LIPASE PRODUCTION BY
CANDIDA RUGOSA

A THESIS SUBMITTED
TO THE FACULTY OF SCIENCE UNDER THE
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY

BY

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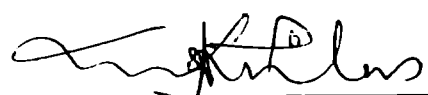UNDER THE SUPERVISION OF

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JUNE, 1997

*Dedicated
to the
Ignorant Poor
on Earth*

DECLARATION

I, Sailas Benjamin do hereby declare that the thesis entitled **Lipase Production by** *Candida rugosa*, is an authentic work accomplished by me under the supervision of **Dr. Ashok Pandey**, Senior Scientist, Regional Research Laboratory (Council of Scientific and Industrial Research), Trivandrum, and also declare that the contents in this thesis have not previously formed in any form for the award of any degree, diploma, associateship, fellowship or other similar title or recognition.

SAILAS BENJAMIN

Dr. Ashok Pandey
Project Leader

23rd June 1997

CERTIFICATE

This is to certify that, to the best of my knowledge and belief, the thesis entitled **Lipase Production by** *Candida rugosa*, is a record of bonafide research carried out by **Mr. Sailas Benjamin**, Research Scholar, under my supervision, and also certified that the contents in this thesis have not previously formed in any form for the award of any degree, diploma, associateship, or other similar title or recognition.

Dr. ASHOK PANDEY

ACKNOWLEDGEMENTS

Sailas Benjamin

Trivandrum
23rd June 1997

PRELUDE

This thesis contains five parts. First part embodies general introduction, followed by significance and specific objectives of the present study. Literature Survey, the second part, deals with a comprehensive description on the systematic position, fermentation aspects and industrial significance of *Candida rugosa* and its lipases. Part three is the sum and substance of the research work in the Thesis entitled **Lipase Production by *Candida rugosa*,** and is subdivided into nine chapters (III A to I). Part four is devoted to summary, conclusion and prospects of the present work. The fifth and last part consists of bibliography, list of publications and a synoptic bio-data.

All the tables and figures that appear in each part or chapter are inserted at the appropriate or nearby locations of their reference in the text. If the chapter contains a photograph (Figs.IIIA 4-7; IIIC 3; IIID 2,3,5 & 8; IIIF 3; IIIG 3 and IIIH 3) that is attached at the end of the corresponding chapter. Numbering system adopted to designate tables and figures is in accordance with the part/chapter in which they appear. Roman letters are used to denote the respective Part and the suffixed English alphabets represent Chapters, followed by Arabic numerals, which designate specific tables/figures.

Since diverse materials and methods were employed for experimentation, to avoid repetition and confusion, materials and methods, once described in any one of the preceding chapters is simply neglected in the succeeding chapters. For instance, microorganism, medium for maintenance, assay methods, etc.

For literature citation, the standard system observed in the international journal **Yeast,** is followed throughout the text and in structuring the bibliography.

CONTENTS

PART - I

GENERAL INTRODUCTION
AND
SIGNIFICANCE

GENERAL INTRODUCTION

Multifaceted microbial lipases [triacylglycerol hydrolase (EC.3.1.1.3)] have an invincible domain in swift-growing modern biotechnology. They have wide-spread occurrence throughout the flora and fauna, though more abundant in the microflora comprising bacteria fungi and yeast. Lipases have manifold features that favour their use as an excellent biocatalyst. They impart specificity to a reaction where a chemical process is typically more unspecific. Furthermore, enzymes may be well cost-effective by comparison with traditional downstream processing, where energy consumption and toxic by-products might often pose problems. Enzymes perform their reaction at 'mild and neutral conditions', thus the burden on the environment from by-products of enzymatically catalyzed industrial reactions will typically be significantly less harmful. Industrial deployment of lipases can either be *in situ* by growing the desired microbe directly in the substrate (especially in food and tanning industry) or by *ex situ* using purified commercial lipases, particularly in fine chemical making. Solid-state or Submerged fermentation technology is mostly employed in the former, while the latter prefers immobilized enzymes in abundance.

Though lipases have been studied for many years and can be produced on large scale by growing microorganisms in fermenters (in contrast to other major groups of hydrolytic enzymes such as proteases and carbohydrates), the use of lipases were mainly confined to the products and processes of oleo-chemistry and dairy based industries. In fact, the last quarter of the present century witnessed an unprecedented stride of lipases into the novel horizons of biotechnology in tandem with already established pastures of tanning, pharmaceuticals, pesticides and of late,

in the production of single cell proteins, cosmetics, waste disposal and in biosensor modulations. The main reason for this steady growing interest in lipases, reflected by an average of 1000 publications appearing per annum, is the biotechnological versatility of these enzymes including their potential to catalyse the hydrolysis and also the synthesis of esters, which was also recognized nearly 70 years ago.

As a matter of fact, in contrast to bacterial lipases, most of the commercial applications of lipases concern enzymes of either fungal or yeast origin by dint of their proven GRAS status (generally regarded as safe). In as much as, literature survey shows, among all yeasts, *Candida rugosa* is the widely studied lone member in terms of lipase production. Conventionally, submerged fermentation or suspension culture is being employed for its cultivation. However, emerging trends emphasize that semi-solid and solid-state fermentation systems could offer much potentialities for the judicious management of *C. rugosa* in industry. Being a pseudo-filamentous, unicellular and nonpathogenic yeast, *C. rugosa* (synonymous as *C. cylindracea*), synthesises and secretes a mixture of lipase iso-enzymes that have been studied by several authors starting from commercial preparation of the enzyme and goaled at the zenith of its purification and characterization to venture on the growing needs in the modern LIP-TECH bio-industry.

SIGNIFICANCE

OF

PRESENT STUDY

Soon after the discovery of lipase secretion by *Candida rugosa* in 1963, *C. rugosa* was patented in the United States of America for commercial production of lipases. Henceforth, an average of one-fourth of a hundred publications appearing every year in the world literature tokens the inevitability of *C.rugosa* and its lipases in biotechnology. This world-wide enthusiasm enabled *C. rugosa* and its lipases for ushering into novel frontiers of biotechnology and to establish a new era in lipase industry.

Nevertheless, statistics on *C. rugosa* lipases (CRL) production over the world is so miserable. Most of the production formulae were focused on submerged fermentation (SmF) under controlled conditions. Notwithstanding, maximum production reported so far is only around 12 U ml^{-1}, using SmF technolgoy. Exploitation of the promising Solid-State Fermentation (SSF) technology could enhance the yield to 36 U g^{-1} rice bran, which was found as much less than the yield obtained from bacterial counterpart. Though industrial production conditions are in vogue, the quest for adopting efficient methodologies are going on in the laboratories. Except a few isolated attempts, thus far, no active involvement of India is witnessed. In India, being predominantly an agrarian country, the recycling of agricultural refuses and its utility through much economic SSF technology has to be diversified with great thrust. The least exploited area is the whole-cell immobilization strategy. This technology has already gained much advancement for the production of valuable microbial products employing living cells of both plant and animal origin for the production of primary as well as secondary metabolites. However, only very little knowledge is available on the immobilization of *C. rugosa* whole cells for the overproduction of lipases. These lacunae in CRL production are the basic motivation behind the present study.

Strain improvement is one of the major objectives for maximizing the microbial production of industrially significant primary and secondary metabolites. This goal can be achieved by judicious tuning of the organisms by monitoring its growth parameters and optimizing adequate supply of micro and macro nutrients, inducers, pH, temperature and other factors which control fermentation. Though *C. rugosa* has been under extensive studies for lipases, maximum world production is only 36 units. In fact, in India, enhanced production conditions for lipases have not yet been initiated. *C. rugosa* has been cultivated in diverse environments like liquid, semi-solid, solid-state and immobilized conditions, though major emphasis is on SmF or suspension culture. Hence the present investigations mainly focused on increasing the yield by adjusting the physico-chemical growth parameters and to characterize the lipase isoforms secreted by *C. rugosa* in the culture medium. Maximum possible improved methods were investigated to achieve these objectives.

Within this under-optimised background, enhancement of lipase production and its characterization were investigated, employing modified liquid, semi-solid, solid-state and immobilized fermentation strategies, keeping the following objectives in view:

(a)   to optimize growth conditions for *Candida rugosa*

(b)   to study lipase production by submerged fermentation

(c)   to study lipase production under immobilized condition

(d)   to study lipase production by semi-solid fermentation using coconut cake extract

(e)   to study lipase production by solid-state fermentation on coconut cake and wheat bran

(f)   isolation, purification and characterisation of lipase isoforms

PART - II

LITERATURE REVIEW

Introduction

Extracellular lipases (EC. 3.1.1.3)[92] produced by microorganisms are actively being investigated by dint of their panoramic use in biotechnological processes. There has been a cosmopolitan spurt in the screening of lipase producing organisms and their utilization in the surmount biotechnological processes for human welfare[21,22]. It is this enthusiasm which led the investigators to introduce *Candida rugosa* lipases (CRL) in bioindustry. In as much as literature survey picturises, *C. rugosa* has been extensively studied microorganism in view of its lipase secretion, employing both submerged as well as solid-state fermentation technologies[94,136].

Isolation and purification of lipases from different sources (animals, plants and mostly microorganisms) have been reported, owing to their wide spectrum of applications in biotechnology[21,48]. Unprecedented interest towards this biocatalyst can be attributed to its inherent role in the hydrolysis, esterification and trans-esterification reactions, especially in food and flavour synthesis, tanning, detergent and oil based industries, optical resolution of fine chemicals for pharmaceuticals and of late for biocides, biosensors, waste disposal, etc[22,197]. In fact, microbial lipases are easily obtained on large scale making them particularly attractive for industrial applications[8]. In the microflora, the unicellular yeast, *Candida rugosa* was proved to be a potent producer of lipases[174,254]. The non-specificity of *C. rugosa* lipases with respect to acyl position, and its high stereospecificity coupled with substrate specificity have become an urge for its maximum production[27,175].

CRL has been used in a wide range of catalytic reactions in both aqueous and water-restricted environments which include non-specific and stereospecific hydrolyses to reversal of hydrolysis *via* esterification, trans-esterification and inter-esterification[136,192]. So far, no lipase is available as much specificities (substrate positional, fatty acid and stereopreference) as attributed to CRL[22]. It has strong indiscrimination towards the position of ester bonds in the glycerides or primary and secondary esters, and accepts different saturated as well as unsaturated esters with varying chain-length of acid and alcohol moieties[24,27]. As a matter of fact, to compensate the exponential hike in the market value, CRL is mostly employed in the immobilized form, being entrapped in durable and cheap supports with prolonged life. *In toto*, modern knowledge on the ultrastructure and ever rising kinetic and characterisation studies of CRL in anticipation with brilliant hydrolysis *versus* synthesis, fascinating specificities and fatidical entrapment techniques have greatly revolutionized the CRL-based bio-industry[24].

