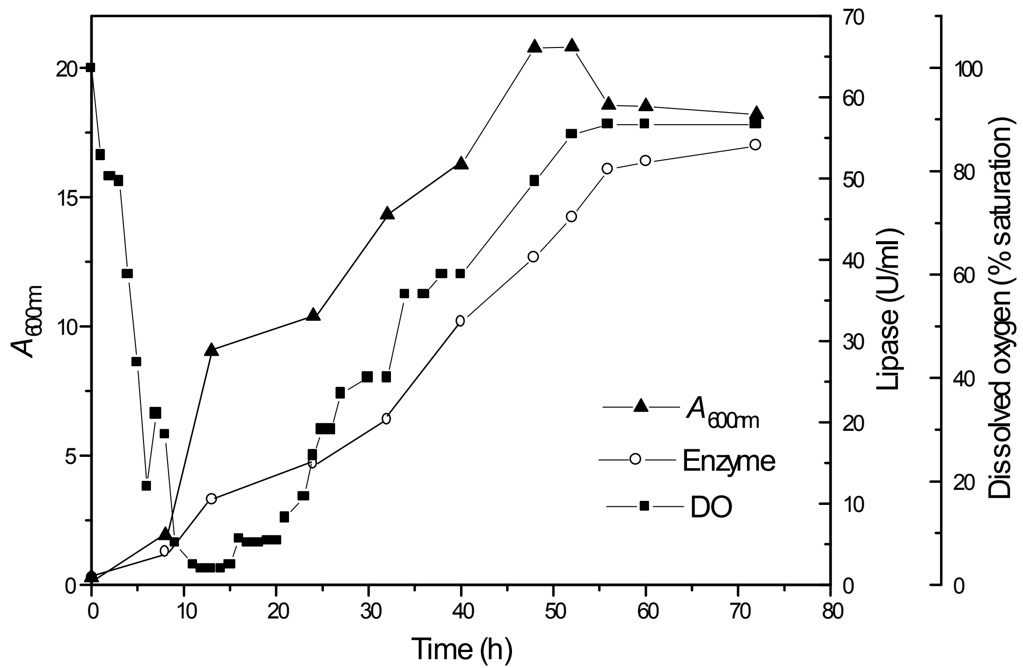


Figure 6.6 Cell growth and enzyme production at 400 rpm agitator speed

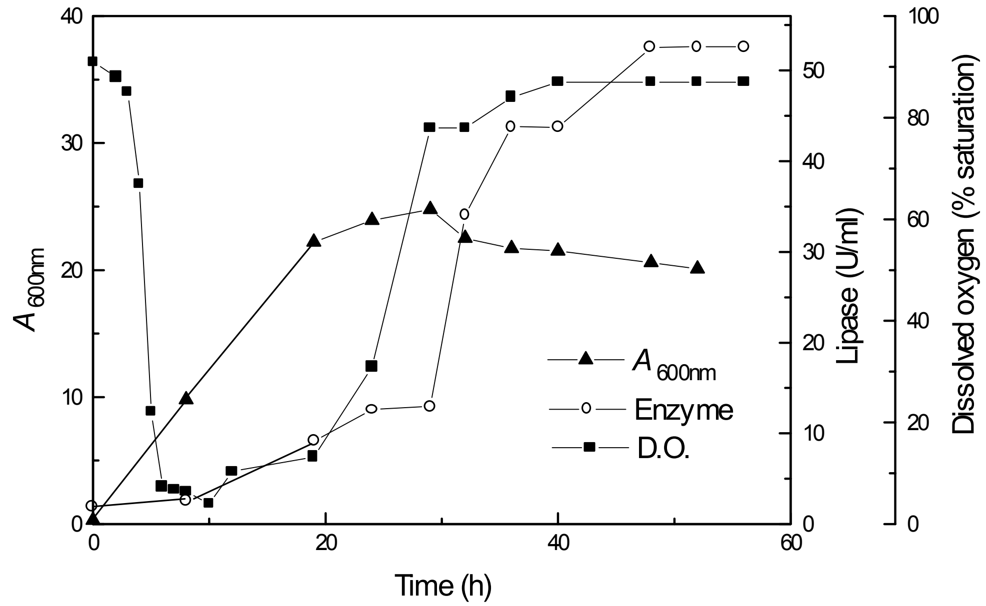


Figure 6.7 Cell growth and enzyme production at 500 rpm agitator speed

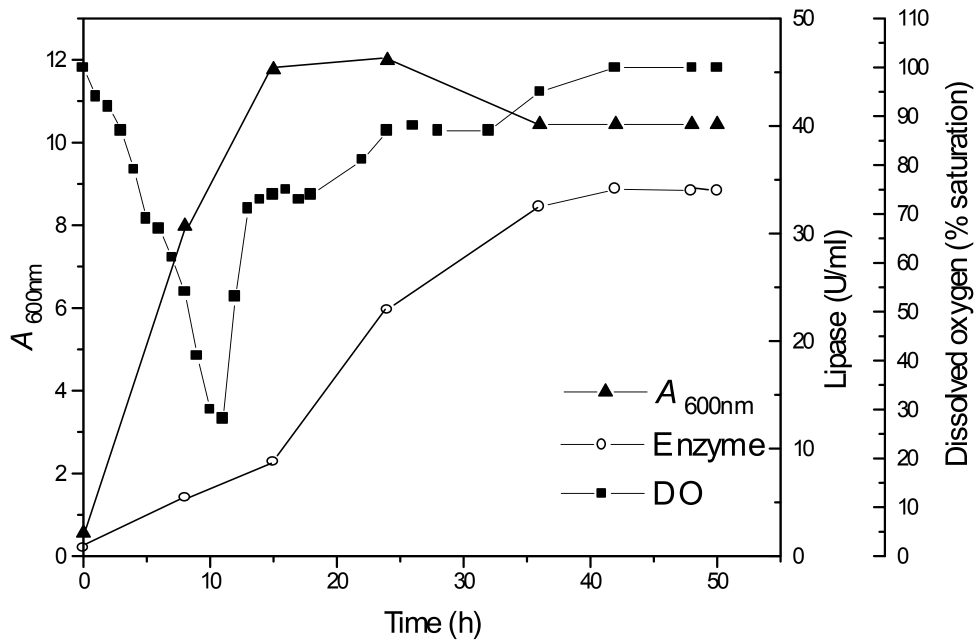
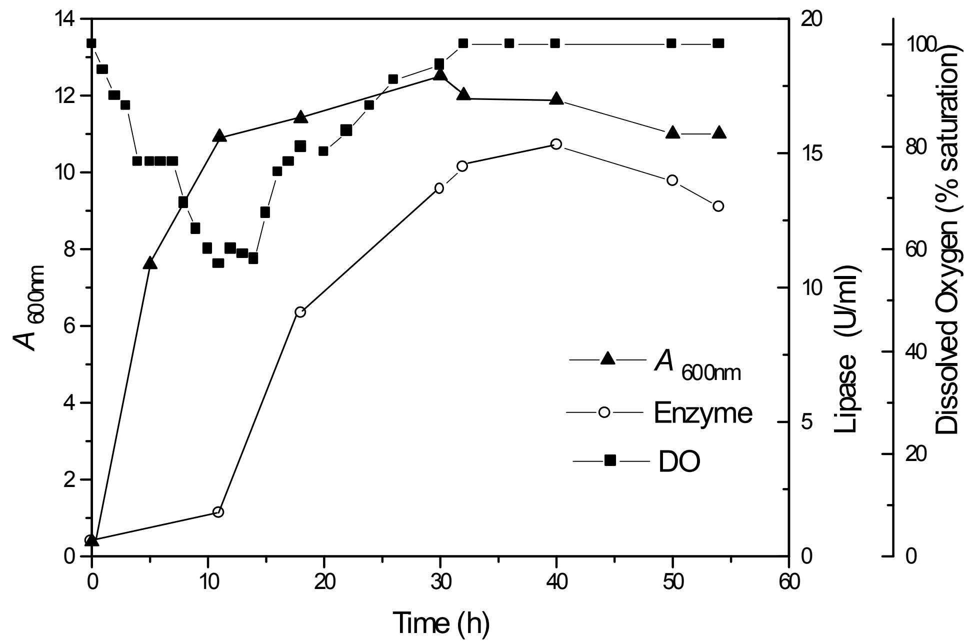


Figure 6.8 Cell growth and enzyme production at 600 rpm agitator speed



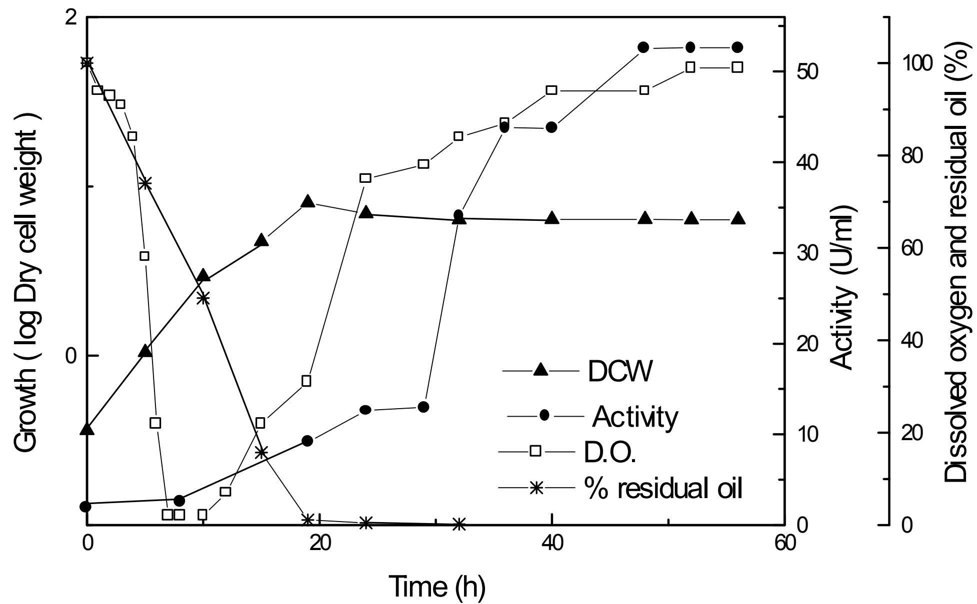
It was observed that after about 8 h of growth bacterial cells stick to oil and form large globules that stick to the sides of fermenter. This resulted in removal of part of oil from the medium. At higher agitation speeds such removal of oil appeared to be more. This might be the reason of lower cell concentration and lipase production at 500 and 600 rpm. The agitation speed of 400 rpm was used in further experiments.

Aeration of culture has been reported to have variable effect on lipase production and the degree of aeration was found to be critical (Alford and Elliott 1960). The amount of aeration needed varies among different organisms. Rowe and Gilmour (1982) showed that reduction in oxygen tension in a synthetic-milk medium increased lipase synthesis by *Pseudomonas fluorescens* strain. Chen *et al*. (1999) have studied effect of oxygen transfer

on lipase production by *Acinetobacter radioresistens*. They observed that increase in oxygen transfer rate either by aeration or agitation resulted in increase in lipase yield.

The consumption of groundnut oil was studied at 400 rpm agitator speed in 14 l NBS fermenter. The oil slowly decreased and there was no residual oil in the medium after 32 h of incubation. It was observed that the disappearance of oil from medium was followed by shoot up in lipase activity in the culture broth after 29 h of incubation. The lipase secretion reached maximum in the stationary phase (Figure 6.9).

Figure 6.9 Lipase production and groundnut oil consumption profile 400 rpm agitator speed



Conclusions

The parameters for lipase production by *Pseudomonas fluorescens* NS2W were optimized in 1 l and 14 l fermenters. The maximum lipase obtained in 1 l fermenter was 68.6 U/ml, which was comparable to the enzyme obtained in shake flask cultures, using optimized medium. Thus, the production of lipase was successfully scaled up to 1 l and 14 l fermenters.

CHAPTER 7

Product formulation and application studies:
A. Preparation of detergent additive and immobilization of lipase
B. Detergent compatibility and castor oil hydrolysis studies

Summary

The alkaline lipase produced by *Pseudomonas fluorescens* NS2W was spray dried to check its suitability as a detergent additive. The crude enzyme was spray dried and the effect of spray drying conditions on the recovery of active lipase was studied. The effect of several compounds as texture modifiers was studied to get a free-flowing powder. Among different compounds tested, the addition of sodium sulfate and sodium silicate resulted in the product with free-flowing character. Although the maximum (98%) lipase activity was recovered when the enzyme was spray dried in the absence of any texture modifier, their addition to the enzyme solution, before spray drying, yielded an enzyme product that had desirable physical characteristics.

The enzyme from the enzyme concentrate (foamate) was directly immobilized on Celite by adsorption and co-precipitation using chilled acetone. Using this immobilized preparation, 50 °C was found to be the optimal temperature for the castor oil hydrolysis. The immobilized lipase caused hydrolysis of castor oil efficiently and resulted in 65% hydrolysis in 24 h. The hydrolysis efficiency was found to decrease to 45% during recycle. Immobilization of the lipase increased the efficiency of hydrolysis of castor oil by 23% as compared to the free enzyme.

Introduction

Lipases play an important role in biotechnology because of their extreme versatility with respect to substrate specificity and stereo-selectivity. Application areas include detergent additives, flavor production in dairy industry, lipid biotransformation in the oleochemical industry and biocatalysis of a wide variety of synthetic reactions in organic chemistry. A

number of review articles have covered the biotechnological applications of lipases (Jaeger *et al*. 1994, Reetz and Jaeger 1998, Pandey *et al*. 1999).

The removal of oil stains from cloths requires highly alkaline conditions and higher washing temperatures, which are normally harmful to the clothes. Due to the new trend of using lower washing temperature and stringent regulations of detergent preparation, lipases find use in detergent industry. Lipases can remove oily stains under milder washing conditions. To be a suitable additive in detergents, lipases should be both alkaline and thermopstable and capable of functioning in the presence of the various components of washing powder formulations (Jaeger *et al.* 1994). *Pseudomonas* lipase preparations have been used as additives during manufacture of detergents.Lipases from *Pseudomonas medocina* (Lumafast®) and *Pseudomonas alcaligenes* (Lipomax®) were manufactured by Genencor international, USA, as detergent additive (Jaeger *et al*1994, Reetz and Jaeger 1998).

At present, the industrial hydrolysis of fat and oil is usually done at high temperature and high pressure, using a process called Colgate-Emery process. Although the process is simple, it has several drawbacks like large energy consumption, induction of polymerization, colour development and requirement of subsequent purification by distillation. On the other hand, hydrolysis of oils and fats using lipases is advantageous due to low energy consumption, the colourless product and decreased quantities of wastes as compared with Colgate-Emery process (Sharon *et al*. 1999).

Castor oil contains about 90% 12-hydroxy-cis-9-octadecanoic acid also called ricinoleic acid. Owing to the presence of two reactive functional groups, ricinoleic acid finds application in a number of cosmetic and food industries (Yamamato and Fujiwara 1995).

It is also used to synthesize water-soluble lubricants, gelatinizer and poly vinyl chloride (PVC) stabilizers.

In this chapter, preparation of a lipase formulation by spray drying the crude lipase and immobilization of the enzyme on Celite is described. An application of the Celite-immobilized lipase for hydrolysis of castor oil is also presented.

Part A

Product formulation

The product formulation involved preparation of two different products, 1) Detergent additive-The crude enzyme was spray dried to get dry powder, which can be used as a detergent additive and 2) Celite-immobilized enzyme preparation.

1. Spray drying

Materials

AR grade lactose and sodium sulfate, sodium lauryl sulfate and buffer ingredients were from S. D. fine chemicals Boisar, India. AR grade trehalose was procured from Sigma (USA). Commercial grade sodium silicate was purchased from Recasil Industries Mumbai, India.

Methods

Bacterial strain

The alkaline lipase producing strain of *Pseudomonas fluorescens* NS2W (NCIM 5145), isolated from a soil sample as described in Chapter 3 was used in this study.

Lipase production

The lipase was produced by growing *Pseudomonas fluorescens* NS2W in shake flasks for 52 h, in medium optimized by factorial design, as described in chapter 3. The cells were removed by centrifugation at 20,000 *g* for 20 min. The cell-free broth was used for spray drying studies.

Spray drying was performed using a Büchi mini spray drier (Model 190). Cell-free culture broth was atomized using a nozzle (0.5 mm) with the help of compressed air. The flow rate of air was controlled at 600 l/h. The cell-free broth was pumped into the spray drier using a peristaltic pump (Watson and Marlow, UK) at a suitable rate (2 ± 0.5 ml/min) in order to maintain the desired outlet temperature.

In the initial studies, nozzle temperatures were varied between 75 and 110 °C. The effect of different additives on the lipase activity retention and the product recovery was studied by spray drying the enzyme broth, in which different compounds (used as texture modifiers) were dissolved (1% w/v), separately. The additives used were lactose, trehalose, sodium sulfate and sodium silicate.

Lipase assay

Lipase activity was estimated by GC method as described in Chapter 2.

