KINETICS OF OLIVE OIL HYDROLYSIS BY FREE AND IMMOBILIZED *CANDIDA RUGOSA* LIPASE

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ABSTRACT

KINETICS OF OLIVE OIL HYDROLYSIS BY FREE AND IMMOBILIZED *CANDIDA RUGOSA* LIPASE

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Lipase catalyzed hydrolysis of olive oil has been studied in oil-in-water emulsion. The fraction of pure lipase in the commercial enzyme preparation was 1.1 %. The molecular weight of monomeric enzyme was determined as 56 000 Daltons. The amount of fatty acids produced was linearly proportional to an enzyme concentration up to 11 µg enzyme/ml. The specific enzyme activity was 2037 units/mg enzyme, at 37°C in 25 mM phosphate buffer, pH 7.0 at 25 % (v/v) olive oil concentration. The hydrolytic reaction obeys Michaelis-Menten kinetics with $V_{max,app}$ and $K_{m,app}$ of 3472 units/mg enzyme and 16.7 %, v/v (0.457 M), respectively. Maximum activity was obtained at pH 7.0, at low buffer ionic strength, 25 mM and 40°C. The enzyme was found to be inhibited by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and o-phthaldialdehyde in a biphasic manner.

Candida rugosa lipase was immobilized by two techniques. Entrapment in polyacrylamide gel and adsorption onto celite changed the optimum pH and optimum temperature in the same manner being 6.5 and 45°C, respectively. Entrapment in polyacrylamide gel resulted in large losses of lipase activity. Immobilized lipase exhibited higher productivity toward tributyrin compared to olive oil whereas free enzyme gave higher rate when olive oil was the substrate under the same experimental conditions.

The enzyme loading used for adsorption on celite was high, being 20 mg protein/gr celite. Adsorption of lipase in a side-on fashion is calculated to give a monolayer at approximately 0.7 m^2/gr celite which is less than the surface area of celite. The specific activity of celite immobilized lipase preparation was lower than that of free enzyme (η = 0.90). Under optimum experimental conditions 90 % hydrolysis was reached at 12 hours reaction time. The activity retained was found to be low during repetitive use. Lipase adsorbed on celite gave higher activity compared to polyacrylamide gel entrapped enzyme.

Keywords: olive oil, Candida rugosa lipase, hydrolysis, immobilization

ÖZET

SERBEST VE TUTUKLANMIŞ CANDIDA RUGOSA LİPAZI İLE ZEYTİNYAĞI HİDROLİZİNİN KİNETİĞİ

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Lipazla katalizlenen zeytinyağı hidrolizi yağ-su emülsiyonunda çalışıldı. Ticari enzim preparatında saf lipaz %1.1 miktarında bulundu. Monomerik enzimin molekül ağırlığı 56 000 Dalton olarak belirlendi. Oluşan yağ asitlerinin miktarı 11 µg / ml enzim derişimine kadar lineerdi. Özgül enzim aktivitesi %25 (h/h) zeytinyağı derişiminde 25 mM fosfat tamponu, pH 7.0 içerisinde ve 37 °C de 2037 ünite / mg enzim idi. Hidrolitik reaksiyon 3472 ünite / mg enzim olarak belirlenen maksimum hız ve %16.7 (h/h) K_m değerleri ile Michaelis-Menten kinetiğine uyum gösterdi. Maksimum aktivite pH 7.0, iyonik gücü düşük tampon, 25 mM ve 40 °C' de elde edildi. Enzim 1-etil -3 - (3-dimetilaminopropil) karbodiimid ve o-fitaldialdehid tarafından iki aşamalı inhibe oldu.

Candida rugosa lipazı iki yöntemle tutuklandı. Poliakrilamid jel içerisinde tutuklanan ve selit üzerine adsorbe edilen enzimin optimum pH' sı ve optimum sıcaklığı benzer şekilde etkilenip, sırası ile 6.5, 45 °C olarak değişti. Poliakrilamid jel içerisine tutuklanan enzim aktivite kaybına uğradı. Tutuklanmış enzim zeytinyağına kıyasla tribütirin ile daha yüksek aktivite verirken, aynı şartlarda serbest enzimle zeytinyağı daha yüksek tepkime hızı verdi.

Selit üzerine adsorpsiyon ile yapılan yöntemde enzim miktarı yüksek tutuldu (20 mg protein / gr selit) . Lipaz moleküllerinin yan yana tekli tabaka oluşturarak

adsorbe olmaları durumunda 1 gr selit üzerinde 0.7 m^2 yüzey kaplayacakları ve bu yüzeyin selit yüzey alanından az olduğu hesaplandı. Selit ile tutuklanmış enzim preparatının özgül aktivitesi serbest enzim aktivitesinden düşüktü ($\eta = 0.90$). Optimum şartlarda 12 saatlık sürede %90 hidroliz sağlandı. Tekrarlamalı kullanımda kalan aktivitenin düşük olduğu bulundu. Selit üzerine adsorplanan lipaz, poliakrilamid ile tutuklanan enzimden daha yüksek aktivite verdi.

Anahtar kelimeler : zeytinyağı, Candida rugosa lipazı, hidroliz, tutuklama

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ABBREVIATIONS

AOT	Sodium bis-(2-ethylhexyl)sulfosuccinate
APS	Ammonium persulfate
CHBP	4-Cyano-4'-heptylbiphenyl
CMB	Chloromercuribenzoate
CNBr	Cyanogen bromide
DEAE	Diethylaminoethyl
DMP	2,3-Dimercaptopropan-1-ol
DTNB	5-5'-Dithiobis(2-nitrobenzoic acid)
EDAC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediamine-tetraacetic acid
ENT	Polyethylene glycol
ENTP	Polypropylene glycol
FA	Fatty acid
FFA	Free fatty acid
HLA	Hydroxylamine
HMPC	4-Hydroxy-3-methyl-2-(2-propynyl)-cyclopent-2-enone
KP	Potassium phosphate
MAG	Monoacylglycerol
MBBA	4-Methoxybenzilidine-4'-n-butylaniline
NBS	N-Bromosuccinimide
PAG	Polyacrylamide gel
PAGE	Polyacrylamide gel electrophoresis
PDA	Phythaldialdehyde
PEG	Polyethylene glycol
PNP	Paranitrophenyl
PVA	Poly(vinyl alcohol)
PVC	Poly(vinyl chloride)
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-Tetramethylethylenediamine
TRIS	2-Amino-2(hydroxymethyl)-1,3-propandiol
Ve	Elution volume
Vo	Void volume

CHAPTER 1
INTRODUCTION

Lipase (EC 3.1.1.3) is defined to be an enzyme hydrolyzing glycerol esters of long chain fatty acids (water insoluble), and is different from esterase (EC 3.1.1.3), which hydrolyzes esters of short chain fatty acids (water soluble) [1]. This property is the basis of an old definition of lipases as "long-chain fatty acid ester hydrolases" or "esterases capable of hydrolyzing esters of oleic acid" [2].

Lipases are widely distributed in animals, plants and microorganisms, and have wide range of properties depending on their sources with respect to positional specificity, fatty acid specificity, thermostability, pH optimum etc. These suggest that one could probably find a suitable lipase from nature that would be suitable for a desired application.

Since microbial extracellular lipases are usually more thermostable than animal or plant lipases, they have received much attention for their potential use in industry and diagnostics [3].

1.1. Microbial Lipases

A large number of microbial lipases have been screened for application in medicines (digestive enzymes), food additives (flavor-modifying enzymes), clinical reagents (glyceride-hydrolyzing enzymes) and cleaners (detergent additives).

The usefulness of microbial lipases is due to their physiological and physical properties. In particular,

1) a large amount of purified lipase is usually available,
2) microbial lipases are generally more stable than animal or plant lipases, and
3) microbial lipases have unique characteristics compared with plant and animal lipases [4].

A number of lipase-producing microorganisms belonging to genera Aspergillus, Rhizopus, Mucor, Candida, Sclerotinia and some

other yeasts or molds have been reported. However, only a few species of bacteria were reported to produce lipase, which are Pseudomonas, Staphylococcus and others [5]. Some of the sources of microbial lipases are tabulated in Table 1.

Table 1. Sources of Microbial Lipases

Source	References
Bacterial	
Chromobacterium viscosum	6
Lactobacillus sp.	7
Micrococcus caseolyticus	8
Pseudomonas fluorescens	9-11
Pseudomonas fragi	12
Pseudomonas spp.	13
Streptococcus faecalis	14
Streptococcus thermophilus	15
Fungal	
Aspergillus niger	16-18
Candida curvata	19
Candida cylindracea	20-22
Candida deformans	19,23
Candida quillermondi	19
Fusarium oxysporum	24
Geotrichum candidum	25-27
Geotrichum cyclopium	16
Humicola lanuginosa	28-30
Mucor miehei	31,32
Penicillium camemberti	25
Penicillium crysogenum	33
Penicillium cyclopium	34
Phycomyces nitens	35
Rhizopus arrhizus	36,37
Rhizopus delemar	25,38,39
Rhizopus oligosporus	40
Saccharomycopsis lipolytica	8,41
Yarrowia lipolytica	19

Candida cylindracea, now named Candida rugosa, is a well-known lipase producer microorganism [19,20]. Lipase from Candida rugosa has been used in the studies of fat hydrolysis [36], interesterification [42] and resolution of chiral compounds [43]. For splitting of fats and oils this yeast lipase is the most recommended because of its random specificity [44] and somewhat inexpensive price [45].

1.2. Properties of Microbial Lipases

1.2.1. Amino Acid Composition

Adsorption of lipases to the lipid-water interface precedes catalysis. The hydrophobic nature of this interaction makes the amino acid composition of microbial lipases important for determining possible structure-function relationships.

For lipases in general, it has been suggested that a hydrophobic region plays an important role in catalysis and that these proteins may have a relatively high content of hydrophobic amino acid residues and lipid compared to other proteins [46,47]. In contrast, lipase B from Chromobacterium does not contain a high content of hydrophobic amino acid residues (Val,Ile,Leu,Tyr,Trp) and any lipid components or carbohydrate [48]. Thus, its affinity for interfaces must arise from the tertiary structure of the protein giving the molecule an amphipathic structure such as found with detergents. The same is true for the Pseudomonas lipase which has only amino acid constituents and a normal content of hydrophobic residues [49].

The results of amino acid analysis given in Table 2 indicate that the enzyme from Bacillus sp. and Geotrichum candidum contains only small amounts of sulfur-containing amino acids, such as cystein and methionine, and has no disulfide bonds [3,50].

Phycomyces lipase contained four residues of half-cystine in the form of disulfide, neither sugar nor lipid. The amino terminal residue was alanine [35]. Candida cylindracea lipase have two disulfide bonds and alanine occupied the amino-terminal position of this enzyme [51].

1.2.2. Nonprotein Moiety

Carbohydrate content of the crystalline lipase preparation from Geotrichum candidum was estimated to be 7.5% - 8.8% as xylose, arabinose, galactose and mannose [26,50]. Also in this enzyme preparation, very small amount of lipid was detected. However, it is questionable whether the lipid plays an essential role on the enzyme action as in the case of partially purified preparation of ricinus lipase [50]. Also the lipase A from Chromobacterium contained carbohydrate, but its possible role in catalysis has not been determined [48].

Phycomyces lipase contained neither sugar nor lipid [35]. Saccharomycopsis lipolytica which is known as a lipase-producing and hydrocarbon assimilating yeast produces two types of cell-bound

lipases containing 8% and 16% carbohydrate moiety [41]. The carbohydrate content of the lipase I and lipase II from Aspergillus niger was estimated as 6% and 9%, respectively [52].

Table 2. Amino Acid Composition of Microbial Lipases

Amino acid residue	Bacillus sp. [3]	P.nitens [35]	C.viscosum [48]		G.candidum [50]	C.rugosa [51]
			A	B		
Phenylalanine	4	10	21	6	28	26
Tyrosine	10	13	29	8	18	17
Leucine	22	16	96	26	40	41
Isoleucine	7	11	33	8	18	23
Methionine	1	2	17	1	0	12
Valine	18	21	87	23	24	24
Half-cystine	1	4	2	0	0	5
Alanine	26	17	120	31	34	43
Glycine	24	17	112	18	40	46-47
Proline	9	10	34	8	26	28
Glutamic acid	16	20	89	19	30	33
Serine	14	18	80	22	28	39
Threonine	22	17	105	28	22	31-32
Aspartic acid	22	36	110	26	54	58
Arginine	7	8	34	9	20	12
Histidine	6	5	27	7	10	4
Lysine	4	11	51	4	16	17
Tryptophan	2	2	3	3	8	5
Total	215	238	1050	247	416	464-466

Amino acid residues were expressed as residues/mole enzyme

1.2.3. The Molecular Weight

Lipases are generally acidic proteins with molecular masses ranging from 20,000 to 60,000 Da. However, there is a great variation in the relative molecular mass of lipases reported from different sources. The Pseudomonas lipase has a molecular weight of 33 kDa. However, the Chromobacterium lipase A has a molecular weight of 120 kDa and consists of two subunits with molecular weights of 80 and 50 kDa. Chromobacterium lipase B is a single subunit enzyme with a molecular weight of 26 kDa [4].

Penicillium roqueforti produces three lipases with relative molecular masses of 7930, 9100 and 11,420 Da. Candidum cylindracea lipase has a molecular mass of 120 kDa. The high molecular weight of

lipase may be due to self association of low-molecular weight protein molecules [53]. The electrophoretic pattern of C.cylindracea lipase showed that the enzyme has a single subunit with a molecular mass of 50 kDa [51] or 67 kDa [54]. Thus from the point of view of molecular weight, microbial lipases do not show common features. The molecular weights of some microbial lipases are summarized in Table 3.

Table 3. Molecular Weights of Lipases from Microbial Sources

Microorganism	Mwt (kDa)	Method	References
P. fluorescens	32.0	Gel filtration	55
Bacillus sp.	22.0	Gel filtration SDS-PAGE	3
A. japonicus	155.0	Gel filtration	53
G. candidum	53.0 61.6	Gel filtration SDS-PAGE	50
H. lanuginosa	39.0	Gel filtration SDS-PAGE	28
P. nitens	26.5	Gel filtration SDS-PAGE	35
C. deformans	207.0	PAGE	23
S. faecalis	20.9	Gel filtration	14
C. cylindracea	67.0	SDS-PAGE	54
	50.0	Gel filtration SDS-PAGE	51
Lipases of all specified sources are monomer except C.deformans in which homogeneity was not specified.			

1.2.4. The Isoelectric Point (pI)

Similar to molecular weight, the isoelectric point of microbial lipases differ between species. Isoelectric points of lipase from two microbial species, Bacillus sp. [3] and Geotrichum candidum [50], were found to be 5.1 and 4.33, respectively. Isoelectric pH of microbial lipases from various sources are given in Table 4.

Table 4. Isoelectric pH of Lipases from Various Sources

Source	pI	References
H.lanuginosa	6.6	28
P.nitens	5.9	35
G.candidum	4.4-4.8	26
S.faecalis	3.6	14
P.fluorescens	4.8	11
S.lipolytica	5.2 (Lipase I) 4.6 (Lipase II)	41
A.niger	4.0 (Lipase I) 3.5 (Lipase II)	52

1.2.5. Substrate Specificity

Lipases are able to act on esters of fatty acids in the aggregated state, for example emulsions, but they act very slowly on monomers of these esters in an aqueous system. This property provides a convenient criterion for differentiating lipases from ordinary esterases which act readily on substrate monomers.

Substrate specificity of a lipase acting in a heterogenous system may depend on both the chemical structure and physical state of substrate molecules. For example, substrate specificity of Chromobacterium lipase B changed from unsaturated or medium-chain saturated fatty acids (C8-C12) to long-chain fatty acids as the reaction temperature increased [56]. Iwai et.al was also found that the rate of enzymatic reaction of the lipase from P.cyclopium depended on the phase of the substrate at the temperature examined, i.e, whether the substrate was in a solid or liquid state [28].

1.2.5.1. Specificity on Synthetic Triglycerides

Microbial lipases show unique specificity towards the triglycerides. F.oxysporum lipase exhibited specificity toward saturated fatty acids, irrespective of length of the carbon chain [24]. The lipase from Streptococcus thermophilus which is utilized as a starter organism in a number of cheeses and yogurt was relatively more active toward tributyrin than toward natural lipids [15].

A high preference for oleic acid over saturated fatty acids was found for lipases from several strains of the Geotrichum candidum but is not a property intrinsic to all Geotrichum candidum lipases [26]. The chain specificity of Candida cylindracea lipase is in the order of oleic > lauric > palmitic > myristic > stearic [57]. Also, A.japonicus lipase is specific for fatty acids of chain length C18 and an unsaturation in the cis-9 position. This preference may be due to the orientation of the carboxy group and olefinic group, which is best in the cis-9 position [53].

Specificity of some microbial lipases towards synthetic triglycerides are summarized in Table 5.

Table 5. Substrate Specificity Towards Triglycerides

Source	Triglycerides (preference for)	References
C.viscosum	C4-FA	5
Bacillus sp.	C8-FA	3
G.candidum	Δ^9-C18-FA C8-FA C16-FA	16,26 58 16
H.lanuginosa	C12-FA	28
P.nitens	C8-FA	35
S.lipolytica	Δ^9-C18-FA	8
C.cylindracea	Δ^9-C18-FA	59
P.cyclopium	Δ^9-C18-FA C16-FA	 16

1.2.5.2. Specificity on Natural Lipids

Lipases from different sources also hydrolyze various natural lipids although the extent differed each other.

The lipase from Chromobacterium viscosum was more active on lard and butter than olive oil [5]. Coconut oil which contains a relatively high proportions of lauric acid was hydrolyzed most rapidly than palm oil, corn oil and olive oil by Humicola lanuginosa lipase [28].

Candida cylindracea lipase gave the highest degree of hydrolysis with edible lard which contains higher content of unsaturated fatty

acids (oleic and linoleic) than inedible beef tallow and inedible pork lard. This result indicates that Candida lipase exhibits some selectivity toward fatty acids with a cis double bond in position 9, although it is non-specific with respect to the fatty acid positions in the triacylglycerol [59].

1.2.6. Positional Specificity

The positional specificities of lipases were examined firstly by Okumura et al. using purified lipases from G.candidum, P.cyclopium, R.delemar and A.niger [16]. They showed that these enzymes can be classified into two groups from the viewpoint of positional specificity: lipases from G.candidum and P.cyclopium cleave all the ester bonds in triglyceride indiscriminately (nonspecific), while those from Candida deformans [23], A.niger and Humicola enzyme [28] hydrolyzed 1- and 3- positioned ester bonds in preferance to a 2-positioned ester bond (1,3- specific) [16]. However, other workers pointed out that the positional specificity of lipases could not be divided clearly into two, but rather continously changed from rigorously specific to nonspecific. Due to this classification, the lipases from Chromobacterium [60] and Pseudomonas [55] are rigorously nonspecific. These enzymes catalyze the hydrolysis of the three ester bonds of triglycerides at comparable rates. This specificity is very different from that exhibited by pancreatic lipase [61] and Bacillus sp.[3] but similar to that of the Candida cylindracea lipase [57].

1.2.7. Stereospecificity

The stereospecificity of lipases depends on the fatty acid chain length of the substrate. For some lipases, the stereospecificity even changed from one to the other enantiomer of the substrate [62]. The rate of hydrolysis by lipase increased almost linearly with chain length from acetyl to lauryl and decreased with the oleyl moiety [25]. The rate of enantioselective hydrolysis by Arthrobacter lipase increased with chain length from acetate to caprylate but decreased with caprate [63]. Lipases from microbial sources preferentially hydrolyzed the l-form of the ester, but as the concentration of the d-form increased, this enantiomer began to split [25]. The interesterification reaction was found to be considerably more enantioselective than either the corresponding hydrolysis reaction or the equivalent esterification

reaction [31]. The stereospecificity of lipases may also depend on the surface pressure of the substrate [64,65]. An increase of the lipid density at the air/water interface decreased the stereospecificity of several lipases. Long-term continuous production of optically active 2-(4-chloro-phenoxy)propanoic acid, the (R)-isomer of which is an important herbicide, by selective esterification with n-tetradecanol was achieved in organic solvent by Candida cylindracea lipase. The stereospecificity of the continuous reaction toward (R)-2-(4-chlorophenoxy)propanoic acid was as high as or higher than that of the batch reaction [66].

1.2.8. Active Site

Determination of the three-dimensional structures of different lipases has confirmed their classification as "serine hydrolases". Their active site is composed of three residues : a serine residue hydrogen-bonded to a histidine residue, and a carboxylate-residue hydrogen bonded to this same histidine residue. The carboxylate may be either an aspartate or a glutamate residue.

The enzyme nucleophile, which may be a serine hydroxyl group [67], will attack the carbonyl carbon atom of the ester bond [68] leads to the formation of a tetrahedral intermediate. The histidine assists in increasing the nucleophilicity of the serine hydroxyl group. The histidine imidizole ring becomes protonated and positively charged. The positive charge is stabilized by the negative charge of the acid residue. The tetrahedral intermediate is stabilized by two hydrogen bonds formed with amide bonds of residues which belong to the oxyanion hole. Finally the alcohol is liberated leaving behind the acyl-enzyme complex. By nucleophilic attack of a hydroxyl ion, the fatty acid is liberated and the enzyme regenerated (Fig.1).

An unusual and interesting feature of the structure of lipases is that the active site is completely buried under a lid-like structure composed of one or two α-helices.

Therefore, triacylglycerol lipases undergo a conformational change in response to adsorption at the oil-water interface. The lid moves, thereby allowing the active site to become accessible for the substrate. Another result of this conformational change is a significant increase in the hydrophobic surface of the enzyme, thus enhancing hydrophobic interactions between the enzyme and the lipid surface.

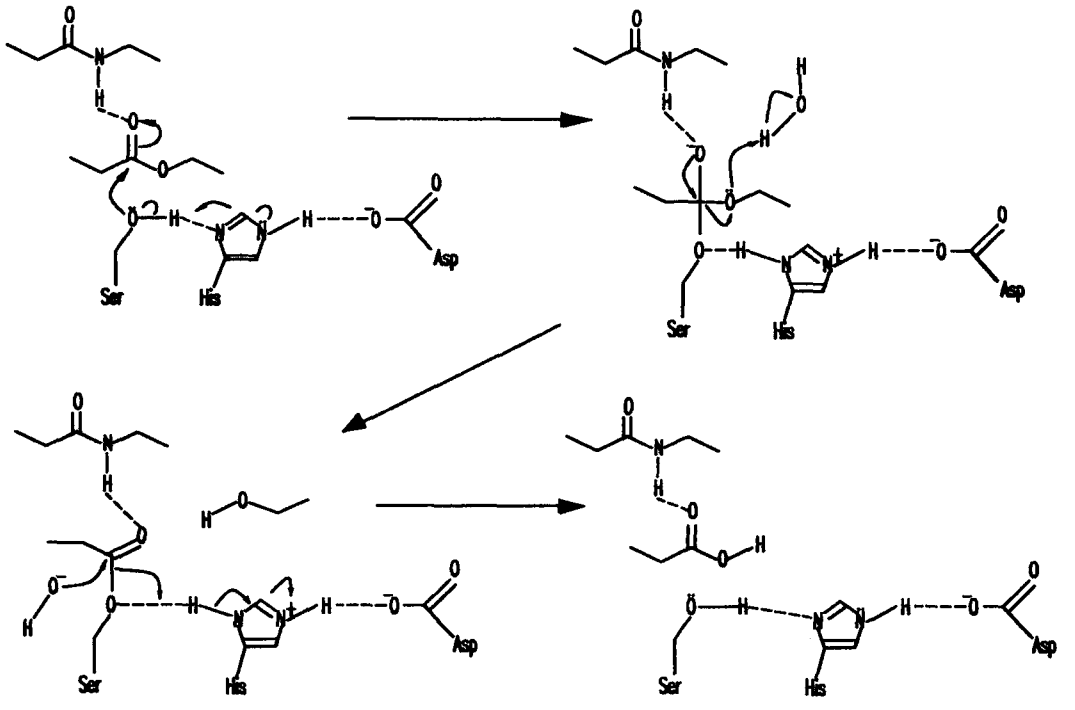

Figure 1. Reaction Mechanism of a Lipase Catalyzed Hydrolysis Reaction [67]

At present, X-ray structures of lipases from fungal sources namely Rhizomucor miehei, Geotrichum candidum, Candida rugosa have been elucidated. The fact that the structures of the lipases from Candida rugosa and Geotrichum candidum are very similar since these two lipases share a high level of sequence homology [67,69]. Geotrichum candidum lipase has a peptide segment of -Ile-Phe-Gly-Glu-Ser-Ala-Gly-Ala- which coincides with the common sequence, -Gly-X-Ser-X-Gly-, for other lipases. It was believed that this segment containing serine forms part of the interfacial lipid binding site [1].