Enchanting positionally non-specific biocatalysis of fat and lipid containing substrates attracted CRL towards the core of dairy-based food industry, mainly for the synthesis of flavour and aroma as valuable food additives. However, the scope of CRL broadened when it entered into the detergent formulations, as bio-surfactants and in tanning industry along with other major hydrolytic enzymes[150]. Modern eco-friendly concept, which attained tremendous momentum in this quarter of the present century has fetched new avenues for CRL by ascertaining its potentialities in fine chemical making especially in biopharmaceuticals and biopesticides[33,65]. Optical resolution of fine chemicals is an important aspect, which could be extended

to the synthesis of pure enantiomers with maximum optical purity and stereospecific hydrolysis and synthesis of valuable compounds[197]. Moreover, in recent years, the same principle is being employed for the production of efficient carbohydrate esters and amino acid derivatives[117,184]. Quantitative estimation of lipids in clinical samples and organic synthesis, led to the development of yet another bio-microelectronic devices called the biosensors, which contains a lipase, especially non-specific CRL[79,195]. As an alternate technology, lipases can be infused (infiltrated) into the intact organisms or cells to hydrolyse excess lipids a challenging approach to be tested in patients, and to extract cell-bound lipids from industrial organisms and oil seed[140]. In the bioremediation of lipid-tainted water, factory and restaurant effluents, lipases are used either in the *ex situ* or *in situ* form and useful products are obtained from these wastes, which is a bypass technology[64,125]. Growing of whole cells (*in situ*) on effluent wastes intending to produce single cell proteins (SCP) offers a promising area with great prospects[123].

Detailed discussion that appear in the succeeding part of this review would impart an ample insight into the biotechnological versatilities of *C. rugosa* and its lipases.

Taxonomy of *Candida rugosa*

Candida rugosa (Anderson) Diddens *et* Lodder var. elegans nom. nud. is a twin word descended from the Latin route; *Candidus* = white and *rugosa* = wrinkle, *ie*, white wrinkled cells[114]. *C. rugosa* is synonymous as *Mycoderma rugosa* (Anderson); *Azymocandida rugosa* (Anderson) Novak *et* Zsol, and lastly *Candida*

cylindrica[40]. The generic name, *Candida* was first coined by Berkhout[114], and he diagnosed it as: a few hyphae, prostrate, breaking up into shorter or longer pieces, conidia arising by budding from the hyphae or on top of each other, are small and hyaline. However, now-a-days, the binomial *Candida rugosa* is invariably used in all parts of the world[22,114].

C. rugosa belongs to the major group Yeast, and can be defined as a unicellular eukaryotic schizoycetous fungus reproduced by budding or fission. The yeasts are - taxonomically - a heterogeneous assemblage, and embrace members of both ascomycetes and basidiomycetes of Fungi. A systematic group - the imperfect yeast - clubs both ascomycetous and basidiomycetous characters. Kreger van Rij[114] included *Candida* under the imperfect yeast family, Cryptococcaceae, and described the *Candida* genus as the budding yeast having pseudomycelium, true mycelium and sometimes arthrospores, cells hyaline or red, orange or yellow due to carotenoid pigments but very seldom brown or black.

Species identification of *Candida* is rather an irksome process. Notwithstanding, lipase activity has been taken to pool some related species like *C. lypolytica*, *C. steatolytica*, *C. rugosa*, etc. together. A host of methods have been proposed for the detection of lipolytic activity in microorganisms; for species identification using various substrates such as: trybutyrin, olive oil, tallow, Tween-40, 60, 80, as well as partial esters of higher fatty acids[114].

Van Uden and Buckley have put-forth an easy identification procedure for *C. rugosa* through various fermentation routes[114]. The cells would become ovoid to

elongate, sometimes sausage shaped or like a pellicle, after 3 days of growth on glucose-yeast extract-peptone-water medium. After one month, on glucose-yeast extract-peptone-agar streak medium, the colour of the culture ranged from dirty white to greyish yellow. The surface of the cell was wrinkled in most strains, but partially or wholly smooth in others. The wrinkled strains were dull-moist. *C. rugosa* produced a primitive much branched pseudo-mycelium[20].

The quest to adopt a novel and simple method to odd out lipase producing microbes continues in the 1990s too. The chlamydospore formation test put forth by Milikhina *et al.*[147] for rapid identification of *C. rugosa* by using soluble starch as the carbon source and sulphanilic acid as the chlamydospore formation stimulant was found to have superb precision. Hou and Johnston[88] have made a comprehensive screening of lipase activities with cultures from the Agricultural Research Service Culture Collection, and Lima and Teixeira[127] developed a wonderful deep-agar diffusion test for the preliminary screening of lipolytic activity of fungi. Very recently, Kim and Rhee[109] carried out a successful device for the rapid screening of microorganisms with phospholipase A_1, A_2 and C activities using agar plate and gas chromatography (GC) method.

Microbial Production of Lipases

Both SmF and SSF are now widely being employed for the exploitation of cell-bound or secreted microbial metabolites[25,177]. Lipases are one of the primary metabolites abundant in the microbial world[24]. In the past, interest in microbial lipases resulted from investigation of food spoilage, especially of dairy products[37]. In contrast, free fatty acids (FFA) in some dairy products, notably cheese, contribute

to desirable flavours. For instance, *Pennicillium roqueforti* spores are deliberately added during the preparation of Roqueforti cheese, so that action of the lipases resulting from mold growth can contribute to the flavour[37]. Many other foods utilize microbial lipases either *in situ* or *ex situ* to obtain desired flavours and textures. This has led the industrial application of lipases in the modern technologies, especially in fats and oil industry[22].

Major Lipase Producing Microorganisms

A broad galaxy of microorganisms are in vogue in the production of lipases. Among them, the most promising bacteria (Table II 1), fungi (Table II 2) and yeasts (Table II 3) are enlisted here. Nevertheless, *C. rugosa* was recognised as a potent producer of lipases and is economically more viable than other microbial lipases.

Table II 1. Important bacterial cultures producing lipases

Name of bacteria	Ref.	Name of bacteria	Ref.
Achromobacter lipolytium	94	*Pseudomonas fragi*	235
Acinetobacter calcoaceticus	35	*P. glumae*	53
Aeromonas hydrophila	94	*P. plantarii*	146
Bacillus subtilis	81	*P. putida*	124
Brevibacterium ammoniagenes	94	*Psychrobacter immobilis*	9
Chromobacterium viscosum	2	*Rhizobium japonicum*	252
Corynebacterium acnes	83	*Serratia marcescens*	94
Leptospira biflexa	94	*Staphylococcus aureus*	82
Micrococcus fruedenreichii	94	*S. carnosus*	244
Mycoplasma gallisepticum	94	*S. epidermidis*	94
Propionibacterium acnes	76	*S. hyicus*	239
Pseudomonas aeruginosa	227	*S. warneri*	230
P. alcaligenesis	150	*Streptomyces coelicolor*	164
P. cepacia	57	*Thermus aquaticus*	28
P. fluorescens	257	*Xenorhabdus luminescens*	245

Table II 2. Important fungal cultures producing lipases

Name of fungi	Ref.	Name of fungi	Ref.
Aspergillus niger	183	Penicillium citrinum	139
A. oryzae	183	P. roqueforti	199
A. tamari	204	Puccinia graminis	37
Fusarium oxysporum	87	Rhizomucor miehei	55
F. solani	240	Rhizopus arrhizus	70
Humicola lanuginosa	199	R. chinensis	159
Mucor javanicus	69	R. delemar	52
M. miehei	135	R. microsporus	52
Penicillium camembertii	39	R. niveus	218
P. candidum	199	R.rhizopodiformis	208
P. cyclopium	122	Torulopsis ernobii	122
P. simplicissimum	226	Utilago maydis	118

Table II 3. Important yeast cultures producing lipases

Name of yeasts	Ref	Name of yeasts	Ref.
Candida albicans	111	Candida rugosa	17
C. antartica	99	C. utilis	126
C. boidini	246	Geotrichum candidum	29
C. curvata	165	Pichia burtonii	224
C. deformans	165	Rhodotorula glutinis	183
C. guilliermondii	165	Saccharomyces cerevisiae	161
C. injens	5	Sporotrichum thermophila	100
C. lipolytica	165	Trichosporon fermentans	42
C. paralipolytica	114	Yarrowia lipolytica	165

SmF vs SSF

With the work of Yamada et al.[254], there has been a resurgence in the cultivation of C. rugosa. The main focus on cultivation is founded upon the submerged fermentation technology[17]. Hundreds of publications are available to

point out the growth of *C. rugosa* in liquid media. A lion's share of this work was done in the medium in which olive oil or any other lipid was added as carbon source[237]. This was because of the increasing role of lipid materials like sterols and olive oil in lipase production[54]. Due to the increasing need of *C. rugosa* non-specific lipase in the industry, it is required to produce it on large scale and compelled the scientists to work in the already established field, the SmF technology[22].

It would be worthwhile to think on an alternate methodology to cultivate *C. rugosa*, which is the SSF system[20]. This technology is getting more and more attention to give heed to the cultivation of *C. rugosa*. For the first time, Rao *et al.*[196] employed SSF using rice bran as the solid substrate for cultivating *C. rugosa*. Wheat bran and coconut cake are some of the potent solid substrates to be exploited[23]. If a competent technology is formulated in SSF against SmF, being cheap and convenient, SSF will dominate SmF to solve the scarcity of *C. rugosa* lipases[20].

Inducers in Lipase Production

Unless a gene is induced or activated, it will not be expressed. The influence of lipids such as fats and sterols in extracellular lipase production by *C. rugosa* is well established[22]. Though fatty acids are the catalytic products of lipases, they act as the best inducers in lipase production; for instance, oleic acid, caprylic and capric acid could induce overproduction of lipases[18]. Moreover, Wu *et al.*[250] found that the addition of small amount of the surfactant, Tween-80 and oils such as soybean oil, sesame oil, linseed oil and olive oil increased the lipase production to a great extent.