Protein estimation

Protein content was estimated according to Lowry *et al*. (1951) using bovine serum albumin (BSA) as a standard.

Results and Discussion

With the increase in the spray drier nozzle temperature, a decrease in the recovery of lipase was observed. At 150 °C, the lipase recovery was around 50% (Table 7.1). However, operation at a lower nozzle temperature resulted in a product with higher moisture content. The nozzle temperature was maintained at 90 °C during further experiments.

Table 7.1 Effect of nozzle temperature on recovery of lipase

Nozzle Temperature (°C) ±2		Lipase recovery %
Inlet	Outlet	
75	52	98.6
80	54	98.2
90	60	97.68
100	61	91.75
110	71	88.74
150	75	49.65

The effect of different additives on the lipase recovery by spray drying is listed in Table 7.2. The recovery of lipase was maximal when no additive was used, but the product was very hygroscopic. Use of lactose as the texture modifier resulted in a product with more moisture content. Addition of trehalose made product very sticky and it was not possible to remove sufficient amount of the product from the spray drier equipment, even for analysis. Addition of sodium sulfate or sodium silicate improved the properties of the product making it more free- flowing, though with lesser recovery of lipase. The product prepared by addition of sodium silicate retained its properties and the enzyme activity even after 24 h incubation, at room temperature. The product formulation prepared by

spray drying at nozzle temperature 90 °C and without addition of the additives gave maximum recovery of the lipase and was therefore used for further studies.

Table 7.2 Effect of additives on recovery of lipase

Additive	Lipase recovery (%)
Control	97.68
Lactose	83.80
Sodium sulfate	52
Sodium silicate	41

2. Immobilization of lipase on celite

Materials

AR grade Celite '545', acetone and buffer ingredients were from S. D. fine chemicals Boisar, India.

Methods

The crude enzyme was prepared as described above. The enzyme from the culture supernatant was concentrated by foam flotation using apparatus described in chapter 5. The conditions for foam separation were as follows. Temperature 30 °C, volume of feed 162 ml, air flow rate 75 ml/min, pool height 19.5 cm, foam collection at 22.5 cm. The lipase from the foamate was immobilized on Celite by adsorption or by co-precipitation, using chilled acetone. One g of Celite was mixed with 10 ml of foamate and the mixture was incubated at 30 °C for 2 h, on a rotary shaker, at 220 rpm. The immobilized preparation was recovered by filtration, using Whatman filter paper number 1. The unbound lipase was removed by washing the immobilized preparation with 10 ml phosphate buffer (0.05 M, pH 6.4), twice. The immobilized preparation was then dried in

a vacuum desiccator. For immobilization by co-precipitation, the lipase was adsorbed on Celite as described above and then treated with 20 ml chilled acetone over an ice bath. The precipitate was allowed to settle overnight at –20 °C. The precipitate was recovered by filtration, as described above and dried in vacuum desiccator.

The lipase activity and protein content of foamate and washings was estimated as described above. The amount of protein bound to Celite was determined from the difference between protein content of foamate and washings.

Results and Discussion

The efficiency of lipase immobilization was more when it was immobilized by adsorption on Celite followed by co-precipitation with chilled acetone. With simple adsorption, only 30% of lipase was immobilized. As compared to this, the co-precipitation not only immobilized 80% of the lipase but also increased the activity 3.92 fold as compared to 1.6 fold increase obtained by simple adsorption of enzyme (Table 7.3). The preparation formed by immobilization using co-precipitation with acetone was used for further experiments.

Several researchers have used Celite for immobilization of lipases (Bornscheuer and Yamane 1994, Plou *et al*. 1996, Fadiloglu and Soylemez 1998, de Castro *et al*. 1999, Chang *et al*. 1999, Akova and Ustun 2000). The lipase from *Candida rugosa* was immobilized on Celite by acetone precipitation and adsorption (Fadioglu and Soylemez 1998) and it was found that adsorption on celite gave better results.

Table 7.3 Comparison of lipase adsorption yield on celite using buffer and acetone as immobilization media

Immobilization medium	Protein bound (%)	Specific activity (U/mg protein)	Fold increase in activity
Control (crude enzyme)	-	13.85	0.0
Buffer	28.45	22.30	1.60
Buffer + acetone	80	54.43	3.92

In contrast to this, in the present work, it was observed that the immobilization of lipase by acetone precipitation resulted in better product, in terms of effectiveness, stability and reuse. de Castro *et al*. (1999) have immobilized porcine pancreatic lipase by adsorption or precipitation with solvent. They observed that immobilization by adsorption showed lesser specific activities as compared with lipase deposited from buffer by solvent precipitation. Tweddell *et al*. (1999) immobilized a lipase from Rhizopus niveus using various carriers, including different types of Celite, Spherosil and Duolite by physical adsorption. The highest recovered hydrolytic activity was observed when the lipase was immobilized on Celite Hyflo-Supercel. Immobilization was reported to enhance lipase hydrolytic activity, as observed in the present study.

Part B

Application

1. Stability of spray dried lipase preparation in the presence of commercial detergents

Materials

The commercial washing powders sold by two different companies in India, were procured locally.

Methods

The spray-dried enzyme powder was prepared as described above, without the texture modifier. Two ml of the solution of the spray-dried enzyme powder (10 mg/ml) was mixed with 3 ml of detergent solution (10 g/l) and incubated at 25 °C for 24 h. The residual lipase activity was estimated at 1, 2, 4 and 24 h, as described above.

Results and discussion

The enzyme lost all of its activity in the presence of sodium lauryl sulfate in four hours. In the presence of detergent 1 (*RIN*, Hindustan Lever Limited, Mumbai, India) it retained more than 40% activity after 4 h and more than 10% activity after 24 hours of incubation. In the presence of detergent 2 (*Surf*, Hindustan Lever Limited, Mumbai, India) and 3 (*Shudh*, Tata Chemicals Limited, Pithampur, India) after 2 h incubation, it retained more than 10 and 20% activities, respectively. The results are summarized in Table 7.4.

Table 7.4 Effect of detergents on lipase activity of the spray dried product

Sample	Time (h)				
	0	1	2	4	24
Control	100	100	100	100	83.8
SDS	0.50	0.47	0	0	0
Detergent 1 (RIN)	70	50.06	43.5	40.7	12.0
Detergent 2 (Surf)	41.0	28.43	14.09	0.5	0
Detergent 3 (Tata - Shudh)	74.2	63.1	24.0	3.42	0

2. Castor oil hydrolysis studies

Materials

Refined Castor oil (Indian Pharmacopoeia grade) was purchased locally. AR grade buffer ingredients were from S. D. fine chemicals, Boisar, India. The HPLC grade solvents were procured from Qualigens Fine Chemicals, Mumbai, India.

Methods

The enzymatic hydrolysis of the castor oil was investigated by incubating 0.5 g of castor oil with 0.25 g of immobilized preparation in 24.5 ml of 0.05 M glycine- NaOH buffer pH 9, at different temperatures, for 24 h at 160 rpm. At the end of incubation, the immobilized preparation was separated from reaction mixture by decantation. The reaction mixture was treated with 50 ml of acetone: ethanol (1:1 v/v) mixture and the fatty acids released were estimated by titrimetry using 0.05 N NaOH. The reaction

mixtures were incubated at temperatures in the range 30 -50°C during the study. The immobilized preparation recovered after decantation was used once to study the recycling efficiency, by hydrolysis of castor oil, as described above.

Definitions

1) Hydrolysis % = Acid value /saponification value

2) Acid value = (ml of base X molarity of the base X Molecular weight)/ weight of oil

The saponification value of castor oil used was 181, as given in Trader's guide to fermentation media formulation (Trader's protein, 1980).

Analysis of ricinoleic acid

The ricinoleic acid released during the hydrolysis of castor oil by the purified enzyme was identified and quantified by HPLC analysis. The reaction mixture was composed 0.1 g of castor oil, 0.9 ml 0.05 M glycine -NaOH buffer (pH 9.0) and 3 U of the enzyme. The pure enzyme was obtained by the method described in Chapter 4. The HPLC conditions used were as described in Chapter 4.

Results and Discussion

Effect of temperature on hydrolysis of castor oil

The immobilized enzyme preparation caused a maximum (65%) hydrolysis of the castor oil at 50 °C in 24 h. It was observed that the efficiency of hydrolysis increased with increase in the incubation temperature from 30 to 50 °C. The hydrolytic efficiency at 50 ° C, decreased to 45% from 65% after recycling (Table 7.5).

The hydrolysis of castor oil by crude enzyme and immobilized enzyme were compared and it was observed that with the crude enzyme preparation, only 42% hydrolysis of castor oil was achieved as compared to 65% obtained by the immobilized enzyme.

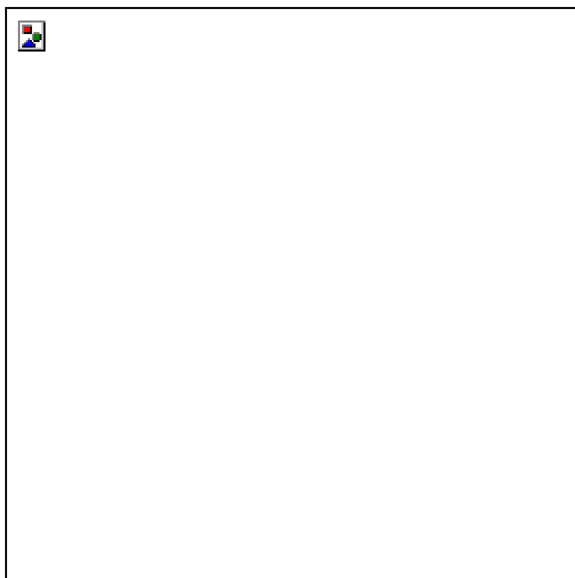
Table 7.5 Hydrolysis of castor oil by immobilized lipase

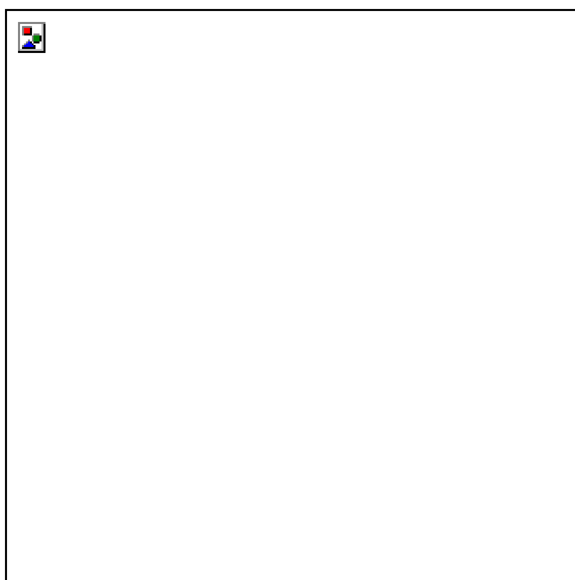
Temperature °C	Hydrolysis %	Hydrolysis in recycle %
30	44.19	27.6
40	52.5	35.8
50	65	45

For the purpose of product analysis, the hydrolysis of castor oil was performed using purified lipase. The HPLC analysis of hydrolysis products of castor oil is shown in Figure 7.1.

Figure 7.1 HPLC analysis of castor oil hydrolysis products a) Castor oil blank

Figure 7.1 HPLC analysis of the products of castor oil hydrolysis b) Ricinoleic acid standard (R. T. 5.10 min), c) Hydrolysis products after 18 h reaction of castor oil with pure enzyme.





The enzyme was found to produce ricinoleic acid as the major product of hydrolysis, with 52.8% hydrolysis at 30 °C, in 18 h.

Yamamato and Fujiwara (1995) have reported optimization of hydrolysis of castor oil using *Pseudomonas* sp. f-B-24 purified lipase preparation. The reaction mixture was composed of 1 g castor oil, 9 ml 0.1 M sodium phosphate buffer pH 7.0 and 100 U of enzyme. They obtained 80% hydrolysis after 72 h of incubation at 40 °C. Sharon *et al*. (1999) studied castor oil hydrolysis by partially purified lipase from *Pseudomonas aeruginosa* KKA-5. The reaction mixture was composed of 0.25 g castor oil, 1 ml 0.01 M $CaCl_2$, 10 ml 10 mM potassium phosphate buffer (pH 8.07) and 300 U of lipase preparation. They have observed that with crude enzyme, only 63% hydrolysis was obtained in a period of 216 h, whereas with purified lipase, 81% hydrolysis was achieved in 96 h. They also observed that the crude enzyme gave only 50% hydrolysis after 96 h of incubation. In the present study, the reaction mixture was composed 0.1 g of castor oil, 0.9 ml 0.05 M glycine -NaOH buffer (pH 9.0) and 3 U of the enzyme. The present enzyme resulted in 52.8% and 42% hydrolysis in 18 and 24 h using pure and crude enzyme, respectively. This is quite high as compared to the previous reports.

Goto *et al.* (1998) immobilized a lipase in the gel beads prepared by polymerization of poly (N-isopropylamide) and N,N'-methylene-bis-acrylamide. The thermal inactivation was found to be decreased because of the immobilization into resin, compared with the free lipase. They observed that ricinoleic acid productivity was also increased during hydrolysis of castor oil. Goto *et al*. (2000) immobilized lipases from *Candida cylindracea* or *Rhizopus* sp. in gel beads prepared by copolymerization of N-isopropylacrylamide, N-N'-methylenebisacrylamide, and acrylamide. They carried out the hydrolysis reaction of castor oil was at 37°C by using the immobilized lipase or free lipase. The optimal condition for immobilization of lipase and the productivity of ricinoleic acid was

investigated. They found that the thermal inactivation of enzyme was suppressed and the formation of byproducts such as estolide decreased when a lipase immobilized into the gel was used, as compared with the free lipase. Immobilization of the present lipase was also found to enhance the hydrolytic ability of enzyme.

Conclusions

The alkaline lipase from a newly isolated strain of *Pseudomonas fluorescens,* NS2W, was spray dried to give a product in the form of dry powder, which can be added in washing powders for laundry. The lipase retained its activity in the presence of commercial detergents in solution and hence such a preparation will be very useful as detergent additive. The lipase was immobilized on Celite and used for the hydrolysis of castor oil. It hydrolyzed castor oil with good efficiency and hence can also be used for the production of ricinoleic acid from castor oil.

CHAPTER 8

General conclusions and future work

General conclusions and future work

Lipases find use in many industries including detergent, chemical, pharmaceutical, leather and food processing. Lipases show different substrate and positional specificities and can catalyze various reactions like hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. Alkaline, thermophilic lipases find use in detergent and oleochemical industry. Several plants, animals, fungi and bacteria are known to produce lipases. Among microorganisms, bacteria have higher specific growth rate as compared to fungi and hence have greater potential as industrial lipase producers.

Lipases act on a variety of substrates including triglycerides, cholesterol esters and wax esters. There are several types of assay methods available for analysis of lipase activity. These assay methods differ from each other in the substrates they employ as well as the techniques of end product detection and quantification. In the present work, a new, simple and sensitive method was developed for estimation of lipase activity. The method uses a synthetic triacylglycerol, tributyrin, as substrate. The assay is a micro-assay requiring small amount of enzyme and substrate as compared to the conventional titrimetric assay. The new assay is very sensitive as it can estimate lipase activity as low as 0.04 IU. The assay has an excellent accuracy and precision.

An alkaline, thermophilic lipase-producing organism was isolated from a soil sample. The organism was identified taxonomically as *Pseudomonas fluorescens*. Production of the lipase from this organism was optimized in shake flasks, using a statistical experimental design. Two-level factorial design was constructed with four variables *viz*. ammonium di-hydrogen orthophosphate, groundnut oil, calcium chloride and magnesium sulfate. Groundnut oil was selected as it gave the maximum lipase production among the carbon sources tested and is easily available, cheap source of oil in India. The optimized medium resulted in about 5-fold increase in the enzyme production, as compared to that obtained in the basal medium. Among different inducers tested, oleic acid was found to be the best inducer. Feeding oleic acid to shake flask cultures at 8, 16 and 24 h increased lipase production by 12%.

The extracellular lipase produced by this organism was purified to homogeneity by foam flotation, acetone precipitation and gel filtration chromatography. The purified

enzyme was characterized in terms of its molecular weight, isoelectric point, pH optimum and stability, temperature optimum and stability, effect of metal ions, substrate specificity and regioselectivity. The enzyme was active at alkaline pH and was stable over range 3-11. It had temperature optimum at 55°C and retained more than 80% activity at 55 °C for 2 h. Thus, this lipase can find application in the detergent industry. The lipase showed marked regioselectivity for 1-3 positions of triolein and hence, can also be useful in chiral synthesis.

The lipase was selectively concentrated using a novel technique of foam separation. The process variables were optimized to obtain the best enrichment of lipase in the foamate. The process was optimized for batch mode of operation and successfully applied to continuous mode of operation. The technique is very easy and cost effective and can be used for the downstream processing of lipase on large scale.