Lipase of Candida cylindracea when subjected to chemical modification with phenylglyoxal lost its activity. When aspartic acid and/or glutamic acid residues were modified with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, the activity of enzyme decreased significantly. These results suggested that arginine residues and carboxyl groups might be involved in catalytic site or substrate binding site of this enzyme [51].

1.2.9. Isoenzymes

Five isoenzymes (pI 4.40, 4.47, 4.58, 4.67 and 4.72) were detected by isoelectric focusing after the purification of commercial G.candidum by hydrophobic interaction chromatography on octyl sepharose [27].

Two lipase isoenzymes having somewhat different substrate specificities were isolated and purified from Aspergillus niger by a combination of ion-exchange chromatography, gel filtration and hydrophobic chromatography methods [52].

Two types of cell-bound lipases were purified from S.lipolytica with molecular weights of 39,000 and 40,000. There were little difference between lipase I and II concerning enzymological properties [41].

1.2.10. Optimum Temperature and Heat Stability

Lipases are known to be active over a wide range of temperature. Candida curvata lipase is thermoresistant and loses only 20% of its activity when heated at 50°C for 30 min. A.japonicus lipase was also stable over a wide range of temperatures and lost 50% of its activity at 40°C for 10 min [53].

The heat stability of enzymes are effected by at least two fundamentally different factors alone or in combination. The first one is the primary structure of the enzyme. A high content of hydrophobic amino acids in the enzyme molecule provides a compact structure which is not denatured easily by a change in the external environment. In addition, disulfide bridges and other bonds provide a high resistance both to heat inactivation and chemical denaturation. Secondly, specific components such as polysaccharides and divalent cations, if any, can stabilize the molecule [11].

The optimum temperature and thermal stability of some microbial lipases are summarized in Table 6.

1.2.11. Optimum pH

Almost all microbial lipases can be regarded as acid lipases or neutral lipases when they are classified by their optimum pH values. Most bacterial lipases exhibit maximum stability in the neutral pH range. Pseudomonas lipase is stable at pH 5.0-10.5, Chromobacterium lipase A at pH 5.0-8.0, and Chromobacterium lipase B at pH 4.0-11.0.

Table 6. Optimum Temperature and Thermal Stability of Microbial Lipases

Source	Optimum Temp.,°C	Stability,°C	References
P.fluorescens 27	45	n.d.	10
P.nitens	40	40°C (10 min)	35
Bacillus sp.	60	65°C (30 min)	3
C.deformans	40-50	n.d.	23
G.candidum	40	55°C (15 min)	50
S.thermophilus	45	45°C (1 week)	15
H.lanuginosa	45-55	65°C (60 min)	28-30
C.viscosum	65	60°C (10 min)	5
S.faecalis	40	40°C (10 min)	14
C.rugosa	30-35	n.d.	70

n.d. not determined

The pH optima of these enzymes are also generally in the neutral range. For example, Pseudomonas lipase is optimally active at pH 7.0, Chromobacterium lipase A and B at pH 6.5-7.0, and Streptococcus lipase at pH 6.0-8.0. However, for the Bacillus lipase the optimum is at pH 3.8. Because of the adaptable nature of the bacteria, the pH dependencies of activity and stability may depend on culture conditions. If the organisms are grown at alkaline pH, the lipase produced may have an alkaline pH optimum [4]. Recently, the lipase from Achromobacter sp. was reported to have an optimum pH at 10.0. No alkaline lipase producers were found in fungi. Although there were some weak alkaline lipase producers in actinomycetes, the strong producers were all bacteria [71,72]. Most fungal lipases also show the highest activity at the neutral pH range. The pH optima of some microbial lipases are given in Table 7.

1.2.12. Catalytic Parameters

Lipases are enzymes that preferentially adsorb at hydrophobic interfaces. Depending on the quality of the interface, the enzyme finds its optimal active conformation to hydrolyze water-insoluble ester

Table 7. pH Optimum of Microbial Lipases

Source	Substrate	Optimum pH	References
C.cylindracea	Olive oil	5.0-7.0	
	Tributyrin	7.0	45
P.fluorescens	Sesame oil	7.0	55
Bacillus sp.	Olive oil	5.5-7.2	3
G.candidum			
Lipase I	Olive oil	8.0	
Lipase II	Olive oil	6.0	58
H.lanuginosa	Olive oil	7.0	28,29
P.nitens	Olive oil	6.0	35
C.rugosa	Olive oil	7.1	70
S.thermophilus	Tributyrin	9.0	15
S.faecalis	Butter oil	7.5	14
Pseudomonas sp.	Corn oil	8.5	73

compounds.

Lipase catalyzed reactions are often examined in emulsion systems. Benzonona and Desnuelle [74] have pointed out the importance of interfacial area rather than the bulk concentration of substrate, and have treated the lipase reaction by a Michaelis-Menten type equation. Resultantly, Michaelis-Menten constant were expressed as interfacial area per volume. The apparent Michaelis constant and the tributyrin emulsion interfacial area covered by Candida rugosa lipase estimated from adsorption were reported as 0.78 cm^2/cm^3 and 2.05×10^{11} $cm^2/mole$, respectively [75].

Most of the kinetic studies involving triglyceride hydrolysis by lipase were conducted in the presence of emulsifier [13,27], in biphasic organic-aqueous system [76] and in reversed micelle [70,77]. Lipase activity was influenced by the presence of emulsifiers and stabilizers [13].

The kinetic parameters for hydrolysis reaction catalyzed by some microbial lipases are tabulated in Table 8.

Table 8. Kinetic Parameters for Hydrolysis Reactions

Source	Substrate	Km	Vmax	Ref.
H.lanuginosa	Trilaurin	14.2 mg/ml	----	28
A.japonicus	Olive oil	1.53 % (v/v)	27.6 units[a]/mg prot.	53
C.rugosa	Olive oil	0.717 M	67.1 mole/min.mg E	77
	Trilaurin	7.8×10^{-3} M	2.2×10^{-4} M min^{-1}	42
	Olive oil	3.7 mg/ml	-----	76
	Olive oil	1 % (v/v)	1020 units[a]/mg prot.	70

[a]One unit of enzyme was the amount of lipase that liberated 1 mole fatty acids/min under the assay conditions.

1.3. Inhibitors and Activators

Various metal ions and reagents were screened for their influence on lipase activity. It has been found that heavy metal ions inhibit the lipase activity while most alkali metal ions enhance the activity.

In general, calcium ion stimulates the lipase-catalyzed lipolysis rate of olive oil (but not tributyrin). The following mechanisms has been proposed for this activation [45].

1) by activating the lipase through enzyme conformation change such as pancreatic lipase,
2) by enhancing the adsorption of lipase to the interface of oil and water, and
3) by removing the fatty acids from oil-water interface so that lipase can act on other oil molecules.

Calcium does not affect the tributyrin hydrolysis since butyric acid is readily soluble in water even in the absence of calcium [45]. Some of the inhibitors and activators are given in Table 9.

1.4. Chemical Modification

In general enzymes are soluble and active in aqueous solution and not in organic solvents due to denaturation. Therefore lipase from various sources has been modified to make them soluble in organic solvents as well as in their hydrophobic substrates.

Table 9. Inhibitors and Activators of Microbial Lipases

Source	Inhibitors	Activators	References
H.lanuginosa	Co^{2+},Ni^{2+},Cu^{2+},Sn^{2+},Hg^{2+}	Li^{+},K^{+},Ca^{2+}	28
P.nitens	Ag^{+},Hg^{2+},Pb^{2+},Cu^{2+},$KMnO_4$, NBS	Mammalian bile	35
C.deformans	Cu^{2+},Zn^{2+},pCMB,EDTA	Ca^{2+},Mg^{2+},Co^{2+}	23
A.niger	Ag^{+}, iodoacetamide	------	52
C.rugosa	Hg^{2+},Fe^{2+},Ca^{2+},Mg^{2+},Cu^{2+}, Co^{2+}	------	70,78
Bacillus sp.	Cu^{2+},Zn^{2+},Hg^{2+}	------	3
P.fluorescens	I_2,Fe^{3+},NBS	Ba^{2+},Ca^{2+}	55
C.viscosum	Cu^{2+},Hg^{2+},Sn^{2+}	Ca^{2+},Mg^{2+},Mn^{2+}, Bile salts	5
B.bassiana	Fe^{2+},Cu^{2+},Tween-80	Mg^{2+}	79

A lipase from Pseudomonas fluorescens was modified with polyethylene glycol (2,4-bis(o-methoxypolyethylene glycol)-6-chloro-s-triazine) [80,81].This modification made lipase suitable for ester exchange reactions which is not possible without modification. The modified enzyme catalyzed these reactions not only in organic solvents, but also in straight hydrophobic substrates [80]. Also modified enzyme was extremely stable at elevated temperature. The heat stable nature as well as the solubility in straight hydrophobic substrates make the modified lipase extremely useful for many practical applications. Candida cylindracea lipase was also modified with polyethylene glycol so it could be dissolved in benzene and catalyzed the interesterification of phospholipids [82-84]. The PEG-lipases were very soluble in aqueous solution (about 40-50 mg in 5 ml solution) and organic solvents (30-40 mg in 5 ml solvent) [84].

Generally, increasing the hydrophobicity of modifier used increased the esterification activity. However, a lower activity was observed when PEG-5000 instead of PEG-1900 was used [84]. The mechanism of the activity enhancement is not clear but it appears that following modification, the microenvironment at or near the active site was altered which facilitated the interaction of the substrate with

the enzyme and the release of the product.

PEG-modified C.cylindracea lipase was 10 times more stable in water than native lipase, but less stable in benzene. The selectivity of the modified lipase was also altered to favor reaction with oleic acid versus stearic acid [83].

1.5. Lipase Reactions

The microbial lipases have been used as biocatalysts for a variety of reactions. Unlike other hydrophilic enzymes, lipases from different origins are uniquely stable in nonpolar organic solvents and can accept a broad range of substrates of varying size and stereochemical complexities. Their flexible protein backbones, which assume a variety of conformations, give them the ability to carry out many profitable reactions such as hydrolysis, ester synthesis, interesterification, transesterification, amidation, biosurfactant production and resolution of racemic mixtures to produce optically active compounds by manipulating the reaction condition in apolar media [37,76].

1.5.1. Hydrolysis of Esters

The present industrial process for fats and oils hydrolysis, Colgate-Emery process, requires a pressure of about 700 psi and temperature of 240-260°C or higher. For the sake of energy conservation and minimizing thermal degradation of the products, researchers have set out to study the hydrolysis of fats and oils catalyzed by the lipase enzyme [77].

The effects of temperature, pH, surfactants, chemical additives, and shear force on the hydrolysis reaction have been investigated in various substrates and lipases. Linfield et al.[85] were used C.rugosa lipase to evaluate the effects of temperature, pH and concentration of lipase on the lipolysis rate of olive oil, coconut oil and tallow in an aqueous system. Among these substrates, olive oil is the best one under the conditions investigated. The differences in hydrolysis rate may depend on the chemical structure of these oils. Although the C.rugosa lipase is considered to be nonspecific, it favors the hydrolysis of unsaturated lipids. No significant increase in the olive oil hydrolysis rate was found in the temperature range 26-46 °C. Some additives such as calcium or sodium ions and albumin had no

significant beneficial effect on the reaction. In addition hydrocarbon solvents and nonionic surfactants showed an adverse effect [76,85].

Researchers have found some problems in performing lipolysis in aqueous media. First, the hydrolysis rates are low due to limitation of interfacial area. This is more evident for materials such as beef tallow or palm oil, which are in a solid form at normal temperatures. Therefore, in order to increase the interfacial area of the oil in water emulsion, emulsifiers have been added [76]. However, if lipases were applied in industry, an emulsifier should be avoided because it would increase the cost of product separation [45]. Additionally, other systems using a lipase-entrapped membrane, chemical-modified lipases, lipase-filled reversed micelles, and immobilized lipases with or without organic solvents were also suggested [51,70,78].

Chemical modification of amino acid residues improves the catalytic activities of enzymes besides it allows to investigate the active sites of lipases. At least one arginine residue and one carboxyl group was considered to be present in the active site of C.rugosa lipase in which lost all catalytic activity, that is hydrolysis, esterification and/or ester exchange, upon modification of these residues. However, the modification of lysine residues and disulfide bonds was effective on the esterification activity without affecting the hydrolysis rate [51].

A relatively new approach is the reversed micelle system that protects lipase from the nonpolar environment by a layer of water and surfactant cells [78,86].

Recently, biphasic aqueous-organic systems have been suggested to accelerate the enzymatic hydrolysis of oils and fats with the addition of suitable amounts of water-immiscible solvents. Biphasic systems seem very attractive, where there is no surfactant contamination as in reversed micelles and diffusional limitation as in immobilized or membrane reactors [76,77]. Of course, some particular problems especially the deactivation of the enzyme in the presence of organic solvents and effect of water content on oil hydrolysis rate may lead to some limitations for these systems.

1.5.1.1. Effect of Organic Solvents on Oil Hydrolysis Rate

For the practical application, the solvent should be dealt with cautiously because of the safety problem, and loss of solvent caused

by the evaporation should be minimized. Also, the cost for separating the solvent from the product should be considered [87].

Immiscible organic solvents facilitate the lipase-catalyzed hydrolysis of lipid, because it serves as a reservoir for the substrate and product. Amoung the various kinds of solvent tested, it has been found that isooctane is the most suitable organic solvent for olive oil splitting by Candida rugosa lipase in biphasic organic-aqueous system and reversed micelle system [39,76]. These results are consistent with those of Virto and co-workers [59] who showed that 90% of hydrolysis of animal fats were obtained only in the presence of isooctane and n-heptane. These solvents were also most effective for the hydrolysis of beef tallow by H.lanuginosa lipase. A solvent-buffer mixture (1:1 (v/v)) accelerated the hydrolysis rate compared to the sole buffer [30]. Isooctane was also found to be most effective organic solvent in the hydrolysis of olive oil by C.rugosa lipase in reversed micelles. The water miscible organic solvents such as ethanol and methanol are not suitable for the formation of reversed micelles [70,78].

Although polar solvents enhance the hydrolysis, at high concentrations they remove essential water of hydration from the enzyme leading to deactivation [12].

1.5.1.2. Effect of Water Content on Oil Hydrolysis Rate

Water plays several roles in enzyme structure and function [88]:

a) action on enzyme structure by contribution to all non-covalent bonding,
b) alteration of protein structure by disruption of hydrogen bonds,
c) facilitation of reagent diffusion and
d) participation in the equilibrium constant (water can be substrate or product).

Wang et al. has been studied the effect of the volume ratio of water to oil on the rate of lipolysis. The hydrolysis rate reached a maximum at a water to oil volume ratio of 1.0. The reaction rate decreased thereafter presumably due to the decreases of the interfacial area and the amount of water available for the oil hydrolysis reaction [45].

The optimum water content for hydrolysis of olive oil in a PVA-emulsion system by H.lanuginosa lipase was found to be 60%. However, esterification by this lipase was adversely affected by the increased

water content and maximum rate was attained at a water content of 7% [30].

In a reversed micelle system, the water content also determines the hydrolysis rates. Han and Rhee [70] were used R values (molar ratio of water to surfactant (AOT)) to express water content in the reversed micelles. At low R values, the reaction proceeded very slowly indicating that a large fraction of enzyme molecules were directly exposed to the organic solvent and denatured. The reaction rate was reached to optimum level at R=10.5 due to an increase in interfacial area as water content increases. However a sharp decrease on reaction velocity on further increase of R seemed to be due to decreased solubility of the lipid in the water pools.

1.5.2. Synthesis of Esters

The hydrolysis of fats and oils (triacylglycerols) is an equilibrium reaction and therefore it is possible to change the direction of the reaction to ester synthesis by modifying the reaction conditions (Fig.2).

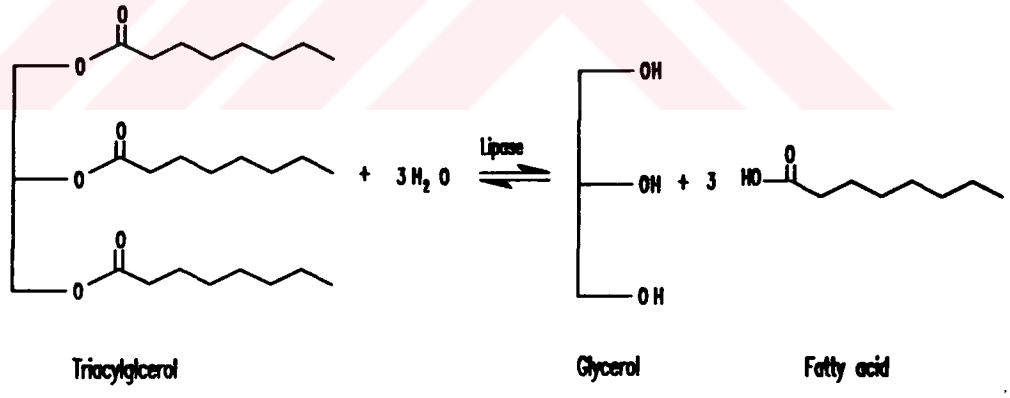

Figure 2. Enzymatic Reaction of a Lipase Catalyzing Hydrolysis or Synthesis of a Triacylglycerol Substrate

The equilibrium between forward and reverse reactions is controlled by the water content of the reaction mixture, so that in a non-aqueous environment lipases catalyze ester synthesis reactions [30,67]. An addition of molecular sieves to the reaction system increases the degree of esterification by the removal of water from the

system [89].

Different types of synthesis reactions can be distinquished: common ester synthesis from glycerol and fatty acids and biotechnologically more important transesterification reactions in which the acyl donor is an ester [67].

A number of fatty acid esters that are important components of natural aroma used in the food industry were produced by the direct esterification catalyzed by lipases. Acetic acid esters were more difficult to obtain due to catalyst inactivation. This inactivation was the result of acetic acid adsorption on the hydrophilic lipase preparation [90].

Esterification catalyzed by various microbial lipases always resulted in mixtures of glycerides, with yield and composition of the mixture depending on the source of lipase [91,92]. C.viscosum was one of the 1,3 selective lipases producing desired glycerides with high yield [93]. Also lipase from R.delemar catalyzed the synthesis of glycerides in reverse micelles in which the 1-monoglycerides and 1,3-diglycerides were the essential products. This is due to 1,3-positional specificity of R.delemar lipase. Temperature and concentrations of substrates and products (including water) were determined the percent conversion. In the same system, lipase from C.cylindracea was not catalyze glyceride formation. The reason may be glycerol-enhanced deactivation or unfavorable localization within the micellar microstructure [38].

1.5.3. Transesterification

Ester synthesis catalyzed by lipases is also observed by transesterification reactions, for instance, when the acyl donor is an ester (instead of an acid like in the esterification reaction). The main advantages of this reaction compared to direct synthesis are as follows [90];

a) no water molecules are formed or consumed in the reaction,

b) the enzyme stability is usually high under these conditions.

Although, most lipase preparations were more active in such systems than in direct synthesis, the yield of reaction was limited by reaction equilibrium and ester hydrolysis. Therefore an excess of the ester donor was used to shift the equilibrium [90].

Transesterifications involving fats and oils can be specified depending on the type of acyl acceptor. Glycerolysis and alcoholysis refer to the transfer of an acyl group from a triglyceride to either an alcohol or a glycerol as shown in Figure 3.

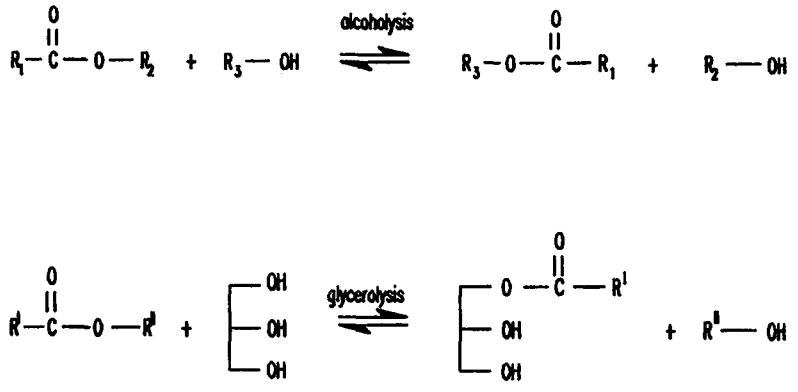

Figure 3. Alcoholysis and Glycerolysis Reactions [67]

1.5.3.1. Glycerolysis

Chemical glycerolysis of fats and oils is used for the commercial production of monoglycerides, which are applied as emulsifiers in a wide range of foods, cosmetic and pharmaceutical products [67]. Typical industrial production of these surfactants is achieved by alkaline catalysis above 200°C in the presence of glycerol. Glycerolysis under these conditions can yield up to 60% MAG but may also yield reaction by-products of undesirable colors and flavors [94]. However, yields of MAG from edible fats and oils have approached 90% when glycerolysis was mediated by lipases of various sources in microaqueous media [95].

R.delemar lipase was catalyzed the glycerol-fatty acid esterification in reverse micellar media with the major product being 1-monoglyceride, a useful food emulsifier. 1,3-Diglycerides were also produced but at relatively low amounts. 1,3-Positional specific R.delemar lipase gave as high as 50-60% conversions while this conversion reached to 80-90% with positionally nonspecific C.viscosum lipase under the same reaction conditions.This difference was due to ability of latter strain to utilize 1.5 times as many free glycerol

hydroxyls [39].

Recently, high yields of monoacylglycerol were obtained from gel-entrapped Pseudomonas lipase mediated glycerolysis of butter oil by optimizing reaction parameters. Optimum conditions for glycerolysis of butter oil were 35-40°C, acyl group:glycerol molar ratio 0.30-0.70 and 4-6% water in the glycerol phase [96].

1.5.3.2. Alcoholysis

Using acyl acceptors other than glycerol, additional monoacylcompounds can be synthesized. For example, alcoholysis of sugar alcohols with various plant and animal oils has been shown to yield sugar monoesters of fatty acids. Among various lipases, the enzyme from C.viscosum showed good catalytic properties [6,83].

Although, chemical synthesis of sugar esters is far cheaper than enzyme technology, refinement of oils containing highly unsaturated fatty acids may lead to enzyme application on a commercial scale, because polyunsaturated fatty acids are easily subject to decomposition in the chemical process, yielding undesirable oxidation products and polymers. Since fish oils possess polyunsaturated fatty acids predominantly at the 2-position and this bond is relatively resistant to lipase attack, 1,3-specific lipases can be particularly useful in the concentration of polyunsaturated fatty acids in monoglycerides [67].

Porcine pancreatic and Chromobacterium lipase were catalyzed transesterification reactions between a number of sugar alcohols and various plant and animal oils in dry pyridine. A number of sorbital esters were produced in this way. The industrially used alternative procedure is very harsh and involves high temperatures and concentrated sulfuric acid as catalyst, consequently, sorbitol undergoes dehydration prior to acylation, and the resultant products are sorbitan monoesters [97]. Also enzymatically formed surfactants were more potent at a much lower concentration than their chemically produced counterparts [6].