Valero et al.[237] reported that olive oil (which has 70% triolein) and cholesterol as powerful lipase inducers. Ferrer and Sola[62] observed that the addition of gum arabic appeared to be a good inducer of immobilized cell mass growth and also of lipase activity. In addition, it did not significantly affect the hydrodynamic stability of the system. The role of ascorbic acid and glycerol in growth stimulation and the yield of yeast biomass during cultivation was reported by Peckurkio et al.[186]. This growth stimulation activity is effective in the case of single cell protein (SCP) production employing microbes as the fodder yeast, C. utilis.

On growth, from the point of industrial production view, oleic acid has been reported as a good inducer of lipase production by C. rugosa. In order to know whether the enzyme is induced by oleic acid itself or by a metabolite, different short chain fatty acids were tested by Obradors et al.[167]. Butyric acid was the best carbon source for the growth of C.rugosa but it did not induce lipase production. Although caprylic and capric acid are the best inducers of lipase production at concentrations upto 0.5 gL^{-1}, they have toxic effect when the concentration exceeds this limit.

Del Rio et al.[54] described the mechanism involved in lipase secretion by C. rugosa in the medium containing olive oil. Accordingly, the initial presence of a small quantity of lipase (with an activity in the medium of about 0.54 U ml^{-1} that could be provided by the inoculum) would give way to the hydrolysis of the triglyceride to produce glycerol and fatty acids. The yeast would preferably be using glycerol, which would repress the use of fatty acids. Therefore, the production of lipase at higher levels would be associated with the consumption of fatty acids. The

addition of gum arabic appeared to be a good enhancer of immobilized cell growth and also of lipase activity. In addition, it did not significantly affect the hydrodynamic stability of the system[62].

These studies indicate that unless a natural substrate or the catalytic product itself is not supplied, as an inducer to 'switch on' the gene responsible for lipase production, it will remain in the 'switched off' condition with no lipase production, and hence it is imperative to add a required quantity of suitable inducer in the production medium[20].

Isolation and Purification of Lipases

Tomizuka *et al.*[37] purified *C. rugosa* lipase for the first time by extraction with water and centrifugation of the aqueous extract and precipitated with ammonium sulphate. The precipitate, after various treatment, was eluted through SE-Sephadex with sodium chloride solution. The elute was concentrated and fractionalised with Sephadex G-100. A concentration of 33 fold with a yield of 18% was obtained. However, extreme pure form of two lipases from *C. rugosa* was isolated by Rua and Ballesteros[202].

At present, there are many sophisticated means to characterise the lipases. Wu *et al.*[250] purified the enzymes by absorption and elution on DEAE-cellulose, butyl Toyopearl-650 hydrophobic chromatography and Sephadex G-100 gel filtration chromatography. The purity increased by 67 fold. In the meantime, Rubin *et al.*[203] purified *C. rugosa* lipase and crystallised in a form suitable for X-ray

crystallography. Many protein fractions with lipolytic activity having isoelectric points from 4.22-5.8 were separated by iron exchange chromatography. As a whole, the purification studies of *C. rugosa* lipase revealed that *C. rugosa* has a 'lipase mini-family' and the genes encoded in this family code for a mixture of lipase isoforms with an apparent molecular weight (MW) of 60 kDa[38,41,132].

Lipase Specificity

Enzymes work to modify specific chemical bonds usually at specific sites on a molecule in contrast to ordinary chemical reactions that occur at random in response to the laws of thermodynamics[59,190]. Enzymes permit control of products produced and also increase yield by reducing side products, coupled with mild reaction conditions and low waste treatment costs[128].

CRL has a broad spectrum of specificities. Specificity of lipases is controlled by the molecular properties of the enzymes, structure of the substrate and factors affecting binding of the enzyme to the substrate[4,98]. Types of specificity are: (I) Substrate: (a) different ratio of lipolysis of TG, DG and MG by the same enzyme or separate enzymes from the same source for TG, DG and MG; (II) Positional: (a) primary esters; (b) secondary esters; (c) all three esters or non-specific hydrolysis; (III) Fatty acid: preference for specific fatty acids (IV) Stereospecificity: faster hydrolysis of one primary *Sn* ester as compared to its counterpart and (V) Combination of I-IV. Industrial viability of CRL has widely been exploited in all the aforesaid areas.

Substrate specificity of CRL is mainly due to the occurrence of multiforms of lipases, one indication for better adaptive value for maximum utilization of the available substrate[132]. Rua and Ballesteros[202] studied the behaviour of two forms of lipases (A and B) on the rate of hydrolysis using p-nitrophenyl butyrate as substrate and observed that both the lipases showed striking variations in their Km values. The behaviour of lipase-B could be consistent with an enzyme which still obeys Michaelis-Menten kinetics but with a Km value for p-nitrophenyl butyrate much higher than the ester solubility at the experimental conditions. With triolein, both the forms showed relatively similar and slow activity upto 20 mM substrate, but higher substrate concentration showed much enhancement of hydrolysis by Lip-B. Studies of Shaw and Chang[217] on the effect of specificity on different acyl chain length with p-nitrophenol ester hydrolysis also confirm the findings of Rua and Ballesteros[202].

The non-specificity of CRL is well documented [59,102,255]. Benzonona and Esposito[27] found that CRL was very active towards long chain triglycerides at pH 8.0 and 30°C. Under these conditions, the three chains of the glycerol moiety were completely hydrolyzed (Fig.II 1). A comparison between the proportions of the fatty acid chains in intact olive oil and coca butter and the proportions of the fatty acids liberated after a limited hydrolysis of these lipids shows that the CRL liberate all types of acyl chains, regardless of their position in the glycerol[27]. However, palmitic acid and oleic acid chains liberated before stearic acid when they are present together in a given glyceride. Since 1,3-dihexadecyl-ether-2 oleoyl-glycerol is readily hydrolyzed, it can be concluded that CRL able to attack secondary ester

groups of glycerol without the help of an isomerase. The positional specificity on a wide range of substrates like trioleoyl glycerol[173], vegetable oil[137], milk and creams[102] was also reported with CRL.

Triglyceride (fat)   + 3H₂O   lipase   Glycerol   Fatty acid
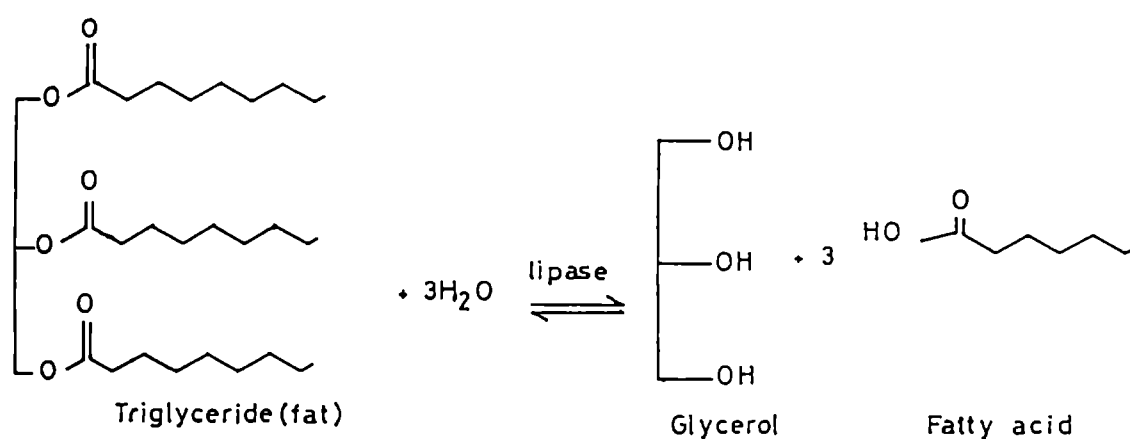
Fig.II 1: Enzymatic reaction of CRL catalyzing hydrolysis or synthesis of a triglycerol substrate

Fatty acid preference of CRL is widely employed in lipase biotechnology. Many studies revealed that CRL has much preference for short chain fatty acids[96,120,125]. Liu et al[131] deduced the selectivity from transesterification of two or more different fatty acids, fatty alcohols and fatty acid esters with another acyl ester or fatty alcohol. Butyl stearate and butyl palmitate were used as acyl donors trans-esterified by lauryl, and products were separated by HPLC. The lipase tended to select short-chain fatty acids. Osada et al.[171] synthesised triglycerides using fatty acids C18:1, C18:2, C18:3, C18:4, C18:5, C18:6 were compared. Higher chain length FA with more double bonds produced less triglycerides. In ester synthesis, CRL preferred short chain fatty acids and alcohols viz. propionic acid, butyric acid,

butanol, hexanol and pentanol, which impart quality flavours in food stuffs[96]. Janssen and Halling[97] studied the esterification of sulcatol and several saturated fatty acids catalyzed by CRL. Measurements made in toluene (based on *Vm/Km*) showed a high preference for C4, C8, C10 and C12 fatty acids.

In the stereospecific esterification of racemic naproxen with trimethylsilyl methanol in isooctane by CRL, improvements in (S) naproxen ester productivity and enzyme solubility were demonstrated by adding bis-(2-ethylhexyl)sodium sulfosuccinate as the best surfactant[234]. In another study, stereospecific esterification of racemic methanol in a solvent system (hexane, isooctane, n-heptane, cyclohexane, benzene, toluene, methylene chloride and chloroform) was investigated using acid anhydrides (acetic propionic or butyoic acid) as acylating agents. The (-) methanol was preferentially esterified with a yield as high as 64%[250]. S-stereopreference was also employed in the synthesis of a number of optically active non-steroidal antiinflammatory drugs: naproxen, Ibuprofen, suprofen[197]. Haalck *et al.*[80] noticed that triglycerols and 2-o-alkyl analogs were affected by the chain length of the *sn*-1 and *sn*-3 acyl groups. As a whole, multifacetedness of CRL in industries lies in its broad specificities.