The production of lipase was studied in 1 l and 10 l fermenters using optimized medium. The effect of temperature, pH and agitation speed on enzyme production was investigated. The maximum lipase obtained in 1 l fermenter was 68.6 U/ml, which was comparable to the enzyme obtained in shake flask cultures, using optimized medium. Thus, the production of lipase was successfully scaled up to 1 l fermenter.

In order to study the use of lipase as detergent additive, the lipase was spray dried to get it in a free-flowing, dry powder form. The suitability of this product as detergent additive was investigated. The lipase retained its activity in the presence of commercial detergents in solution and hence such a preparation will be very useful as detergent additive. The lipase was also immobilized on Celite and used for the hydrolysis of castor oil. It hydrolyzed castor oil with good efficiency and hence can also be used for the production of ricinoleic acid from castor oil.

Future work

The work presented in this thesis is part of the ongoing work in Biochemical Engineering Division, National Chemical Laboratory. There is much more to be done which is listed below.

1. The present strain produces about 6900 U/l of lipase in shake flask. The productivity can be further increased by mutagenesis of this stain. The culture produces a yellow fluorescent pigement. Attempts can also be made to isolate unpigmented mutants, which will be helpful in the down stream processing of the enzyme.
2. In the present study, the lipase was purified to homogeneity. The lipase can be further studied with respect to its amino acid sequence, and three-dimensional structure.
3. The feeding of oleic acid was shown to increase lipase yield at shake flask level. The production of lipase can be further improved in fermenters by fed-batch culture.
4. The lipase was spray dried to test its suitability as detergent additive. The enzyme can be immobilized by encapsulation to get a formulation, which can be incorporated in laundry detergents.
5. The lipase cleaves ester bond at 1 and 3 positions of triacylglycerols, selectively. This property of lipase can be applied in chiral synthesis of important drug or pesticide precursors.
6. During my study, it was observed that the lipase strongly adsorbed to hydrophobic membranes that are commonly used for microfiltration of fermentation broth. The lipase can be immobilized on such hydrophobic membrane and used to design a membrane-bioreactor, which can be used for several applications like oil hydrolysis or chiral synthesis.

CHAPTER 9

References

- Abdelraham A. A. and Yousef N. E. (1997) Production and optimization of different microbial lipases. *Al-Azhar J. Microbiol.* **38**, 121-130. **CA 130:51376**

- Adamezyk M., Gebler J. C. and Mattingly P. G. (1994) Lipase mediated hydrolysis of rapamycin 42-hemisuccinate benzyl and methyl esters, *Tetrahedron Lett*. **35**, 1019–1022.

- Adamitsch B. F. and Hampel W. A. (2000) Formation of lipolytic enzymes by *Brevibacterium linens*. *Biotechnol. Lett*. **22(20)**, 1643-1646.

- Aires-Barros M. R. and Cabral J. M. S. (1991) Selective separation and purification of two lipases from *Chromobacterium viscosum* using AOT reversed micelles. *Biotechnol. Bioeng*. **38**, 1302-1307.

- Aisaka K. and Terada O. (1980) Studies on lipoprotein lipases from microorganisms. Part II. Purification and Properties of lipoprotein lipase from *Rhizopus japonicus*. *Agric. Biol. Chem*. **44(4)**, 799-805.

- Akatsuka H., Kawai E., Omori K., Komatsubara S., Shibatani T. and Tosa T. (1994) The lipA gene of *Serratia marcescens* which encodes an extracellular lipase having no N-terminal signal peptide. *J. Bacteriol*. **176(7)**, 1949-1956.

- Akatsuka H., Kawai E., Omori K. and Shibatani T. (1995) The three genes lipB, lipC, and lipD involved in the extracellular secretion of the *Serratia marcescens* lipase which lacks an N-terminal signal peptide. *J. Bacteriol*. **61**, 6381-6389

- Akhtar M., Waheed M. A. Q. and Chughtai M. I. D. (1980) Lipase induction in *Mucor hiemalis*. *Appl. Environ. Microbiol*. **40(2)**, 257-63.

- Akhtar M., Waheed M. A. Q. and Saleem M. (1976) Influence of nature of triglycerides on the amount and specificity of lipase produced by fungi. *Pak. J. Biochem.* **9(1)**, 1-4. **CA 87:196959**

- Akita H., Chen C. Y. and Uchida K. A. (1995) Formal total synthesis of nikkomycin B based on enzymatic resolution of a primary alcohol possessing two stereogenic centers. *Tetrahedr. Asymm.* **6**, 2131–2134.

- Akita H., Umezawa I. and Matsukura H. (1997) Enzymic hydrolysis in organic solvents for kinetic resolution of water-insoluble a-acyloxy esters with immobilized lipases. *Chem. Pharm. Bull*. **45**, 272–278. **CA 126:251049**

- Akova A. and Ustun G. (2000) Activity and adsorption of lipase from *Nigella sativa* seeds on celite at different pH values. *Biotechnol. Lett.* **22**, 355-359.

- Alford J. A. and Elliott L. E. (1960) Lipolytic activity of microorganisms at low and intermediate temperatures. I. Action of *Pseudomonas fluorescens* on lard. *Food Res.* **25**, 296-303.

- Alhir S., Markakis P. and Chandan R. C. (1990) Lipase of *Penicillium caseicolum. J. Agric. Food Chem*. **38(3)**, 598-601.

- Al-Saleh A. A. and Zahran A. S. (1999) Synthesis of extracellular lipase by a strain of *Pseudomonas fluorescens* isolated from raw camel milk. *Fd. Microbiol.* **16**, 149-156.

- Arima K., Liu W. -H. and Beppu T. (1972) Isolation and identification of the lipolytic and thermophilic fungus. *Agric. Biol. Chem.* **36(11)**, 1913-1917.

- Arpigny J. L. and Jaeger K. -E. (1999) Bacterial lipolytic enzymes: classification and properties. *Biochem. J.* **343**, 177–183.

- Avdagic A., Cotarca L., Ruzic K. S., Gelo M. and Sunjic V. (1994) An efficient chemoenzymic synthesis of S-(-)-fenpropimorph. *Biocatalysis* **9**, 49–60.

- Bachmatova I., Marcinkeviciene L. and Brazenas G. (1995) Lipase of *Pseudomonas mendocina* 3121-1 and its substrate specificity. *Biologija*, **(1-2)**, 57-9. **CA 124:224682**

- Ballot C., Favre-Bonvin G. and Wallach J. M. (1984) Lipase assay in duodenal juice using a conductimetric method. *Clin. Chim. Acta* **143**, 109-114.

- Banerjee R., Agnihotri R. and Bhattacharya B. C. (1993) Purification of alkaline protease of *Rhizopus oryzae* by foam fractionation. *Bioprocess Eng.* **9(6)**, 245-248.

- Baral A. and Fox P. F. (1997) Isolation and characterization of an extracellular lipase from *Pseudomonas tolaassi*. *Fd.. Chem*. **58**, 33-38.

- Bashkatova N. A. and Severina L. O. (1980) Isolation and characterization of intracellular lipase from *Serratia marcescens* 345. *Prikl. Biokhim. Mikrobiol*. **16(3)**, 315-26 (Russian). **CA 93:90807**

- Beisson F., Tiss A., Riviére C., and Verger R. (2000) Methods for lipase detection and assay: a critical review. *Eur. J. Lipid Sci. Technol*. 133-153.

- Belov A. N. and Umanskii M. S. (1977) Comparative characteristics of the major biochemical properties of mesophilic lactic-acid streptococci differing in lipase activity. In: Biol. Mikroorg. Ikh Ispol'z. Nar. Khoz., **pp.** 28-35. Edited by: Grinevich, A. G. Irkutskii Univ.: Irkutsk, USSR. (Russian). **CA 90:117958**

- Bhattacharya P., Ghosal S. K. and Sen K. (1991) Effect of physiochemical parameters on the separation of proteins from human placental extract using continuous foam fractionating column. **26(10 &11)**, 1279-1293.

- Bianchi D., Cesti P., Golini P., Spezia S., Garavaglia C. and Mirenna L. (1990) Chemoenzymic synthesis and biological activity of both enantiomeric forms of tetraconazole, a new antifungal triazole. *J. Agric. Food Chem.* **39**, 197–201.

- Bianchi D., Cesti P., Golini P., Spezia S., Filippini L. and Garavaglia C. (1992a) Chemoenzymic synthesis and fungicidal activity of the four pure stereoisomers of a new morpholine derivative. *J. Agric. Food Chem.* **40**, 1989–1992.

- Bianchi D., Cesti P., Golini P., Spezia S., Garavaglia C. and Mirenna L. (1992b) Enzymic preparation of optically active fungicide intermediates in aqueous and in organic media. *Pure Appl. Chem.* **64**, 1073–1078.

- Birkeland S. E., Stepaniak l. and Soerhaug T. (1984) Quantitative analysis of proteinases and lipase from psychrotrophic fluorescent *Pseudomonas* strains with enzyme-linked immunosorbent assay (ELISA). *Spec. Publ.- R. Soc. Chem.* **49**, 335. **CA 101: 53461.**

- Bisignano G. (1980) Lipase of *Staphylococcus aureus* cultured in a medium containing glucose. II. Synthesis and secretion of the enzyme in cultures supplemented with glucose and cyclic AMP. *Boll. - Soc. Ital. Biol. Sper*. **56(11)**, 1169-74 (Italian). **CA 93:234751**

- Blum H., Bejer H. and Gross H. J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93-99.

- Boel E. and Huge-Jensen I. (1989) Birgitte Manufacture of *Humicola lanuginosa* lipase by recombinant *Aspergillus* (Novo Industri A/S, Den.). Eur. Pat. Appl. EP 305216 A1. (European Patent Organization) **CA 112:6094**

- Boel E., Huge-Jensen B., Christensen M., Thim L. and Fiil N. P. (1988) *Rhizomucor miehei* triglyceride lipase is synthesized as a precursor. *Lipids* **23(7)**, 701-6.

- Bompensieri S., Mahler G. F., Castaneda N., Miranda M. V., Cascone O. and Nudel B. C. (1998) Rapid purification of a lipase from *Acinetobacter calcoaceticus* by temperature-induced aqueous two-phase systems. *Biotechnol Tech.* **12(8)**, 611-613.

- Bornscheuer U., Reif O. -W., Lausch R., Freitag R., Scheper T., Fragiskos K. and Menge U. (1994) Lipase of *Pseudomonas cepacia* for biotechnological purposes: purification, crystallization and characterization. *Biochim. Biophys. Acta* **1202(1)**, 55-60.
- Bornscheuer U. T. and Yamane T. (1994) Activity and stability of lipase in the solid-phase glycerolysis of triolein. *Enzym. Microb. Biotechnol.* **16**, 864-869.

- Box G. P., Hunter W. G. and Hunter J. S. (1978) Factorial designs at two levels In: Statistics for experiments: An introduction to design, data analysis and model building. **pp.** 306-351. John Wiley and Sons Inc, New York, USA.

- Bozoglu F., Swaisgood H. E. and Adams D. M. (1984) Isolation and characerization of heat-stable lipse from *Pseudomonas fluorescens* MC50. *J. Agric. Food. Chem.* **32**, 2-6.

- Bradoo S., Saxena R. K. and Gupta R. (1999) Two acidothermotolerant lipases from new variants of *Bacillus* spp. *World J. Microbiol. Biotechnol.* **15(1)**, 97-102.

- Brahimi-Horn M. C., Mickelson C. A., Gall A. M., Guglielmino M. G. and Sparrow L. G. (1991) Lipolytic activity by *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus* strains grown in Wool-Scour effluent. *Enzym. Microb. Technol.* **13(9)**, 740-746.

- Briand D., Dubreucq E., Grimaud J. and Galzy P. (1995) Substrate specificity of the lipase from *Candida parapsilosis*. *Lipids* **30(8)**, 747-54.

- Brown L. K., Narasimhan G. and Wenkat P. C. (1990) Foam fractionation of globular proteins. *Biotechnol. Bioeng.* **36**, 947-959.

- Brown A. K., Kaul A. and Varley J. (1999a) Continuous foaming for protein recovery: Part I. Recovery of β- casein. *Biotechnol. Bioeng.* **62**, 278-290.

- Brown A. K., Kaul A. and Varley J. (1999b) Continuous foaming for protein recovery part II. Selective recovery of proteins from binary mixtures. *Biotechnol. Bioeng.* **62**, 291-300.

- Bycroft N. L. and Byng G. S. (1990) Detergent formulations containing alkaline lipase derived from *Pseudomonas plantarii*. (Miles, Inc., USA). U.S. US 4950417 A 21 **CA 120:296800**

- Carlile K., Rees G. D., Robinson B. H., Steer T. D. and Svensson M. (1996) Lipase-catalyzed interfacial reactions in reverse micellar systems. Role of water and microenvironment is determining enzyme activity or dormancy. *J. Chem. Soc. Faraday Trans.* **92**, 4701–4708. **CA 126:141283**

- Carrasco M. S., Scarinci H. E. and Simonetta A. C. (1995) Technological properties of strains of lactic acid bacteria for cheese starter: Proteolytic and lipolytic activities. *Microbiol. Aliments Nutr*. **13(3)**, 249-254. **CA 125:56682**

- Castellar M. R., Taipa M. A. and Cabral J. M. S. (1997) Kinetic and stability characterization of *Chromobacterium viscosum* lipase and its comparison with *Pseudomonas glumae* lipase. *Appl. Biochem. Biotechnol.* **61**, 299-314.

- Chakrabarti S., Chanda S. and Matai S. (1987) Effect of some oils, fats and bile salts on growth and lipase production by *Streptomyces* sp. L4. *J. Food Sci. Technol.* **24(3)**, 135-136.

- Chander H., Sannabhadti S. S., Elias J. and Ranganathan B. J. (1977) Factors affecting lipase production by *Penicillium chrysogenum. Food Sci*. **42(6)**, 1677, 1682.

- Chander H., Ranganathan B. and Singh J. (1979) Purification and some properties of lipase from *Streptococcus faecalis*. *J. Food Sci.* **44(6)**, 1747-1751.

- Chander H., Batish V. K., Ghodekar D. R. and Srinivasan R. A. (1981a) Factors affecting lipase production in *Rhizopus nigricans J. Dairy Sci*., **64(2)**, 193-6.

- Chander H., Batish V. K. and Parkash O. (1981b) Role of lipids in growth and lipase production by *Rhizopus stolonifer. J. Food Prot*., **44(5)**, 353-4.

- Chander H. and Klostermeyer H. (1982a) Factors affecting lipase production in *Mucor mucedo Zentralbl. Bakteriol., Mikrobiol. Hyg., Abt. 1, Orig. C,* **3(3)**, 427-31. **CA 97:214468**

- Chander H. and Klostermeyer H. (1982b) Production of lipase by *Penicillium funiculosum* under various growth conditions. *Arch. Lebensmittelhyg*. **33(2)**, 42-4. **CA 97:53936**

- Chander H. and Klostermeyer H. (1983) Production of lipase by *Byssochlamys fulva. J. Food Prot*. **46(8)**, 707-9.

- Chang Q. –L., Lee C. -H. and Parkin K. L. (1999) Comparative selectivities of immobilized lipases from *Pseudomonas cepacia* and *Candida Antarctica* (fraction B) for esterification reactions with glycerol analogues in organic media. *Enzym. Microb. Biotechnol.* **25**, 290-297.

- Chang R. –C., Chou S. –J. and Shaw J. –F. (2001) Synthesis of fatty acid esters by recombinant *Staphylococcus epidermidis* lipases in aqueous environment. *J. Agric. Food Chem.* **49(5)**, 2619-2622.

- Charm S. E., Morningstar, J., Mtteo C., and Paltiel B. (1966) The separation and purification of enzymes through foaming. *Anal. Biochem*. **15**, 198

- Charmoille L., Andre C., Hazbon M. H., Cornelis P. and Dhaese P. (1997) Recombinant enzyme expression system including plasmid vectors for secretion signal-mature enzyme fusion product production, especially *Pseudomonas wisconsinensis* lipase production. (Genencor International, USA). PCT Int. Appl. WO 9713847 A1. **CA 126:339668**

- Chartrain M., Katz L., Marcin C., Thien M., Smith S., Fisher E., Goklen K., Salmon P., Brix T., Price K. and Greasham R. (1993) Purification and chracterization of a novel bioconverting lipse from *Pseudomonas aeruginosa* MB 5001. *Enzym. Microb. Technol.* **15**, 575-580.

- Chen J., Shimura S., Kirimura K. and Usami S. (1992) Lipase production from petroleum products by *Trichosporon fermentans* WU-C12. *Hakko Kogaku*, **70(3)**, 185-9. (Japanese). **CA 117:46631**

- Chen S. -J., Cheng C. -Y. and Chen T. -L. (1998) Production of an alkaline lipase by *Acinetobacter radioresistens*. *J. Ferment. Bioeng.* **86(3)**, 308-312.

- Chen J. –Y., Wen C. –M. and Chen T. -L. (1999) Effect of oxygen transfer on lipase production by *Acinetobacter radioresistens*. *Biotechnol. Bioeng.* **62(3)**, 311-316.