On the other hand, C.cylindracea lipase was catalyzed the esterification reaction between fatty acids and alcohols in reversed micellar media. However, the rapid loss of enzyme activity occured due to factors that affect the microstructure of the media. The chain length and molecular structure of the alcohol affect micellar structure and hence enzyme activity. Polar alcohols such as methanol, propanol and

tert-butanol have no significant amphilicity and so they do not partition strongly to the interface but remain inside the water pools and/or the bulk organic solvent. Their presence in the aqueous pseudophase may deactivate the enzyme. The rate of reaction was strongly affected by the ratio of fatty acid/alcohol in the media. For all types of alcohol, the larger the value of this ratio, the larger the rate. When more alcohol was added to the media, it creates a more fluid interface and so displaces the fatty acid from the surfactant layer resulting in low concentration of fatty acid in the vicinity of lipase [38].

Sonnet and Moore have been evaluated esterification reactions of typical aliphatic alcohols with several acids and their esters using commercial lipase preparations. It has been found that nature of solvent can influence both stability and reactivity. Also reactivity of esters changed amoung lipases of different sources. In fact, the activated esters were more reactive with C.rugosa lipase, especially the acetates [98].

1.5.4. Interesterification

Fats are important components of a wide variety of foods. It is often desirable to alter the fatty acid composition of naturally occuring triglycerides to obtain fat with the required melting characteristics [81,99,100]. A number of processes are used industrially to modify and manipulate the composition of triglycerides. One of these processes is interesterification.

The interesterification reaction involves interchanging (exchanging) the fatty acids (also called acidolysis) or a fatty acid ester within and amoung the triacylglycerols of fats and oils (Fig.4).

Commercial interesterification is done by heating perfectly dry and purified oils or lipid mixtures free of fatty acids and peroxides with chemical catalyst such as sodium alkoxide. This catalyst is used to promote the uncontrolled migration of fatty acid groups between glyceride molecules [101]. Interesterification of fats and oils can also be achieved by using biocatalysts such as lipases. Enzymatic interesterification is of great interest since it has several advantages over the chemical method, including selective and positionally specific fatty acid exchange, milder reaction conditions and tolerance of water and free fatty acids [102,103].

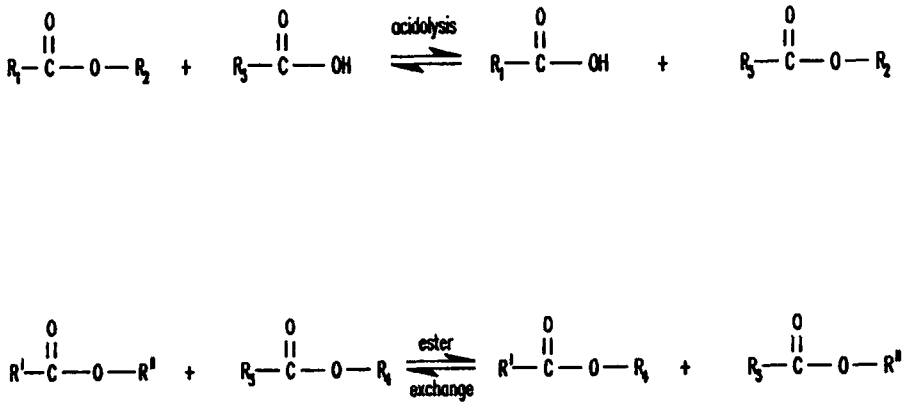

Figure 4. Interesterification Reactions [67]

Under normal physiological conditions, lipases hydrolyze triacylglycerols to fatty acids and glycerol, with di and mono acylglycerols being the intermediates. However, under appropriate conditions the reaction can be reversed so that ester synthesis, rather than hydrolysis becomes the dominant reaction [104,105].

Interesterifications require a small amount of water, in addition to the amount needed for the enzyme to maintain an active hydrated state. Therefore the use of biocatalysts in organic media has attracted much interest in recent years [106,107].

The role of water content and the effects of organic solvents on interesterification selectivity over hydrolysis have been studied using triacetin and tributyrin as model compounds. In the absence of an organic solvent, a buffer to enzyme weight ratio of 1 gave maximum selectivity to interesterification over hydrolysis [42,106]. To evaluate the effects of polar and nonpolar solvents, pyridine and hexane were choosen. Hexane enhanced interesterification and suppressed hydrolysis while pyridine at low concentrations shifted the reaction towards hydrolysis. In reversed micellar media, interesterification completely dominated hydrolysis at low values of molar ratio of water to surfactant (W_0). This result may be due to binding ability of surfactant to the water molecules, thus inhibiting hydrolysis. At higher W_0 values, hydrolysis predominated [106].

1.5.4.1. Acidolysis

Esterification of dilaurin and lauric acid was achieved by C.cylindracea lipase in cyclohexane. It has been shown that catalytic turnover number for esterification is three times larger than that for hydrolysis. The main reason for the slower rate of hydrolysis than esterification may be the low water content which is tightly bound to the enzyme and unavailable for hydrolysis and the existence of water-immiscible solvent, cyclohexane, that make the structure of enzyme more rigid. A more rigid structure could favor esterification over hydrolysis [42].

Structured triglycerides are also a potential product of lipase catalyzed interesterification reaction. These are glycerides containing medium chain fatty acids on the 1- and 3- positions and an essential fatty acid on the 2-position. They can form an alternative for the medium-chain triglycerides which are currently used to meet the nutritional needs of patients with malabsorption problems, because shortage of essential fatty acids in these patients can easily occur due to sole consumption of medium-chain lipids [108].

1.5.4.2. Ester-Exchange Reactions

From the viewpoint of food technology, it is sometimes necessary to reform of fat or oil, introduction of unsaturated fatty acid to triglycerides and change of melting temperature. This can be achieved by ester-exchange reaction within and among the triacylglycerols of fats and oils [9,85,99,109,110].

PEG-modified Pseudomonas fluorescence was catalyzed ester exchange reactions between olive oil and beef fat and lowered the melting point from 32-36°C to 19-23°C [81].

C.cylindracea lipase catalyzed interesterification within the butter fat was markedly changed the triglyceride composition of butter fat which may affect melting and crystallization characteristics [99].

A commercial scale application of interesterification is the transformation of low-value oils, like the palm-oil mid fraction, into high value cocoa-butter triglycerides. The main components of cocoa butter are 1,3-dipalmitoyl 2-oleine, 1-palmitoyl 2-oleoyl 3-stearine, and 1,3-distearoyl 2-oleine. A typical method for producing a cocoa butter like fat was accomplished by interesterification reaction between palm mid fraction (1,3-dipalmitoyl 2-oleine) and ethyl stearate by using 1,3

specific lipase of Rhizopus species. The composition of gained fats was similar to that of cocoa butter and had good properties for chocolate [109]. Cocoa butter like fat was also prepared from completely hydrogenated cottonseed and olive oils by enzymatic interesterification with 1,3 specific Mucor miehei lipase. The melting point of this product was estimated to be 39°C which is comparable to the 36°C melting point of natural cocoa butter [110].

Recently, many reports have been published in relation to the interesterification of fats and oils using microbial lipases. Some important processes were summarized in Table 10.

Table 10. Interesterification Reactions Catalyzed by Microbial Lipases

Process	Origin of Lipase	Reference
Incorporation of stearate ester into palm-mid fraction	Rhizopus sp.	109
Incorporation of myristic acid into palm-mid fraction	Aspergillus sp.	101
Incorporation of heptadecanoic acid into phosphatidylcholine	Mucor miehei Rhizopus arrhizus	82
Incorporation of palmitic acid into triolein	Rhizopus arrhizus	111
Incorporation of octanoic, decanoic or lauric acid into olive oil	Mucor miehei	32

1.6. Production of Microbial Lipases

The amount of lipase produced by each microbial strain is dependent on environmental factors, such as cultivation temperature, composition of the nitrogen, carbon and lipid sources, the concentrations of inorganic salts, and the availability of oxygen. In particular, culture conditions influence the properties of the enzymes produced as well as the ratio of exocellular to endocellular lipases [4,19,112].

In general, lipase production is stimulated by lipids such as butter, lard, olive oil and fatty acids. However, common optimum conditions for lipase production have not been identified [4,40].

The best results in growth and lipolytic activity were obtained when the P.fluorescens strain was incubated in a medium with tributyrin or olive oil as a carbon source while soybean and rapeseed oil have an inhibitory effect on the growth of bacterial cells [113].

On the other hand, olive oil was shown to induce lipase production by the fungus, Beauveria bassiana. However, the addition of fatty acids inhibited both growth and lipase production [79]. There are no common rules about what kinds of lipids are effective for producing microbial lipases.

Carbon and nitrogen as well as inorganic ions such as Na^{+}, PO_4^{3-}, and Ca^{2+} are also fundamental substances for the growth of bacteria itself. But their influence on lipase production is unknown. For example, maximum amount of enzyme was produced by Penicillium crysogenum when glucose is the sole carbon source, followed by maltose, mannitol, galactose, sucrose, lactose and fructose in decreasing order [33]. However, Iwai and Tsujisaka were found no significant differences in lipase production with different carbon sources by Rhizopus delemar [114].

The yeast, Candida rugosa, was produced extracellular lipase in the presence of lipid material, especially when a fat and steroid are present simultaneously [21]. Cholesterol was found to be most effective sterol in lipase induction [20]. The production of lipase by C.rugosa in a medium containing olive oil was observed in two stages in which the first stage was glycerol depletion without lipase formation and the second stage was fatty acid consumption with the lipase production [115]. Also it has been found that oxygen deficiency restricts lipase production by this microorganism, while a shortage of nitrogen source seems to have slight influence on lipase production when glucose and olive oil are used jointly as carbon sources [22]. Amoung the substrates tested, olive oil was found to be best carbon source for this strain [21,22]. The addition of urea and maltose have a pronounced effect on the production of C.rugosa lipase in a solid state fermentation using rice bran as a solid substrate [20].

1.7. Purification of Microbial Lipases

Microbial lipases are purified by a combination of ion exchange and gel filtration chromatographies in the same manner as for the purification of other proteins. However, if microbial lipases are

cultivated in a lipid-abundant medium [5,71] the first purification step must be extraction or precipitation with organic solvents such as ethanol, acetone or butanol [12]. Such treatments to dissociate bound lipids are very important to further purification.

A four step purification of Pseudomonas fluorescens lipase by the classical methods including $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography, Sephadex G-75 gel filtration and DEAE-cellulose rechromatography was resulted in 521 fold purification with a yield of 13.7% [55]. By a similar procedure, Geotrichum candidum and Phycomyces lipase were purified 40 fold and 1200 fold with a yield of 20% and 17% respectively [35,50].

1.8. Lipase Assay Systems

A number of assays to determine lipase activity have been developed and the majority of methods for determining lipase activity are based on the estimation of the rate of release of FFA from triglycerides [67,116].

1.8.1. Assay of Free Fatty Acids

The FFA liberated due to lipolytic reaction can be assayed titrimetrically. Although tributyrin [6,52] and triolein (or olive oil) [27,51] are widely used as substrates, triolein is the most common one for lipase assay due to its long chain fatty acids. Tributyrin can be dispersed in water simply by shaking, however, some emulsifying and/or stabilizing agents may be required with triolein [35,40,78]. The released FFA can either be titrated after termination of the reaction [3,55] or titrated automatically, the pH stat technique [11,26,57,83,117], against alkali to maintain a constant pH. The rate of addition of alkali was taken as a measure of activity in units of mequiv. $min^{-1}ml^{-1}$ of the enzyme solution.

Alternatively, FFA was determined by conversion to copper soaps and reaction with chromogenic reagent such as sodium diethyl dithiocarbamate [10].

Labelling of carbon in the fatty acids of triolein was another way used for lipase assay. Although the method is very sensitive, thin layer chromatography is required to separate the released radioactive FFA from the unreacted radioactive triglyceride [118,119].

Recently, the released FFA containing the chromophore could be

measured spectrophotometrically, when a substrate like acylglycerol esters of trinitrophenyl aminolauric acid was used in lipolysis reaction [116].

Thioester substrates like 1-S hexadecanoyl-1-mercapto 2,3 propanediol have been prepared for lipase assay and made possible to measure released SH groups spectrophotometrically by reacting them with 4,4'-dithiobispyridine to form a coloured complex [116].

1.8.2. Use of Chromogenic Phenolic Esters

Various synthetic esters that liberate a fluorescent or coloured phenolic residue on hydrolysis have been applied to the assay of microbial lipases.

Commercially available esters of ß-naphthol have been used to measure lipase activity. The ß-naphthol released by lipolysis was reacted with tetra-azotized orthodianisidine (Fast blue) to form a coloured complex and estimated with a spectrophotometer [120].

2,3-dimercaptopropane-1-ol tributyroate using DTNB as a chromogenic reagent is an alternative substrate for lipase assay [121].

Para-nitrophenyl esters of various chain-length fatty acids were also used as substrates. However, these compounds are not suitable for specific lipase assays because they are also cleaved by esterases [34].

1.8.3. Use of Fluorogenic Substrates

Fluorescent compounds have also been used for lipase assay. The oleic acid ester of the fluorescent phenol 4-methylumbelliferone (4-MU) was a suitable substrate for most microbial lipases. In the simplest form of the assay, an emulsion of 4-MU ester was added to a buffered solution containing lipase, and lipase activity was measured directly as a rate of increase of fluorescent intensity [122,123]. It is also possible to use triacylglycerols having one of the alkyl groups substituted with a fluorescent groups (e.g. pyrenyl) [124,125].

1.9. Industrial Application of Lipases

Microbial transformation performed by microorganisms or catalyzed by enzymes has given a high potential to prepare pharmaceuticals, fine chemicals, food additives and commodity chemicals. Mucor miehei lipase has been used for the preparation of esters of optically active secondary alcohol in high enantiomeric purity [31].

An efficient process was developed by using microbial lipases for the conversion of racemic HMPC to (S)-HMPC, which is an important alcohol for preparation of an insecticidally-active synthetic pyrethroid [63].

Another important application of lipase was for flavor development in food such as Italian cheeses [116]. Fungal lipases are also known to accelerate the ripening of cheddar cheese and to improve the flavor of processed blue cheeses [126].

Controlled lipolysis of milk fat with microbial lipase is important in the production of lipolyzed milk fat. Production conditions are set to allow controlled release of specific fatty acids, minimizing the release of those that could cause off flavors [36,127].

Lipases are already used in industry although the number of applications and therefore their importance to the enzyme manufacturing industry was rather small [128]. The reason for this low interest is most likely the limited availability and relatively high costs of these enzymes, especially for the potential larger applications such as the detergent industry.

However, as a result of recombinant DNA technology, it is possible to construct microbial strains which produce different types of lipases in an economically attractive way.

Remarkably, all commercial applications of lipases concern enzymes of fungal or yeast origin. This can be explained by the fact that the food applications, which until recently exceeded the number of non-food applications, prefer the use of lipases from fungal origin because of their proven GRAS-status (Generally Regarded As Safe).

Recently, many new potential applications of lipases have been presented including their use in the paper manufacturing industry, in the preparation of structured lipids and in organic chemical processing [128]. The glucoside esters of FA's synthesized under the catalytic activity of Candida antarctica B lipase exhibited surfactant properties and might represent an attractive alternative to existing products in areas ranging from cosmetics, to household detergents, to industrial de-greasing of metals, electronics and leather.

1.10. Immobilization of Lipases

Immobilization of lipase can offer several advantages including repeated usage of enzyme, improvement of enzyme stability, ease of

product separation, high purity and product yields, and continous operation in a packed-bed reactor [129,130].

In practical applications the repeated use of the enzyme is important for the process economy. Therefore, immobilization of the lipase is considered to be one possible way to facilitate enzyme recovery for reuse and to setup a continous process.

There are many factors which influence the performance of an immobilized enzyme preparation. Some of the most important factors are the choice of a carrier and the selection of an immobilization strategy [107].

1.10.1. Support Materials

The choice of a support for the lipase is dependent upon several characteristics which are important in industrial processing applications. These factors are mechanical strength, microbial resistance, thermal stability, chemical durability, chemical functionality, hydrophobic/hydrophilic character, ease of regeneration, loading capacity and cost [131]. However no single substance fulfills all these requirements in an optimal fashion.

Some of the support materials commonly employed are porous glass and derivatives [29], diatomaceous earth or kieselguhr [32,132,133], cellulose and derivatives [134], acrylic copolymers [134], sephadex [78], polypropylene and derivatives [17,18], alginate [29], etc. Synthetic support materials have been manufactured or obtained in a number of forms and shapes, including membranes, fibers, granules, and powders.

Some of the important support materials and related immobilization techniques are summarized in Table 11.

The lipase catalyzed oil hydrolysis is an interfacial reaction involving the adsorption of enzyme to the surface of oil droplet. For the immobilized lipase to express its activity, the emulsified oil droplets of submicron size have to reach the enzyme molecules within the porous support. Therefore, pore size of the support used was an important criteria for the coupling efficiency of lipase and the specific lipolytic activity of the immobilized lipase.

The effect of pore size on lipase immobilization was examined by using cross-linked Sepharose of three different pore size. The amount of lipase coupled to all three cross-linked Sepharose was same.

However, the lipolytic activity of the biocatalyst decreased as the pore size reduced suggesting a greater mass transfer resistance for the substrate in the smaller pores [130].

Wisdom et al. were compared three grades of diatomaceous earth (Celite 560, Filtercel and Hyflo supercel) for their suitability as support materials for lipase catalysing the interesterification of fats.

Table 11. Support Materials for Immobilization of Lipase

Source	Support	Immobilization technique	References
C.cylindracea	Acrylic	Adsorption	134
C.cylindracea	Alginate	Entrapment	135
A.niger	Polypropylene	Adsorption	17
A.niger	Polypropylene	Adsorption	18
M.miehei	MBBA, CHBP	Entrapment	136
C.cylindracea	Polypropylene	Adsorption	137,138
H.lanuginosa	ENTP,Amberlite	Entrapment	29
H.lanuginosa	OctylSepharose	Adsorption	29
M.miehei	Celite	Adsorption	32
C.cylindracea	Nylon 6	Cross-linking	139
C.cylindracea	Sephadex	Adsorption	78
C.cylindracea	Celite	Adsorption	66

Lipolytic activities which gave a measure of the amount of lipase immobilized, were nearly same for the preparations immobilized on the three grades of Celite. However, a much greater variation was observed in their interesterification activities. Hyflo supercel which was flux-calcinated gave the highest lipolytic and interesterification activity due to its larger pores and so uniform location of protein in this material [101].

On the other hand, the amount of lipase immobilized was affected by the length of the hydrocarbon chain attached to the matrix. Hydrocarbons longer than 6 carbon lengths probably increased the

local surface concentration of lipase for immobilization and favors the immobilization of enzyme on support but has no significant effect on the specific activity of the immobilized lipase [130].

Type of carrier was also an important criteria which determines hydrolysis versus esterification selectivity. Lie and Molin were studied the hydrolysis and esterification with C.cylindracea lipase immobilized on hydrophobic and hydrophilic zeolites. The hydrophilic zeolite blocked the hydrolysis but mediated the esterification while hydrophobic zeolite were better than hydrophilic zeolite in hydrolysis [140].

In general, hydrophilic supports are generally characterized by high losses of lipase activity upon immobilization [131]. These losses have been attributed to;

1) a change in the conformation of the lipase upon adsorption to a form which has reduced activity
2) a situation in which only a small amount of lipase is immobilized
3) a decrease in the ability of hydrophobic substrates to reach the active site of the lipase; or
4) the existence of steric hindrance imposed by the carrier matrix which constrains flexibility of the lipase molecule.

1.10.2. Immobilization Techniques

The selection of an immobilization technique is based on process specifications for the catalyst, including such parameters as overall enzymatic activity, effectiveness of lipase utilization, deactivation and regenaration characteristics, cost of immobilization procedure, toxicity of immobilization reagents, and the desired final properties of the immobilized lipase.

The various immobilization methods used to date may be subdivided into two main categories [141]: i)chemical methods, where covalent bonds are formed with the lipase (e.g lipase attachment to a matrix by covalent bonds and formation of a cross-linked lipase matrix); and ii) physical methods, where weaker interactions or mechanical containment of the lipase is utilized (e.g entrapment, encapsulation, and adsorption).

Although there is no universal binding technique, covalent methods, adsorption and entrapment have been considered to be standard immobilization techniques.

1.10.2.1. Covalent Attachment

For industrial applications, noncovalent methods seem to be very attractive. However, these methods often involve covalent bonding. For example crosslinking and copolymerization has also been used in conjuction with adsorption and entrapment methods. Therefore covalent methods were limited on bonding between specific, identifiable groups of both the enzyme and the carrier. Covalent attachment involves derivatization of the support to provide a chemical group capable of reacting with available side groups of the lipase [131]. Therefore, type of reactions in covalent attachment depends on the reactivity of the functional groups and the relative concentrations of the amino acids carrying the functional groups in the protein [107]. The cyanogen bromide (CNBr) and carbodiimide with reactive groups of -OH and -COOH respectively were extensively used to couple enzymes to various carriers. Lipases have been successfully immobilized to cyanogen bromide activated agarose beads, cellulose and sephadex. One of the key advantages of covalent binding is the stability of the enzyme-carrier bond, which prevents elution of protein into the product stream.

In the case of immobilization of enzyme to the carrier directly, the high molecular weight of the substrates may cause the steric hinderance effect. It can be overcome by attaching an alkyl chain between the enzyme and carrier as a spacer. When Mucor miehei lipase was immobilized directly on the Florisil (magnesium silicate, an inorganic matrix) by glutaraldehyde, the interesterification activity was only 35 fold higher than that of free lipase. However, when lipase was immobilized on the Florisil by various hydrophobic spacers, the interesterification activity was increased 67-88 fold depending on the length of the alkyl chain. These results may be due to hydrophobic and flexible nature of adopted spacers which overcome diffusional limitation of hydrophobic substrate. Therefore, local concentration of the substrates around the lipase may be higher than that of free lipase, and the substrates are more accessible to the active site of the lipase since the substrates of the lipase are hydrophobic and of large molecules [107].

Effect of amounts of glutaraldehyde as a cross-linking agent was studied on the covalent attachment of pancreatic lipase on porous glass. Results showed that an increase in the amount of glutaraldehyde

results in a decrease in enzyme activity while an increase in the amount of enzyme bound onto carrier [142].

1.10.2.2. Adsorption

Noncovalent immobilization of enzyme molecules can be achieved by precipitation of the enzyme from an aqueous solution by the addition of chilled acetone [101,142]. An alternative method of noncovalent immobilization is adsorption. It is generally regarded as the simplest and most economical procedure for binding an enzyme to a solid support. Normally, immobilization can be accomplished by equilibrating an enzyme solution with the support followed by washing of the enzyme preparation. In most cases the enzyme solution is added to a solid support and then the water is removed by evaporation. The enzyme gets forced onto the support and physically "dried" onto it [111,142].

When the interesterification and lipolytic activity of pancreatic lipase immobilized on Amberlite IRA 938 by acetone precipitation and adsorption were compared, the best result was obtained with lipase immobilized by adsorption [142].

A variety of inorganic supports have been used to adsorb enzymes, including clay, alumina, titania, sand, glass and silica. These carriers generally result in rather low amounts of bound protein and continous elution of the enzyme. Therefore, a strong hydrophobic or electrostatic interaction is needed between the enzyme and the support for immobilization by adsorption to be successful. Strong electrostatic interaction can be achieved by using highly charged supports or charged enzymes. Strong hydrophobic interactions can be achieved by using hydrophobic supports or hydrophobic enzymes. For example, C.rugosa lipase modified with several hydrophobic modifiers adsorbed more strongly onto the various polymers as compared with the native lipase. The catalytic power of lipase immobilized in this manner was found to be affected by the type of modifiers, degree of modification and type of supports used [84].

Generally, both native and hydrophobic lipase derivatives showed higher esterification activities when immobilized on polar polymers compared with non-polar polymers. This may be due to the ability of hydrophilic supports to absorb the water that was produced in the esterification reaction with result that more ester was formed

[84].