Hydrolysis *vs* Synthesis

Potential industrial application of CRL include production of fatty acids and glycerol via hydrolysis of oils and fats, modification of composition and physical properties of triglyceride mixtures by interesterification and transesterification (Fig.II 2), synthesis of chemicals in organic solvents[50,52,95]. The non-hydrolytic

(esterification) properties can occur mainly in water restricted environment, Lipases bind at the interface between aqueous and organic phases, and catalyze hydrolysis at this interface[78,149]. This binding not only places the lipase close to the substrate, but also increases the catalytic power of the lipase, a phenomenon called interfacial activation[107,251]. In the environment of organic solvents, CRL catalyze the synthesis of new esters and also modification of saccharides, peptides or the formation of optically active enantiomers for fine chemical making[96,260].
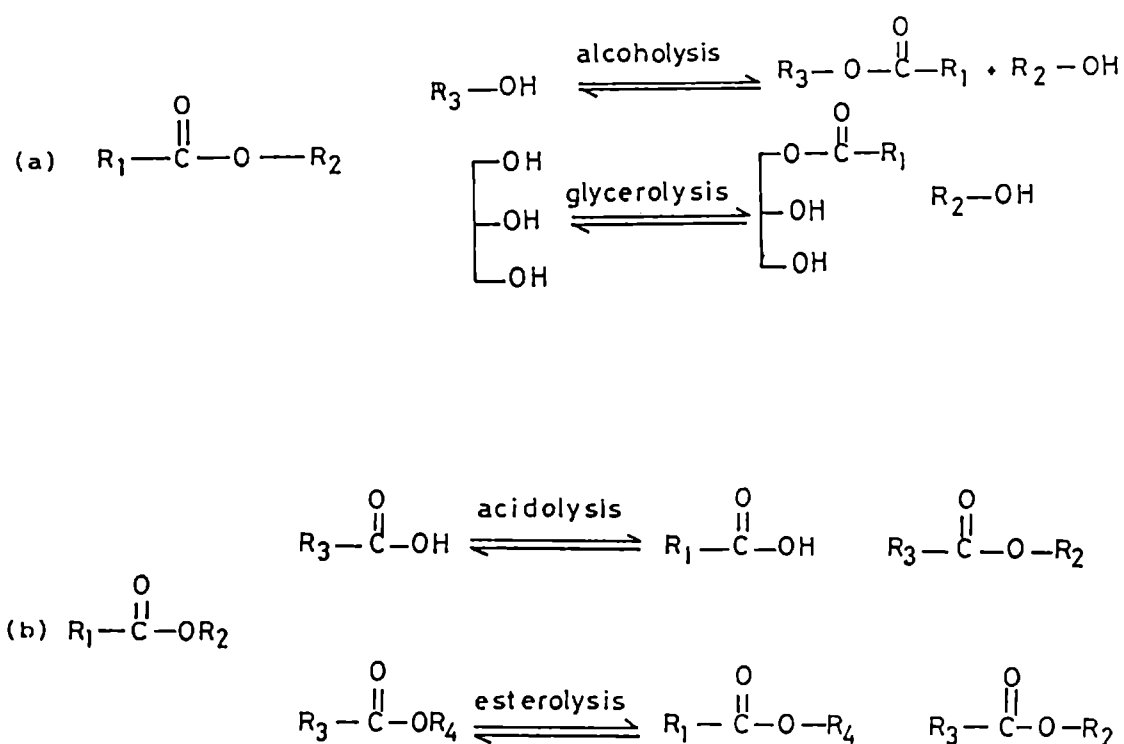
Fig.II 2: Industrial important reaction catalysed by CRL
(a) Transesterification involves the transfer of an acyl group to an alcohol (alcoholysis) or glycerol (glycerolysis);
(b) Interesterification describes the transfer of an acyl group to a fatty acid (acidolysis) or a fatty acid ester (esterolysis).

The hydrolysis of fats and oils is an equilibrium reaction, and therefore it is necessary to change the direction of the reaction to ester synthesis by modifying the reaction conditions[94,122] (Fig.II 1). The equilibrium between to and fro reactions in this case is controlled by the water content of the reaction mixture so that in a non-aqueous environment lipases catalyse ester synthesis. Another potent role is to deprotect (by hydrolysis) the ester-protecting groups in synthetic intermediates so as to exploit the ability of lipases to catalize hydrolysis under mild conditions[97,107]. In fact, CRL was found to catalyze the hydrolysis of a wide range of unnatural esters, and many of these hydrolyses are enantioselective, thus enabling chemists to generate pure enantiomers in organic synthesis (Figs.II 3a&b). These starting

(a)
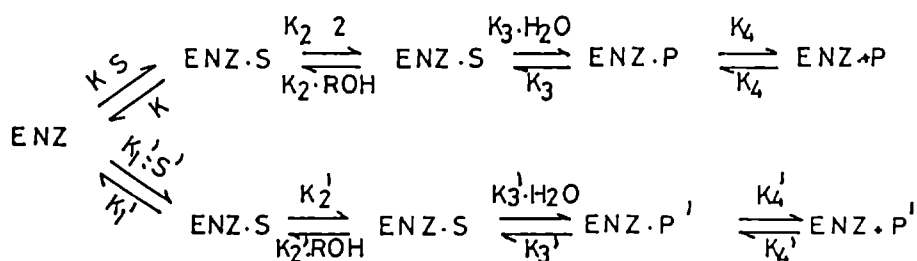
(b)
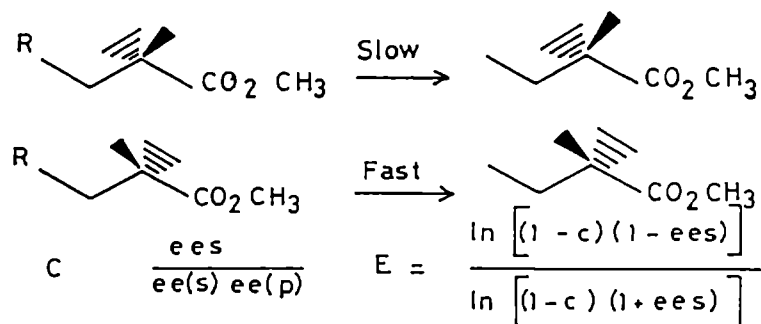
Fig.II 3: General mechanism in the CRL mediated catalytic resolution of fine chemicals: (a) asymmetric catalysis and (b) kinetic resolution of enantiomers.

materials are required for the next generation of pharmaceuticals, which will be enantiomerically pure to maximize their potency and to minimize side products, for instance, production of optically pure ibuprofen[108].

Advent of inter and transesterification reactions has revolutionized lipase biotechnology[80,90]. In transesterification, the acyl donor is an ester (Fig.II 2a) Transesterification involving fats and oils can further be specified depending on the type of acyl acceptors. Alcoholysis and glycerolysis refer to the transfer of an acyl group from a triglyceride to either an alcohol or glycerol respectively[94]. In interesterification, the acyl group is exchanged between a triglyceride or a fatty acid ester [more specifically (tri)glyceride], the former is acidolysis and the latter being esterolysis (Fig.II 2b). Interesterification requires controlled quantity of water, in addition to the amount needed for the enzyme to maintain the active hydrated state. As the presence of too much water will decrease the amount of ester synthesis products, the water content should be carefully adjusted (where upon interesterification predominates hydrolysis), to achieve accumulation of desired reaction products[21,137].

Immobilized CRL

Entrapment technology could prefer to soluble enzymes owing to its proven advantages, which include: (a) enzymes can be reused, (b) process can be operated continuously and can be readily controlled, (c) products are easily separated, (d) effluent problems and material handling are minimized, (e) enzyme properties like

activity, thermostability can be altered favourably and (f) after all, it is much cost effective[19,215].

Table II 4. Commonly used: agents for entrapping CRL and types of reactions with products

Agent	Reaction	Product	Ref.
Celite	Stereoselective esterification	2-(4-chlorophenoxy) propanoic acid	67
Nylon fiber	Stereospecific hydrolysis	Methyl methoxyphenyl glycidate	36
Co-polymer of methyl acrylate and methyl methacrylate	Hydrolysis	Free fatty acids	258
Polymethyl methacrylate	Esterification	Fatty ester	15
Lyotropic liquid crystal	Esterification	Butyl butyrate	66
Glass bead	Esterification	Citronellol ester with valeric acid	90
Kieselguhr	Esterification	Ester of isoamyl alcohol with butyric acid	106
Colloidal liquid aphrons	Hydrolysis	p-nitrophynil derivatives	134
Chitin	Hydrolysis	Free fatty acid	248
Silica gel	Hydrolysis	Glycerol	209
Cross-linked enzyme crystals	Hydrolysis/synthesis	Esters	188,243

The costs of producing lipases necessary to catalyze versatile reactions which contribute much to the biotechnological processes often prohibitive[13,138]. Hence processes that do not require the physical presence of lipase in the final product and that use feedstocks that are fluids (or that can be treated as such) are subjected to dramatic improvements in process economics if the lipase is employed in

immobilized form[253]. A vivid illustration on the applications of immobilized enzymes was presented by Katchalski-Katzir unveiling immobilization techniques, immobilized enzyme reactors and immobilized enzymes in food, pharmaceutical and chemical industries[104].

Entrapment of enzymes is based on the coupling of enzymes to the latices of a polymer matrix or enclosing them in semi permeable membranes, tight enough to prevent the leakage of protein, while allowing the diffusion (mass transfer) of substrate and products to and fro[58,61]. Natural and synthetic polymers are in vogue as matrices in many immobilizing techniques. A list of commonly used modern immobilizing agents for entrapping CRL and the reaction involved are enumerated in Table II 4 Gel entrapment involves entrapping the enzyme within the meshes of cross-linked polymeric network can be obtained from monomeric, oligomeric or polymeric precursors by changing the solubility variables such as solvents, temperature, enzyme strength and pH are taken into account during the cross-linking reactions[36]. Use of pure and lasting lipase has been very limited due to their low stability and high cost. Cross-linked enzyme crystals (CLEC) offer not only a pure but also very active and stable form of the enzyme[241]. The mucleophile (alcohol) selectivity studies showed that lipase immobilized on XAD_7 and celite were more accessible to 3-12C alcohols. Lipase immobilized on polymethylmethacrylate (PMMA) had a marked preference towards 3-10C alcohols[15]. Immobilization in lyotropic liquid crystals consisting of polyoxyethylene-n-alkyl ester-type nonionic surfactant offer another potentialities for esterification reactions[66]. Lipase entrapped in methyl-substituted organic silicates formed on Kieselguhr such as Celite-545 and

Hyflo Super-Cel, revealed the thermostability of lipase upto 65°[106]. This findings of Kawakami were very significant in detergent industry, which needs non-specific thermotolerant lipases[106].

In view of the reusability of the enzyme, many different methods of immobilization were applied. Covalent methods were used to entrap lipase to polysaccharides, carboxymethyl cellulose and polyacrylamide gels[192]. Another method applied for immobilization of lipases is photo cross-linkable resins[188]. Finally, adsorption was used to immobilize lipase to amphiphilic gels and to different types of membranes, like cellulose membranes reactors[114]. More recent attraction is on the surfactant-coated lipases for better and lasting activities.

CRL in Food and Flavour Synthesis

Versatile CRL have an invincible role in modern food technology[21,47]. The use of enzymes to improve traditional chemical processes of food manufacture has been developed in the past few years, owing to economic and technological advantages it offer. Diverse products and processes ranging from juices, baked food, vegetable fermentation, dairy enrichment, desirable flavours in cheese and allied stuffs, and for the interesterification of fats and oils to produce modified glycerides unobtainable by chemical interesterification have been manufactured by free or immobilized *C. rugosa* lipases[25,222].