- Cho A. -R., Yoo S. -K., Kim E. -J. (2000) Cloning, sequencing and expression in *Escherichia coli* of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiol. Lett*. **186(2)**, 235-238.

- Choo D., Kurihara T., Suzuki T. Soda K. and Esaki N. (1998) A cold adapted lipase of an alaskan psychrotroph, *Pseudomonas* sp. strain B11-1: Gene cloning and enzyme purification and characterization. *Appl. Environ. Microbiol.* **64(2)**, 486-491.

- Chopra A. K. and Chander H. (1983) Factors affecting lipase production in *Syncephalastrum racemosum*. *J. Appl. Bacteriol*. **54(2)**, 163-9.

- Chopra A. K., Chander H., Batish V. K. and Ranganathan B. (1981) Factors affecting lipase production by *Mucor racemosus. J. Food Prot*. **44(9)**, 661-4.

- Chopra A. K., Chander H., Singh J. and Ranganathan B. (1980.) Lipolytic activity of *Aspergillus wentii. Milchwissenschaft* **35(4)**, 228-30.

- Chou C. C., Ho F. M. and Tsai C. S. (1988) Effects of temperature and relative humidity on the growth and enzyme production by *Actinomucor taiwanensis* during sufu pehtze preparation. *Appl. Environ. Microbiol*. **54(3)**, 688-92.

- Chuang Y. C., Chiou S. F., Su J. H., Wu M. L. and Chang M. C. (1997) Molecular analysis and expression of the extracellular lipase of *Aeromonas hydrophila* MCC-2. *Microbiology* (Reading, U. K.), **143(3)**, 803-812. **CA 126:272995**

- Chung G. H., Lee Y. P., Yoo O. J. and Rhee J. S. (1991) Overexpression of a thermostable lipase gene from *Pseudomonas fluorescens* in *Escherichia coli*. *Appl. Environ. Microbiol.* **35**, 237-241.

- Colomina J., Villar J., Buesa J. and Borras R. (1997) Enzymic activities in *Campylobacter jejuni*, *C. coli* and *C. lari*. *Rev. Argent. Microbiol*. **29(2)**, 68-74 (Spanish) **CA 127:202649**

- Commenil P., Belingheri L., Sancholle M. and Dehorter B. (1995) Purification and properties of an extracellular lipase from the fungus *Botrytis cinerea. Lipids* **30(4)**, 351-6.

- Converse C. A., Cooper A. and Nutley M. A. (1981) A radial-diffusion assay for serum lipase. *Biochem. Soc. Trans*. **9**, 320-321.

- Cooper A. B. and Morgan H. W. (1981) Improved method to assay for soil lipase activity. *Soil Biol. Biochem.* **13(4)**, 307-311. **CA 95:202515**

- Cordenons A., Gonzalez R., Kok R., Hellingwerf K. J. and Nudel C. (1996) Effect of nitrogen sources on the regulation of extracellular lipase production in *Acinetobacter calcoaceticus* strains. *Biotechnol. Lett.* **18(6)**, 633-638.

- Costa M. L., Herrera V. C., Romero A. M. and Carrasco E. R. (1997) Purification and characterization of proteases and lipases produced by *Pseudomonas fluorescens* in milk at 4 °C. *Alimentos* **22(1-2)**, 73-82 (Spanish). **CA 129:132902**

- Cruz H., Perez C., Wellington E., Castro C. and Servin-Gonzalez L. (1994) Sequence of the *Streptomyces albus* G lipase-encoding gene reveals the presence of a prokaryotic lipase family. *Gene* **144**, 141–142.

- Dahot M. U. and Memon A. R. (1989) Lipase activity of *Penicillium expansum. J. Pure Appl. Sci.*, **8(1)**, 7-13. **CA 111:152063**

- Danda H., Machara A. and Umemura T. (1991) Preparation of (4S)-4-hydroxy-3-methyl-2-(2'-propynyl)-2-cyclopentenone by combination of enzymatic hydrolysis and chemical transformation *Tetrahedron Lett*. **32**, 5119–5122.

- Davies L. (1993) The optimization of processes and products. In: Efficiency in research, development, and production: The statistical design and analysis of chemical experiments. **pp**. 123-132. Royal Society of Chemistry, Cambridge. UK

- De Angelis M., Gobbetti M. and Corsetti A. (1999) Esterase and lipase activities of *Lactobacillus sanfranciscensis* strains used in sourdough fermentation*. Ital. J. Food Sci.* **11(2)**, 167-172. **CA 131:271131**

- de Castro H. F., deOliveira P. C., Soares C. M. F. and Zanin G. M. (1999) Immobilization of porcine pancreatic lipase on celite for application in the synthesis of butyl butyrate in a nonaqueous system. *J. Am. Oil. Chem. Soc.* **76(1)**, 147-152.

- DeMoraes J. and Chandan R. C. (1982) Factors influencing the production and activity of a *Streptococcus thermophilus* lipase. *J. Food Sci*. **47(5)**, 1579-83.

- Deploey J. J., Fortman B. and Perva L. (1981) Daily measurements of *Mucor miehei* lipase activity. *Mycologia* **73(5)**, 953-8.

- DeShazer D., Brett P. J., Burtnick M. N. and Woods D. E. (1999) Molecular characterization of genetic loci required for secretion of exoproducts in *Burkholderia pseudomallei*. *J. Bacteriol*. **181(15)**, 4661-4664.

- Deveer A. M. T. J, Dilkman R., Leuveling-Tjeenk M., Van den Berg L., Ransac S., Batenburg M., Egmond M., Verheij H. M., and De Haas G. H. (1991) A monolayer and bulk study on the kinetic behaviour of *Pseudomonas glumae* lipase using synthetic pseudoglycerides. *Biochem.* **30(41)**, 10034-10042.

- Dharmsthiti S. and Ammaranond P. (1997) Purification and characterization of lipase from a raw-milk yeast (*Trichosporon asteroides*). *Biotechnol. Appl. Biochem.* **26(2)**, 111-116.

- Dharmsthiti S. and Luchai S. (1999) Production purification and characterization of thermophilic lipase from *Bacillus* sp. THL027. *FEMS Microbiol. Lett.* **179**, 241-246.

- Dighe A.S., Shouche Y. S. and Ranade D. R. (1998) *Selenomonas lipolytica* sp. nov., an obligately anaerobic bacterium possesing lipolytic acitivity. *Int. J. Syst. Bacteriol.* **48**, 783-791.

- Diogo M. M., Silva S., Cabral J. M. S. and Quireoz J. A. (1999) Hydrophobic interaction chromatography of *Chromobacterium viscosum* lipase on polypropylene glycol immobilized on sepharose. *J. Chromatogr.* **849**, 413-419.

- Dring R. and Fox P. F. (1983) Purification and characterization a heat stable lipase from *Pseudomonas fluroscens* strain AFT 29. *J. Dairy Res.* **50**, 77-89.

- Dünhaupt A., Lang S. and Wagner F. (1992) *Pseudomonas cepacia* lipase: studies on aggregation, purification and on the cleavage of olive oil. *Biotechnol. Lett*. **14**, 953-958.

- Dupuis C., Corre C. and Boyaval P. (1993) Lipase and esterase activities of *Propionibacterium freudenreichii* subsp. *freudenreichii*. *Appl. Environ. Microbiol.* **59(12)**, 4004-9.

- El Khattabi M., Van Gelder P., Bitter W. and Tommassen J. (2000) Role of the lipase-specific foldase of *Burkholderia glumae* as a steric chaperone. *J. Biol. Chem*. **275(35)**, 26885-26891.

- El-Gammal S. M. and Rizk M. I. (1989) Effect of cations, sodium chloride and sodium deoxycholate on lipase production by some yeasts. *Egypt. J. Bot.* **29-30 (1-3)**, 1-9. **CA 113:112148**

- El-Sawah M.M.A., Sherief A.A. and Bayoumy S. M. (1995) Enzymic properties of lipase and characteristics production by *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Antonie van Leeuwenhoek* **67(4)**, 357-62.

- El-Shirbiny E. S. A. and Ghaly M. F. (1992) Optimization of lipase biosynthesis by *Streptomyces parvulus* under various growth conditions. *Delta J. Sci*. **16(1)**, 229-43. **CA 119:179262**

- Elwan S. H., Mostafa S. A., Khodair A. A. and Ali, O. (1978) Identity and lipase activity of an isolate of *Thermoactinomyces vulgaris. Zentralbl. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt. 2: Mikrobiol. Landwirtsch., Technol. Umweltschutzes*, **133(7-8)**, 713-22. **CA 90:117588**

- Elwan S. H., El-Hoseiny M. M. Ammar M. S. and Mostafa S. A. (1983) Lipase production by *Bacillus circulans* under mesophilic and osmophilic conditions. Factors affecting lipase production. *G. Bacteriol.*, *Virol. Immunol.* **76(7-12)**, 187-199. **CA 103: 19500**

- Elwan S. H., El-Hoseiny M. M., Ammar M. S. and Mostafa S. A. (1985) Lipase production by *Bacillus circulans* under mesophilic and osmophilic conditions. A. Factors affecting lipase production. *Egypt. J. Microbiol.* **20(1)**, 103-114. **CA 105: 187316**

- Elwan S. H., Ammar M. S. and Mohawed S. M. (1986) Lipases from *Aspergillus sydowi. Zentralbl. Mikrobiol.* **141(3)**, 233-9. **CA 105:111671**

- Englard and Siefter (1990) Precipitation techniques. *Methods in Enzymol.* **182**, 285.

- Fadiloglu S. and Soylemez Z. (1998) Olive oil hydrolysis by celite-immobilized *Candida rugosa* lipase. *J. Agric. Food Chem.* **46(9)**, 3411-3414.

- Farrell A. M., Foster T. J. and Holland K. T. (1993) Molecular analysis and expression of the lipase gene from *Staphylococcus epidermidis*. *J. Gen. Microbiol.* **139**, 267-277.

- Feller G., Thiry M., Arpigny J. L., Mergeay M. and Gerday C. (1990) Lipases from psychrotrophic antarctic bacteria. *FEMS Microbiol. Lett.* **66**, 239-244.

- Fernandez S., Ferrero M., Gotor V. and Okamura W. H. (1995) Selective acylation of A-Ring precursors of vitamin D using enzymes in organic solvents. *J. Org. Chem.* **60**, 6057–6061.

- Finkelstein A. E., Strawich E. S. and Sonnino S. (1970) Characterization and partial purification of a lipase from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta* **206(3)**, 380-91.
- Fonchy E., Morin A., Rodrigue N., Müller B. And Chalier P. (1999) Effect of growth temprature on hydrolytic and esterifying activities from *Pseudomonas fragi* CRDA 037 grown on whey. *Appl. Environ. Micrbiol.* **65(7)**, 3114-3120.

- Fossati P., Ponti M., Paris P., Berti G. and Tarenghi G (1992) Kinetic colorimetric assay of lipase in serum. *Clin. Chem.* **38**, 211-215.

- Fox P. F. and Stepaniak L. (1983) Isolation and some properties of extracellular heat-stable lipases from *Pseudomonas fluorescens* strain AFT 36. *J. Dairy Res.* **50(1)**, 77-89.

- 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*. **63-65**, 409-421.

- Frich A. S., Jensen B. and Olsen J. (1994) The simultaneous production of amylase and lipase by the thermophilic fungus, *Thermomyces lanuginosus. Prog. Biotechnol*., 9(ECB6: PROCEEDINGS OF THE 6TH EUROPEAN CONGRESS ON BIOTECHNOLOGY, 1993, PT. 1), 299-302. **CA 121:250853**

- Fuji T., Tatara T., and Minagwa M. (1986) Studies on application of lipolytic enzyme in detergency I. *J. Am. Oil Chem. Soc.* **63(6)**, 796-799.

- Fujimoto T., Saeki A., Obara M., Nagai T., Yamashita F. and Kenmasa G. (1996) Preparation and characterization of lipase of *Candida entomophila*. (Deii Eichi Ee Kodo Seisei Chus, Japan). Jpn. Kokai Tokkyo Koho JP 08066186 A2. **CA 124:283165**

- Fusetti F., Brocca S., Porro D. and Lotti M. (1996) Effect of the leader sequence on the expression of recombinant *C. rugosa* lipase by *S. cerevisiae* cells. *Biotechnol. Lett*. **18(3)**, 281-6.

- Gao X. -G., Cao S. -G. and Zhang K. -C. (2000) Production, properties and application to nonaqueous catalysis of lipase from a newly isolated *Pseudomonas* strain. *Enzym. Microb. Technol.* **27**, 74-82.

- Gargouri Y., Piéroni G., Rivière C., Sauniére J. F., Lowe P. A. Sarda L. and Verger R. (1986) Kinetic assay of human gastric lipase on short- and long-chain triacylglycerol emulsions. *Gastroenterology* **91**, 919-925. **CA 105:186528**

- Gaskin D. J. H., Turner N. A. and Vulfson E. N. (1994) Expression of *Mucor miehei* lipase in *E. coli*. Appl. Biocatal., Three-Day Symp., 74-6. Inst. Chem. Eng.: Rugby, UK. **CA 121:253789**

- Gawande B. N. and Patkar A. Y. (1999) Application of factorial design for optimization of cyclodextrin glycosyltransferase production from *Klebsiella pneumoniae* AS-22. *Biotechnol. Bioeng.* **64(2)**, 168-173.

- Gawel L. J. and Chen C. -S. (1977) Bacterial production of lipase and pyocynins from n-paraffins. (Continental Oil Co., USA) U.S. US 4119959. **CA 87:4043**

- Gehle R. D., Schügerl K. (1984) Protein recovery by continuous flotation. *Appl. Microbiol. Biotechnol.* **20**, 133-138.

- Gerritse G., Hommes R. W. J. and Quax W. J. (1998) Development of a lipase fermetation process that uses a recombinant *Pseudomonas alcaligenes* strain. *Appl. Environ Microbiol.* **64**, 2644-2651.

- Ghanem E. H., Al-Sayed H. A. and Saleh K. M. (2000) An alkalophilic thermostable lipase produced by new isolate of *Bacillus alcalophilus*. *World. J. Microbiol. Biotechnol*. **16(5)**, 459-464.

- Gilbert E. J., Drozd J. W. and Jones C. W. (1991a) Physiological regulation and optimization of lipase activity in *Pseudomonas aeruginasa* EF2. *J. Gen. Micobiol.* **137**, 2215-2221.

- Gilbert E. J., Cornish A. and Jones C. W. (1991b) Purification and properties of extrcellular lipase from *Pseudomonas aeruginosa* EF2. *J. Gen. Microbiol.* **137**, 2223-2229.
- Glenza A. and Ben Jaballah L. (1985) Study on the lipolytic system of *Penicillium verrucosum* var. *cyclopium* (Westling). II. Conditions for lipase system production. *Arch. Inst. Pasteur Tunis*, **62(1-2)**, 69-89 (French). **CA 104:87076**

- Gobbetti M., Fox P. F., Smacchi E., Stepaniak L. and Damiani P. (1996) Purification and characterization of a lipase from *Lactobacillus plantarum* 2739. *J. Food Biochem.* **20(3)**, 227-246.

- Goetz F., Popp F., Korn E. and Schleifer K. H. (1985) Complete nucleotide sequence of the lipase gene from *Staphylococcus hyicus* cloned in *Staphylococcus carnosus*. *Nucleic Acid Res*. **13(16)**, 5895-906.

- Goodman L. P. and Durgan L. R. (1969) The effect of sonication on lipase activity. *Lipids* **5**, 362-364.

- Gopinath S., Hilda A. and Ramesh V. M. (1996) Detection of enzymes in *Geotrichum candidum* and *Trichothecium roseum. J. Indian Bot. Soc*. **75(3-4)**, 309-310.

- Goto M., Hatanaka C. and Haraguchi T. (1998) Hydrolysis reaction of castor oil by lipase immobilized within the N-isopropylacrylamide gel beads. *Kitakyushu Kogyo Koto Senmon Gakko Kenkyu Hokoku* **31**, 141-143 (Japanese). **CA 128:206133**

- Goto M., Hatanaka C. and Haraguchi T. (2000) Selective production of ricinoleic acid by hydrolysis of castor oil using lipase immobilized in N-polyisopropylacrylamide gel. *Kagaku Kogaku Ronbunshu* **26(3)**, 402-405 (Japanese). **CA 133:103985**

- Gowland P., Martin K. and Trichur K. S. (1987) Thermophilic bacterial isolates producing lipase. *FEMS Microbiol. Lett.* **48**, 339-343.

- Guillou J. P. and Chevrier L. (1977) Study of activity on tribytyrin of lipase from anaerobic bacteria by the liquid gas chromatographic method. *C. R. Hebd. Seances Acad. Sci., Ser. D.* **284(1)**, 97-99. **CA 86:117347**

- Guillou C., Merieau A., Trebert B. and Guespin M. J. F. (1995) Growth temperature is involved in the regulation of extracellular lipase at two different levels in *Pseudomonas fluorescens* strain MF0. *Biotechnol. Lett.* **17(4)**, 377-382.