Most of the adsorbents such as celite, cellulose, silica gel, kieselguhr, clay, alumina, carbon, etc. were found to decrease the lipase activity upon immobilization. However, the hydrophobic microporous materials were found to provide better performances. Therefore Ruckenstein et al. were performed the immobilization of lipase from C.rugosa on the internal surface of hydrophobic microporous poly(styrene-divinylbenzene) supports. It has been concluded that hydrophobic interactions as well as the interactions between the enzyme and the head group of the surfactant molecule gave high stability. This favorable interactions was also made the immobilized enzyme molecule more active than the free one [129].

Some proteins (such as albumin, gelatin and casein) and polyethylene glycol were found to be effective additives to avoid activity loss by protecting the enzyme during the immobilization. The stabilizing effect of additives might be due to increase surface covarage that otherwise the enzyme and the support material were not fully compatible at low enzyme loadings. The additive made the properties of the support more favorable to retention of the enzyme activity. The addition of additive had a minor stabilizing effect even at higher enzyme loadings that was enough to achieve self-stabilization [143].

Since lipase adsorbed onto solid supports was quite unstable in an aqueous system, Phutrakul et al. was screened various carrier materials namely Celite 545, Dowex 50, Silica gel, Sephadex LH-20 and Sephadex LH-60 for the adsorption of lipase from C.cylindracea to work in organic solvents. Sephadex LH-60 and Celite 545 were found to be best supports due to higher activity and stability in organic solvents. In the case of hydrolytic reactions, more polar organic solvents might be considered [144].

In a similar study, lipase from C.rugosa immobilized on Celite was enhanced esterification reaction over hydrolysis by manipulating the microenvironment of the catalyst. It was concluded that the addition of solvents like NN-dimethylformamide that share same properties with water (high dielectric constant and low log P values) was advantageous in order to favour transesterification over hydrolytic process [145].

1.10.2.3. Entrapment

Entrapment, the capture of the enzyme within a matrix of a crosslinkable resin, is another way for enzyme immobilization. A variety of lipases have been trapped using photo-crosslinkable resin prepolymers [29,127], urethane prepolymers [29] and Ca-alginate [135]. The immobilization process consists of mixing the prepolymers with a photosensitizer (e.g., benzoin ethyl ether), melting, mixing with a lipase solution, and gelling by exposure to near ultraviolet radiation. An alternative method of entrapment consists of freezing a monomer solution containing the lipase in the form of small beads. Polymerization is then initiated using gamma radiation.

Lipases can also be immobilized by polyacrylamide gel formation at room temperature. In this case, the enzyme is mixed in a buffered aqueous solution of acrylamide monomer and a cross-linking agent, often N,N'-methylenebisacrylamide. Polymerization may be initiated by N,N,N',N'-tetramethylenediamine, followed by addition of ammonium persulphate.

Omar et al. were compared the stability of H.lanuginosa lipase immobilized by entrapment in resin prepolymers or Ca-alginate, adsorption to different resins or by covalent binding methods. Although, the resin immobilized lipase exhibited excellent stability to repeated batch hydrolysis of triglycerides, adsorption on Amberlite XAD-7 followed by crosslinking with glutaraldehyde was superior to several points. Firstly, the adsorption efficiency for lipase was relatively high and technique employed was simple. Secondly, the optimal temperature was about 10°C higher than that for the free enzyme which is vital for solid fat hydrolysis [29].

Alginate was evaluated as an immobilization matrix for enzyme-catalyzed reactions in organic solvents. In hydrophobic solvents, the alginate gel (with its high content of water (>95%)) provided a stable, two phase liquid system. Thus calcium alginate retains the enzyme inside the gel matrix and protects the biocatalyst against the denaturing influence of the organic solvent.

C.cylindracea lipase immobilized in alginate beads catalysed esterification and transesterification in n-hexane under both batch and continous-flow conditions. The entrapped lipase was found to be possess greater stability than the free enzyme after repeated use which is important for a continuous process [135].

The hydrolytic activities of photocrosslinkable resins ENT and ENTP-entrapped C.cylindracea and R.arrhizus lipase were investigated in relation to the pH, temperature, prepolymer chain length and gel hydrophobicity. Lipases in hydrophobic gels with the longest chain lengths exhibited highest activity and stability. The surface area of immobilized lipase preparations was also effective on reaction rate. The initial rate was linearly increased as the particle size decreased providing less diffusional resistance [127].

Ultrafiltration membranes which has no diffusional restrictions for large molecular weight or insoluble substrates was another alternative system for entrapment. Rhizopus sp. immobilized directly into the interior of poly(vinyl chloride) ultrafiltration membranes was shown better time stability as compared with those of lipase immobilized by adsorption into PVC [146].

Several reactor configurations have been designed in studies of immobilized lipases. Two types of phases are invariably present: a solid phase (i.e., the carrier on which the lipase is immobilized), and one or two liquid phases (depending on whether both reactant and product species are dissolved in an organic solvent, or the oil and aqueous phases maintain their respective identities).

Continous hydrolysis of triglycerides of melted butterfat for the production of lipolyzed butter flavors was performed by a lipase from A.niger immobilized by adsorption on a microporous, polypropylene flat-sheet membrane [17].

A similar system was designed for the continous hydrolysis of olive oil by C.cylindracea lipase [147]. These systems have many advantages relative to conventional batch process. Mainly,

a) no need for the addition of emulsifiers coupled with vigorous stirring
b) contamination of the product by residual lipase was avoided by retention of lipase within the pores of the membrane
c) operational life of lipase was extended
d) the reactor was regenarated easily with fresh lipase
e) autooxidation of fatty acid was avoided because the reactor has no free space to hold water when it is filled with reactants
f) product was obtained in a single phase with no component of another phase

Yang et al. were screened various enzyme carriers (DEAE-

Sephadex A50, Sephadex G50, Sephadex LH20, and Amberlite XAD-7) for their suitability in packed-bed reactor. The enzyme immobilized with DEAE-Sephadex A50 was found to be most effective for continous hydrolysis of olive oil in isooctane. Continous packed bed reactor eliminated the depletion of water and accumulation of glycerol that may be the main problems in batchwise operation [87].

1.11. Aim of This Study

Lipase from Candida rugosa has been subjected to intensive study during last thirty years with the aim of elucidating the molecular properties of the enzyme, its substrate and positional specificities, and the nature of interaction with either substrates or modifiers. The commercial preparations of the enzyme with different levels of purity are now available. The kinetics of hydrolysis by the enzyme seem to be dependent on factors such as pH, temperature, ionic strength and most importantly on the type of substrate used. Among microbial lipases, the enzyme from Candida rugosa is an interesting lipase because of its high activity in hydrolytic as well as synthesis reactions. In addition, this yeast lipase is the most recommended due to its random specificity and somewhat inexpensive price [44,45].

Recently, a method has developed for lipase assay, in which the reaction mixtures containing water and oil were emulsified either by sonication or magnetic stirring [45,85]. The results showed that the rate of oil hydrolysis could be determined reproducibly in the absence of any added emulsifier. From an industrial viewpoint, bioconversion of water-insoluble compounds in the absence of emulsifier and hydrocarbon solvent is most important, because they would increase the cost of product separation in addition to their adverse effect on the reaction rate [85].

The aim of the present work was to investigate the kinetics of olive oil hydrolysis in the absence of any added emulsifier. The effects of ionic strength, pH, temperature and active site-directed covalent inhibitors on the activity of enzyme were also considered.

To study the catalytic effect of celite- and polyacrylamide-immobilized Candida rugosa lipase, the hydrolysis of olive oil was chosen as a model reaction. The activity of the immobilized lipase was compared to that of free enzyme. The hydrolysis was studied as a

function of time, substrate concentration, repeated use, pH and temperature. Being a cheap material and having high resistance to most severe conditions, celite should be an ideal support. In addition, the support particle size does not seriously affect productivity [66]. Acrylamine seems to be the most popular monomer for gel polymerization and is used for entrapping enzymes. The immobilization process is affected greatly by monomer concentration and cross-linker/monomer ratio. Since increasing the monomer concentration increases bound protein, as well as the rate of denaturation, optimum concentration and cross-linker/monomer ratio needs to be determined experimentally.

CHAPTER 2
MATERIALS AND METHODS

2.1. Enzyme and Substrates

Lipase isolated from Candida rugosa (900 units/mg solid) was purchased from Sigma Chemical Co. (St Louis. MO) and used without purification. A unit of lipase activity was defined as that which would hydrolyze 1.0 microequivalent of fatty acid from a triglyceride (triacetin as substrate) in 1 hr at pH 7.4 at 37°C.

Commercial olive oil was purchased from local market (Komili, riviera) and purified with charcoal before use. p-Nitrophenyl-fatty acid esters and triglycerides were obtained from Sigma. The common reagents used were all of reagent quality (Merck or Riedel de Haan) and solutions were prepared using double glass-distilled water.

2.2. Purification of Olive Oil

Commercial olive oil was purified with charcoal as described by Benzonana et al. [57]. 50 g of olive oil was dissolved in 500 mL of petroleum ether and 50 g of charcoal was added to it. After shaking for 30 mins., charcoal was removed by filtration and washed twice with 15 mL of petroleum ether. Solvent was evaporated under nitrogen at 35-40°C. Purified olive oil was completely free of coloured material and free fatty acids. Yield of purification was about 58 %.

2.3. Protein Assay

Protein content was determined by the method of Lowry et al. [148] with bovine serum albumin as the standard.

2.4. Molecular Weight Determination

2.4.1. Gel Filtration

A 1 mL of enzyme solution containing 20 mg enzyme preparation in 25 mM KP buffer, pH 7.0, was applied to the Sephadex G-100 column

(1.3x25)cm which had already been equilibriated with the same buffer. The enzyme was eluted with the same buffer. In the collected 1.5-mL fractions, protein concentration and activity were determined. After washing the column overnight, 1 mL of marker proteins (12 mg) bovine serum albumin (66 000 D), carbonic anhydrase (29 000 D), cytochrome c (12 400 D) and aprotinin (6 500 D) were applied and 1.5-mL fractions were collected with the same flow rate (24 mL/hr). The protein concentration of each fraction was analyzed. Under the same column conditions, blue dextran (2 000 000 D) was allowed to run for the void volume determination and for checking the homogeneity of the bed.

2.4.2. SDS-Polyacrylamide Gel Electrophoresis

Gel buffer contained 1.5 M Tris-HCl buffer, pH 8.8, plus 0.4 % (w/v) SDS. 30 % acrylamide stock solution was prepared in water. 14x16 cm uniform slab gel (thickness, 0.75 mm; 7.5 % acrylamide) was polymerized at room temperature (25°C). 25 mM Tris/200 mM glycine buffer, pH 8.3 plus 0.1 % SDS was used as electrophoresis buffer. Electrophoresis was performed at 150 V and 13-17 mA for about 4 hr at room temperature. The gel was stained for protein with Coomassie Blue. Marker proteins (Sigma Chemical Co.): Bovine serum albumin (66 000 D), chicken egg albumin (45 000 D) and carbonic anhydrase (29 000 D); tracking dye: bromophenol blue.

2.5. Initial Rate Assays

Reactions with olive oil as substrate were carried out in 100 mL conical plastic-stoppered flasks at 37°C by immersing in a water bath and with shaking at 120 strokes per min. The enzymatic reaction was initiated by adding enzyme solution or immobilized enzyme into the reaction mixture (4 mL) containing a known amount of olive oil and 25 mM Tris-HCl, pH 8.0 unless otherwise stated. Before incubation at 37°C, 1 min of wortex mixing was applied [45]. The reaction was terminated by the addition of 5 mL ethanol followed by titration (Metrohm titration system) with 0.5 N KOH solution using phenolphthalein as the indicator. One unit of lipase activity was defined as the amount which liberated 1 micromole of fatty acid per min at 37°C.

To investigate the effect of pH on the activity of lipase, the buffers (25-100 mM) used were as follows: pH 4.0-5.5 with acetate buffer; pH 6.0-8.0 with potassium phosphate buffer; pH 7.0-9.0 with

Tris-HCl buffer. Enzyme free blanks were obtained by titrating the mixture of reactants. Blanks were also obtained by the same procedure after the enzyme had been inactivated. Similar results were observed. All data are averages of at least duplicate runs and are reproducible within ±10 %.

Activity measurements with p-nitrophenyl (PNP)-fatty acid esters were done in 100 mM KP-buffer, pH 7.0 at 25°C. A Beckman model 24 double-beam spectrophotometer with a Beckman recorder model 24-25 AC was used for these measurements and the temperature of the cell compartment was maintained with Haake circulator KT 33. The initial rates of reactions were taken from the slope of the linear progress curves generated on the recorder. In all measurements, the volume of the assay mixture was 2 mL and the sample was assayed against a reference which contained all components except enzyme. In place of enzyme solution the same amount of water was added to the reference cell. The formation of p-nitrophenolate ion was followed at 405 nm in an assay mixture containing 5 % acetonitrile (v/v).

DMP-tributyrate activity was followed at 412 nm in an assay solution containing DTNB as indicator, based on the technique described by Kurooka et al. [149] for lipases. Several DTNB concentrations were tested to ascertain that the enzymatic reaction was rate-limiting.

2.6. Inactivation of Enzyme

Inactivation was carried out at 37°C in Tris-HCl buffer, pH 7.0, using 25 µg protein/mL and olive oil as substrate. The process was initiated by the addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) or o-phythaldialdehyde (o-PDA). o-PDA stocks were prepared daily in methanol. Residual activity was determined at pH 7.0 after dilution of the inactivation mixture into the assay media described above. Respective control runs were carried out without inhibitors. Reversibility of the effect of EDAC was tested [150] by diluting a fraction of the inactivation mixture 1:1 with 0.5 M hydroxylamine (HLA), pH 7.0, keeping the concentration of Tris-HCl and EDAC constant.

2.7. Estimation of Hydrolysis

The hydrolysis ratio was calculated from the acid value of the

oil in the mixture after hydrolysis based on the saponification value of the oil used. The saponification value of the purified olive oil was determined according to TS 342. The saponification value of purified olive oil was 176 (average of four replicates). Density of olive oil was found as 0.8731 gr/mL.

$$\%\text{Hydrolysis} = \frac{\mu\text{mole FA liberated}}{176 * \frac{1000}{56.1} * \text{gr oil}} * 100$$

2.8. Immobilization

2.8.1. Entrapment by Polyacrylamide Gel

Candida rugosa lipase was immobilized within uniform slab gel (thickness 1.5 mm) derived from acrylamide/bisacrylamide polymer in a final gel concentration of 12 %. Different amounts of lipase (45-225-450 mg) was dissolved in gel buffer (0.65 M Tris-HCl, pH 8.8) and centrifugated to remove insoluble debris. Then acrylamide/bisacrylamide was added to clear supernatant and degassed for 1 min. Finally polymerization was initiated by adding TEMED and APS and allowed to polymerize at room temperature. Gel and slice dimension was adjusted to obtain an enzyme concentration of 2 mg enzyme/slice unless otherwise stated. Water was removed by lyophilization for 1.5 hours and slices were obtained in dry form unless otherwise stated.

2.8.2. Adsorption on Celite

2.8.2.1. Precipitation by Acetone

Candida rugosa lipase was dissolved in 5 mL 0.1 M KP-buffer, pH 7.0 and 10 mg carrier was added. The enzyme was precipitated by the addition of chilled acetone (20 mL) at -20°C. The immobilizate was left at -20°C for 15 min and then acetone was removed by decantation.

2.8.2.2. Immobilization by Adsorption

Candida rugosa lipase was dissolved in distilled water (2 mL). The enzyme solution was added to celite hyflo supercel (4 mL solution/g support) and mixed slowly. Water was removed by evaporation under vacuum at room temperature (20-25°C) for 7 hrs. The immobilized enzyme was then desiccated and stored in a

refrigerator. The moisture content of the preparate was determined as 0.88 % using an oven drying at 105°C.

2.9. Estimation of Molar Concentration

The conversion of olive oil concentration in the reaction mixture into a concentration unit of molarity, based on the ester bond in olive oil, was defined as follows:

$$S_0(M) = \frac{176\ S_0(gr/ml)}{56.1}$$

2.10. Estimation of Effectiveness Factor

The effectiveness factor, η, was estimated as the ratio of the rate of reaction for an immobilized enzyme to the rate of reaction for the same amount of enzyme in solution. Initial reaction rates were considered in order to make a reliable comparison.

CHAPTER 3
RESULTS

3.1. The Purity of the Commercial Preparation

The commercial enzyme preparation was examined for protein content. The reference protein was bovine serum albumin. A calibration curve was plotted for every determination (Fig.1). The protein content of the commercial preparation was nearly 10 % by mass.

Gel filtration yielded a single active peak (Fig.2) across which 65% of olive oil hydrolyzing activity and 10.8 % of protein were conserved. Specific activity was increased from 220 U per mg protein to 1310 U per mg protein (olive oil, 25 % (v/v) in 25 mM potassium phosphate buffer, pH 7.0, T=37°C).

Polyacrylamide gel electrophoresis yielded one main lipase component (fraction number 15). Commercial enzyme contained low molecular weight protein components (Fig.3). The fraction of pure lipase in the mixture was determined as 1.1 % by mass.

3.2. The Molecular Weight of the Enzyme

The molecular weight of C.rugosa lipase was determined by gel filtration (Fig.4) and SDS-PAGE (Fig.5). The purified enzyme preparation was homogeneous as judged by the presence of a single protein band on SDS-PAGE. The enzyme exhibited one band corresponding to a molecular mass of 56 kDa. The molecular mass of the native enzyme was estimated to be 56 kDa from gel filtration on Sephadex G-100 column. Thus the enzyme was found to be a monomeric protein.

3.3. Substrate Specificity

Substrate specificity was tested with p-nitrophenyl-fatty acid monoesters. The enzyme showed low activities toward esters of long chain (> C12) fatty acids and also caproate acid ester. Table 1 lists relative hydrolytic activities toward a variety of PNP-fatty acid esters

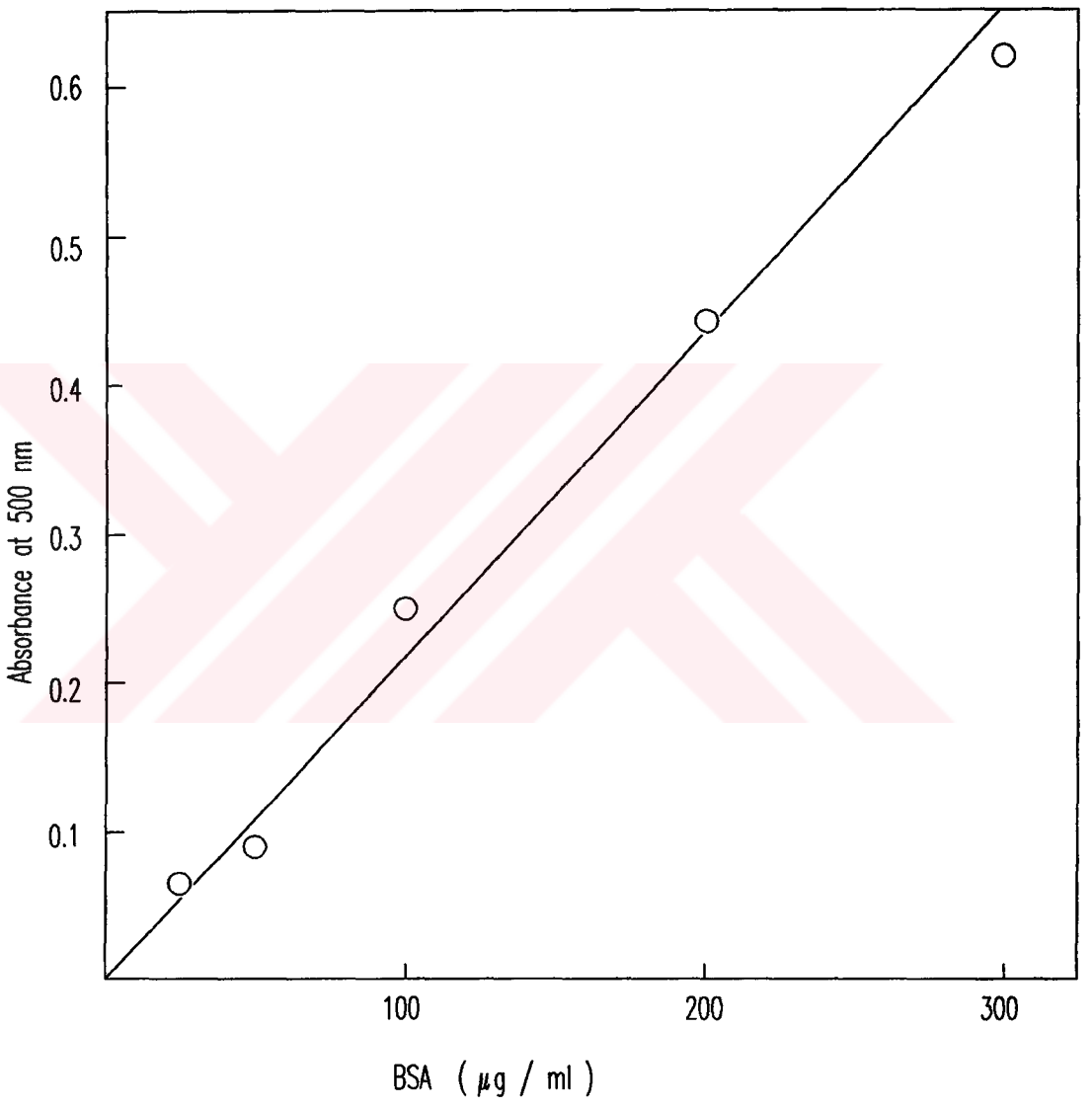

Figure 1. Calibration Curve for Protein Determination. Bovine serum albumin is used as the standard

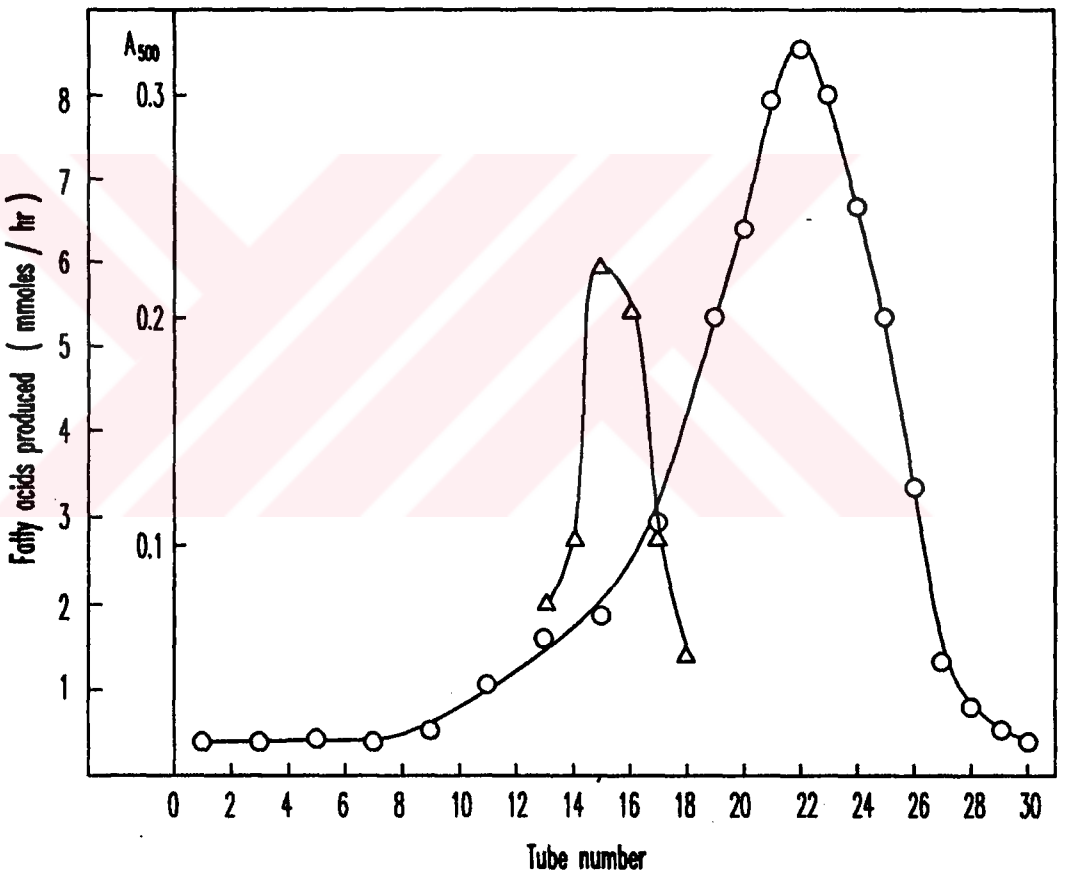

Figure 2. Gel Filtration of Lipase from C.rugosa on Sephadex G-100. Flowrate 24 ml/hr. Absorbance at 500 nm, o--o; lipase activity, ▲--▲