(a) Fats and Oils: A lion's share of lipase mediated food industry embodies lipid compounds, especially fats and oils[128,221]. During storage, one of the most important

changes that occurs in the lipid fraction is the hydrolysis of triglycerides catalyzed by lipases retaining free fatty acids, which are very important for the characteristic flavour of these products[120]. Likewise, desired moiety of the triglyceride can be deleted or replaced under controlled esterification and ester interchange reactions[102].

Fragrance development in dairy products is dependent on the release of volatile fatty acids. The catalytic functions of CRL on cattle, sheep and goat milk fats reveal that CRL yielded high amount of volatile branched chain fatty acids non-selectively as against only small quantities of volatile straight chain fatty acids[93,129]. Considerable efforts have been made, notably in Japan and Europe, to commercialise lipase catalyzed transesterification and interesterification of relatively valuable food products. Ester exchange for vegetable oil modification involving *C. rugosa* lipase suggests that it can effectively convert triolein in vegetable oil with high industrial qualities. Unilever obtained a series of patents for the preparation of interesterification of fats and glycerides. These processes afford efficient means for the inter-esterification fats suitable for use in emulsion and other fat based food products such as margarine, artificial creams and ice creams[22,142].

(b) Fermented Foods: Traditionally fermented foods from fruits, vegetables, cereals, root crops, legumes and oil seeds have been used all over the world as baked or cooked stuffs[63]. An important result of the fermented fruits and vegetables is that products so obtained can be stored and used as food supply during off-seasons[259]. The bioconversion caused by fermentation contribute very much to the character and organoleptic properties of the fermented products. In many cases also fermentation

contributes to the digestibility and nutritional value of the final products[21]. For instance, soyabeans can be stored relatively well in the dry state, but as such they are not readily consumable by humans, even when cooked. When converted to temp, they become a base material for several delicious, easily digestible and nutritional food items, which provide many millions of people with a valuable and affordable source of proteins[201].

(c) Ice Cream: Flavour and fragrance rich ice creams have large market in the modern life[185]. Production of flavour esters which contained short chain or medium chain fatty acids to be used in ice cream was investigated by many authors[120,229]. However, the flavour compounds produced in solvent-free system could be advantageous of being safe for delicious creams[168]. Ester-interchange reactions are now employed to get desired flavour esters[21,141].

Single Cell Proteins (SCP)

Exploitation of microbes for the production of valuable products has age old history, but production of single cell protein is an emerging technology by which microbes can be grown in valueless waste to produce value-added products[186]. Lee and Lee[123] of Hoseo University have very recently isolated a thermotolerant C. rugosa strain from Sudan which was used for the continuous production of SCP from sugar beet stillages, a by-product of ethanol production process. The yeast contained 45.1% crude protein, 36.5% actual protein and 5.6% RNA. The yeast protein had adequate essential amino acids except for sulphur containing amino acid cysteine using recombinant-DNA technology. These protein can be enriched with

cysteine and be used as a staple food source. In many fermentation industries, especially fat and oil industry, the treatment of waste is of crucial significance[21,119]. In the treatment of high strength molasses waste-water, molasses stillage, brewery waste, pharmaceutical wastes much attention is needed, though *C. rugosa* was a failure in these treatments[126]. However, growth of *C. rugosa* in oil rich wastes was very much luxuriant. So far, the focus on *C. rugosa* was mainly on its lipase producing capacity[52]. Potentiality of *C. rugosa* for the copious secretion of protein including lipases would gradually pave an avenue for the large scale production of SCP using this yeast[22,43].

Pharmaceuticals

One of the challenging targets of a biopharmacists is to build lipase catalyzed bioactive components by hydrolysis *vs* synthetic reactions (Fig.II 1). In addition to *in situ* racemization for optically pure enantiomers (Fig.II 3), CRL should also be capable of catalyzing synthetic reactions which led to the production of innumerable life saving drugs (Table II 5). Efficient kinetic resolution processes are in vogue for the preparation of optically active non-steroidal antiinflammatery drugs (ibuprofen, naproxen, ketoproten), potential virucide carbovir (which can be applied against HIV) and for the enantiospecific synthesis of alkaloids, secondary alcohols, antibiotics, biochemical inhibitors and prodrugs (Table II 5).

Conventional chemical synthesis of drugs containing a chiral center generally yields an equal mixtures of enantiomers[219]. During the past decade, many studies have shown that racemic drugs usually have the desired therapeutic activity

Table II 5. Important CRL-catalyzed pharmaceuticals

Targets	Precursor	Reaction	Reference
Ibuprofen	Fatty alcohol	esterification	74
Trifluorophenanthryl ethanol	Chloroacetate ester	alcoholysis	105
Aspirin-like pro-drugs	Acetoxy and phenethylbenzoates	hydrolysis	45
Acetic acid derivatives	α-substituted acetic acid methyl esters	hydrolysis	166
Androstane	Acetylated androstane	hydrolysis	14
Prostaglandin	Polyunsaturated fatty acid containing oil	hydrolysis	247
Naproxen	Trimethyl silyl methanol	esterification	197,223
Carbapenem antibiotics	Pyrolidine dione derivatives	esterification	210
Ketoprofen	(R,S)chloroethyl ketoprofen	hydrolysis	117
Inhibitor drugs	Tricarboxylic acid alkenyl ester	esterification	216
Propanolamines	epoxide and 2-proplamine	esterification	101
1Solketal	Trifluoroethyl butyrate	esterification	24
Aconitium alkaloid	Vinyl acetate and mesodiols	esterification	89
Anti-HIV carbovir	Azabicylo hept-en-ones	esterification	158

residing mainly in one of the enantiomers, and the other enantiomer may interact with different receptor sites, which will create untoward side effects CRLs have made a significant mettle in pharmaceutical as well as agrochemicals, mainly due to its S-stereospecificity[128]. Goto et al.[74] observed that S-(+)-ibuprofen was selectively transferred to the ester form by esterification reactions held in dry homogeneous

organic media by CRL. Similar reports were available on a number of allied drugs[21].

Stereospecific esterification of racemic naproxen with trimethyl silyl methanol in isooctane showed improvements in (S)-naproxen ester productivity by CRL[137]. Yasufuku and Ueji[255] have observed that alteration of temperature during esterification reaction would drastically change the E-values and also stereopreferance. Ihara and his co-wrkers[89] while studying on the products of chiral building blocks for the synthesis of Aconitium alkaloids with vinyl acetate and meso-diols found that CRL could yield the alkaloids over 96% ee. Enantioselective interesterification and transesterification have great significance in pharmaceuticals for selective acylation and deacylation[187,216,223].

Carbohydrate Ester Synthesis

Lipase mediated organic synthesis finds new avenues in the production of carbohydrate esters of fatty acids, glyceroglycolipids and glycosylated products like sphingomyelins, cerobrosides or gangliosides[6,46,157]. Utilisation of carbohydrate esters has become a significant event in fine chemicals making. Asahi-Electrochem of Japan produced maltose and lactose like sugar fatty acid esters using CRL[10]. Production of these esters involve reacting the disaccharides or their analogs (with atleast one primary alcohol group) and a 8-22C fatty acid like myristic, stearic, linolenic, hydroxy stearic acids etc. The sugars include acetyl or amino or acetyl amino maltose/lactose. Lion group obtained a new process for the esterification/ transesterification involving the synthesis of methylglyceride fatty acid monoester suppressing the formation of diesters and higher esters[130]. The methyl glyceride is

widely used as a surfactant in many biotechnological formulations. The stereospecificity of CRL is used for the stereospecific resolution of a mixture of carbohydrate monoesters (CMS), which attracted the industrial arena by dint of its involvement in the pharmaceutical, food or cosmetic product formation[6]. Broad substrate specificity towards various monosaccharides (glucose, galactose, fructose) and methyl alpha-glucoside, sorbitol, xylitol, N-acetyl, D-glucosamine, glucosamine, glucuronic and galacturonic acid, and ascorbic acid[184].

Amines and Amides

A novel enzymatic method was developed for protecting amines as carbamates as substrates for lipase[170]. The symmetrical structure of the homocarbamates gave unambiguously a single carbamate product, making the process very simple. The carbamate was easily deprotected or converted to the n-methyl derivative using LiAlH$_4$, providing a new method for chemoenzymatic amine methylation. Rakels *et al.*[194] reported that lipase-catalysed simultaneous hydrolysis and amidation led to significantly improved amide yield and ee in racemic methyl 2-chloropropionate hydrolysis and aminolysis. Lalonde[117] had made a pure and well defined cross-linked enzyme crystals (CLEC) for the production of enantiomerically pure amines, amino acids and peptides.

Biocides

As shown in Table II 6, CRLs are very frequently used for racemic mixture resolution, via. both hydrolysis and acylation reactions. The lipase catalyzed resolution of 2-(4-isopropyloxy)phenyl)propionic acid, an intermediate in the

synthesis of a wonderful chiral acaricide was described by Bosetti et al.[33] with

hydrolytic reactions in a stirred emulsion of the oily substrate, over 95% ee

steriospecificity with (S)-enantiodiscrimination. Optically pure (S)-1,3,4-

oxadiazole-2 (3H)-ones obtained showed high ovicidal activity against Tetranychus

urticae. and insect juvenile hormone analogs useful as insecticides was obtained in

high purity by enzyme-mediated hydrolysis of the respective cis and

trans-2-(4-methoxybenzyl)-1-cyclohexylacetates[260].

Table II 6. Important CRL-catalyzed pesticides and their sources

Product	Precursor	Catalysis	Ref.
Nikkomycin-B	p-siloxybenzaldehyde	Esterification	3
Perhydrofuro (2,3b)furan	2-methoxy tetrahydrofuran-3-carboxylate	Alcohlysis	65
1,3,4-oxadiazole-2(3H)-ones	2-(4-(isopropoxy)phenyl) propionic acid methyl ester	Hydrolysis	33
Hormone analogs	2-(4-methoxybenzyl)-1-cachohexyl acetate	Hydrolysis	260
Vinyl glycine	Racemic Vinyl glycine	Hydrolysis	86
Morpholines and triazole	Chiral alcohol intermediate	Hydrolysis	31
Pyrethroids	Racemic ester	Hydrolysis	151
Fenpropimorph	4-tert-butylbenzyl bromide and and methyldiethyl malonate	Acylation	12
Venturicidin	Aglycone	Hydrolysis	156

Enzymatic synthesis of a chiral building blocks for the synthesis of potent

pesticides is one of the successful methods in CRL mediated biocide (pesticide)

industry[2,156]. Franssen et al.[65] have succeeded in the resolution of

2-methoxy-3-carbomethoxy tetrahydrofuran, a chiral building block for

perhydrofuro-furan insecticides. Optically pure enantiomers of triazole and

morpholine fungicides could be produced by tranesterification of enzymatically prepared chiral alcohol intermediates[31]. Both triazole and morpholines (R-isomer) were active against many agricultural fungal pathogens.