- Gulati R., Saxena R. K., Gupta R., Yadav R. P. and Davidson W. S. (2000) Parametric optimization of *Aspergillus terreus* lipase production and its potential in ester synthesis. *Process Biochem.* **35 (5)**, 459-464.

- Haalck L., Hedrich H. C., Hassink J. and Spener F. (1992) Modification of waste fats by lipase-catalyzed reaction in solvent-free substrate blends. *Prog. Biotechnol*. **8**, 505–512.

- Hadeball W., Konieczny B. and Heide G. (1991) Optimization of lipase manufacture with *Yarrowia lipolytica*. (Akademie der Wissenschaften der DDR, Fed. Rep. Ger.). Ger. (East) DD 289062 A5 18 3 pp. **CA 115:90683**

- Haferburg D. and Kleber H. P. (1983) Regulation of extracellular lipase of an alkane-utilizing *Acinetobacter* strain. *Acta Biotechnol*. **3(2)**, 185-6 (German). **CA 99:19444**

- Hamed N.A.A. A. (1996) Production of lipase by certain fungal strains. (I) Effect of some cultural conditions. *Egypt. J. Microbiol*. **31(1)**, 101-114. **CA 127:16534**

- Hamed N. A. A. A. (1997) Production of lipase by certain fungal strains. II. Effect of some additives to the growth medium. *Egypt. J. Microbiol*. **31(2)**, 139-154 **CA 127:134738**

- Harris P. L., Cuppett S. L. and Bullerman L. B. (1990) Optimization of lipase synthesis by *Pseudomonas fluorescens* by response surface methodology. *J Food Prot.* **53 (6)**, 481-483.

- Henderson C. (1971) A study of the lipase produced by *Anerovibrio lipolytica*, a rumen bacterium. *J. Gen. Microbiol.* **65**, 81-89.

- Hirayama S. and Halkier T. (1996) (Novo Nordisk A/s, Den.) Alkaline lipase of *Botryosphaeria* or *Guignardia* with optimum activity at pH 10 and enzyme use as detergent additive for cleaning stain from cloth or textile. PCT Int. Appl. WO 9630502 A1. **CA 125:303867**

- Holmes P. E. (1990) Lipase SD2 and its manufacture with novel *Pseudomonas alcaligenes*. (Olin Int. Corp., USA) PCT Int. Appl. WO 9010695 A1. **CA 114: 205549**

- Holmes P. E. and Kornacki J. A. (1993) A novel lipase from *Pseudomonas alcaligenes* and its manufacture for use in laundry detergents. (Olin Corp., USA). U.S. US 5227300 A 13. **CA 119:220517**

- Holmström B. (1968) Foam concentration of streptokinase from crude culture filtrates. *Biotechnol. Bioeng.* **10**, 551-553.

- Hong M. C. and Chang M. C. (1998) Purification and characterization of an alkaline lipase from a newly isolated *Acinetobacter radioresistens* CMC-1. *Biotechnol. Lett.* **20(11)**, 1027-1029.

- Hoong L. W. and Omar I. C. (1993) Production and characterization of lipoprotein lipase from a newly isolated strain of *Cunninghamella echinulata. J. Biosci.* **4(1)**, 69-83.

- Horiuti Y. and Imamura S. (1977) Purification of lipase from *Chromobacterium viscosum* by chromatography on palmitoyl cellulose. *J. Biochem.* **81(6)**, 1639-1649.

- Hoshino T., Mizutani A., Shimizu S., Hidaka H. and Yamane T. (1991) Calcium ion regulates the release of lipase of *Fusarium oxysporum. J. Biochem*. **110(3)**, 457-61.

- Hoshino T., Ohgiya S., Shimanuki T. and Ishizaki K. (1996) Production of low temperature active lipase from the pink snow mold, *Microdochium nivale* (syn. *Fusarium nivale*). *Biotechnol. Lett*. **18(5)**, 509-510.

- Hostacka A. (2000) Influence of some antibiotics on lipase and hydrophobicity of *Acinetobacter baumannii*. *Cent. Eur. J. Public Health* **8(3)**, 164-166. **CA 133:278605**

- Hube B., Stehr F., Bossenz M., Mazur A., Kretschmar M. and Schafer W. (2000) Secreted lipases of *Candida albicans*: cloning, characterisation and expression analysis of a new gene family with at least ten members. *Arch. Microbiol.* **174(5)**, 362-374.

- Hughes D. L., Song Z., Smith G. B., Bergan J. J., Dezeny G. C. and Grabowski E. J. J. (1993) A practical chemoenzymic synthesis of an LTD4 antagonist. *Tetrahedr. Asym*. **41**, 865–874.

- Ihara F., Kageyama Y., Hirata M., Nihira T. and Yamada Y. (1991) Purification, characterization and molecular cloning of lactonizing lipase from *Pseudomonas* sp. *J. Biol. Chem.* **266(27)**, 18135-18140.

- 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.

- Imamura S. and Kitaura S. (2000) Purification and characterization of a monoacylglycerol lipase from the moderately thermophilic *Bacillus* sp. H-257. *J. Biochem.* **127**, 419-425.

- Inagaki M., Hiratake J., Nishioka T. and Oda J. (1992) One-pot synthesis of optically active cyanohydrin acetates from aldehydes via lipase-catalyzed kinetic resolution coupled with in situ formation and racemization of cyanohydrins. *J. Org. Chem*. **57**, 5643–5649.

- Ingham E., Holland K. T., Gowland G. and Cunliffe W. J. (1981) Partial purification and characterization of lipase from *Propionibacterium acnes*. *J. Gen. Microbiol.* **124(2)**, 393-401.

- Inoue S., Kimura Y., Takei A. and Fushimi K. (1987) Lipase manufacture with *Pseudomonas* KSM-16. (Seitai Kino Riyo Kagakuhin Shinseizo Gijutsu Kenku Kumiai, Japan) Jpn. Kokai Tokkyo Koho JP 62210987 A2. **CA 108:73818**

- Ishida R., Suzuki M., Kotsuka T. and Sakimoto K. (1995) Novel alkaline lipase and its preparation from *Pseudomonas*. (Showa Denko K. K., Japan) PCT Int. Appl WO 9506720 A1. **CA 122: 309812**

- Ishihara K., Suzuki T., Yamane T. and Shimizu S. (1989) Effective production of *Pseudomonas fluorescens* lipase by semi-batch culture with turbidity dependent automatic feeding of olive oil and iron ion. *Appl. Microbiol. Biotechnol.* **31**, 45-48.

- Ishii T. (2001) *Burkholderia cepacia* lipase expression activation genes. Jpn. Kokai Tokkyo Koho JP 2001008683 A2. **CA 134:96290**

- Ishikawa H., Ogino H.and Myamoto K. (1995) Lipase, *Pseudomonas aeruginosa* producing the lipase, and the method to obtain the *P. aeruginosa*. (Cosmo Sogo Kenkyusho Kk; Cosmo Oil Co. Ltd, Japan) Jpn. Kokai Tokkyo Koho JP 07227276 A2. **CA 123: 312336**

- Izumi T., Tamura F., Akutsu M., Katou R. and Murakami S. (1997) Enzymic transesterification of 3,7-dimethyl-4,7-octadien-1-ol using lipases. *J. Chem. Technol. Biotechnol*. **68**, 57–64.

- Jäeger K. -E. and Winkler U. K. (1984) Interaction between hyaluronate and the cell surface of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **21**, 33-38.

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

- Jansen A. E. M., Vaidya A. M. and Halling, P. J. (1996) Substrate specificity and kinetics of *Candida rugosa* lipase in organic media. *Enz. Microb. Technol*. **18**, 340–346.
- Jensen R.G. (1983) Detection and determination of lipase (acylglycerol hydrolase) activity from various sources. *Lipids* **18**, 650-657.

- Jimenez O., Bosch M. P. and Guerrero A. (1997) Lipase-catalyzed enantioselective synthesis of Methyl (R)- and (S)-2-Tetradecyloxiranecarboxylate through sequential kinetic resolution. *J. Org. Chem.* **62**, 3496–3499.

- Johri B. N., Alurralde J. L., Kressdorf B. and Klein J. (1991) Production of lipase by *Sporotrichum* (Chrysosporium) thermophile Apinis immobilized in alginate and porous glass beads. *GBF Monogr*. **16**(Lipases), 353-9. **CA 116:17571**

- Jorgensen S., Skov K. W., and Diderichesen B. (1991) Cloning, sequence and expression of a lipase from *Pseudomoans cepacia*: lipase production in heterologus hosts requires two *Pseudomoans* genes*. J. Bacteriol.* **173**, 559-567.

- Kamaly K. M., Takayama K. and Marth E. H. (1990) Acylglycerol acylhydrolase (lipase) activities of *Streptococcus lactis*, *Streptococcus cremoris*, and their mutants. *J. Dairy Sci*., **73(2)**, 280-90.

- Kamimura E. S., Mendieta O., Sato H. H., Pastore G. and Maugeri F. (1999) Production of lipase from *Geotrichum* sp. and adsorption studies on affinity resin. *Braz. J. Chem. Eng.* **16(2)**, 103-112. **CA 132:49062**

- Kamini N. R., Fujii T., Kurosu T. and Iefuji H. (2000) Production, purification and characterization of an extracellular lipase from the yeast, *Cryptococcus* sp. S-2. *Process Biochem*. **36(4)**, 317-324.

- Kashyap M. L., Meelies M. J. Brady D. Hnd B. A. and Robinson K. (1980) A micromethod using gas-liquid chromatography from measuringindividual fatty acids liberated during interaction of triglyceride -rich lipoproteins and lipoprotein lipase. *Anal. Biochem.* **107(2)**, 432-435.

- Kennedy M. B. and Lennarz W. J. (1979) Characterization of the extracellular lipase of *Bacillus subtilis* and its relationship to a membrane-bound lipase found in a mutant strain. *J. Biol. Chem*. **254(4)**, 1080-9.

- Kim H. -K. and Oh T.-K. (1998) Characterization of an alkaline lipase from *Proteus vulgaris* K80 and the DNA sequence of the encoding gene. In: Proc. World Conf. Oilseed Edible Oils Process., Meeting Date 1996, Volume **2**, 346-349. Edited by: Koseoglu, S. S.; Rhee, K. C.; Wilson, Richard F. AOCS Press: Champaign, Ill**. CA 130:77772**
- Kim K. K., Song H. K., Shin D. H., Hwng K. Y. and Suh H. W. (1997) The crystal structure of a triglyceride lipase from *Pseudomonas cepacia* reveals a highly open conformation in the absence of bound inhibitor. *Structure* **5**, 173-185.

- Kim H. K., Park S. Y., Lee J. K. and Oh T. K. (1998) Gene cloning and characterization of thermostable lipase from *Bacillus stearothermophilus*. *Biosci. Biotechnol. Biochem*. **62(1)**, 66-71. **CA 128:240102**

- Kojima Y., Yokoe M. and Mase T. (1994) Purification and characterization of an alkaline lipase from *Pseudomonas fluorescens* AK102. *Biosci. Biotechnol. Biochem*. **58(9)**, 1564-1568.

- Kokusho Y., Machida H. and Iwasaki S. (1982) Studies on the alkaline lipase. Part II. Production and properties of alkaline lipase from *Alcaligenes* sp. strain No. 679. *Agric. Biol. Chem*. **46(7)**, 1743-50.

- Konstantinou P. and Roussis I. G. (1998) Some properties of extracellular lipase from *Pseudomonas* 92. *Mlichwissenschaft* **53(11)**, 622-624.

- Kordel M., Hofmann B., Schomburg D. and Schmid R. D. (1991) Extracellular lipase of *Pseudomonas* sp strain ATCC 21808: purification, characterization, crystallization and preliminary X-ray diffraction data. *J. Bacteriol.* **173**, 4836-4841.

- Kornacki K., Stepaniak L., Adamiec I., Grabska J. and Wrona K. (1980) Production of lipases and proteases by molds of *Penicillium roqueforti* and *Penicillium candidum* under selected conditions of surface and submerged cultivation. *Acta Aliment. Pol*. **6(4)**, 281-8. **CA 95:130926**

- Koseki M., Tsuji K., Nakagawa Y., Kawamura M., Ichigawa T., Kazama M., Kha batake N. and Doi E. (1989) Effect of gum arabic and pectin on the emulsification, the lipase reaction, and the plasma cholesterol level in rats. *Agric. Biol. Chem.* **53(12)**, 3127-3132.

- Kosugi Y. and Suzuki H. (1998) Hydrolusis of bef tallow by lipase from *Pseudomonas* sp. *Biotechnol. Bioeng.* **31**, 349-356.

- Kotsuka T., Tanaka K. and Sakimoto K. (1996) Novel thermostable lipase of *Pseudomonas solanacearum* and its preparation and characterization. (Showa Denko K. K., Japan) PCT Int. Appl. WO 9627659. **CA 125:269267**

- Kotting J., Jürgens D. and Huser H. (1983) Separation and characterization of two isolated lipases from *Staphylococcus aureus* (TEN 5). *J. Chromatogr.* **281**, 253-261.

- Kottwitz B., Upadek H. and Carrer G. (1994) Zeolite A and co-builders in non-phosphate detergents. *Chim. Oggi* **12**, 21–24.

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

- Kugimiya W., Ootani Y. and Hashimoto Y. (1989) (Fuji Oil Co., Ltd., Japan). Cloning and expression of *Rhizopus* lipase gene. Jpn. Kokai Tokkyo Koho JP 01080290 A2 Heisei, 11 pp. (Japan) **CA 112:93238**

- Kulkarni N. and Gadre R. V. (1998) Simple gas chromatography method for lipase assay. *Biotechnol Tech.* **12**, 627-628.

- Kulkarni N. and Gadre R. V. (1999) A novel alkaline, thermostable, protease free lipase from *Pseudomonas* sp. *Biotechnol. Lett.* **21**, 897-899.

- Kumura H., Mikawa K. and Saito Z. (1993) Purification and characterization of lipase from *Pseudomonas fluorescens* No. 33. *Milchwissenschaft* **48(8)**, 431-434.

- Kundu M., Basu J., Guchhait M. and Chakrabarti P. (1987) Isolation and characterization of an extracellular lipase from the conidia of *Neurospora crassa*. *J. Gen. Microbiol*. **133(1)**, 149-53.

- Kunimoto M., Kaminishi Y., Minami K. and Hatano M. (1996) Lipase and phospholipase production by *Aspergillus repens* utilized in molding of "Katsuobushi" processing. *Fish. Sci*. **62(4)**, 594-599. **CA 125:166080**

- Kurooka S., Okamoto S. and Hashimoto M. (1977) A novel and simple colorimetric assay for human serum lipase. *J. Biochem.* (Tokyo) **81**, 361-369.

- Kuznetsov V. G. and Bagryantsev V. N (1992) Detection of intestinal *Yersinia* lipase activity. *Klin. Lab. Diagn*. **(9-10)**, 57-9 (Russian). CA 119:89572
- Labourdenne S., Gaudry-Rolland N., Letellier S., Lin M., Cagna A., Esposito G., Verger R. and Riviere C. (1994) The oil-drop tensiometer: potential applications for studying the kinetics of (phospho)lipase action. *Chem. Phys. Lipids*, **71(2)**, 163-73.
- Labuschagne R. B., Tonder A.V. and Litthauer D. (1997) *Flavobacterium odorum* lipase: isolation and characterization. *Enzym Microb. Technol.* **21**, 52-58.
- Lalchev Z., Dimitrova L., Tzvetkova P., and Exerowa D. (1982) Foam separation of DNA and proteins from solutions. *Biotechnol. Bioeng*. **24**, 2253-2262.
- Lamberet G. and Menassa A. (1983) Purification and properties of an acid lipase from *Penicillium roqueforti*. *J. Dairy Res*. **50(4)**, 459-68.
- Laemmli (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Lang S., Katsiwela E., Kleppe F. and Wagner F. (1991) *Ustilago maydis* lipolytic enzymes: characterization and partial purification. *GBF Monogr*., **16**(Lipases), 361-4. **CA 116:17572**
- Lang D., Hofmann B., Haalck L. Hecht H. J., Spener F., Schimd R. D. and Schomburg D. (1996) Crystal structure of a bacterial lipase from *Chromobacterium viscosum* ATCC 6918 refined at 1.8 Å resolution. *J. Mol. Biol.* **259**, 704-717.
- Large K. P., Mirjalili N., Peacock M. O. L. M., Zormpaidis V., Walsh M., Cavanagh M. E., Leadlay P. F. and Ison A. P. (1999) Lipase activity in Streptomycetes. *Enzym. Microb. Technol*. **25(7)**, 569-575.
- Lawler D. and Smith S. (2000) Enzyme- producting strain of *Bacillus* bacteria. (Roebic Laboratories, Inc., USA) U. S. US 6162634 A. **CA 134:41190**
- Lebedeva Z. D. (1977) Lipolytic activity of the cells of *Mycobacterium rubrum* immobilized in polyacrylamide gel. *Mikrobiologiya*, **46(4)**, 651-5 (Russian). **CA 87:148409**
- Lechner M., Märkl H. and Götz F. (1988) Lipase production of *Staphylococcus carnosus* in a dialysis fermentor. *Appl. Microbiol. Biotechnol.* **28**, 345-349.
- Lee Y. K. and Choo C. L. (1989) The kinetics and mechanism of shear inactivation of lipase from *Candida cylindracea. Biotechnol. Bioeng*. **33(2)**, 183-90.