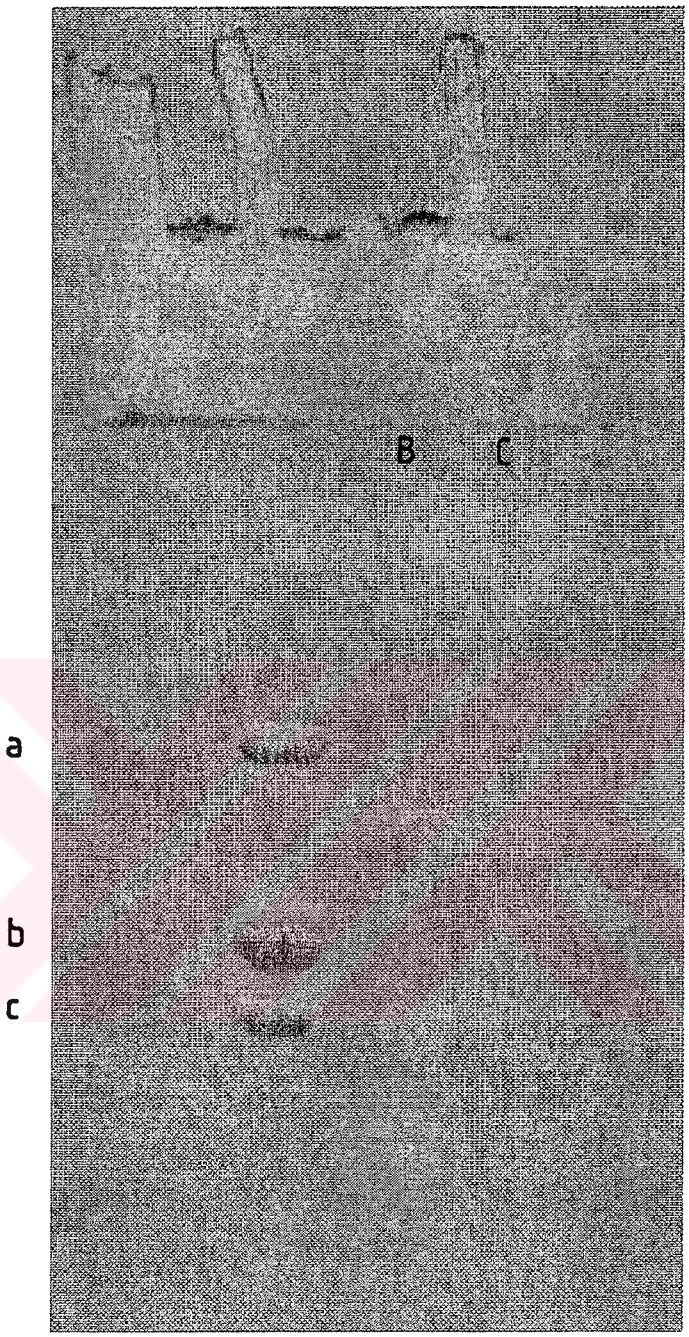

Figure 3. SDS-Polyacrylamide Gel Electrophoresis of Candida rugosa Lipase. Commercial enzyme (Lane B), fractionated enzyme (Lane C). Marker proteins (a) bovine serum albumin, (b) chicken egg albumin, (c) carbonic anhydrase

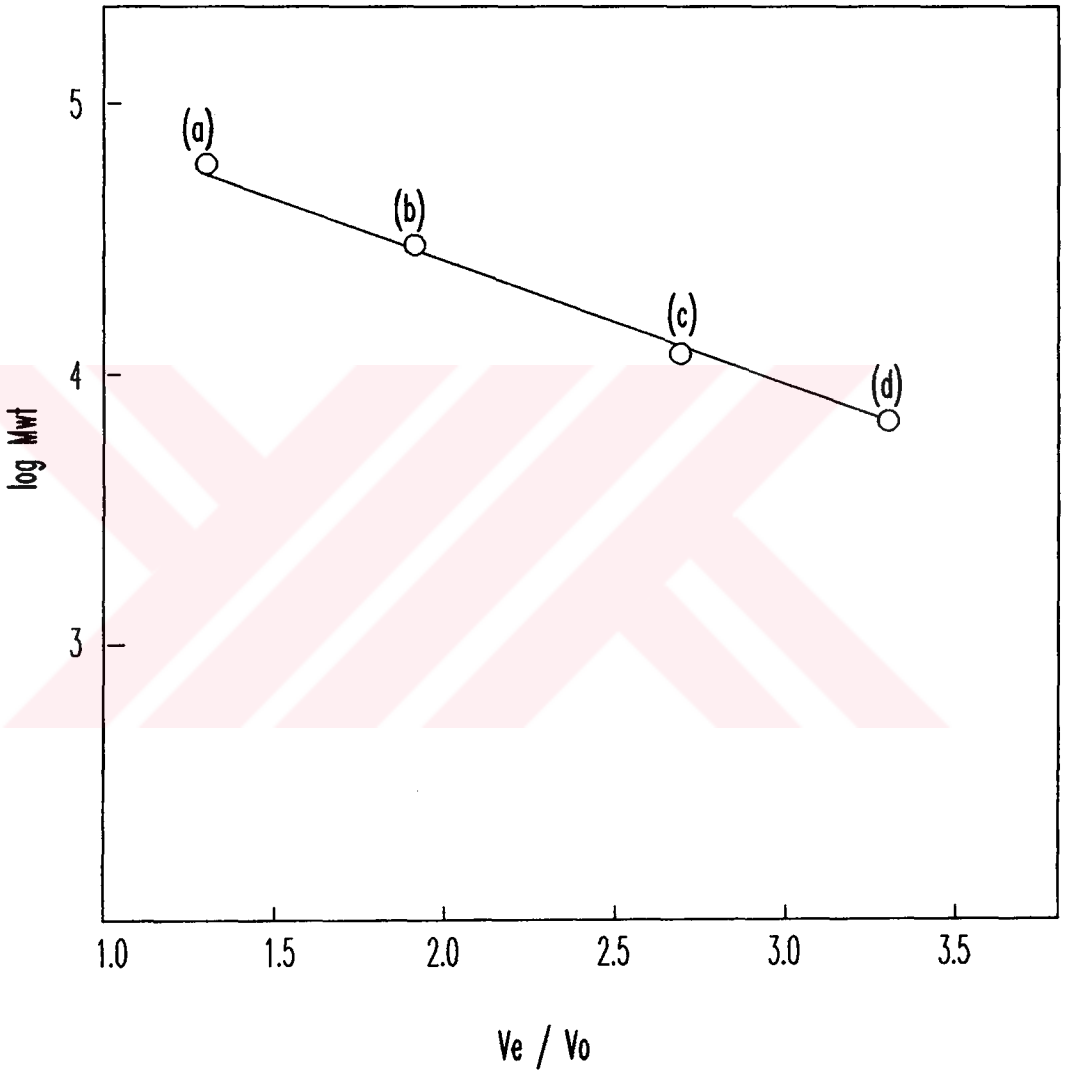

Figure 4. Molecular Weight Estimation of C.rugosa Lipase by Sephadex G-100 Gel Filtration. Marker proteins: bovine serum albumin (a), carbonic anhydrase (b), cytochrome c (c) and aprotinin (d). The molecular weight of marker proteins is plotted versus the elution volume

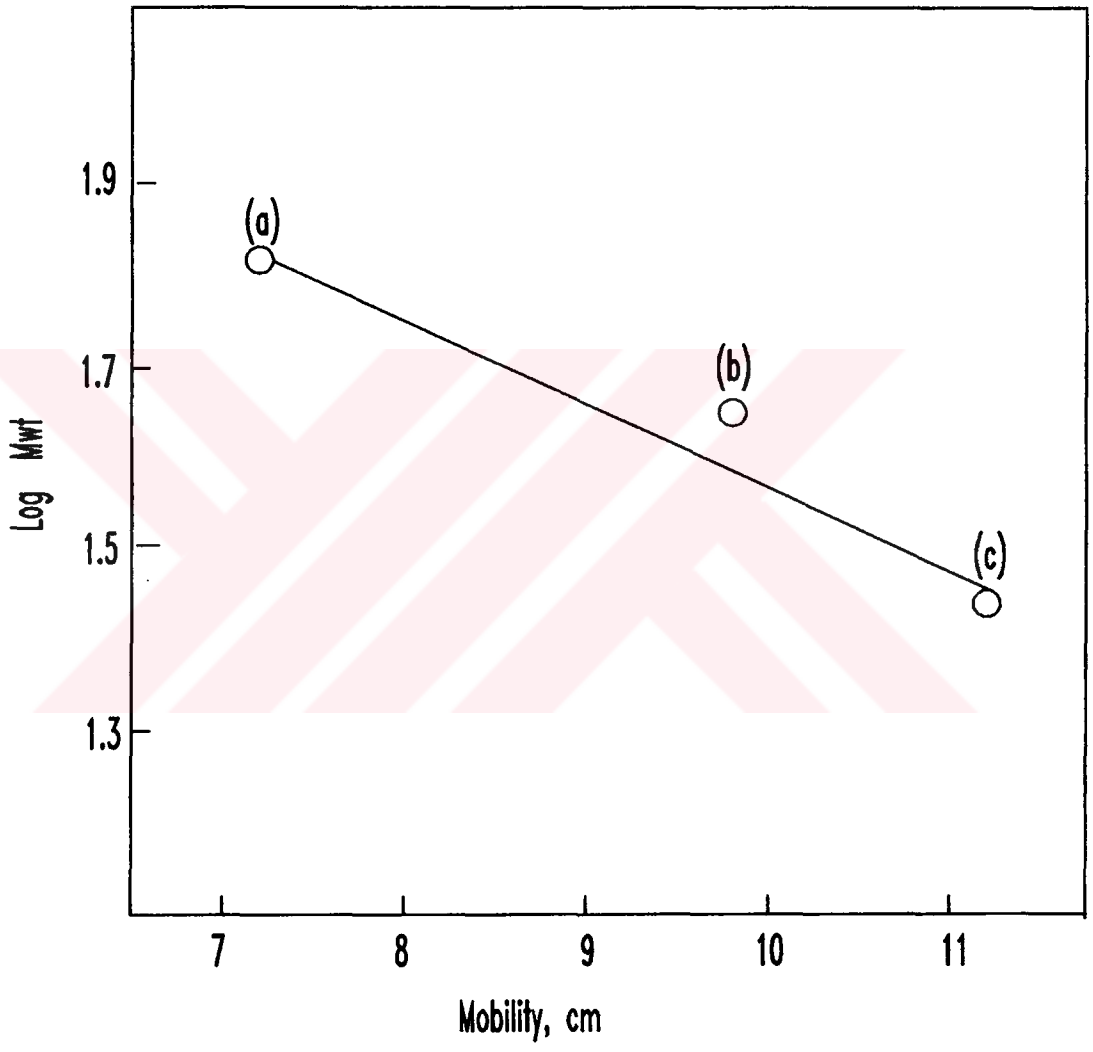

Figure 5. Molecular Weight Estimation of C.rugosa Lipase by SDS-PAGE. Marker proteins: bovine serum albumin (a), chicken egg albumin (b) and carbonic anhydrase

examined at 25°C.

Table 1. Influence of Substrate on C.rugosa Lipase

Carbon Number	Relative Hydrolytic Activity
C6-FA	8.2
C10-FA	100.0
C12-FA	18.3
C14-FA	14.8
C16-FA	9.7
C18-FA	1.4

3.4. The Effect of Enzyme Concentration

The extent of hydrolysis was calculated from the amount of fatty acids. The fatty acid formation as a function of enzyme concentration at a fixed substrate concentration (25 %,v/v) is shown in Figure 6. The amount of fatty acid produced seemed to be linearly proportional to the enzyme concentration of 0.1 mg protein/ml (E_t= 0.4 mg) within 10 min reaction time showing that the system was in steady-state. Figure 7 shows the fatty acid formation as a function of reaction time at fixed substrate (25 %,v/v) and enzyme (25 µg protein/ml) concentrations. The progress curve of the lipase-catalyzed hydrolysis shows a typical hyperbolic form. The product formation was linearly proportional to the reaction time up to 10 min during which the hydrolysis was about 4.5%.

3.5. Inactivation of Lipase by EDAC and o-PDA

Figure 8 shows the effects of EDAC and o-PDA on lipase activity. The enzyme in 25 mM Tris-HCl buffer, pH 7.0 was incubated with 10 mM of each of the covalent inhibitors for 10 min and then the enzyme activity was measured after 10 min reaction time. Methanol concentration was held constant at 4 %(v/v) during inactivation with o-PDA. Modification of the Candida lipase resulted in significant decreases in activities (nearly 60 %) (Fig.8). The ability of hydroxylamine to reverse the inhibition by 1 mM and 10 mM EDAC was tested (Table 2). In both concentrations of EDAC, introduction of 0.25 M hydroxylamine into the inactivation medium caused noticeable return

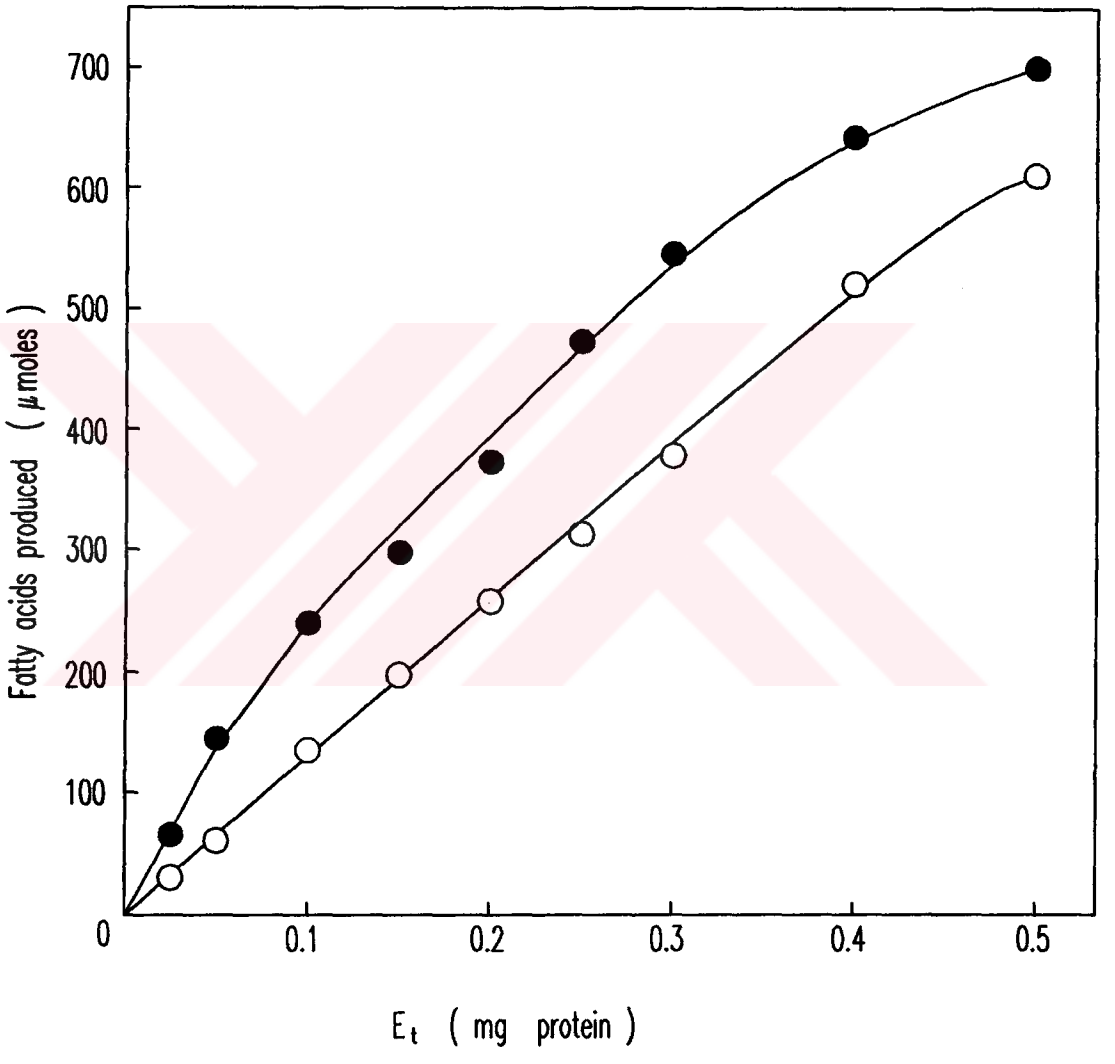

Figure 6. Fatty Acid Formation as a Function of Enzyme Concentration. Reaction conditions: olive oil, 25 % (v/v); temperature, 37°C; buffer, 25 mM Tris.HCl, pH 8.0; reaction time (●) 30 min; (○) 10 min; reaction mixture, 4 ml.

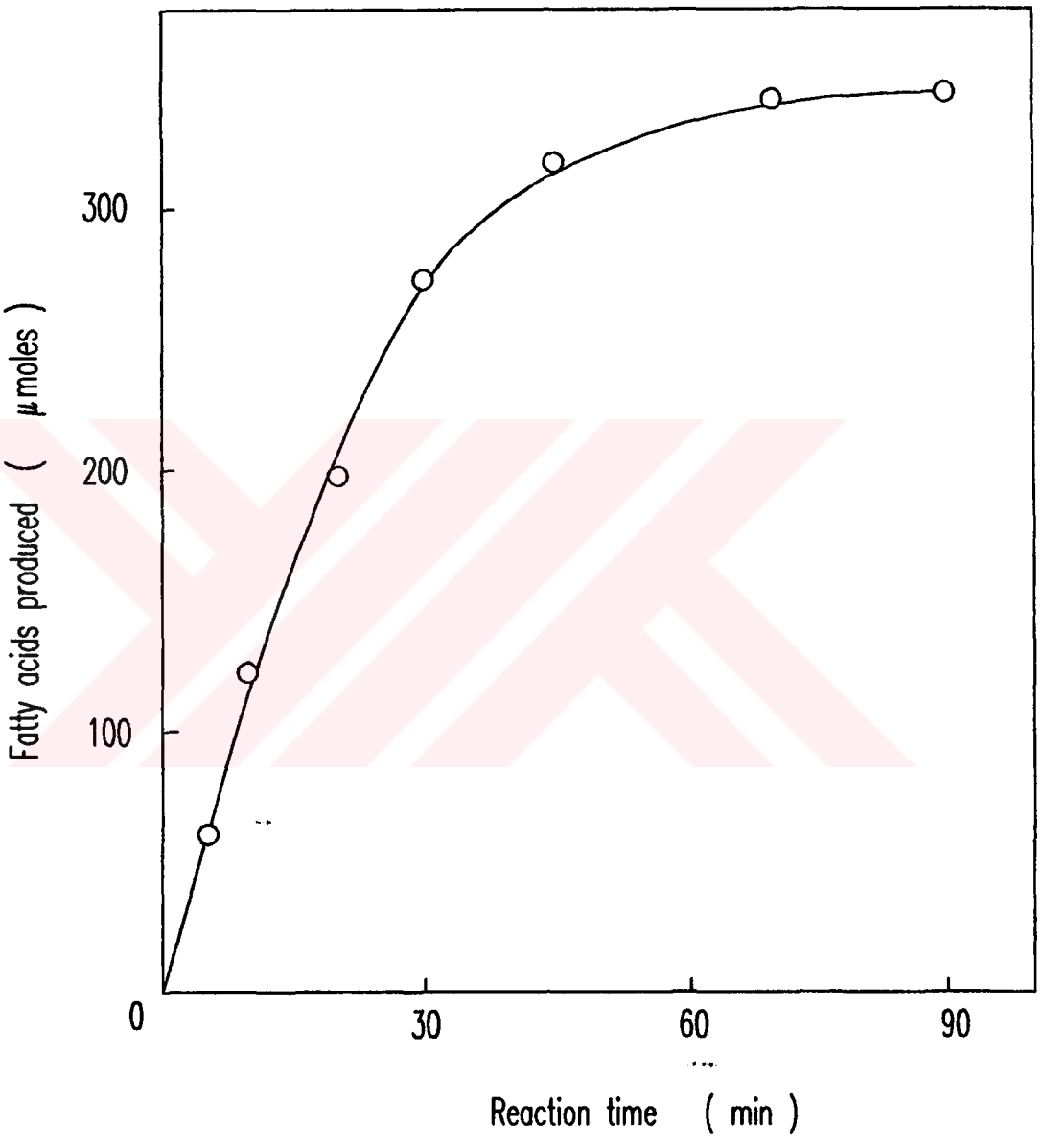

Figure 7. Amount of Fatty Acids Produced as a Function of Reaction Time at a Fixed Concentrations of Enzyme and Substrate (25 μg protein/ml). Conditions as in Figure 6

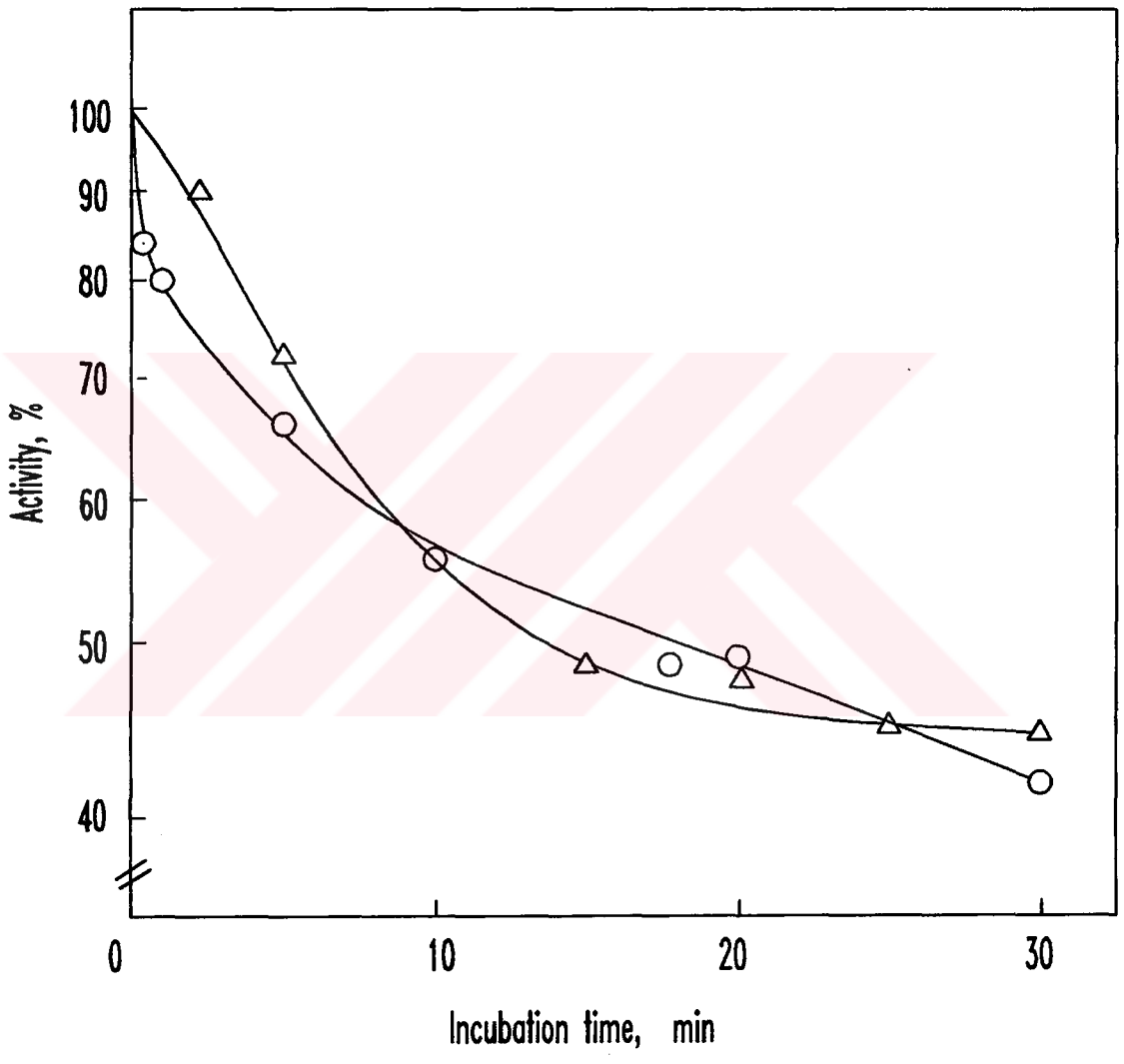

Figure 8. Semilogarithmic Plots for Inactivation of Lipase by EDAC and o-PDA. Percentage residual activity measured at 25 µg protein/ml; 37°C; 25 % (v/v) olive oil; 25 mM Tris.HCl, pH 7.0. (o) 10 mM EDAC, (▲) 10 mM o-PDA

of activity (21-24 %). In the modification with o-PDA, the catalytic site and/or the substrate binding site was protected at 1 mM o-PDA. The biphasic nature of the semilogarithmic plots for EDAC and o-PDA remains to be determined.