Fine and intermediate chemical makers emphasize in new products and processes with new possibilities to make valuable and effective biocides[223]. In the field of biotechnology, much attention has been focused on the use of lipases as enantioselective biocatalysts in organic media, however the flexibility of the lipase molecule is restricted in organic solvents. Therefore, probably the resulting enzyme conformation recognizes one of the enantiomers in the mixture more selectively. A new process for producing chiral compounds of high optical purity comprises: reacting chiral and non-chiral reactants in super critical CO_2 in the presence of lipase so that principally only one of the optically active isomers of the chiral starting material reacts to produce a new critical compound with more optical purity[145]. A Kazlauskas active site kinetic model was validated for CRL-catalyzed alcoholysis, esterification and acidolysis reactions in organic medium[65]. Crude CRL lipase was used to resolve C_3-sterioisomers of the furo-furan building block methyl-2-methoxytetrahydrofuran-3-carboxylate by alcoholysis using n-butanol in octane, and the substrate was converted into a mixture of cis- and trans butyl esters and unconverted methyl esters without significant inactivation[65]. The enantiomeric ratio was well over 100, allowing isolation of product with over 98% ee at 45% conversion. The enantioselectivity of CRL depended considerably on the used substrates and the water content of the system, but it did not depend on the degree of purity of lipase[173].

Biosensors

Biological micro-devices or biosensors with enzymatic intensification are available for the quantitative determination of triglycerides in the fats and oil industry, in food and in chemical diagnosis[79,195]. A promising new method involves the use of lipase, where the glycerol liberated during lipid hydrolysis is oxidized by glycerol-dehydrogenase[213]. NADH formed during the reaction is measured by fluorescence spectroscopy. Non-specific lipase with high specific activity have been selected to allow rapid liberation of glycerol[213].

An important analytical use of lipases in the determination of lipases is notably for clinical purposes. As far as the physicians are concerned, this principle enables them to diagnose the patients with cardiovascular complaints very precisely[151]. Lipase biosensors are flourished not only in the diagnosis of clinical samples but it is being penetrating into new horizons like food and drinks industry, pollutant analysis, especially pesticide contamination, and pharmaceutical industry as well[256]. Very recently a CRL biosensor has been developed as a an enzyme labelled probe by Pittner et al.[186], which is useful as a bioassay reagent, as components of teat trips and biosensors or as DNA probes.

The major objective in constructing a lipase biosensor should that the substrate has to be presented in a manner, which meets the interfacial constraints of lipase activity. Secondly, to facilitate the repeated reuse of the biocatalyst by immobilization. In fact, immobilization of lipase as CLEC was found to be very useful for detecting an analyte in a fluid[188,233]. Furthermore, the advent of modern

computer boom has revolutionized the biosensor technology too, from the oldest

electrode to modern miniaturized optically active models through calorimetric

determination[113,121].

Bioremediation

Bioremediation for waste disposal is new in lipase biotechnology. Oil spills

during rigging and subsequent refining, oil wet night soil and shore sand,

lipid-tinged wastes in lipid processing factories and restaurants and lipid-tainted

clothes efficiently managed at the cost of lipases[21,51]. In fact, CRL are used as a

mixture along with other hydrolytic enzymes like proteases, amylases and cellulases.

For waste management CRL are used either in *ex situ* form (purified lipases) or *in*

situ (growing whole cells in the target waste) state[22].

Bioremediation not only aims at the eradication of wastes but its useful

management as well. Nisshin Oil Mills of Japan could utilize waste edible oil into

valuable transparent liquid soap by hydrolyzing it with CRL in the presence of an

emulsifier[162]. *C. rugosa* cells can be mixed with lipid-containing waste water,

containing animal or vegetable fat and oil, or higher fatty acids, and the lipid is

assimilated[64]. The yeast has high lipase activity, hydrolyses animal or vegetable

lipid, and has superior oxidation capacity on higher fatty acids. The yeast may be

used for direct treatment of lipid containing waste water released from oil

manufacturing, the food industry or restaurants[64]. Kurita-water devised a simple

process for treating waste water containing lipids and oils[115]. Biotechnological

methods for upgrading and modification of animal waste fats from fowl lipid or beet

tallow can be achieved by *in situ* utilisation of *C. rugosa* or by enzymatic glycerolysis[16]. Degradation of unsaturated polyester waste after slicing into small particles by CRLs is yet another area[21]. The low MW reusable products so obtained may be used especially in moulding articles reinforced with glass fibres *eg.* vehicle parts, bathroom equipments, casings for electrical equipment, kitchen sinks, boat hulls, etc[22].

Biodetergents

Detergents comprise surfactants, *ie*, surface active agents and builders, which are inorganic compounds, and creates favourable chemical background for the actions of surfactants[44,150]. The old organic surfactants are now replaced by much active biosurfactants, which are proteinaceous in nature[39]. The marketing success of surfactants with trypsin led to the development of other types of surfactant proteases, and the introduction of amylases, cellulases, and lipases for various detergent applications[39]. However, the cost of enzymes remains a major obstacle for their substantial use in the surfactant industry[211]. Intensive screening programmes have resulted in the introduction of highly thermostable biocatalysts capable of performing at evaluated temperature for hundreds of hours without substantial loss of activity, making enzymatic processing cost-efficient in manufacturing of many speciality products[211]. To be a suitable additive in detergents, lipases should be both thermotolerant(~60°C) and alkalophilic (pH 7-11) and capable enough to function in the presence of the varying components of washing formulations such as proteins which cleave lipase proteins and surfactants - the known lipase inhibitors without

losing its viability and stability. Moreover, it should also be stable at storage in the surfactants and during its activity in the washing machine[73,150].

The advent of Recombinant DNA technology and protein engineering greatly influenced the biosurfactant industry too. Uniliver, Cosmo Oil and Procter & Gamble are prominents in this field[21,49]. The activity, stability, priority and efficiency of enzymes can be improved via. genetic engineering. The application of genetic engineering may lead to a significant ecological advantage, i.e. the energy demand of a genetically engineered enzyme was reduced by over 60%, compared to the neutral enzyme. Utilisation of genetic engineering tools should lead to a further 50% reduction of environmental pollution[112].

Tanning

An enzyme preparation for leather processing is new in tanning industry[103]. As in detergents, a mixture of hydrolytic enzymes which contains lipase, pepsin, chymotrypsin, elastase, papain, aminopeptidase, choline esterase, and amylase is also employed for the production of ready-to-tan hides[200]. The enzymatic process for the production of hides and skins ready for tanning involves the steps of soaking, washing, dehairing and bating in aqueous bath containing the enzyme mixture. In this alternate technology, no tenside is used in soaking stage and in subsequent washing, dehairing and bating. This method suggests minimum use of detergents in the tanning industry[200]. A Russian patent[154] describes another technology for sheepskin which improves the quality of intermediate sheepskin products by increasing the strength and elasticity of the skin and reducing rigidity, with a

reduced use of surfactants. One of the prerequisites for the biological detergents in tanning is that it should work under alkaline conditions[75]. Genetic engineered non-specific lipases, which are functionally stabilised could offer much in this regard[60,124].

Cosmetics and Perfumery

Overwhelming interest of technocrats in tuning of lipases for cosmetic industry is mainly due to its activity as softener and aroma production, which are the main ingredients in cosmetics[153,172]. Glyceride esters, polyunsaturated fatty acids, mixed acid type polyesters are the main components in CRL mediated cosmetics[247]. McCrae *et al.*[141] formulated esterification reactions for the synthesis of costly cosmetic esters from fatty acids and miscible primary and secondary alcohols.

In toto, publications on *C. rugosa* focus mainly on the increasing demand for fascinating CRL to be employed in the emerging biotechnological processes. It has attracted new investigators towards *C. rugosa* to explore and to bring its efficacy into limelight. It is this great imbalance between less availability and high demand along with its non-pathogenicity, makes *C. rugosa* an interesting specimen for active research and over-production of its versatile lipases.

MATERIALS, METHODS, RESULTS AND DISCUSSION

CHAPTER - III A

GROWTH PROFILE

IN

LIQUID MEDIA

Introduction

Different media formulations have been reported for the growth and maintenance of *Candida rugosa*[236]. In all the formulae, carbon, nitrogen and phosphorus are the inevitable macro-nutrients, coupled with major micro-nutrients and some vitamins. Each medium has its own composition and characteristic effect on the behaviour of the growth of micro-organisms. Apart from the ingredients, optimum pH, temperature and aeration are important parameters which influence the growth[17]. Optimisation of these growth parameters is an important prerequisite for better growth of the organism. With this view, the opening section of experimental part is intended to spot out the most suitable medium for the growth and maintenance of *C. rugosa* along with standardization of optimum temperature, pH and agitation, which are essential parameters required for the subsequent studies.

Materials and Methods

Chemicals Used

All the chemicals used throughout the whole experiments were of analytical grade with maximum available purity supplied by prominent dealers of fine chemicals in India [BDH (E-Merk, Bombay), CDH (Central Drug House, New Delhi), Hi-media (Bombay), Loba Chemie (Bombay), Nice (Cochin), Sd-fine Chem. (Bombay), Sisco (Bombay), Spectrochem (Bombay) etc.] or abroad [Sigma-Aldrich (USA) and Bachem (Switzerland)].

Microorganism

The yeast, *Candida rugosa* (DSM- 2031) obtained from DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen HmbH, Germany, has been employed for the whole experiments described in this thesis[56].

Maintenance of Culture

Medium-A (Universal Yeast Medium) with agar was used for the short-term maintenance (2-4 weeks) of *C. rugosa* on slants or in petri-plates. For the preparation of slants, 10 ml of this medium was taken in a culture tube (25 ml), plugged with cotton, autoclaved, inoculated with a loopful of cells of *C. rugosa* and kept in an incubator for 2 days (28°C). The slants were preserved at 4°C in a refrigerator. Glycerine stocks were prepared for long term storage (6-12 months). For making glycerine stocks, 24 h old culture was centrifuged in a refrigerated centrifuge at 8000 g for 10 min at 5°C in a sterile tube. The sterile pellet was resuspended in sterile distilled water and was mixed with pre-autoclaved 50% glycerol (1:1) contained in a screw-capped polythene tube. This mixture was sealed and preserved at -20°C.