- Lee S. Y. and Lee B. H. (1989) Production and characterization of esterase-lipase of *Lactobacillus casei* supsp. *pseudoplantarum* LE2. *Biotechnol. Appl. Biochem.* **11**, 552-563.

- Lee S. Y. and Lee B. H. (1990) Esterolytic and lipolytic activities of *Lactobacillus casei-subsp-casei* LLG *J. Food Sci*. **55(1)**, 119-22, 126.

- Lee S. Y. and Rhee J. S. (1993) Production and partial purification of a lipase from *Pseudomonas putida* 3SK. *Enzym. Microb. Technol*., **15(7)**, 617-23.

- Lee D. -W., Koh Y. -S., Kim K. -J., Kim B. -C., Choi H. -J., Kim D. -S., Suhartono M. T. and Pyun Y. -R. (1999) Isolation and characterization of thermophilic lipase from *Bacuillus thermoleovorans* ID-1. *FEMS Microbiol. Lett.* **179**, 393-400.

- Lesuisse E., Schanck K., Colson C. (1993) Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. *Eur. J. Biochem.* **216(1)**, 155-60.

- Lin S. -F., Chiou C. -M. and Tsai Y. –C. (1995) Effect of Triton X-100 on alkaline lipase production by *Pseudomonas pseudoalcaligenes* F-111. *Bitechnol. Lett.* **17**, 959-962.

- Lin S. F., Chiou C. M., Yeh C. M. and Tsai Y. C. (1996) Purification and partial characterization of an alkaline lipase from *Pseudomonas pseudoalkaligenes* F-111. *Appl. Environ. Microbiol.* **62(3)**, 1093-1095.

- Liu W. -H., Beppu T., Arima K. (1977) Studies on the lipase of thermophilic fungus *Humicola lanuginosa*. Part VII. The chemical modification of the lipase of *Humicola lanuginosa* by N-bromosuccinimide in urea solution. *Agric. Biol. Chem.* **41(1)**, 131-5.

- Lockwood C. E., Jay M. and Bummer P. M. (2000) Foam fractionation of binary mixtures of lysozyme and albumin. *J. Pharm. Sci.* **89**, 693-704.

- London M. And Hudson P. (1953) Studies on the purification of acid prostatic phosphatase. *Arch. Biochim. Biophys. Acta* **46**, 141-153.

- Lotrakul P. and Dharmsthiti S. (1997a) Lipase production by *Aeromonas sobria* LP004 in a medium containing whey and soybean meal. *World J. Microbiol. Biotechnol.* **13(2)**, 63-166.

- Lotrakul P. and Dharmsthiti S. (1997b) Purification and characterization of lipase from Aeromonas *sobria* LP004. *Biotechnol*. **54(2)**, 113-20.

- Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

- Lu J. Y. and Liska B. J. (1969) Lipase from *Pseudomonas fragi* I. Purification of the enzyme. *Appl. Microbiol.* **18**, 104-107.

- Majumdar D., Sharan E. and Kamat M. Y. (1998) Studies in production of lipase from a novel lipase producing yeast (*Sterigmatomyces* sp.) isolated from soil. *Res. J. Chem. Environ.* **2(1)**, 11-15. CA 129:119972

- Makhzoum A., Knapp J. S. and Owusu R. K. (1995) Factors affecting growth and extracellular lipase production by *Pseudomonas fluorescens* 2D. *Fd. Microbiol.* **12(4)**, 277-290.

- Manco G., Adinolfi E. P., Francesca M., Ottolina G., Carrea G. and Rossi M. (1998) Overexpression and properties of a new thermophilic and thermostable esterase from *Bacillus acidocaldarius* with sequence similarity to hormone-sensitive lipase subfamily. *Biochem. J.* **332(1)**, 203-212.

- Martinez C. P., Christen P., Farres A. (1993) Medium optimization by a fractional factorial design for lipase production by *Rhizopus delemar.* *J. Ferment. Bioeng*. **76(2)**, 94-7. **CA 119:248138**

- Matselis E. and Roussis I. G. (1992) Influence of culture conditions and hydrogen peroxide on growth and extracellular enzyme production by *Pseudomonas* UICD31. *Fd. Sci. Technol.* **25 (5)**, 433-437.

- Maurich V., Moneghini M., Zacchigan M., Pitotti A. and Lencioni E. (1991a) High-performance liquid chromatograohic assay of Pancereatic lipase activity. *J. Pharm. Biomed. Anal.* **9**, 453-431.

- Maurich V., Zacchigna M. and Pitotti A. (1991b) *p*-Nitrophenyllaurate: a substrate for the high-performance liquid chromatograohic determination of lipase activity. *J. Chromatogr.* **566**, 453-459

- McKellar R.C. (1986) Colorimetric assay for lipases. *Dairy Ind. Int.*, **51 (12)**, 19. **CA 107:5822.**

- McKellar R. C. and Cholete H. (1986) Possible role of calcium in the formation of extracellular protease by *Pseudomonas fluorescens*. *J. Appl. Bacteriol.* **60**, 37-44.

- Medcova I. L., Smirnov K. V. and Surinov B. P. (1981) Method for the determination of monoglyceride lipase. *Lab. Delo* **3**, 157-159. **CA 94: 15235**

- Mencher J. R. and Alford J. A. (1967) Purification and characterization of the lipase of *Pseudomonas fragi*. *J. Gen. Micorbiol.* **48**, 317-328.

- Merilainen V. and Uusi-Rauva A. (1976) Lipolytic properties of the propionic acid bacteria. *Meijeritiet. Aikak*. **34**, 137-42 (German). **CA 86:2077**

- Milton J., Brand S., Jones M. F. and Rayner C. M. (1995) Enantioselective enzymatic synthesis of the anti-viral agent lamivudine (3TCTM). *Tetrahedr. Lett.* **36**, 6961–6964.

- Mirza A. Q., Akhtar M. W., Chughtai M. I. D. (1979) Effect of different triglycerides on lipase production by various *Mucor* species. *Pak. J. Biochem*. **12(1)**, 10-17. **CA 94:154927**

- Mitsuda S., Umemura T. and Hirohara H. (1988) Biological preparation of an optically active alcohol. Part I. Preparation of an optically pure secondary alcohol of synthetic pyrethroids using microbial lipases. *Appl. Microbiol. Biotechnol*. **29(4)**, 310-15.

- Mitsuda S., Yamamoto H., Umemura T., Hirohara H. and Nabeshima S. (1990) Biological preparation of an optically active alcohol. Part III. Enantioselective hydrolysis of a-cyano-3-phenoxybenzyl acetate with *Arthrobacter* lipase. *Agric. Biol. Chem.* **54**, 2907–2912.

- Montero G. A., Kirchner T. F., Tanner R. D. (1993) Bubble and foam concentration of cellulase. *Appl. Biochem. Biotechnol.* **39**, 467-475.

- Montet D., Ratomahenina R., Pina M., Graille J., Galzy P. (1985) Purification and characterization of a lipase from *Candida curvata* Lodder and Kreger-van Rij CBS 570. *Fette, Seifen, Anstrichm*. **87(5)**, 181-5. **CA 103:18891**

- Montgomery D. C. (1991) Design and analysis of experiments. 3rd edition. John Wiley & Sons. New York.

- Mourey A. (1981) Lipolytic activity of *Bacillus pumilus*. *Rev. Fr. Corps Gras*, **28(2)**, 55-8 (French). **CA 94:188319**

- Mozaffar Z. and Weete J. D. (1993) Purification and properties of an extracellular lipase from *Pythium ultimum*. *Lipids* **28(5)**, 377-82.

- Mrin M., Pedregosa A., Rios S., Ortiz M. L. and Laborda F. (1995) Biodegradation of diesel and heating oil by *Acinetobacter calcoaceticus* MM5: its possible applications on bioremediation. *Int. Biodeterior. Biodegrad.* **35**, 269–285. **CA 124:109198**

- Muderhwa J. M., Ratomahenina R., Pina M., Graille J. and Galzy P. (1985) Purification and properties of the lipase from *Candida deformans* (Zach) Langeron and Guerra. *J. Am. Oil Chem. Soc*. **62(6)**, 1031-6.

- Mukoyama K. and Umehara K. (1989) Lipase-containing detergents. (Lion Corp., Japan). Jpn. Kokai Tokkyo Koho JP 01161096 A2. **CA 111:235670**

- Nagao H., Shimada J., Sugihara A., Tominaga Y. and Yamamoto K. (1995) Molecular cloning of cDNA for organic solvent-resistant lipase of *Fusarium heterosporum*. (Osaka City; Toyo Boseki, Japan). Jpn. Kokai Tokkyo Koho JP 07231788 A2. **CA 124:3998**

- Nahas E. and De Assis L. C. (1988) Characterization of lipase secreted by *Rhizopus oligosporus. Rev. Microbiol*. **19(3)**, 321-6 (Portuguese). **CA 110:91016**

- Nair C. S. and Bone D. H. (1987) Production of lipase of *Aspergillus foetidus* in a batch stirred reactor. *Biotechnol. Lett*. **9(8)**, 601-4.

- Nakanishi K. and Ikeda M. (1986) lipase production with *Serratia* D94. (Daikin Industries Ltd. Japan) Jpn. Kokai Tokkyo Koho JP 61058586 A2. **CA 105:41284**

- Nantel G., Baraff G. and Proulx P. (1978) The lipase activity of a partially purified lipolytic enzyme of *Escherichia coli*. *Can. J. Biochem*. **56(5)**, 324-8.

- Nawani N., Dosanjh N.S. and Kaur J. (1998) A novel thermostable lipase from a thermophilic *Bacillus* sp.: characterization and esterification studies. *Biotecnol Lett*. **20 (10)**, 997-1000.

- Negre-Salvayre A., Dagan A., Gatt S. and Salvayre R. (1991) New fluorescent pyrene-containing substrates for assaying cellular lipases, cholesterol esterases and carboxyl esterases. *Appl. Fluoresc. Technol*. **3**,1-6.

- Nishio T., Chikano T. and Kamimura M. (1987) Studies on *Pseudomonas fragi* 22.39 B lipase. Part II. Purification and some properties of lipase produced by *Pseudomonas fragi* 22.39 B. *Agric. Biol. Chem*., **51(1)**, 181-6.

- Noble M. F. M., Cleasby A., Johnson L. N., Egmond M. R. and Frenken L. G. J. (1994) Analysis of the structure of *Pseudomonas glumae* lipase. *Protein Eng.* **7**, 559-562.

- Nthangeni B., Patterton H. -G., Tonder A. V., Vergeer W. and Litthauer D. (2001) Over- expression and properties of a purified recombinant *Bacillus licheniformis* lipase: a comparative report on *Bacillus* lipases. *Enzym. Microb*. *Technol*. **28**, 705-712.

- Nury S., Pieroni G., Riviere C., Gargouri Y., Bois A., and Verger R. (1987) Lipase kinetics at the triachlglycerol-water interface using surface tension measurements. *Chem. Phys. lipids*. **45**, 27-37.

- O'Connor C. J., Petricevic S. F., Coddington J. M. and Stanley R. A. (1992) An NMR assay for quantitating lipase activity in biphasic macroemulsions. *J. Am. Oil Chem. Soc*. **69**, 295-300.

- Odera M., Takeuchi K. and Tohe A. (1986) Molecular cloning of lipase genes from *Alcaligenes denitrificans* and their expression in *Escherichia coli*. *J. Ferment. Technol.* **64(5)**, 363-71. **CA 106:1340**

- Oestdal H., Baron C. P., Blom H. and Andersen H. J. (1996) Production, isolation and partial characterization of a lipase-esterase from *Pediococcus pentosaceus* SV61. *Food Sci. Technol*. (London), **29(5 & 6)**, 542-546. **CA 125:220021**

- Ogierman M. A., Fallarino A., Riess T., Wiiliams S. G., Attridge S. R., and Manning P. A. (1997) Characterization of the *Vibrio cholerae* El Tor lipase operon lipAB and a protease gene downstream of the hly region. *J. Bacteriol.* **179(22)**, 7072-80.

- Oh B. -C., Kim H. -K., Lee J. -K., Kang S. -C. and Oh T. -K. (1999) *Staphylococcus haemolyticus* lipase: biochemical properties, substrate specificity and gene cloning. *FEMS Microbiol. Lett*. **179(2)**, 385-392.

- Ohno N. and Matsuno M. (1997) Utilization of fats and oils by imperfect fungi *Fusidium* sp. BX-1. Wayo Joshi Daigaku Kiyo, Kaseikei-hen, **37**, 25-32 (Japanese). **CA 127:47562**

- Okafor J. I. and Gugnani H. C. (1990) Lipase activity of *Basidiobolus* and *Conidiobolus* species. *Mycoses* **33(2)**, 81-5. **CA 113:148472**

- Okumura S., Iwai M. and Tsujisaka Y. (1980) Purification and properties of partial glyceride hydrolase of *Penicillium cyclopium* M1. *J. Biochem*. (Tokyo), **87(1)**, 205-11.

- Olson G. J., Woese C. R. and Overbeek R. (1994) The winds of (evolutionary) change: breathing new life into microbiology. *J. Bacteriol*. **176**, 1-6.

- Omar I.C., Nishio N., and Nagai S. (1987). Fat hydrolysis and esterification by lipase from *Humicola lanuginosa*. *Agric. Biol. Chem*. **51**, 2153-2159.

- Osman M., Mohamed Y. A. H. and Metwally M. (1988) Lipolytic activity of *Alternaria alternata* and *Fusarium oxysporum* and certain properties of their lipids. *Microbios Lett*. **39(155-156)**, 131-5.

- Oxenboell K. M., Borch K. and Patkar S. A. (1996) (Novo Nordisk A/s, Den.). Lipolytic enzyme from *Fusarium culmorum* for use in detergents. PCT Int. Appl. WO 9613579 A1. **CA 125:52379**

- Ozer D. and Elibol M. (2000) Effects of some factors on lipase production by *Rhizopus arrhizus*. *J. Food Sci. Technol*. **37(6)**, 661-664.

- Padmini P., Rakshit S. K. and Baradarajan A. (1990) The fermentative production of lipase. *Indian Chem. Eng*. **32(4)**, 51-4.

- Palleroni N. J. (1984) Family I. Pseudomonadaceae In: Bergey's Manual of Systematic Bacteriology, Volume I, edited by Krieg N. R. and Holt J. G. **pp.** 140-199. Williams and Wilkins, Baltimore.

- Palleroni N. J. (1992) Human- and animal- pathogenic Pseudomonads. In: The Prokaryotes, Volume III, second edition, edited by Balows A., Trüper H. G., Dworkin M., Harder W., Schleifer K. H., **pp.** 3087-3103. Springer- Verlag, New York.

- Pandey A., Benjamin S., Soccol C. R., Nigam P., Krieger N. and Soccol V. T. (1999) The realm of microbial lipases in biotechnology. *Biotechnol. Appl. Biochem.* **29**, 119–131.

- Papaparaskevas D., Christakopoulos P., Kekos D. and Macris B. J. (1992) Optimizing production of extracellular lipase from *Rhodotorula glutinis*. *Biotechnol. Lett*. **14(5)**, 397-402.

- Papon M. and Talon R. (1988) Factors affecting growth and lipase production by meat lactobacilli strains and *Brochothrix thermosphacta*. *J. Appl. Bacteriol*. **64(2)**, 107-15.

- Parmar V. S., Bisht K. S., Singh A. and Jha A. (1996) Potentially useful lipase-catalyzed transesterifications. *Proc. Ind. Acad. Sci.* **108**, 575–583.

- Parmar V. S., Kumar A. P., Pati H. N. and Saxena R. K., Davidson S. and Gupta R. (1998) Potential use of a novel lipase from *Aspergillus carneus* in deacetylation reactions. *Biochim. Biophys. Acta* **1387(1-2)**, 325-330.

- Phillips A. and Pretorius G. H. J. (1991) Purification and characterization of an extracellular lipase of *Galactomyces geotrichum*. *Biotechnol. Lett*. **13(11)**, 833-8.

- Piao Z., Liu C., Feng Y. and Cao S. J. (1998) Lipase production by *Pseudomonas stutzeri. Daxue Ziran Kexue Xuebao*, **(1)**, 97-99 (Chinese) **CA 128:269547**

- Ping L. B. and Omar I. C. (1993) Fermentation behavior of lipase production by *Corynebacterium* sp. grown in a palm oil medium. *J. Biosci*. **4(1)**, 105-9.

- Plou F. J., Barandiráa M., Calvo M. V. Ballesteros A. and Pastor E. (1996) High-yield production of mono-and di-oleylglycerol by lipase-catalysed hydrolysis of triolein. *Enzym. Microb. Biotechnol.* **18**, 66-71.