Table 2. Inactivating Effect of EDAC and o-PDA and Protecting Effect of Hydroxylamine on Lipase Activity

Modifier	Concentration	Relative activity (%)[a]
None	----	100
EDAC	1 mM	61
EDAC	10 mM	55
EDAC+HLA	1 mM+0.25 M	85
EDAC+HLA	10 mM+0.25 M	76
Methanol	4 % (v/v)	79
o-PDA[b]	1 mM	100
o-PDA[b]	10 mM	55

[a]Reaction conditions are the same as described in Figure 8.
[b]Methanol concentration was 4 % (v/v) during inactivation.

3.6. The Effect of pH and Buffer Concentration

The effect of buffer ionic strength was investigated at three different buffer concentrations, 25 mM, 50 mM and 100 mM (Fig.9). At low pH's between 4.0-5.5 (acetate buffer) increase in buffer concentration from 25 mM to 100 mM increased the activity of enzyme. At pH 4.0 the activity increase was 100 % whereas at pH 5.5, it was nearly 10 %. But at higher pH's between pH 6 to 8 (phosphate buffer) and pH 7 to 9 (Tris-HCl buffer) the activity declined as the buffer concentration increased. The cause of this effect was 10-70 % depending on the pH and identity of the buffer used. Figure 10 shows the effect of pH on the lipase activity at zero buffer concentration. The optimum pH was found to be 7.0. The effect of pH on the stability of Candida lipase after preincubating the enzyme at the indicated pH values (Fig.11) exhibited inactivation at pH 6.0 and pH 8.0. Inactivation was irreversible at pH 6.0.

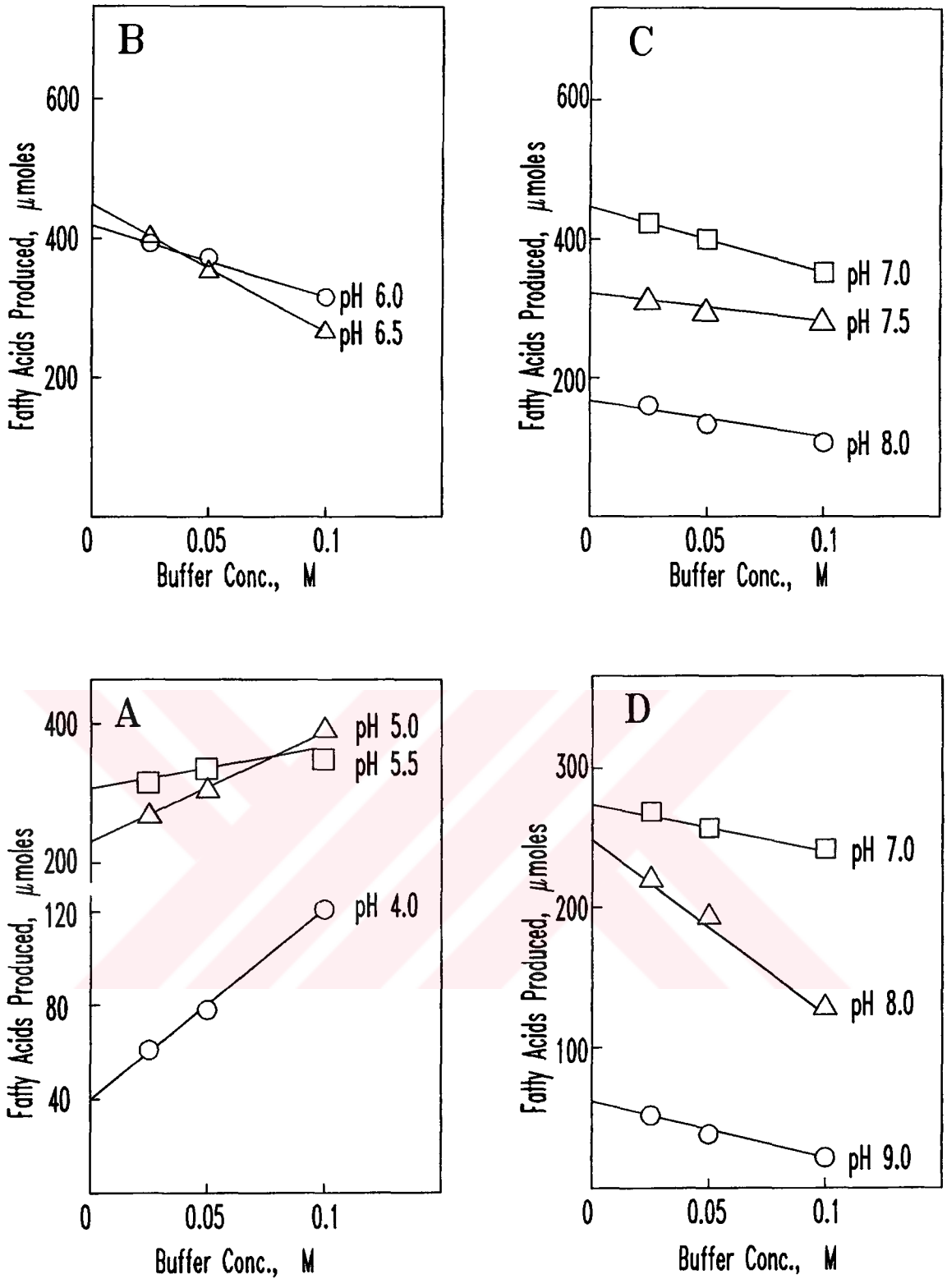

Figure 9. Effect of Buffer Concentration on Activity of Lipase. Buffers (25-100 mM): pH 4.0-5.5 with acetate buffer (A); pH 6.0-8.0 with potassium phosphate buffer (B,C); pH 7.0-9.0 with Tris.HCl buffer (D). Reaction conditions: olive oil, 25 % (v/v); 37°C; reaction time, 30 min; enzyme concentration, 25 μg protein/ml

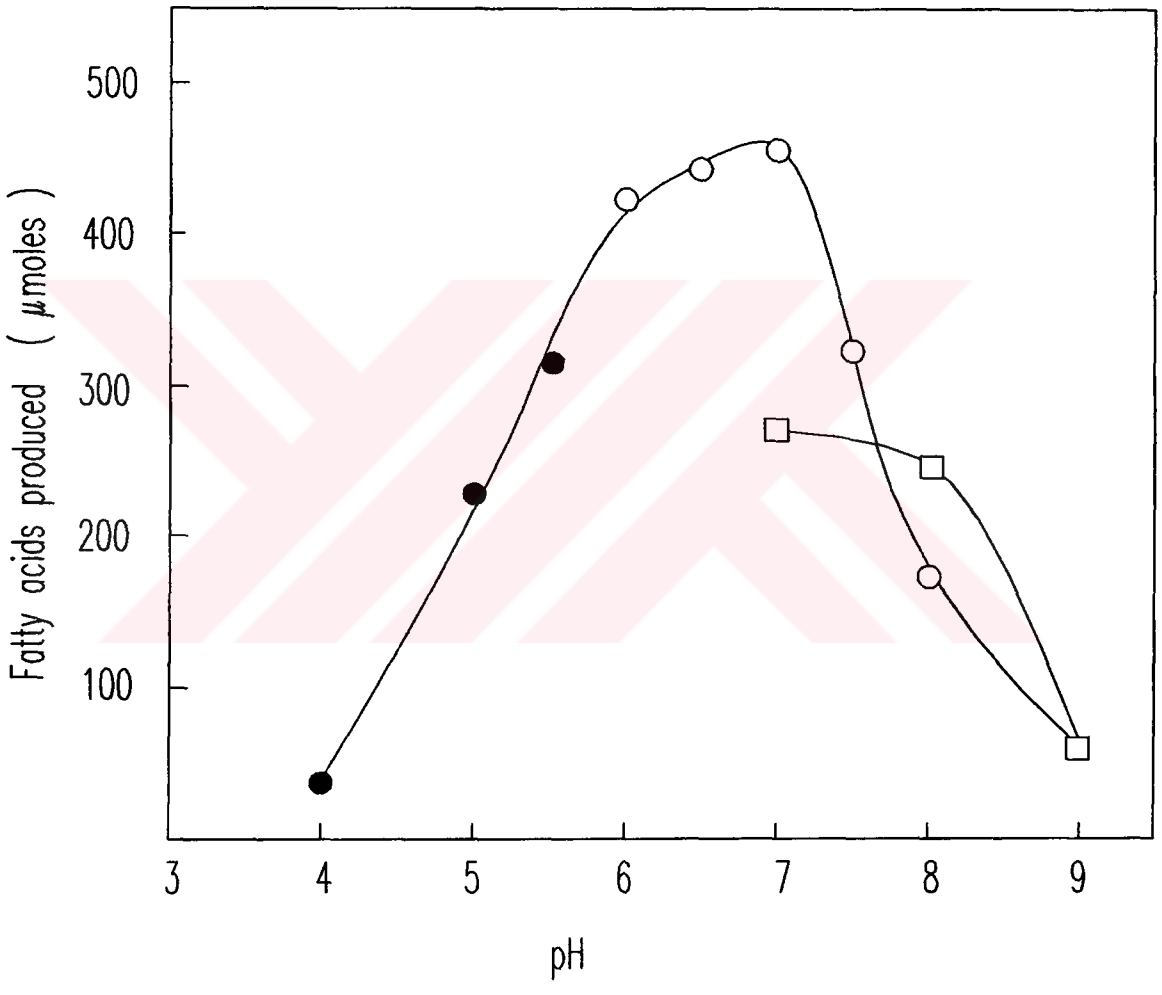

Figure 10. Effect of pH on the Activity of Lipase at Zero Buffer Concentration. (●) Acetate buffer; (o) Phosphate buffer; (□) Tris.HCl buffer. Conditions as in Figure 6.

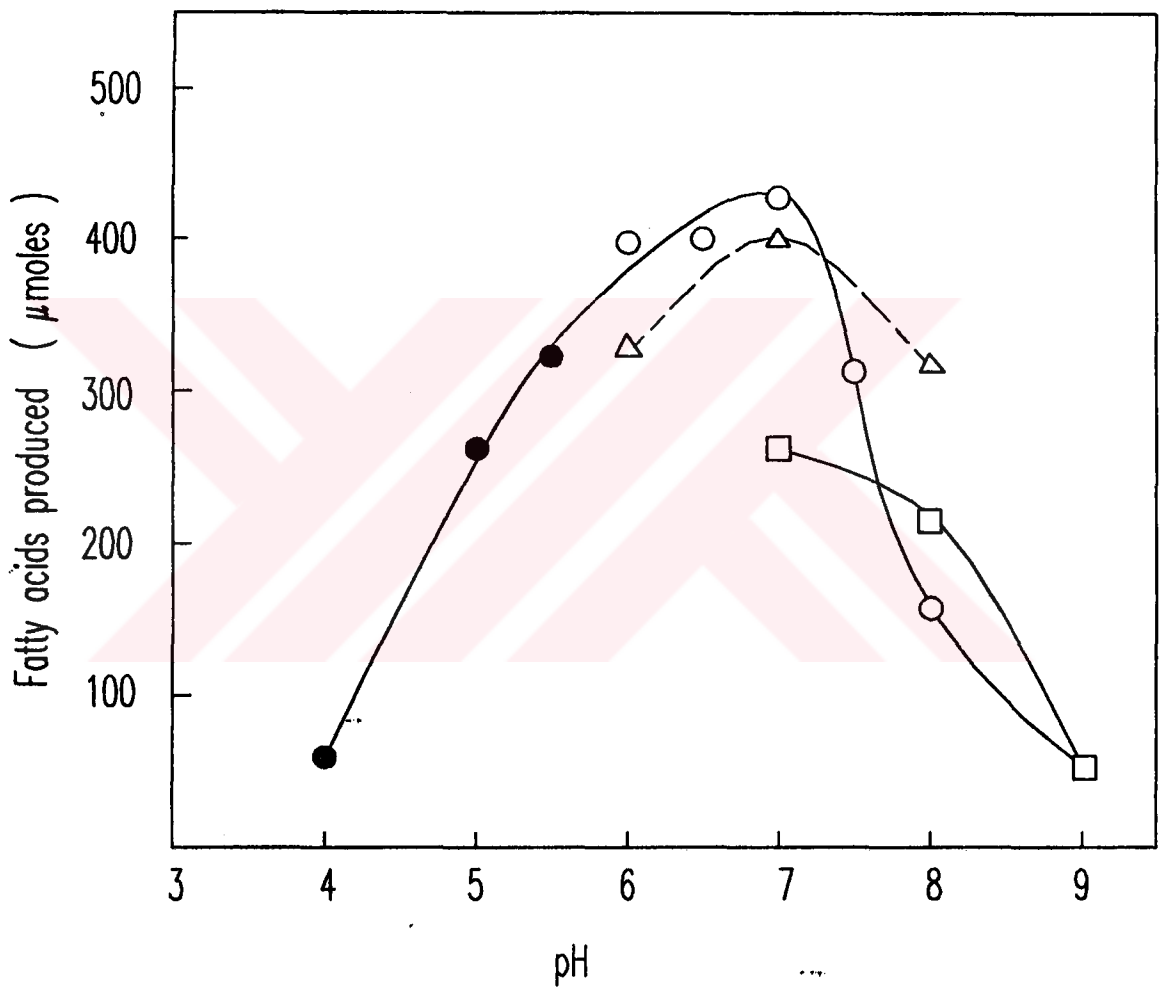

Figure 11. Effect of pH on Activity and Stability of Lipase at 25 mM Buffer Concentration. (●) Acetate buffer; (o) Phosphate buffer; (□) Tris.HCl buffer. The dotted line (Δ--Δ) indicates the fatty acid production at pH 7.0 after preincubating the enzyme at the indicated pH values for 30 min in phosphate buffer. Conditions as in Figure 6.

3.7. The Effect of Temperature

The enzyme exhibited its maximal activity at 40°C when assayed in potassium phosphate buffer, pH 7.0 (Fig.12).

3.8. The Effect of Substrate Concentration

Lipase catalyzed hydrolysis of olive oil followed Michaelis-Menten kinetics (Fig.13,14). Plotting of initial velocity versus substrate concentration indicated that the lipase was not inhibited up to the substrate concentration of 50 % (v/v). The parameters of the Michaelis-Menten kinetic equation were determined from double reciprocal plot of the lipase activities. The values of $K_{m,app}$ and $V_{max,app}$ obtained were 12.1 % (v/v) and 2926 units/mg enzyme in 25 mM Tris-HCl, pH 7.0 and 16.7 % (v/v) and 3472 units/mg enzyme in 25 mM phosphate buffer, pH 7.0 and 19.8 % (v/v) and 2028 units/mg enzyme in 25 mM Tris-HCl buffer, pH 8.0, respectively. Olive oil in Tris-HCl buffer, pH 7.0 has lowest $K_{m,app}$. The quality of the fit for Lineweaver-Burk plots in Figure 13 and 14 is quite good, with average correlation coefficients of 0.9978.

3.9. Immobilized Lipase

3.9.1. Entrapment by Polyacrylamide Gel

Immobilization in polyacrylamide gel (PAG, 12 % (w/v)) was performed at room temperature. Monomer/crosslinker ratio was 36.5. A slab gel (4x4 cm) is prepared with a thickness of 1.5 mm. After polymerization is completed the gel was cut into small pieces (2x2x1.5 mm or 4x4x1.5 mm). Polyacrylamide concentration of 12 % had normally been used to produce structurally stable gels. Initial immobilization of C.rugosa lipase was performed using 450 mg enzyme preparation per gel. In order to maintain maximum level of accessible lipase activity per unit volume of gel, such a high level enzyme concentration was chosen but the resultant gel had poor mechanical properties. The enzyme concentration was lowered to a concentration of 45 mg per gel. The gel was sliced and olive oil hydrolysis was followed for 30 and 60 minutes. A very low activity was obtained (25 µmole FA with 5 slices). Optimum temperature (Fig.15) and optimum pH of the olive oil hydrolysis was found as 45°C and 6.5, respectively. Increase in slice dimensions from 2x2x1.5 mm to 4x4x1.5 mm keeping the enzyme load/slice constant, resulted appreciable decrease in activity. Lipase in wet gel gave the

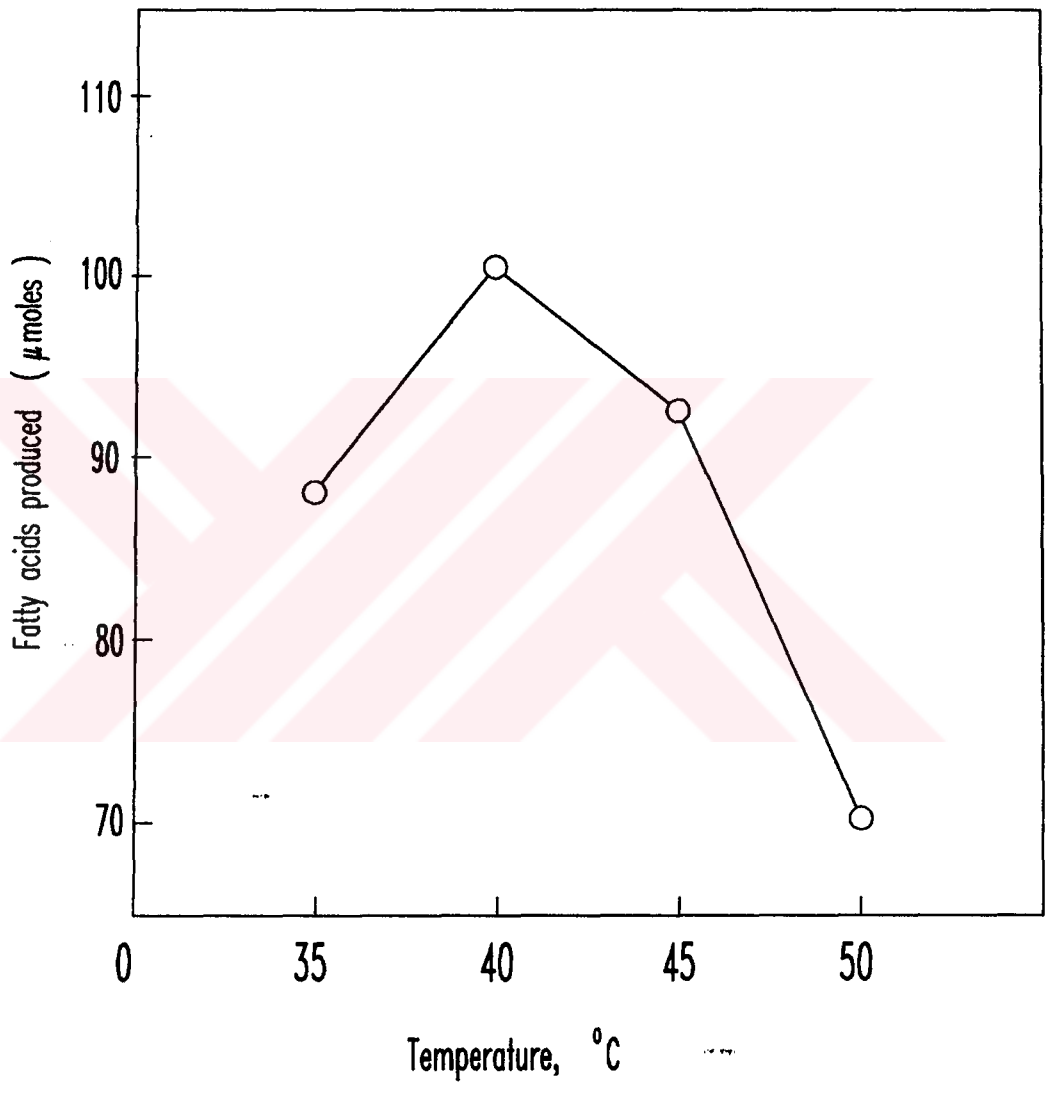

Figure 12. Effect of Temperature on Activity of Lipase. Reaction conditions: olive oil, 10 % (v/v); enzyme, 25 µg protein/ml; buffer, 25 mM potassium phosphate buffer, pH 7.0; reaction time, 10 min