Inoculum preparation

A loopful of *C. rugosa* cells from a freshly raised agar slant was transferred to 50 ml UY medium in 250 ml flask and allowed to grow for 24 h at 28°C and pH 6.8 under 250 rpm on an incubator shaker.

Growth Media

Five defined growth media for the growth and maintenance of yeast were selected from the available literature to choose the best among them for further studies. The nutrients used in each medium and their proportions are shown in Table IIIA1. Among them, media-A[56] (DSM) and B[11] (ATCC) were complex

Table IIIA1. Five media compositions screened for selecting the best for the growth pattern of *C. rugosa*.

Medium -A		Medium -B	
Glucose	10 gL^{-1}	Glucose	5 gL^{-1}
Peptone	5 gL^{-1}	Peptone	5 gL^{-1}
Yeast extract	3 gL^{-1}	Yeast extract	3 gL^{-1}
Malt extract	3 gL^{-1}	Agar	20 gL^{-1}
Agar	15 gL^{-1}		

Medium - C		Medium - D	
Glucose	20 gL^{-1}	Carbon source	Fatty acids
KH$_2$PO$_4$	6 gL^{-1}	KH$_2$PO$_4$	3.9 gL^{-1}
Urea	1 gL^{-1}	K$_2$HPO$_4$	3.3 gL^{-1}
MgSO$_4$.7H$_2$O	1 gL^{-1}	Urea	2.0 gL^{-1}
Biotin	0.008 mgL^{-1}	MgSO.7H$_2$O	1 gL^{-1}
Inositol	0.004 mgL^{-1}	Inositol	0.004 mgL^{-1}
FeCl$_3$.6H$_2$O	0.04 mgL^{-1}	Biotin	0.008 mgL^{-1}
Thiamine-HCl	0.2 mgL^{-1}	Thamine-HCl	0.2 mgL^{-1}
Olive oil	10 gL^{-1}		
Cholesterol	1 gL^{-1}		

Medium - E	
Carbon source (Olive oil)	2%
Nitrogen source (NH$_4$)$_2$SO$_4$	0.5%
Yeast extract	0.5%
MgSO$_4$.7H$_2$O	0.05%
Gum arabic	2%
Tween-80	1%

organic media, which contained complex nutrients like peptone, yeast extract, etc. Media-C[236] and D[167] constitute synthetic media in which simple sugars, oil or fatty acids were used as carbon source. In addition, many inorganic salts and vitamins were also used. Medium-E[242] is a semi-synthetic medium which contained complex organic and simple inorganic constituents.

Selection of Best Medium

Experiments were conducted in 250 ml Erlenmeyer flasks. In each flask, 50 ml of the medium (without agar) (Table IIIA1) was taken, autoclaved (15 *psi* for 15 min) and inoculated with 1% cell suspension under aseptic condition (using Laminar-Flow chamber. It was allowed to grow at room temperature with a constant agitation of 180 revolution per minute (rpm). Growth was estimated by measuring the optical density at 618 *nm* on the UV-spectrophotometer continuously for 48 h with 2 h intervals. The medium which yielded best results was selected for further studies.

Optimization of Temperature, pH and Agitation

Optimum temperature, pH and agitation (rpm) were determined by growing the culture on an incubator shaker at varying initial temperature, pH and rpm by changing one parameter at a time. UY-medium (without agar) was used for these studies. Samples were withdrawn at 2 h intervals and OD was measured at 618 *nm* to assess the growth. pH was measured using a pH meter.

Assay Methods

Soluble proteins and sugars were estimated by the methods of Lowry et al.[133]

and Miller[148], respectively. For the estimation of soluble proteins, 0.2 ml of the

sample (diluted if necessary to a suitable concentration) was made up to 1 ml by

adding distilled water in a 30 ml test tube. To this, 4.5 ml of freshly prepared

alkaline mixture was added, vortexed on a Vortex-Mixer and kept undisturbed for 10

min at room temperature. [Alkaline mixture contained 1% copper sulphate (a); 2%

sodium potassium tartarate (SPT), (b) and 2% sodium carbonate in 0.1 N NaOH (c)

ie. a:b:c in 1:1:98 ratio]. After 10 min, 0.5 ml of Folin's reagent was added, vortexed

and kept undisturbed for 30 min at room temperature. Before use, Folin's reagent

was diluted with distilled water at 1:2 ratio. After 30 min, the mixture was read at

640 nm for obtaining concentration against blank, which did not contain sample

solution. Bovine Serum Albumin (BSA) was used to make a standard graph for

estimating proteins by using the concentrations (mg ml^{-1}): 0.1; 0.2; 0.3; 0.4; 0.5; 0.6;

0.7; 0.8; 0.9 and 1.0. Concentrations (mg ml^{-1}) of soluble proteins were calculated

directly from the spetrophotometric readings.

For the estimation of soluble sugars in the sample, 0.5 ml of the sample was

taken in a 30 ml test tube and 2 ml distilled water was added to it. To this, 3 ml

dinitrosalicylic acid (DNS) reagent was added using a water dispenser. This mixture

was heated in a boiling water bath for 12 min. After cooling to room temperature,

16 ml distilled water was added to dilute the mixture and vortexed for 3 min and

then read at 546 nm for obtaining the concentration. [DNS reagent was prepared by

dissolving 100 g sodium potassium tartarate, 0.25 g sodium sulphite (anhydrous), 1

g phenol and 5 g 3, 5-DNS in 250 ml 2 N NaOH using a magnetic stirrer and diluted to 500 ml with distilled water]. Glucose was used as standard.

Cell Count

Viable cells in the growth medium was determined by plate-count (colony-count) technique, using a colony counter. In this method, 1 ml of an appropriately diluted (10^1-10^8) cell suspension was poured into a pre-sterilized petri-dish. Autoclaved UY-medium containing agar (40°C) was poured into the petri-dish containing the cell suspension and was thoroughly mixed with the inoculum by rotating the plate under an inoculation hood. After the solidification of the medium, the plates were inverted and sealed using parafilm and incubated for 30 h at 28°C. Plates having 1-300 colonies were used for counting the number of yeast cells.

Preparation of Sample for SEM

Culture at the log phase (20 h growth) was centrifuged at 8000 g for 10 min at 4°C in a sterile tube. The pellet was made into a paste with distilled water. 0.1 ml of this paste was mounted on a clean glass slide. Two drops of 10% gluteraldehyde (GD) was added on it to fix the cell and kept undisturbed. After 6-8 min, GD was drained off and washed the sample repeatedly with distilled water (5 times) to remove traces of GD. In the next step, the above preparation was dehydrated with ethyl alcohol using different dilutions (%) for 5 min each (70, 80, 90 and absolute alcohol in succession). The dehydrated sample was then mounted on a brass stud and

coated with gold (100 A° film). This gold coated specimen was scanned using

Scanning Electron Microscope (SEM) at an accelerated voltage of 15 KV.

Instruments Used

Spectrophotometer	Shimadzu, UV-160A, Japan
Laminar Flow Chamber	Thermadyne, HCL-104, India
Cooling Centrifuge	Remi, C-24, India
Microcentrifuge	Remi, RM-12 C, India
Incubator Shaker	MB-Orbit Environ Shaker, SK-1009 R, India
Incubator Shaker	Certomat-MO, Germany
Incubator	MB Laboratory Incubator, DTC-1, India
Weighing Balance	Mettler, PM-200, Switzerland
Water Activity Meter	Novasina, RTD-33, TH-2, Switzerland
Moisture Balance	Mettler, LP-16, Switzerland
pH meter	Systronics, μ pH system - 361, India
Portable CO_2 Indicator	Riken Keiki Co. Ltd., RI-411A, Japan
Colony Counter	Lapiz, India
Hot Air Oven	Kemi, India
Electrophoretic set-up	Biotech. India
Peristaltic pump	Eyela Microtube pump, MP-3, Japan
Water bath	Superfit, India
Magnetic Stirrer	Remi-ZMLH, India
Vortex Mixer	Superfit-VM 301, India
Heating mantle	JSGW, India
Water dispenser	: Dispensette, Switzerland
Refrigerator	Kelvinator, Corona DLX, India
Polarised Optical Microscope	Nikon-HFX, Japan
Scanning Electron Microscope	Jeol, JSM-35C, Japan
Ultrafiltration Unit	Millipore, XX-80 002 30, USA

Photomicrography

Culture broth in UY-medium at different time intervals was mounted on a clean glass-slide and observed through a high resolution polarised optical microscope to study the morphology of *C. rugosa*. Interesting morphological features were photomicrographed.

Results and Discussion

Candida rugosa grew best in various defined media. A few of them are enlisted in Table IIIA1. Studies on optimization of growth parameters were started with 180 rpm at neutral pH (7.0) and at room temperature, which were chosen arbitrarily to choose the best among the five media used (Fig.IIIA1). In all the five media, maximum growth (as monitored by OD) was obtained at around 20 h (Fig.IIIA1).

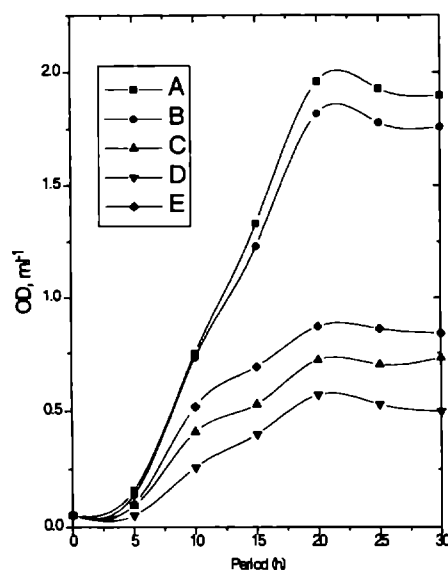Fig.IIIA1. Comparative study of growth (OD) of *C. rugosa* in five media at different time intervals, 180 rpm, pH 7.0 at room temperature. A: MA; B: MB; C: MC; D: MD and E:ME

Both the complex media (A and B) supported the growth of *C. rugosa* to the maximum followed by semi-synthetic medium (Medium-E). Synthetic media (C and D) supported the least growth (Fig.IIIA1). Complex media contained more or less

finished compounds (abundant in peptone/yeast/malt extract), which are the intermediate compounds required for the biosynthesis of proteins, nucleic acids, lipids and derived compounds. On the contrary, synthetic and semi-synthetic media did contain only minimal supply of basic elements. Comparatively, retarded growth in these media could be due to some hindrances in the biosynthetic pathways, which led to the formation of final products for the construction of body[17]. Or else, some biosynthetic pathways, which are highly developed in other higher eukaryotes, may be poorly developed in C. rugosa and made it unsuitable to live in synthetic media[5]. Moreover, the complex media would contain a number of trace and ultra-trace elements (as contaminants), which were not obtained through synthetic media. Between complex media A and B, A was superior to B. Hence, for further studies, medium-A (UY-medium) was used.