- Pokorny D., Friedrich J. and Cimerman A. (1994) Effect of nutritional factors on lipase biosynthesis by *Aspergillus niger*. *Biotechnol. Lett.* **16(4)**, 363-366.

- Poluiaktova S. K., Gol'berg N. D. and Fel'dkoren B. I. (1995) A micromethod for determining tissue lipase activity using gas-liquid chromatography. *Vopr Med Khim*

41(1), 57-59 (Article in Russian). **PMID: 7771096** [PubMed - indexed for MEDLINE]

- Prazeres D. M. F., Garcia F. A. P. and Cabral J. M. S. (1993a) An ultrafiltration membrane bioreactor for the lipolysis of olive oil in reversed micellar media. *Biotechnol. Bioeng.* **41**, 761-770.

- Prazeres D. M. F., Lemos F., Garcia F. A. P. and Cabral J. M. S. (1993b) Modeling lipolysis in a reversed micellar system. 1. conventional batch reactor. *Biotechnol. Bioeng.* **42**, 765-771.

- Queiroz J. A., Garcia F. A. P. and Cabral J. M. S. (1999) Purification of *Chromobacterium viscosum* lipase by hydrophobic interaction chromoatography. *Isoation Purf.* **3**, 1-7.

- Ransac S., Riviere C., Gancet C., Verger R. and de Haas G. H. (1990) Competitive inhibition of lipolytic enzymes. I. A kinetic model applicable to water-insoluble competitive inhibitors. *Biochim. Biophys. Acta* **1043**, 57-66.

- Ransac S., Moreau H., Riviere C. and Verger R. (1991) Monolayer techniques for studying phospholipase kinetics. *Methods Enzymol.* **197**, 49-65.

- Rapp P. (1995) Production, regulation, and some properties of lipase activity from *Fusarium oxysporum* f. sp. vasinfectum *Enzym Microb. Technol*. **17(9)**, 832-8.

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

- Rathi P., Bradoo S., Saxena R. K. and Gupta R. (2000) A hyper thermostable, alkaline lipase from *Pseudomonas* sp. with the property of thermal activation. *Bitechnol Lett.* **22**, 495-498.

- Rawyler A. and Siegenthaler P. A. (1989) A single and continuous spectrophotometric assay for various lipolytic enzymes, using natural, non-labeled lipid substrates. *Biophys. Methods* **23**, 249-253.

- Reddy D. R. (1992) In: Industrial Biotechnology, edited by Malik V. S. and Sridhar P., **pp.** 387–404, Oxford & IBH Publishing Co., New Delhi.

- Reetz M. T. and Jaeger K. -E. (1998) Overexpression, immobilization and biotechnological application of *Pseudomonas* lipases. *Chem. Phys. Lipids* **93**, 3-14.

- Richardson A. C., Smith B. V., Price R. G. and Praill P. F. G. (1989) Chromogenic substituted arylethene derivatives as substrartes for hydrolytic enzyme assays. European Patent WO 89-02473. **CA 112:94609**

- Robertson E. F., Dannelly H. K., Malloy P. J. and Reeves H. C. (1987) Rapid isoelectric focusing in a vertical polyacrylamide mini gel system. *Anal. Biochem.* **167**, 290-294.

- Robinson J. B. Jr., Levinson R. S., Johnson H. W., Mowles D. L. and Swopes H. (1989) Europian patent application EP 297387 A2. **CA 111: 129799**

- Rogalska E., Cudrey C., Ferrato F. and Verger R. (1993) Stereoselective hydrolysis of triglycerides by animal and microbial lipases. *Chirality*, **5(1)**, 24-30. **CA 119:89723**

- Rogel A. M., Stone W. L. and Aldebonojo F. O. (1989) A novel spectrophotometric assay for lipase activity utilizing cis- parinaric acid. *Lipids* **24(6)**, 518-525.

- Rollof J. and Nomark S. (1992) In vivo processing of *Staphylococcus aureus* lipase. *J. Bacteriol*. **174**, 1844-1847.

- Rollof J., Hedstrom S. A. and Nilsson-Ehle P. (1984) A simple turbidometric method for specific measurement of *Staphylococcus aureus* lipase activity. *Acta Pathol. Microbiol. Immunol. Scand.*, Sect B, **92B(3)**, 155-158. **CA 101:186656**

- Rollof J., Hedström S. A. and Nisson-Ehle P. (1987) Purification and chracterization of lipase from *Staphyllococcus aureus*. *Biochim. Biophys. Acta.* **921**, 370-377.

- Rosenstein R. and Götz F. (2000) Staphylococcal lipases: Biochemical and molecular characterization. *Biochimie* **82**, 1005- 1014.

- Rowe M. T. and Gilmour A. (1981) A modified serum lipase assay method for use with microbial lipase. *Chem. Microbiol. Technol. Lebensm.* **7(1)**, 18-20. **CA 95:56885**

- Rowe M. T. and Gilmour A. (1982) Growth, enzyme production and changes in oxygen tension occurring during batch cultivation of psychrotrophic *Pseudomonas fluorescens* strains. *Milchwissenschaft* **37(10)**, 597-600.

- Roy R. N. (1980) Fluorimetric assay of the activity of extracellular lipases of *Pseudomonas fluorescens* and *Serratia marcescens*. *J. Appl. Bacteriol*. **49(2)**, 265-271.

- Rúa M. L., Schmidt- Dannert C., Wahl S., Sprauer A. and Schimd R. D. (1997) Thermoalkalophilic lipase of *Bacillus thermocatenulatus* large-scale production and properties: aggregation behaviour and its effect on activity. *J. Biotechnol*. **56**, 89-102.

- Rúa M. L., Atomi H., Schmidt-Dannert C. and Schmid R. D. (1998) High-level expression of the thermoalkalophilic lipase from *Bacillus thermocatenulatus* in *Escherichia coli*. *Appl. Microbiol. Biotechnol*. **49(4)**, 405-410.

- Ruban E. L., Ksandopulo G. B., Murzina L. P. (1978) Conditions of exolipase biosynthesis by the fungus *Oospora fragrans. Prikl. Biokhim. Mikrobiol*. **14(6)**, 849-57 (Russian) **CA 90:117703**

- Ruiz L. and Roudriguez-Fernandez M. F. C. (1982) Kinetic study of hepatic triglyceride lipase from rat liver soluble fraction. *Enzyme* **27**, 215-219.

- Ruschen S. and Winkler U. K. (1982) Stimulation of the extracellular lipase activity of *Saccharomycopsis lipolytica* by hyaluronate. *FEMS Microbiol. Lett*. **14(2),** 117-21.

- Saad R.R. (1995) Production of lipase by *Aspergillus tamari* and its compatibility with commercial detergents. *Acta Microbiol. Pol*. **44(1)**, 31-8. **CA 105:111671**

- Safarik I. (1991) A spectrophotometric assay for lipase activity utilizing immobilized triacylglycerols. *J. Biochem. Biphys. Methods* **23**, 249-353.

- Sagai H., Ohta H., Suzuki K. and Imamura S. (1989) Cloning and expression of *Chromobacterium* lipase gene Ger. Offen. DE 3908131 A1. **CA 113:18939**

- Saimoku F., Hayano T. and Nakamura O. (1999) Cloning of gene for novel lipase from *Alteromonas* and use for emulsion break agent. (Tonen Co., Ltd., Japan). Jpn. Kokai Tokkyo Koho JP 11169177 A2 Heisei, **CA 131:84837**

- Samad M. Y. A., Salleh A. B., Razak C. N. A., Ampon K., Yunus W. M. Z. W. and Basri M. (1990) A lipase from a newly isolated thermophilic *Rhizopus rhizopodiformis. World J. Microbiol. Biotechnol*. **6(4)**, 390-4.

- Samad M.Y. A., Razak C. N.A., Salleh A. B., Zin Wan Yunus, W. M., Ampon. K. and Basri M. (1989) A plate assay for primary screening of lipase activity. *J. Microbiol. Methods* **9(1)**, 51-56.

- Sandal T., Kauppinen S., Kofod L. V. (1996) Recombinant production and detergent properties of a lipolytic enzyme from *Humicola insolens* DSM 1800 (NovoNordisk A/s, Den.). PCT Int. Appl. WO 9613580 A1 (World Intellectual Property Organization). **CA 125:52380**

- Savitha J. and Ratledge C. (1992) An inducible, intracellular, alkalophilic lipase in *Aspergillus flavipes* grown on triacylglycerols. *World J. Microbiol. Biotechnol.* **8(2)**, 129-31.

- Schmidt-Dannert C., Sztajer H., Stocklein W., Menge U. and Schmid R. D. (1994) Screening, purification and properties of a thermophilic lipase from *Bacillus thermocatenulatus*. *Biochim. Biophys. Acta* **1214(1)**, 43-53.

- Schmidt-Dannert C., Rúa M. L., Atomi H. and Schmid R. D. (1996) Thermoalkalophilic lipase of *Bacillus thermocatenulatus*. 1. Molecular cloning, nucleotide sequence, purification and some properties. *Biochim. Biophys. Acta* **1301**, 105-114.

- Schmidt-Dannert C., Rúa M. L., Wahl S. and Schmid R. D. (1997) *Bacillus thermocatenulatus* lipase: a thermophilic lipase with interesting prooerties. *Biochem. Soc. Trans.* **25(1)**, 178-182.

- Scholefield J., O'Donnell E. T. and Davies G. (1978) Procedure for the isolation of lipases produced by *Pseudomonas* species and *Achromobacter lipolyticum*. *J. Food Technol.* **13(2)**, 129-36. **CA 89:38588**

- Schulte G., Bohne L. and Winkler U. K. (1982) Gycogen and various other polysachharides stimulate the formation of the exolipase by *Pseudomonas aeruginosa*. *Can J. Microbiol.* **28**, 636-642.

- Selezneva A. A. and Kazanina G. A. (1986) Fungal lipases from *Penicillium solitum* and *Geotrichum asteroides*. *Biotekhnologiya* **6**, 13-20 (Russian). **CA 107:2949**

- Servin-Gonzalez L., Castro C., Perez C., Rubio M. and Valdez F. (1997) bldA-dependent expression of the *Streptomyces exfoliatus* M11 lipase gene (lipA) is mediated by the product of a contiguous gene, lipR, encoding a putative transcriptional activator. *J. Bacteriol*. **179(24)**, 7816-7826.

- Shabtai Y. and Daya-Mishne N. (1992) production, purification and properties of a lipase from a bacterium (*Pseudomonas aeruginosa* YS-7) capable of growing in water restricted environments. *Appl. Environ. Microbiol*. **58(1)**, 174-180.

- Sharon C., Furugoh S., Yamakido T., Ogawa H. I. and Kato Y. (1998) Purification and characterisation of *Pseudomoans aeruginosa* KKA-5 and its role in castor oil hydrolysis. *J. Ind. Microbiol. Biotechnol*. **20(5)**, 304-307.

- Sharon C., Nakazato M., Ogawa H. I. and Kato Y. (1999) Bioreactor operated production of lipase: Castor oil hydrolysis using partially purified lipase. *Ind. J. Experimental Biol.* **37**, 481-486.

- Shash S. M. (1998) Regulation of alkaline lipase(s) biosyntheses under bench scale fermentation conditions by *Aspergillus flavus*., S-7 allowed to grow on Nile hyacinth homogenates. *Egypt. J. Biotechnol*. 3, 91-103. **CA 129:329743**

- Shchelokova S. S., Tabak M. Y. and Zakirov M. Z. (1977) Strain of *Oospora lactis* which actively produces lipase. *Mikrobiologiya*, **46(4)**, 656-60 (Russian). **CA 87:148410**

- Sheela P., Amuthan G. and Mahadevan A. (1996) Cloning of extracellular lipase gene from *Xanthomonas campestris* pathovar *sesami* on to *Escherichia coli*. *Indian J. Exp. Biol*. **34(1)**, 27-31.

- Shibata H., Kato H. and Oda J. (1998) Calcium ion-dependent reactiovation of a *Pseudomonas* lipase by its specific modulating protein, Lip B. *J. Biochem.* **123**, 136-141.

- Shimada Y., Sugihara A., Nagao T. and Tominaga Y. (1992) Induction of *Geotrichum candidum* lipase by long -chain fatty acids. *J. Ferment. Bioeng.* **74(2)**, 77-80.

- Silva A. M. G. M., Cabral J. M., Costa M. S. and Garcia F. A. P. (1991) Characteristics of a new lipase from a *Thermus* sp. bacterium. *GBF Monogr.* **16**(Lipases), 417-20. **CA 115:250809**

- Simons J. W., Adams H., Cox R. C., Dekker N., Götz F., Slotboom A. J. and Verheij H. M. (1996) The lipase from *Staphylococus aureus*. Expression in *Escherichia. Coli*, large scale purification and comparison of sustrate specificity to *Staphylococus hyicus* lipase. *Eur. J. Biochem.* **241**, 760-769.

- Simons J. W. F. A., Van Kampean M. D., Riel S., Gotz F., Egmond M. R. and Verheij H. M. (1998) Cloning, purification and characterization of the lipase from *Staphylococcus epidermidis*, comparison of the substrate selectivity with those of other microbial lipases. *Eur. J. Biochem.* **253(3)**, 675-683.

- Smerdon G. R., Walton E. F. and Aves S. J. (1998) Stable production of human gastric lipase by chromosomal integration in the fission yeast *Schizosaccharomyces pombe. Appl. Microbiol. Biotechnol*. **49(1)**, 45-50.

- Sommer P. Bormann C. and Götz F. (1997) Genetic and biochemical characterization of a new extracellular lipase from *Streptomyces cinnamomeus. Appl. Environ. Microbiol.* **63(9)**, 3553-3560.

- Starace C. A. (1983) Detergent enzymes - past, present and future. *J Am Oil Chem Soc.* **60(5)**, 1203-1207.

- Stead D. (1984) Evaluation of fluorimetric assay on the lipases from strains of milk psychrotrophoic bacteria. *J. Dairy Res.* **51 (1)**, 123-130.

- Stepaniack L., Birkeland S. E., Vegias G., and Soerhaug T. (1987) Enzyme-linked immunosorbent assay (ELISA) for monitoring the production of heat stable proteinases and lipase from *Pseudomonas*. *Milchwissenschaft* **42(3)**, 168-172.

- Stuer W., Jaeger K. -E. and Winkler U. K. (1986) Purification of extracellular lipase from *Pseudomonas aeruginosa*. *J. Bacteriol.* **168**, 1070-1074.

- Sugai T., Katoh O. and Ohta H. (1995) Chemo-enzymic synthesis of (R,R)-(-)-pyrenophorin. *Tetrahedron* **51(44)**, 11987-98.

- Sarkar P., Bhattacharya P., Muukharjea R. N., Mukherjea M. (1987) Isolation and purification of protease from human placenta by foam fractionation. *Biotechnol. Bioeng.* **29**, 934-940.

- Sugiura M. and Isobe M. (1975) Studies on the mechanism of lipase reaction. II. Comparative studies on the adsorption of lipases and various proteins at the air-water interface. *Biochim. Biophys. Acta.* **397**, 412-417.

- Sugiura M. (1984) Bacterial lipases. In: Lipases, edited by Borgstrom B. and Brockman H. L. **pp.** 505-523. Elsevier, Amerstadam.

- Sugihara A., Tadaki T. and Yosio T. (1991) Purification and characterization of a novel thermostable lipase from *Bacillus* sp. *J. Biochem.* **109**, 211-216.

- Sugihara A., Senoo T., Enoki A., Shimada Y., Nagao T. and Tominaga Y. (1995) Purification and characterization of a lipase from *Pichia burtonii*. *Appl. Microbiol. Biotechnol.* **43(2)**, 277-81.

- Suzuki T., Mushiga Y., Yamane T. and Shimizu S. (1988) Mass production of lipase by fed-batch culture of *Pseudomonas fluorescens*. *Appl. Microbiol. Biotechnol*. **27**, 417-422.

- Svendsen A., Pathar S. A., Egel-Mitani M., Borch K., Clausen I. G. and Hansen M. T. (1994) *Candida antarctica* lipase and lipase variants. (Novo Nordisk A/S, Den.). PCT Int. Appl. WO 9401541 A1 20. (World Intellectual Property Organization). **CA 120:293005**

- Svetlitshnyi V., Rainey F. and Wiegel J. (1996) *Thermosyntropha lipolytica* gen. nov., sp. nov., a lipolytic, anaerobic, alkalitolerant, thermophilic bacterium utilizing short- and long-chain fatty acids in syntrophic coculture with a methanogenic archaeum. *Int. J. Syst. Bacteriol*. **46(4)**, 1131-1137. **CA 126:4280**

- Svetlov A. K., Krakhmalets I. A., Kryuchkov V. V., Oreshchenko L. I. and Novikova I. A (1984) Removal of pigments from the lipase of *Aspergillus awamori. Otkrytiya, Izobret., Prom. Obraztsy, Tovarnye Znaki* **21**, 203. **CA 101:106523**

- Swaisgood H. E. and Bozoglu F. (1984) Heat inactivation of the extracellular lipase from *Pseudomonas fluorescens* MC50. *J. Agric. Food. Chem.* **32**, 7-10.