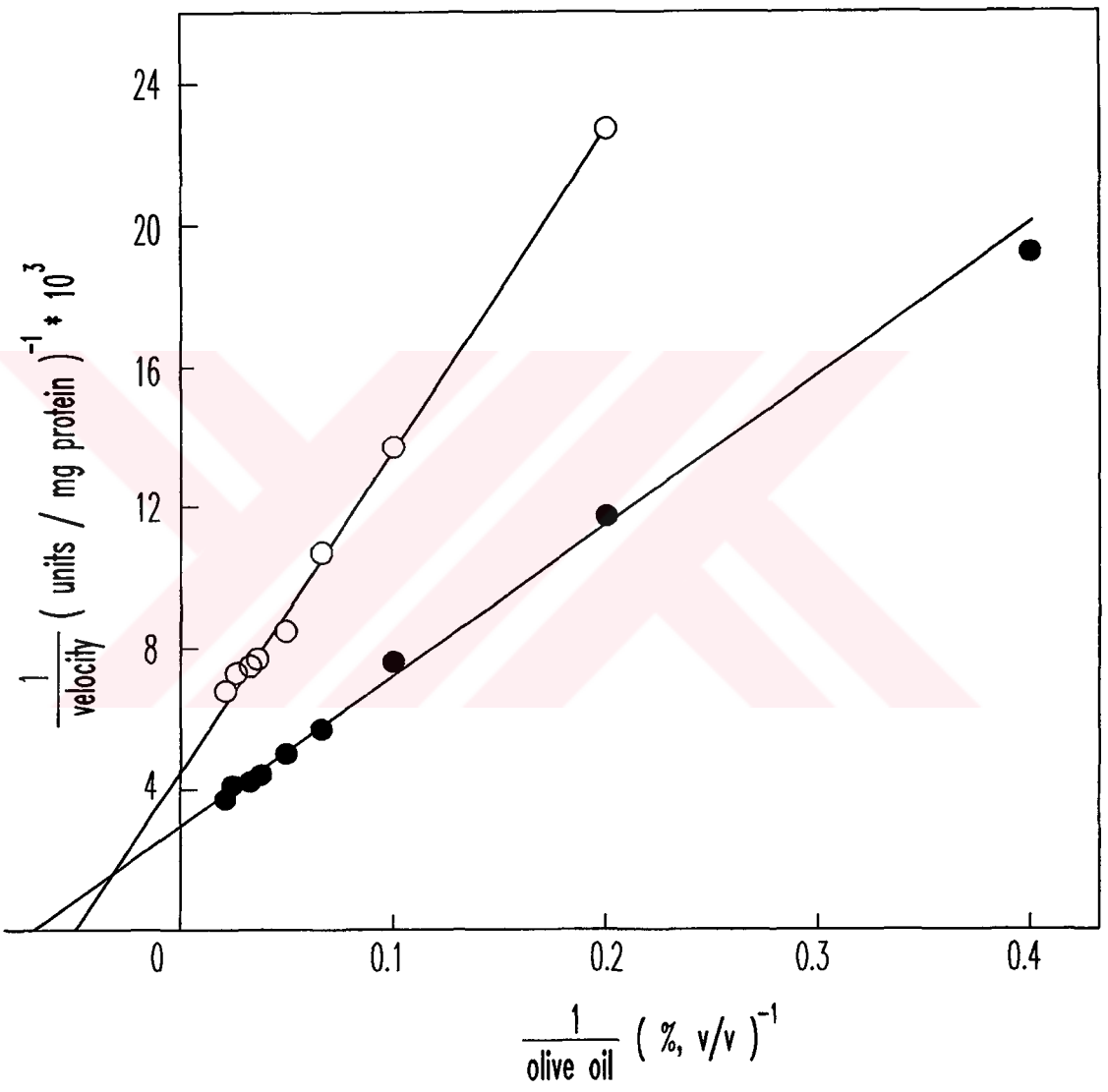

Figure 13. Lineweaver-Burk Plots of Initial Velocity as a Function of Olive Oil Concentration at Two pH Values. Enzyme concentration, 25 µg protein/ml; temperature, 37°C; reaction time, 10 min; buffer, 25 mM Tris.HCl. (o) pH 8.0, (●) pH 7.0

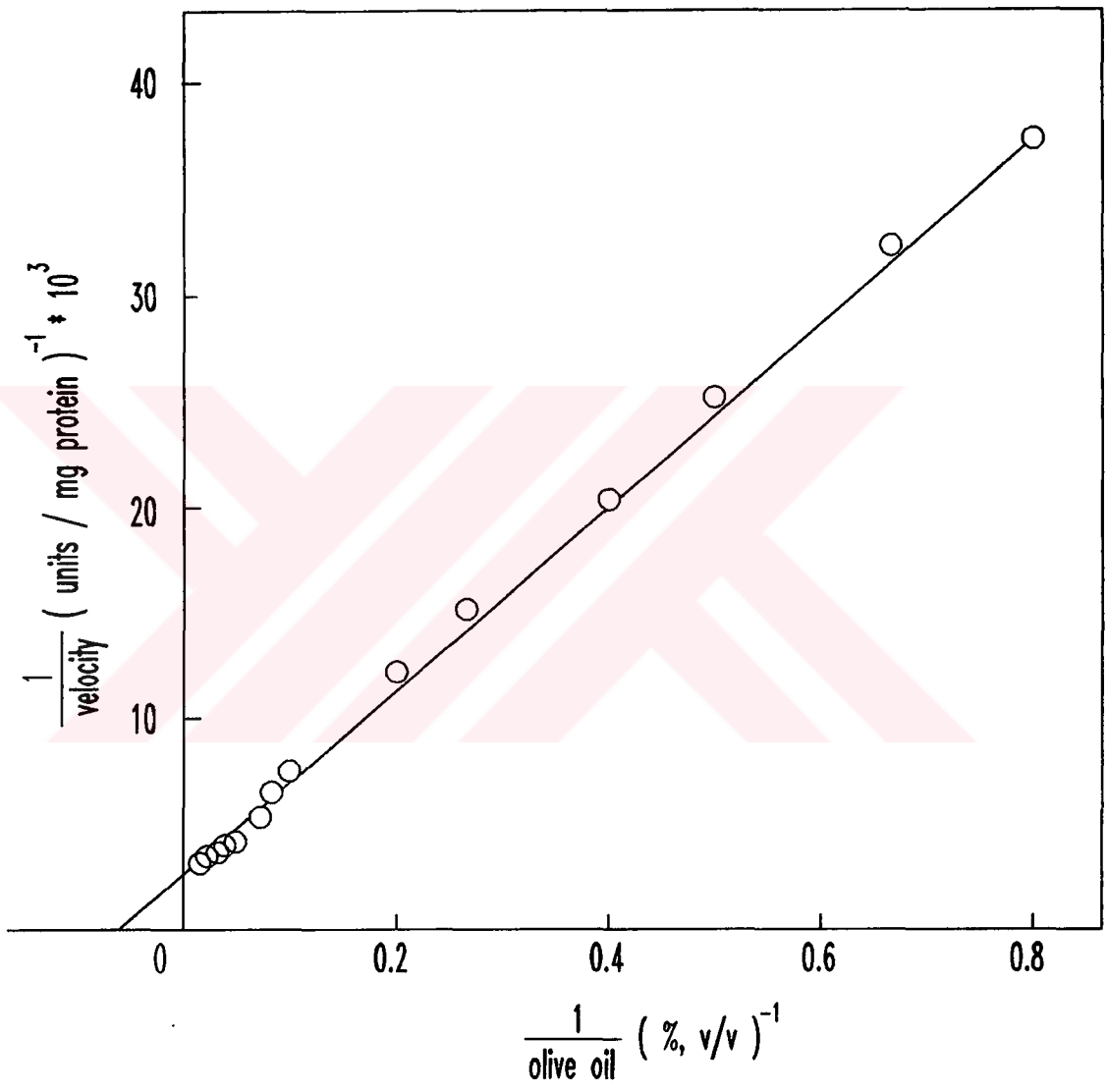

Figure 14. Lineweaver-Burk Plot of Initial Velocity as a Function of Olive Oil Concentration. Conditions as in Figure 13 except buffer (25 mM phosphate buffer, pH 7.0)

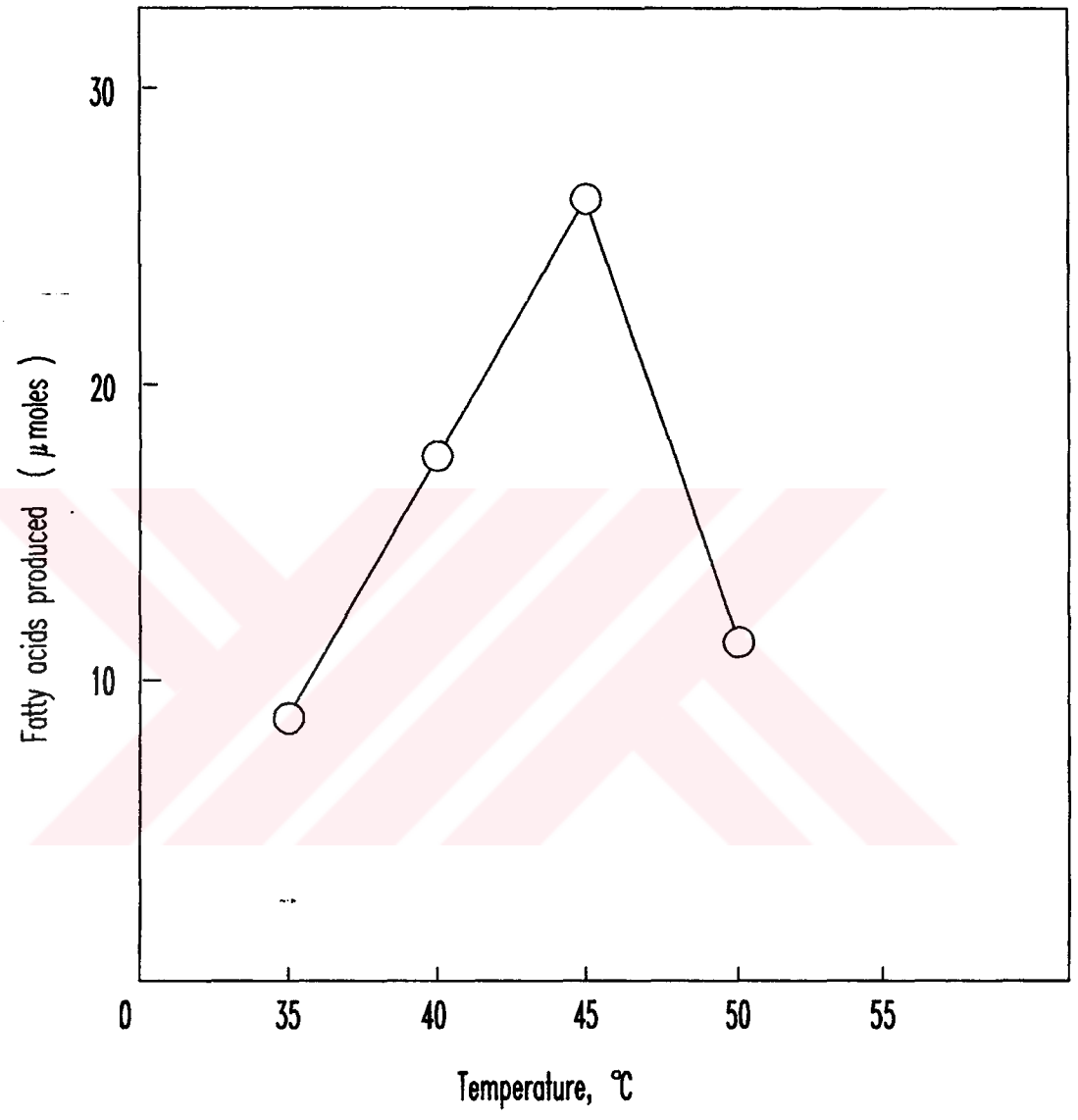

Figure 15. Effect of Temperature on the Activity of Entrapped Lipase. Reaction conditions: olive oil, 25 % (v/v); 0.20 mg protein/slice; 25 mM phosphate buffer, pH 6.5; slice dimension 4x4x1.5 mm; reaction time, 60 min

same activity with the lipase in dry gel.

The apparent difference in diffusion rates was tested by determining effectiveness factor when tributyrin and olive oil was used as the substrate. The effectiveness factor was found to be consistently decreasing with increasing size (C atoms) of the substrate: 0.106 for tributyrin, 0.014 for olive oil. Immobilized lipase exhibited higher rate with tributyrin compared to olive oil (Table 3) whereas free enzyme gave higher rate when olive oil was used as the substrate under the same experimental conditions.

Table 3. Substrate Specificity of Entrapped Lipase

Reaction time (min)	µmole fatty acids produced	
	Olive oil	Tributyrin
30	13.2	39.6
60	18.5	45.7
90	23.3	48.4
Reaction conditions: 25 %, v/v olive oil in 25 mM phosphate buffer, pH 6.5 at 45°C; enzyme load= 2 mg solid/assay; slice dimensions 4x4x1.5 mm, wet gel was used.		

It has been previously reported that the initiator potassium persulfate may reduce enzyme activity by oxidation of sulfhydryl groups [151]. Candida rugosa lipase has one free sufhydryl group [51]. In order to find the origin of the activity loss and the possible interference of free sulfhydryl group, enzyme activity was assayed with DMP-tributyrate as substrate. DTNB at a concentration of 0.3 mM was not rate-limiting. Doubling the concentration of DTNB caused 11 % increase in activity. The presence of a reducing agent such as cysteine at a concentration of 0.25 %, w/v for a persulfate concentration of 0.05 %, w/v might protect against this effect. Therefore PAG was prepared in the presence and absence of cysteine, and no activity difference was observed.

3.9.2. Adsorption on Celite

Immobilization of C.rugosa lipase on celite was done by acetone precipitation of enzyme or simple adsorption of enzyme after removing water by vacuum evaporation. The enzyme got forced onto the support

physically and dried onto it in the latter case.

Noncovalent immobilization of several enzymes was achieved by precipitation of enzyme from an aqueous solution by the addition of chilled acetone [101]. Such enzyme preparation usually show good stability when operated in organic solvents [143]. Under assay conditions of 12.5 % (v/v) olive oil in 25 mM potassium phosphate buffer pH 7.0, at 37°C in the absence of organic solvent, the enzyme preparation (20 mg protein/g celite) exhibited poor stability. Fatty acid release was 198 µmole FA and 18 µmole FA for the first and second use within 60 min reaction time, respectively.

Immobilization of lipase by simple adsorption using vacuum evaporation seemed to be more favorable to retention of the enzyme activity. Fatty acid produced was 340 µmole FA and 154 µmole FA for first and second use within 60 min reaction time, respectively. The enzyme loading used was high, being 20 mg protein/gr celite. The immobilized enzyme preparation gave poor results for repetitive use (Table 4). At 25 %, v/v substrate concentration the effectiveness factor was found as 0.90.

Table 4. Repetitive Use of Candida rugosa Lipase

# of use	µmole FA	Retained activity (%)
1st	295	100
2nd	84	29
3rd	31	11

Reaction conditions: Enzyme, 2 mg solid/assay; olive oil, 25%, v/v; buffer, 25 mM phosphate buffer, pH 6.5; temperature, 45°C; reaction time, 10 min.

The effect of temperature on the activity of celite immobilized enzyme was studied (Fig.16). The optimum temperature was shifted from 40°C to 45°C due to immobilization.

The effect of pH on the activity of immobilized lipase was compared with that of free enzyme (Fig.17). The optimum pH of immobilized enzyme was found as 6.5. A decrease of 0.5 pH unit was observed compared to free enzyme.

Olive oil hydrolysis was affected by the amount of enzyme adsorbed on celite (Fig.18). Fatty acid released versus enzyme

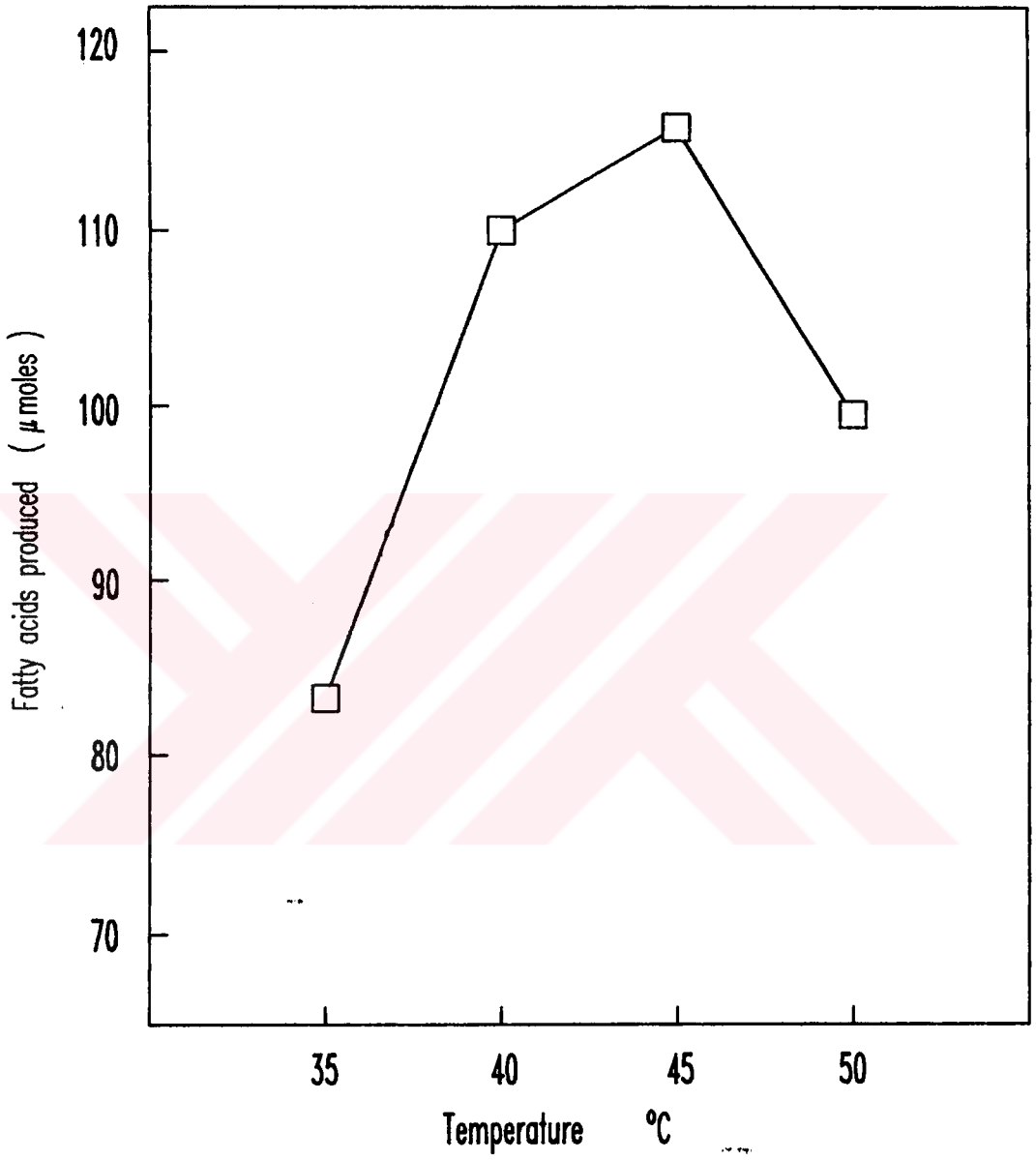

Figure 16. Effect of Temperature on Activity of Immobilized Lipase on Celite. Reaction conditions: olive oil, 12.5 % (v/v); 12.5 µg protein/ml; 25 mM potassium phosphate buffer, pH 7.0; reaction time, 20 min

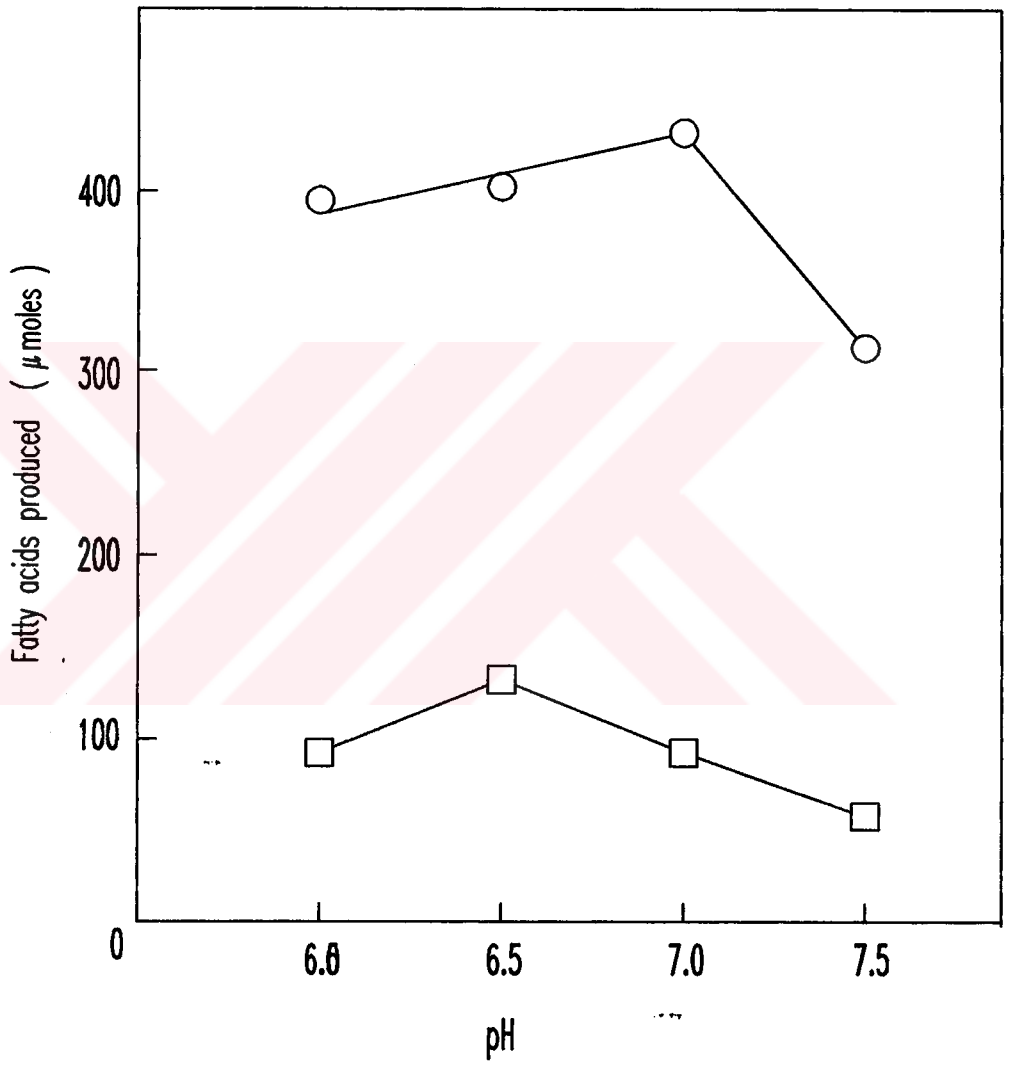

Figure 17. Effect of pH on Activity of Free and Immobilized Enzyme. Reaction conditions: 25 mM potassium phosphate buffer, 37°C; with free enzyme as in Figure 9 (o--o), with immobilized enzyme as in Figure 16 (□--□)

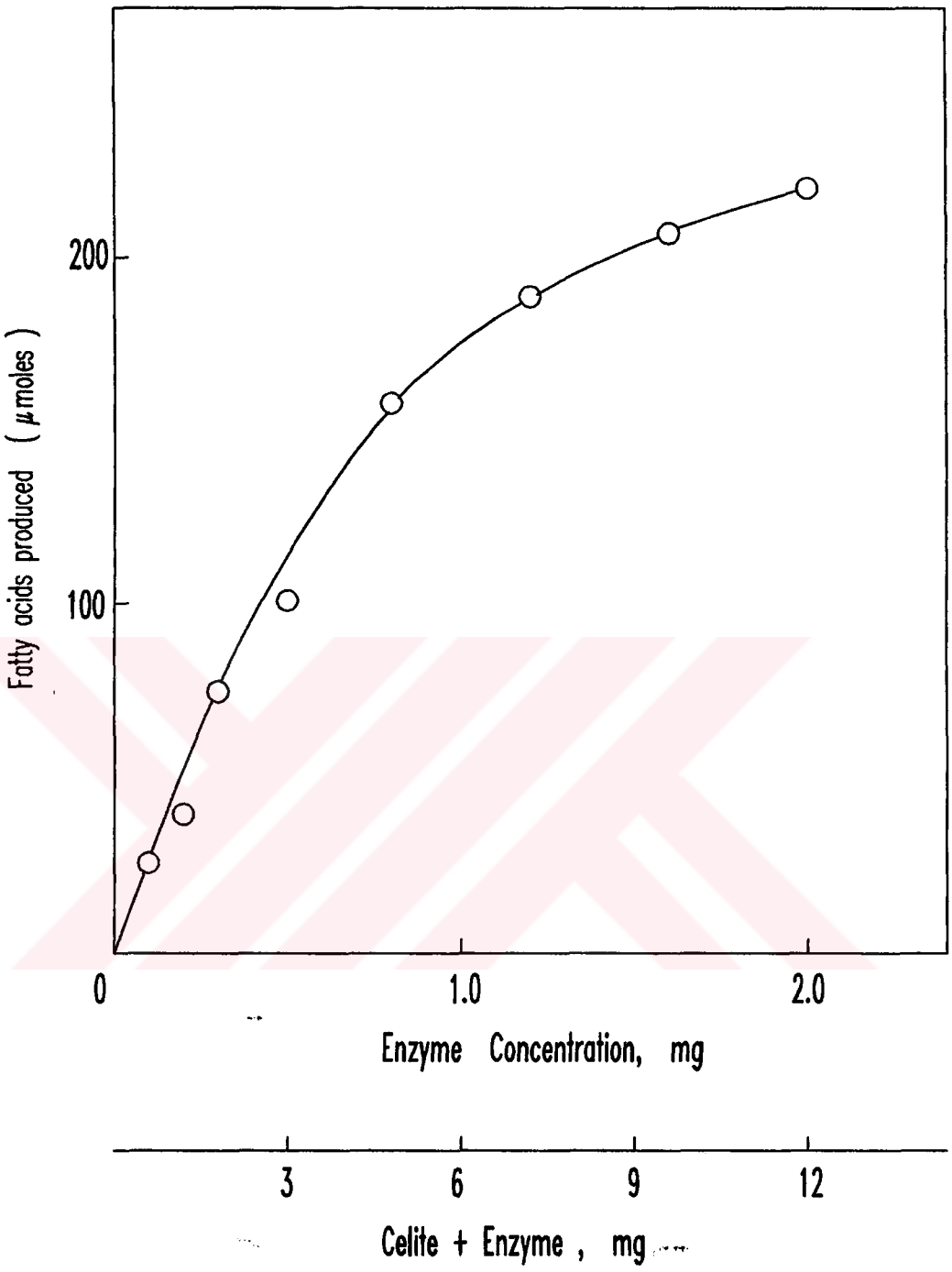

Figure 18. Effect of the Amount of Adsorbed Enzyme on Olive Oil Hydrolysis. Reaction conditions: olive oil, 12.5 % (v/v); temperature, 37°C; buffer, 25 mM potassium phosphate buffer, pH 7.0; reaction time, 20 min; reaction mixture, 4 ml

concentration was linear up to 0.5 mg enzyme (50 µg protein). After this point % hydrolysis exceeds 5 % (Table 5) and reaches 16 % under the catalytic activity of 2 mg adsorbed enzyme preparation.

Table 5. Dependence of Olive Oil Hydrolysis on Enzyme Concentration

E, mg	E+Celite, mg	% Hydrolysis
0.1	0.6	1.9
0.2	1.2	2.9
0.3	1.8	5.5
0.5	3.0	7.4
0.8	4.8	11.6
1.2	7.2	13.8
1.6	9.6	15.1
2.0	12.0	16.1

Figure 19 shows the fatty acid formation as a function of reaction time at fixed substrate (12.5 %, v/v) and enzyme (2 mg/assay = 0.2 mg protein/assay) concentrations. The progress curve of the olive oil hydrolysis gave a typical hyperbolic form. The fatty acid formation was linearly proportional to the reaction time up to 20 min. At the end of 60 min reaction time 24 % olive oil hydrolysis was found. Under optimum conditions (pH 6.5 and T=45°C) with 25 %, v/v olive oil and 0.2 mg protein/assay, the % hydrolysis increased up to a value of 90 % at the end of 12 hours reaction time (Fig.20). The solvent and emulsifier free system used in this study was found to be suitable for the olive oil hydrolysis under the catalytic activity of celite immobilized Candida rugosa lipase.

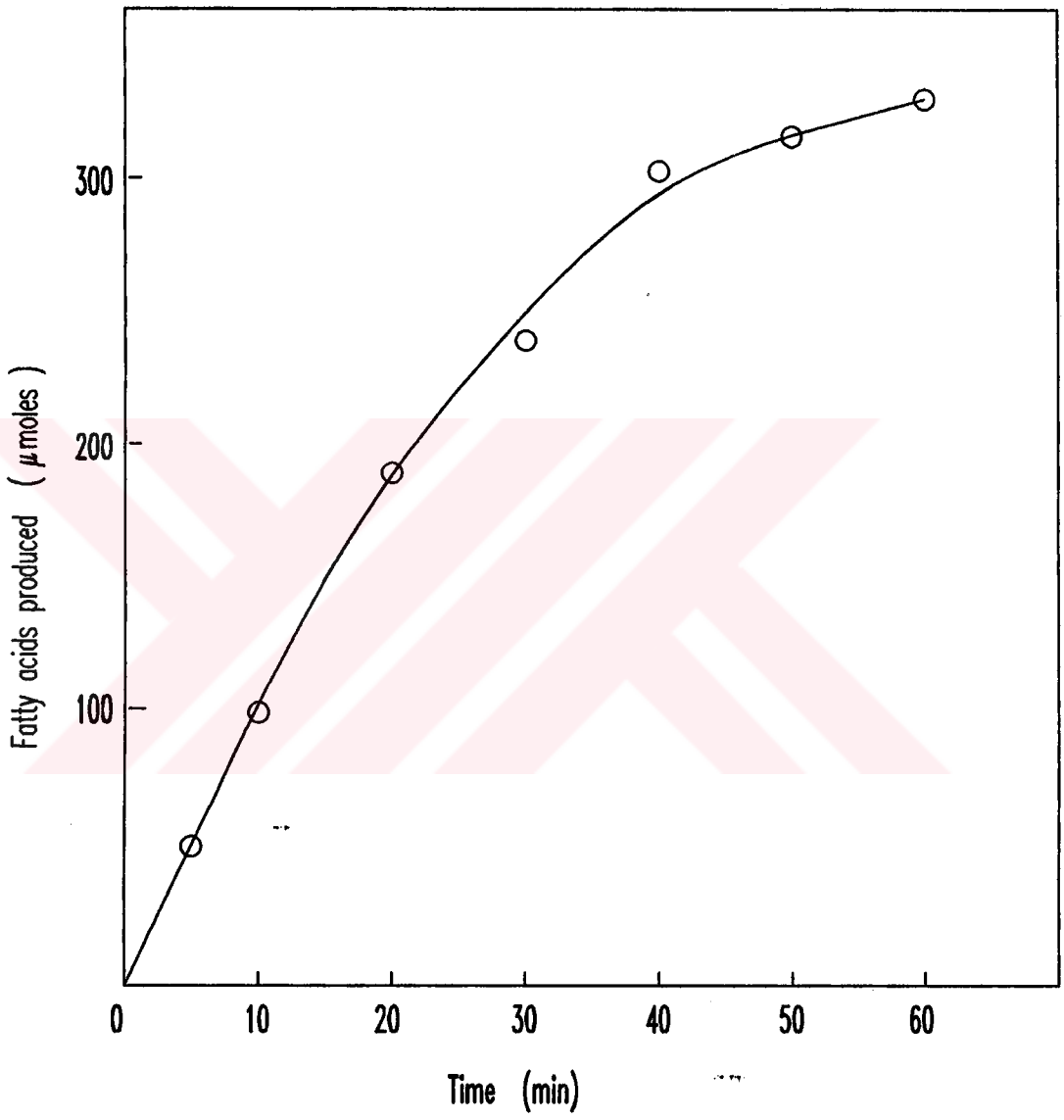

Figure 19. Time Course of Olive Oil Hydrolysis by Immobilized Lipase on Celite. Reaction conditions as in Figure 18. Enzyme concentration is 2 mg solid/assay (12 mg celite+enzyme per assay)

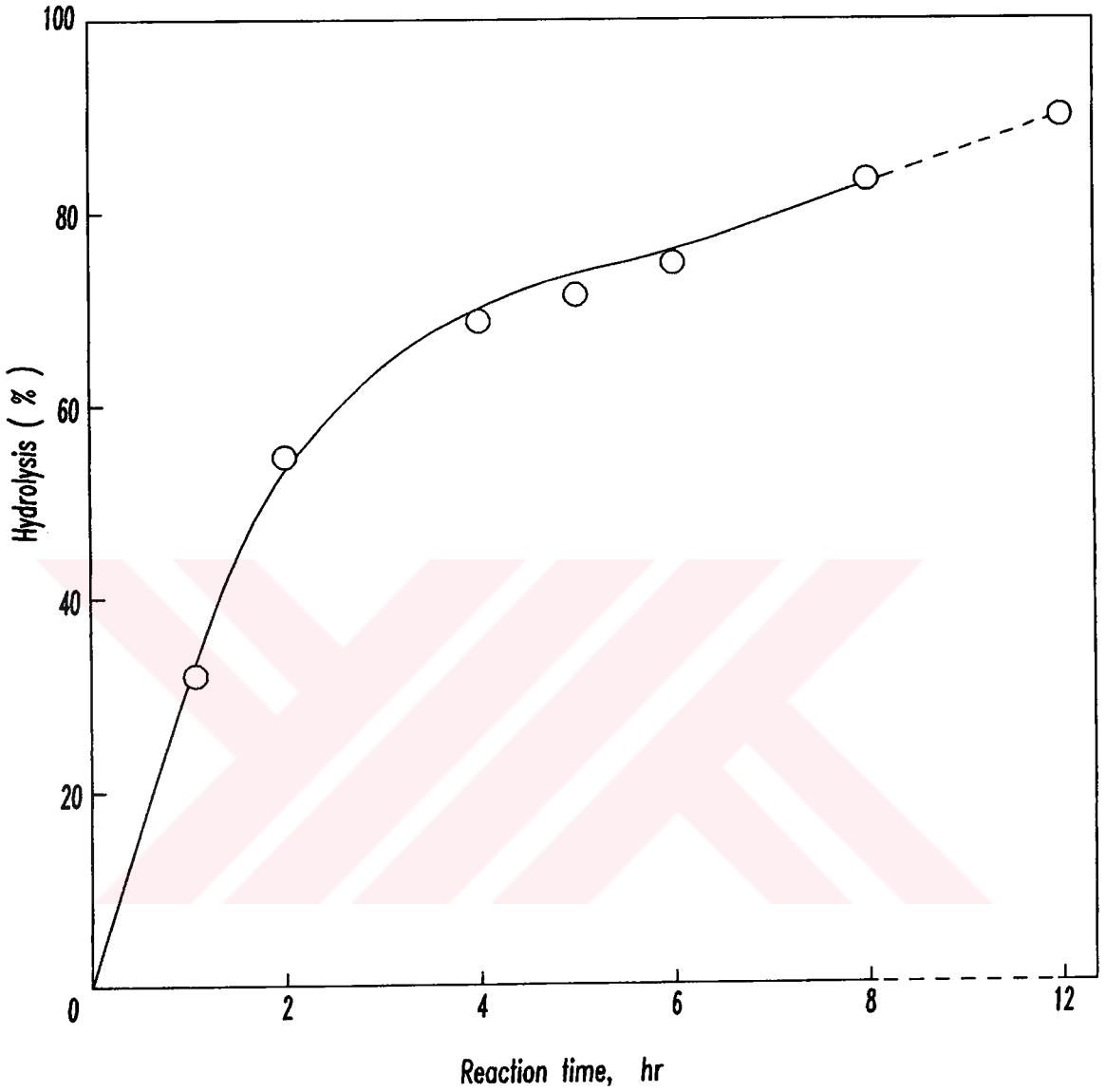

Figure 20. Percentage Hydrolysis as a Function of Time by Celite Immobilized Lipase. Reaction conditions: olive oil, 25 % (v/v); temperature, 45°C; buffer, 25 mM potassium phosphate buffer, pH 6.5, enzyme concentration is 2 mg solid/assay

CHAPTER 4
DISCUSSION

4.1. Enzyme Used

Lipase from Candida rugosa was used in the study of olive oil hydrolysis. The commercial enzyme contained 10 % protein. The enzyme was fractionated by gel filtration on Sephadex G-100 and the enzyme purification was about 6-fold with a yield of 65 % and a specific activity of 1310 U/mg enzyme with 25 % (v/v) olive oil. The fraction of pure lipase in the mixture was determined as 1.1 %. The purified enzyme showed a single protein band on SDS-polyacrylamide gel electrophoresis. Its molecular weight was estimated to be 56 000 on both SDS-PAGE and gel filtration, suggesting that the enzyme was a monomer. The heterogeneity previously reported may be due to proteolytic or degradative breakdown or a conformational change induced during purification [27].

The enzyme was found to be partly inhibited by EDAC and o-PDA. These results suggested that carboxyl groups, lysyl and cysteinyl residues might be involved in the catalytic site or substrate-binding site of this enzyme and also methanol (cosolvent for o-PDA) might protect the catalytic site and/or the substrate binding site from modifier [51]. The identities of the residues modified by EDAC and o-PDA in the two phases of the inactivation processes remain to be determined. At present, the mechanism of lipase catalysis and substrate binding is unknown.

4.2. Assay Method

Recently, the use of lipases for hydrolysis reactions in the absence of emulsifier has received considerable attention [45,85]. This assay system has several advantages. The procedure excludes the step to make a fresh emulsion at every experimental run and also the addition of activators besides emulsifier. Consequently many samples can be simultaneously treated.

In the conventional laboratory assay of lipase, gum arabic is usually added besides emulsifier to emulsify the mixture of oil and water [27]. The reproducibility of the data obtained using this enzyme assay system is relatively poor due to the tendency of the emulsified oil droplets to coagulate after a prolonged period of storage. If lipases were applied in industry, an emulsifier should be avoided because it would increase the cost of product separation. In addition, the kinetic data obtained in the presence of emulsifier could not be applied directly for industrial usage since the emulsifier, by acting as a surface impurity, may change the lipase-catalyzed reaction rate.

The Candida rugosa lipase is a good candidate for industrial lipase application, because of its random specificity and somewhat inexpensive price. Therefore, in this study lipase-catalyzed olive oil hydrolysis was followed in the absence of any added emulsifier.

4.3. Kinetics of Free Enzyme

The kinetic analysis of the lipase-catalyzed hydrolysis reaction was found to be possible in the absence of any added emulsifier. The specific enzyme activity was 220 units/mg protein at 37°C in 25 mM phosphate buffer, pH 7.0 at 25 % (v/v) olive oil concentration. Maximum activity was obtained at pH 7.0 and at low buffer ionic strength, 25 mM. The observed optimal pH value for olive oil hydrolysis in this study is different from that reported by Wang et.al [45]. They found two optimal pH values at pH 5.0 and pH 7.0. Optimum temperature was found as 40°C.

It is well known that lipases are surface-active enzymes that are being adsorbed at the oil-water interface to carry out the lipolytic reaction. For lipid splitting, it is assumed that the enzymatic reaction follows the Michaelis-Menten model [42,70,86]. The hydrolytic reaction preceeds as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \ (P's) \qquad (1)$$

If the interfacial concentration of the substrate is larger than that of the enzyme, the pseudo-steady-state approximation for the enzyme-substrate complex may be used to give

$$K_m = (k_{-1} + k_2)/ k_1 \rightleftharpoons (E)_i (S)_i / (ES)_i \qquad (2)$$

where $(E)_i$, $(S)_i$ and $(ES)_i$ represent the interfacial concentrations of enzyme, oil substrate and enzyme-substrate complex, respectively. Since enzyme can be dissolved only in the aqueous phase, one may assume that

$$b_1 = [(E)_i + (ES)_i] / (E)_a \quad (3)$$

$$b_2 = (S)_i / (S)_0 \quad (4)$$

where b_1 and b_2 are the proportionality constants, and $(E)_a$ and $(S)_0$ are the enzyme concentration in the aqueous phase and total olive oil concentration, respectively. The conservation of enzyme is written as

$$[(E)_i + (ES)_i] A + (E)_a V_a = E_t \quad (5)$$

From eqs. (2), (3) and (5), one obtains

$$(ES)_i A = E_t / (1 + V_a / b_1 A) / (1 + K_m / (S)_i) \quad (6)$$

where A, E_t and V_a represents total interfacial area of the oil-in-water emulsion, total mass of enzyme and volume of aqueous phase, respectively. Thus, combining eqs. (4) and (6) gives the reaction rate as

$$v = k_2 (ES)_i A = V_{max} (S)_0 / (K_m^1 + (S)_0) \quad (7)$$

in which the apparent maximum velocity, V_{max} and the apparent Michaelis-Menten constant, K_m^1 are defined as

$$V_{max} = k_2 E_t / (1 + V_a / b_1 A) \quad (8)$$

$$K_m^1 = K_m / b_2 \quad (9)$$

Under the assumption that all olive oil droplets of the oil-in water emulsion are of the same size and spherical in shape, the total interfacial area can be expressed as the following:

$$A = n4\pi R^2 = 3n(4/3)\pi R^3 \; / \; R = 3V_0 \; / \; R$$

or

$$R = 3V_0 \; / \; A \; ; \quad D = 6 \; V_0 \; / \; A$$

where n, R, V_0 and D are the number of oil droplets, the radius of the oil droplet, volume of the oil phase in the emulsion, and the diameter of the oil droplet in the oil-in-water emulsion.

The rate of olive oil hydrolysis was followed against substrate concentration for 10 min reaction time and 25 µg protein/ml at 37°C. In this reaction, the time has to be limited to the time about 5.0 % of the substrate is utilized. Lipase was found not to be inhibited by the substrate concentrations up to 50 % (v/v), and thus the condition for Michaelis-Menten kinetics that the rate does not change during the period when a small fraction of the substrate is utilized was satisfactorily met. At substrate concentrations below 50 %, v/v (1.37 M, mid point between oil-in-water and water-in-oil emulsion) olive oil, the reaction obeys Michaelis-Menten kinetics, and the Michaelis constant K_m and k_{cat} (k_2) were found to be 16.7 %, v/v (0.46 M) and 3472 µmole per min per mg enzyme, respectively at 37°C in 25 mM phosphate buffer, pH 7.0.

The rate of olive oil hydrolysis against enzyme concentration was linear up to 0.1 mg protein/ml. Ekiz and Caglar have conducted an experiment on the adsorption of Candida rugosa lipase at the tributyrin-water interface [75]. The results showed that monolayer adsorption was predominant when the enzyme concentration was less than 0.286 mg/ml at 600 rpm. Under this condition, a constant ratio of the amount of enzyme adsorbed at the interface (or the initial reaction rate) to the total mass of enzyme might be obtained. Above this concentration multilayer formation starts, resulting in lower rates of hydrolysis owing to undesirable conformational changes in the enzyme molecules and mass transfer difficulties impeding the reaction products as they diffuse away from the interface. In Figure 13, 14, the applied lipase concentrations were low enough to assume monolayer adsorption, yielding the fixed proportionality constant (b_1) defined in eq. (3).

The molecular area occupied by the lipase of Candida rugosa at the tributyrin/water interface was estimated as 1.83×10^{11} cm^2/mole [75]. Since the total interfacial area of oil-in-water emulsion (A=1200 cm^2)

assuming an oil diameter of 50 μm [45] is greater than the molecular area of the enzyme used (35 cm^2) we concluded that mass transfer does not influence the reaction rate. The rate of reaction is solely controlled by enzyme kinetics.

4.4. Immobilization of Enzyme

4.4.1. Entrapment by Polyacrylamide Gel

The diffusion of triglycerides into the gel from the bulk solution will depend on their size; smaller triglycerides containing mainly short- and medium-chain fatty acids will diffuse faster than their larger counterparts [36].

From the kinetic theory of immobilized enzyme systems with internal diffusion effects, the effectiveness factor is a function of the Thiele modulus [152]. This modulus has the physical interpretation of enzymatic reaction rate divided by substrate diffusion rate. The effectiveness factor was shown to increase with decreasing Thiele modulus (or reaction rate), rendering the overall enzyme kinetics less diffusion controlled. Since reaction rate is proportional to enzyme load in the gel (units of enzyme per gram of gel), this factor was expected to influence the diffusion rate and the free fatty acid profile [36].

The effectiveness factor (η) was found to be consistently decreasing with increasing size (# of carbon atoms) of the substrate: 0.106 for tributyrin, 0.014 for olive oil. The optimum pH and optimum temperature of entrapped lipase was 6.5 and 45°C. Preparing entrapment in the presence of cystein did not cause any increase in the activity. Further studies are needed to determine the reason for this low activity.

4.4.2. Adsorption on Celite

The use of organic solvent-free systems in the oils and fat industry is rare, especially for systems employing immobilized enzymes. Organic solvent free systems offer several advantages, including greater safety, the reduction in solvent removal costs.

Important information concerning the activity of lipase from Candida rugosa has been obtained. The immobilization procedure developed was quite simple and easily reproduced and high protein loadings were obtained. From results of hydrolysis reactions, it has been shown that adsorption on celite changes the inherent activity of

the enzyme; for example, the pH profile and optimum temperature were changed. Optimal pH and temperature was found as 6.5 and 45°C, respectively.

An important fact of an immobilized enzyme preparation is, in general, the retention of activity through the immobilization procedure and retention of this activity over the prolonged periods of storage time in refrigerator. Generally, the retention of activity after immobilization of lipase is below 50 % of original activity [131]. Goderis et.al [111] reported about 85-90 % of the activity was retained for a lipase (Rhizopus arrhizus) preparation adsorbed on Celite 535. This activity was retained for several weeks, both in the dry state and in the presence of organic substrate solution. In addition, the specific activity of immobilized lipase preparation was found to be higher than that of the free enzyme [107,134]. The particle size of celite did not seriously affect productivity [66].

The immobilized lipase was quite stable and retained about 56 % of the initial activity during the course of 6 months. Free fatty acid formation in repeated experiments using the same immobilized enzyme catalyst (protein loading=0.2 mg) exhibited a rapid loss of enzyme activity. The most important problem of this application with Candida lipase appears to be the rapid loss of enzyme activity during repetitive use. Further investigations are needed to determine the cause of this activity loss.

Immobilization of lipase on celite affected the olive oil hydrolysis even though immobilized enzyme was never more efficient than free enzyme. The effectiveness factor for Candida rugosa lipase was found as 0.90. This is in agreement with the findings of Lie and Molin [140] but in contrast to those of Cho and Rhee [107] who found that immobilization on a hydrophobic carrier enhances the lipase activity. A probable explanation is that the enzyme may be discharged from the carrier during the course of the experiment.

Lipase immobilized on celite gives relatively higher activity than entrapped lipase. One reason might be that the enzyme immobilized on celite gives selfstabilization and fully retained enzymatic activity due to the smaller surface area of celite [143]. Under experimental conditions optimized in this work the percent hydrolysis up to a value of 90 % at substrate concentration of 25 %, v/v was obtained. These results suggested that Candida rugosa lipase immobilized on celite

could be used in industry due to its relatively high olive oil hydrolysing activity.

Enzymatic hydrolysis of olive oil with lipase has been studied as a low energy alternative to the present high temperature process. But enzyme splitting has not yet gained acceptance because reaction rates are low in comparison to existing industrial processes. Also the capital investment for plant is high, which excludes small or middle scale operation. However it offers the benefit of applying economically a much higher reusable enzyme loading per unit of reactor volume.

The methodology presented in this work might be used to find kinetic parameters for reactor design and scaleup.

CHAPTER 5
CONCLUSIONS

1. The rate of olive oil hydrolysis under the catalytic activity of C.rugosa lipase was reproducibly measured without using any emulsifier and stabilizer.

2. Commercial C.rugosa lipase was impure, containing only 1.1 % pure lipase. Olive oil hydrolysis seemed to be catalyzed by this single lipase component. Therefore the enzyme used throughout the work without further purification except molecular weight determination.

3. The hydrolysis of olive oil obeys Michaelis-Menten kinetics under the experimental conditions in which the steady state assumptions were satisfactorily met.

4. Immobilization of enzyme by adsorption and entrapment increased the optimal temperature by 5°C and optimum pH was lowered by 0.5 pH unit. The increase of optimum temperature to 45°C makes the system applicable for fats also. The change in optimum pH is most likely due to the conformational change during adsorption.

5. Entrapment in PAG changed the substrate specificity toward low carbon fatty acids. This system can be used for selective triglyceride hydrolysis.

6. Adsorption on celite by evaporation gave better results in terms of effectiveness factor, reuse and stability compared to acetone precipitation. The immobilized Candida rugosa lipase thus obtained seemed to be feasible for various applications of triglyceride hydrolysis.

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