- Szell V., Kovacs-Szabo I., Kelemen A., Horvath I., Cseh G., Hegyaljai K. G. and Tolgyesi L. (1977) Enhancement of the lipase production of Rhizopus oryzae microorganisms. Hung. Teljes HU 12817 28. (Hungarian). **CA 86:153971**

- Sztajer H., Maliszewska I., Wieczorek J. (1988) Production of exogenous lipases by bacteria, fungi, and actinomycetes. *Enzyme Microb. Technol*. **10(8)**, 492-7.

- Sztajer H. and Maliszewska I. (1988a) The effect of culture conditions on lipolytic production of microorganisms. *Bitechnol. Lett*. **10(3)**, 199-204.

- Sztajer H., Erdmann H., Isobe K., Morelle G. and Schmid R. (1991a) Production and some properties of partially purified lipase from *Penicillium simplicissimum*. *GBF Monogr*. **16** (Lipases), 339-44. **CA 115:274189**

- Sztajer H., Borkowski J. and Sobiech K. (1991b) Purification and some properties of *Pseudomonas fluorescens* lipase. *Biotechnol. Appl. Biochem.* **13**, 65-71.

- Sztajer H. and Maliszewska I. (1989) The effect of culture conditions on lipolytic productivity of *Penicillium citrinum*. *Biotechnol. Lett*. **11(12)**, 895-8.

- Tahoun M. K., Mostafa E., Mashaly R. and Abou-Donia S. (1982) Lipase induction in *Geotrichum candidum. Milchwissenschaft*, **37(2)**, 86-8.

- Taipa M. A., Liebeton K., Costa J. V., Cabral J. M. and Jaeger K. E. (1995) Lipase from *Chromobacterium viscosum*: biochemical characterization indicating homology to the lipase from *Pseudomonas glumae*. *Biochim. Biophys. Acta* **1256(3)**, 396-402.

- Talon R., Dublet N., Montel M. C. and Cantonnet M. (1995) Purification and characterization of extracellular *Staphylococcus warneri* lipase. *Curr Microbiol*. **30(1)**, 11-6.

- Talon R., Montel M. -C. and Berdague J. -L. (1996) Production of flavor esters by lipases of *Staphylococcus warneri* and *Staphylococcus xylosus*. *Enzym Microb. Technol*. **19(8)**, 620-622.

- Tan K. H. and Gill C. O. (1985) Effect of culture conditions on batch growth of *Pseudomonas fluorescens* on olive oil. *Appl. Microbiol. Biotechnol.* **23(1)**, 27-32.

- Tan Y. and Miller K. J. (1992) Cloning, expression and nucleotide sequence of a lipase gene from *Pseudomonas fluorescens* B52. *Appl. Environ. Microbiol.***58**, 1402-1407.

- Tanaka N. and Imamura S. (1986) Skin cleansing agents containing lipase and *Aspergillus saitoi* extracts. Jpn. Kokai Tokkyo Koho JP 61266499 A2 Showa. (Japan). **CA 106:72728**

- Tatara T., Fujii T., and Minagawa M. (1985). Studies on application of lipolytic enzymes in detergency II. Evaluation of adaptability of various kinds of lipases in practical laundry conditions. *J. Am. Oil Chem. Soc.* **62**, 1053-1058.

- Thuren T., Virtanen J. A., Verger R. and Kinnunen P. K. J. (1987) Hydrolysis of 1 palmitoyl-2[6-(pyren-1-yl)] hexanoyl-sn-glycero-3-phospholipids by phospholipase A2: Effect of the polar head- group. *Biochim. Biopys. Acta* **917**, 411-417.

- Toida J., Fukuzawa M., Kobayashi G., Ito K. and Sekiguchi J. (2000) Cloning and sequencing of the triacylglycerol lipase gene of *Aspergillus oryzae* and its expression in *Escherichia coli. FEMS Microbiol. Lett*. **189(2)**, 159-164.

- Trader's guide to fermentation media formulation (1980), edited by Zabriskie D. W., Armiger W. B., Philips D. H. and Albano P. A. **p. p.** 12 Trader's protein, Memphis, Tennessee, USA.

- Tsereteil M., Kruchua T., Mushkudiani Kh. (1994) Investigation of some properties of exogenous lipase from *Rhizopus cohnii* sp. (II). (Georgia). *Soobshch. Akad. Nauk Gruz*. **150(3)**, 529-532 (Georgian). **CA 125:268871**

- Tweddell R. J., Kermasha S., Combes D. and Marty A. (1999) Immobilization of lipase from *Rhizopus niveus*. A way to enhance its synthetic activity in organic solvent. *Biocatalysis and Biotransformation* **16(6)**, 411-426.

- Tyski S. Waleria H. and Janusz J. (1983) Purification and some properties of the staphylococcal extracellular lipase. *Biochim. Biophys. Acta.* **749(3)**, 312-317.

- Uraizee F. and Narsimhan G. (1990a) Foam fractionation of proteins and enzymes: I. Applications. *Enzym. Microb. Technol.* **12**, 232-233.

- Uraizee F. and Narsimhan G. (1990b) Foam fractionation of proteins and enzymes. II. Performance and modeling. *Enzym. Microb. Technol.* **12**, 315-316.

- Uraizee F. and Narsimhan G. (1995a) Foam-based separation of proteins. In: Bioseparation processes in foods, edited by Singh R. K. and Rizvi S. S. H. **pp**. 175-225. IFT basic symposium series. Institute of food technologists, Chicago, USA.

- Uraizee F. and Narsimhan G. (1995b) A model for continuous foam concentration of proteins: Effect of kinetics of adsorption of proteins and coalescence of foam. *Sepr. Sci. Tech.* **30**, 847 –881.

- Uraizee F. and Narsimhan G. (1996) Effect of kinetics of adsorption and coalescence on continuous foam concentration of proteins: Comparison of experimental results with model predictions. *Biotechnol. Bioeng.* **51**, 384-398.

- Usher J. J., Romancik G., Politino M. and Lowe D. A. (1995) Enzymatic acylation of 3-hydroxymethyl cephalosporins. (Bristol-Myers Squibb Co., USA). Eur. Pat. EP-667396. **CA 123:196753**

- Ushio k., Hirata T., Yashida K., Sukane W., Hirose C., Suzuki T. and Ishizuka M. (1996) Superinducers for induction of thermostable lipase production by *Pseudomonas* sp. NT-163 and other *Pseudomonas* like bacteria. *Bitechnol. Tech.* **10(4)**, 267-272.

- Uyttenbroeck W., Hendriks D., Vriend Gert; D. B. I., Moens L. and Scharpe, S. (1993) Molecular characterization of an extracellular acid-resistant lipase produced by *Rhizopus javanicus. Biol. Chem. Hoppe-Seyler*, **374(4)**, 245-54. **CA 119:3982**

- Valero F., Ayats F., Lopez-Santin J. and Poch M. (1988) Lipase production by *Candida rugosa*: fermentation behavior. *Biotechnol. Lett*. **10(10)**, 741-4.

- van Autryve P., Ratomahenina R., Riaublanc A., Mitrani C., Pian M., Galzy P. (1991) Spectrophotometry assay of lipase activity using Rhodamine 6G. *Oleagineux* **46**, 29-31. CA 115:109034

- van Oort M. G., Deveer A. M. Th. J., Dijkman R. and Tjeenk M. L. (1989) Purification and substrate specificity of *Staphyllococcus hyicus* lipase. *Biochem*. **28**, 9278-9285.

- Vandamme E., Schanck-Brodrueck K. H., Colson C., Hanotier J. D. V. (1987) Cloning of the lipase gene of *Geotrichum candidum* in *Escherichia coli* and yeast. (Labofina S. A., Belg.). Eur. Pat. Appl. EP 243338 A2. **CA 108:199489**

- Vecozola A. and Bekers M. (1978) Optimization of the composition of the medium for the biosynthesis of lipase by *Candida paralipolytica* 739 yeasts. *Tekhnol. Mikrobn. Sinteza*, 81-4 From: Ref. Zh., Biol. Khim., Abstr. No. 21KH158 (Russian). **CA 90:68957**

- Veerraghavan K. (1990) A simple and sensitive method for the estimation of microbial lipase activity. *Anal. Biochem*. **186(2)**, 301-305.

- Verger R. (1980) Enzyme kinetics of lipolysis. *Methods Enymol*. **64**, 341-392.

- Vitolo S., Petarca L. and Bresci B. (1998) Treatment of olive oil industry wastes. *Biores. Technol.* **67**, 129–137.

- Voit H., Wenzig E. and Mersmann A. (1991) Production of a microbial lipase by

Staphylococcus carnosus (pLipMut2) in a bubble column reactor and a centrifugal field bioreactor. *Bioprocess Eng*. **6(5)**, 221-6.

- Vora K. A., Bhandare S. S., Pradhan R. S., Amin A. R., Modi V. V. (1988) Characterization of extracellular lipase produced by *Aspergillus japonicus* in response to *Calotropis gigentea* latex. *Biotechnol. Appl. Biochem*. **10(5)**, 465-72.

- Vorderwülbecke T., Kieslich K. and Erdmann H. (1992) Comparison of lipases by different assays. *Enzym Microb. Technol.* **14**, 631-639.

- Wakelin N. G. and Forster C. F. (1997) An investigation into microbial removal of fats, oils and greases. *Bioresour. Technol.* **59(1)**, 37-43.

- Walde P. and Luisi P.L. (1989) A continuous assay for lipase in reverse micelles based on Fourier transform infrared spectroscopy. *Biochemistry* **28**, 3353-3360.

- Wang T. -J. and Chen T. -L. (1998) Lipase production by *Acinetobacter radioresistens* in a batch fill-and-draw culture. *Appl. Biochem. Biotechnol*. **73(2-3)**, 185-194.

- Wang Y., Srivastava K. C., Shen G. J. and Wang H. Y. (1995) Thermostable alkaline lipase from a newly isolated thermophilic *Bacillus* strain A30-1 (ATCC 53841). *J. Ferment. Bioeng.* **79(5)**, 433-438.

- Watanabe N., Ota Y., Minoda Y. and Yamada K. (1977) Isolation and identification of alkaline lipase producinmg micororganisms, cultural conditions and some properties of crude enzymes. *Agric. Biol. Chem.* **41(8)**, 1353-1358.

- Wei Y., Swenson L., Castro C., Derewenda U., Minor W., Arai H., Aoki J., Inoue K., Servin-Gonzalez L. and Derewenda Z. S. (1998) Structure of a microbial homologue of mammalian platelet-activating factor acetylhydrolases: *Streptomyces exfoliatus* lipase at 1.9 Å resolution. *Structure* **6**, 511–519.

- Wenzig E., Lingg S., Paul K., Zeh G. and Mersmann A. (1993) Comparison of selected methods for downstream processing in the production of bacterial lipase. *Chem. Eng. Technol.* **16(6)**, 405-412.

- Wilton D. C. (1990) A continuous fluorescence displacement assay for the measurement for phospholipase A2 and other lipases that release long-chain fatty acids. *Biochem.J*. **266**, 435-439.

- Wilton D. C. (1991) A continuous fluorescence-displacement assay for triacylglycerol lipase and phospholipase C that also allows the measurement of acylgycerols. *Biochem. J.* **276**, 129-133.

- Wingender J. and Winkler U. K. (1984) A novel biological function of alginate in *Pseudomonas aeruginosa* and its mucoid mutants: stimulation of exolipase. *FEMS Microbiol. Lett*. **21**, 63-69.

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

- Wirth S. J. (1992) Water soluble, dye lablled fatty acid derivatives for preliminary detection of lipolytic microorganisms in agar media. *FEMS Micorbiol. Lett.* **95**, 77-80.

- Woollett L. A., Beitz D. C., Hood R. L. and Aprahamian S. (1984) An enzymatic assay for activity of lipoprotein lipase. *Anal. Biochem.* **143**, 25-29.

- Yamaguchi S., Takeuchi K., Mase T. and Matsuura A. (1997) Efficient expression of mono- and diacylglycerol lipase gene from *Penicillium camembertii* U-150 in *Aspergillus oryzae* under the control of its own promoter. *Biosci. Biotechnol. Biochem*. **61(5)**, 800-805.

- Yamamato K. and Fujiwara N. (1988) Purification and some properties of a castor-oil-hydrolyzing lipase from *Pseudomonas* sp. *Agric. Biol. Chem.* **52**, 3015-3021.

- Yamamato K. and Fujiwara N. (1995) The hydrolysis of castor oil using a lipase from *Pseudomonas* sp. f-B-24: Positional specificity and substrate specificity of the enzyme and the optimum reaction conditions. *Biosci. Biotech. Biochem*. **59(7)**, 1262-1266.

- Yasuda M., Ogino H., Kiguchi T., Kotani T., Takakura S., Ishibashi T., Nakashima T. Fukuda H. and Ishikawa H. (1999) Purification and characterization of lipase from *Rhizopus chinensis* cells. *J. Biosci. Bioeng*. **88(5)**, 571-573. **CA 132:177307**

- Yasuhiro N. (1987) Study on the assay of lipase with emulsified substrate. *Yukagaku*, **36(10)**, 821-5. **CA: 108527**

- Yasuhiro N. and Satoru (1988) Study on the assay method of lipase with emulsified substrate. The effect of poly (vinyl alcohol) as emulsifying agent on the stability of emulsified subtrate and coefficient of variation (C. V.). *Yukagaku* **37**, 133-134. **CA: 163678**

- Yeo S., Nihira T. and Yamada Y. (1998) Purification and chracterization of tert-butyl ester hydrolyzing lipase from *Burkholderia* sp. YY62. *Biosci. Biotechnol. Biochem.* **62(12)**, 2312-2317.

- Yokoe M. and Mase T. (1988) Lipase AML, its manufacture with *Pseudomonas*, and its use in detergent production (Amano Pharmaceutical Co., Ltd., Japan). Jpn. Kokai Tokkyo Koho JP 63039579 A2. **CA 109:72058**

- Yokomatsu T., Nakabayashi N., Matsumoto K. and Shibuya S. (1995) Lipase-catalyzed Kinetic resolution of cis-1-Diethylphosphonomethyl-2-hydroxymethylcyclohexane. Application to enantioselective Synthesis of 1-Diethylphosphonomethyl-2-(5'-hydantoinyl)cyclohexane. *Tetrahedr. Asymm*. **6**, 3055–3062.

- Yoneda T., Takada H., Ohno K. and Sasuga J. (1996) Cloning of gene for novel lipase of *Pseudomonas*, recombinant production of lipase, and its uses. Pat. WO-9627002. **CA 125:269257**

- Yoshinari K., Aoki M., Ohtsuka T., Nakayama N., Itezono Y., Mutoh M., Watanbe J. and Yokose K. (1994) Panclicins, novel pancreatic lipase inhibitors. II. Structure elucidation. *J. Antibiot.* **47(12)**, 1376-84.

- Zahran A. S. (1998) Purification and characterization of a lipase secreted by *Micrococcus varians* CAS4 isolated from Cheddar cheese. *Egypt. J. Dairy Sci*., **26(2)**, 251-261. **CA 131:15535**

- Zeh G., Wenzig E. and Mersmann A. (1992) Enrichment of a bacterial lipase by foam fractionation. *Bioforum* **15(11)**, 407-408. **CA 121: 274936**

- Zou W., Liu H. and Yu W. (1996) Isolation of lipase-producing *Serratia* liquefaciens and characterization of its lipase. *Nanjing Daxue Xuebao, Ziran Kexue* 32(4), 713-716. (Chinese) **CA 126:222127**

- Zuyi L. and Ward O. P. (1993) Stability of microbial lipase in alcoholysis of fish oil during repeated enzyme use. *Biotechnol. Lett*. **15**, 393–398.

Note: **CA** stands for Chemical abstracts. For some references only the abstracts were refered from chemical abstracts. These references have **CA numbers** quoted in the end. **PMID** number is quoted for the references for which abstracts were referred from the Pub-Med (Medline) internet site.

List of publications

Kulkarni N. and Gadre R.V. (1999): A novel alkaline, thermostable, protease free lipase from *Pseudomonas* sp. *Biotechnol Lett.*, 21: 897-899

Kulkarni N. and Gadre R.V. (1998): Simple gas chromatography method for lipase assay. *Biotechnol Tech*, 12: 627-628.

Kulkarni N. and Gadre R.V. (2002) Application of factorial design for optimization of alkaline lipase production from *Pseudomonas fluorescence* NS2W. *J. Ind. Microbiol Biotechnol.* (Manuscript received for corrections)

Kulkarni N. and Gadre R.V. (2002) Separation of alkaline thermophilic lipase from fermentation broth of *Pseudomonas fluorescence* by foam fractionation: optimization of physiochemical parameters. (Under preparation for *Biotechnol. Bioeng.*)

Kulkarni N. and Gadre R.V. (2002) Purification and characterization of alkaline thermophilic lipase from *Pseudomonas fluorescence* NS2W. (Under preparation for *Biotech. Lett.*)