Studies on pH optima in UY medium showed that C. rugosa preferred neither acidic nor alkaline pH, but a pH at around 7.0 supported maximum growth. Growth was highly retarded at pH below 6.0. On the basis of this finding, different initial pH viz. 6.0; 6.2; 6.4; 6.6; 6.8; 7.0; 7.2; 7.4 and 7.6 were set to monitor the growth behaviour. Generally, maximum growth (OD) was observed at 20 h of growth (Table IIIA2). Gradual increment in growth was noticed when the initial pH was changed from acidic (6.0) to neutrality. However, beyond pH 7.0, growth showed further decreasing trend. Maximum OD (1.51) was obtained in the sample with initial pH 6.8. During prolonged fermentation beyond 20 h, OD did not show much variation. These studies confirmed that 6.8 pH was the optimum for better growth of C. rugosa.

Table IIIA2. Influence of different initial pH on the growth (OD) of *C. rugosa* in UY-medium at a constant agitation (180 rpm) and room temperature

Fermentation time (h)	Growth (OD ml^{-1})								
	Initial pH								
	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6
2	0.05	0.01	0.03	0.07	0.11	0.08	0.06	0.03	0.02
4	0.02	0.02	0.06	0.19	0.22	0.12	0.10	0.06	0.04
6	0.06	0.05	0.09	0.35	0.29	0.25	0.18	0.13	0.11
8	0.07	0.08	0.12	0.42	0.36	0.38	0.26	0.15	0.13
10	0.09	0.10	0.16	0.59	0.65	0.61	0.53	0.42	0.25
14	0.22	0.25	0.26	0.93	0.94	0.90	0.95	0.85	0.72
16	0.26	0.29	0.38	0.98	1.40	1.32	0.99	0.92	0.76
18	0.31	0.36	0.42	1.01	1.48	1.39	1.12	1.02	0.82
20	0.30	0.35	0.44	1.03	1.51	1.45	1.15	1.04	0.83
22	0.30	0.34	0.44	1.03	1.51	1.40	1.11	1.02	0.80
24	0.30	0.34	0.43	1.02	1.51	1.41	1.08	1.00	0.81
26	0.29	0.34	0.43	1.03	1.50	1.40	1.08	1.00	0.82
28	0.29	0.32	0.42	1.03	1.48	1.40	1.06	1.01	0.80
30	0.28	0.31	0.42	1.01	1.49	1.30	1.06	1.01	0.80

Standardization of temperature optima studies started on arbitrarily chosen temperature, *ie.* 30±1°C (room temperature). Since optimum temperature is a crucial factor, different initial temperature were set to analyse the most suitable initial temperature (°C) *viz.* 25; 28; 30; and 35. Maximum OD (growth) observed with room temperature was only 1.51, as shown in Table IIIA2. However, corresponding growth with initial temperature (standardised) , 28°C was 1.72 (Table IIIA3). It indicates that 28°C is the optimum initial temperature, against conventional room temperature, for the best growth of *C. rugosa*.

Table IIIA3. Influence of varying temperature on the growth (OD) of *C. rugosa* in the UY-medium at a constant agitation (180 rpm) and initial pH 6.8.

Fermentation period (h)	OD ml^{-1}			
	Temperature (°C)			
	25	28	30	35
2	0.06	0.06	0.90	0.70
4	0.16	0.31	0.32	0.21
6	0.22	0.41	0.45	0.33
8	0.40	0.52	0.61	0.48
10	0.68	0.92	0.95	0.81
12	0.91	0.96	1.02	0.92
14	0.98	1.21	1.15	1.05
16	1.15	1.34	1.23	1.08
18	1.19	1.52	1.32	1.15
20	1.36	1.72	1.51	1.25
22	1.36	1.72	1.50	1.25
24	1.36	1.72	1.50	1.25
26	1.35	1.70	1.50	1.24
28	1.35	1.70	1.50	1.24
30	1.35	1.70	1.50	1.24

Like pH and temperature, agitation (rpm) is also a limiting factor for *C. rugosa* growth. The experiments were started with an arbitrary initial rpm (180). Different initial rpm selected along with optimized pH (6.8) and temperature (28°C) were: 100, 150, 200, 250, 300, 350, 400, 450. Under these conditions, 250 rpm was found to be optimum (Table IIIA4) with maximum growth (1.86), which was the best result observed in the present study. Hence, it could be confirmed that 250 rpm, 28°C and 6.8 pH were the optimum parameters for the maximum growth of *C.rugosa*. These parameters were adopted invariably in all the succeeding experiments, unless otherwise specified.

Table IIIA4. Influence of aeration (agitation) on the growth (OD) of *C. rugosa* in UY-medium at varying rpm (initial pH, 6.8 and temperature, 28°C)

Period (h)	Growth, OD ml^{-1}							
	Agitation (rpm)							
	100	150	200	250	300	350	400	450
0	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
2	0.07	0.10	0.12	0.14	0.10	0.08	0.06	0.06
4	0.12	0.21	0.26	0.27	0.15	0.12	0.10	0.08
6	0.35	0.36	0.38	0.40	0.25	0.21	0.15	0.13
8	0.61	0.66	0.71	0.76	0.76	0.73	0.76	0.71
10	0.65	0.72	0.78	0.95	0.81	0.76	0.76	0.75
12	0.72	0.81	0.89	1.05	0.85	0.85	0.81	0.86
14	0.79	0.93	0.98	1.12	0.96	0.97	0.95	0.92
16	0.85	0.99	1.12	1.35	1.21	1.16	1.01	1.08
18	1.23	1.28	1.36	1.52	1.36	1.23	1.11	1.21
20	1.43	1.48	1.73	1.86	1.73	1.70	1.63	1.42
22	1.40	1.45	1.70	1.84	1.70	1.65	1.63	1.42
24	1.40	1.44	1.71	1.84	1.68	1.65	1.61	1.40
26	1.41	1.44	1.70	1.85	1.68	1.65	1.61	.140
28	1.40	1.43	1.70	1.84	1.68	1.65	1.62	1.40
30	1.41	1.43	1.70	1.84	1.68	1.63	1.61	1.41

Table IIIA5 gives a vivid picture on the combined effect of all the three parameters discussed above. As it describes, the initial soluble sugar concentration was 14.12 mg ml^{-1}. After 20 h growth, it was depleted to 3.11 mg ml^{-1}, showing 78% consumption. Growth (OD) (Fig.IIIA2) and viable cell count (Fig.IIIA3) also showed gradual increase upto 20 h. On the contrary, upto 12 h growth, concentration of soluble protein showed gradual depletion. But, after 12 h, its concentration gradually increased and rose to the level, 6.8 mg ml^{-1} at 20 h of growth. It was a clear evidence for protein secretion in the medium[17]. After 20 h of growth, OD did

not show appreciable change (Fig.IIIA2). A reasonable explanation for this invariability is that both growth and death were equal at this stage (zero growth rate)[17]. However, viable cell count remained more or less standstill upto 48 h of growth, by 60h, 90% of the cells were perished(Fig.IIIA3).Surprisingly, OD did not show much change at this stage. It can be concluded that this stagnancy in OD was due to the decay of the dead cells and simultaneous division of the viable cells utilizing these dead remains[5]. Sharp decline of viable cell count after 48 h was due to the less availability of absorbable nutrients and also growth retarding substances released by the decaying cells[17] (Table IIIA5).

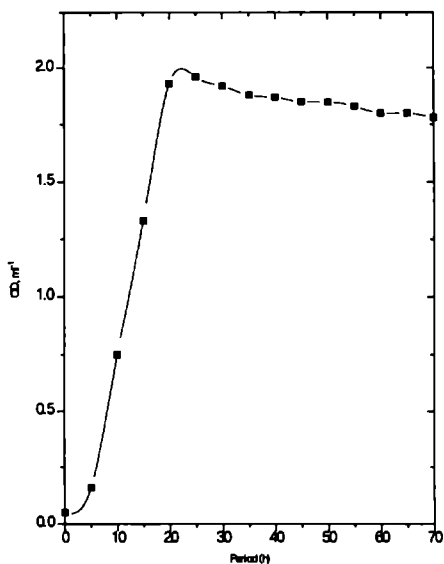

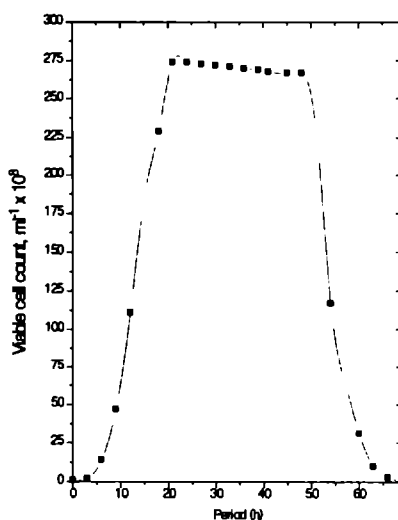Fig.IIA2. Growth curve using OD (0-70 h) at pH 6.8, 28°C and 250 rpm in UY-medium

Fig.IIA3. Growth curve showing viable cell count upto 70 h fermentation

Table IIIA5. Standardized growth behaviour of *C. rugosa* in UY-medium (at 250 rpm, 28°C and initial pH 6.8 showing OD; sugar consumption; protein consumption/secretion, viable cell count and pH variation

Period (h)	OD (ml⁻¹)	Soluble sugar (mg ml⁻¹)	Soluble protein (mg ml⁻¹)	(Viable cell count ml⁻¹) x 10^8 (dilution factor)	pH
0	0.10	14.12	3.16	1	6.8
2	0.37	13.71	3.01	1	6.8
4	0.37	12.51	2.82	3	6.9
6	0.50	12.17	2.62	14	6.9
8	0.86	11.01	2.30	29	6.9
10	1.05	8.32	2.12	56	7.0
12	1.15	6.77	2.52	111	7.1
14	1.22	6.15	3.71	160	7.2
16	1.45	4.71	3.95	201	7.3
18	1.62	3.59	4.61	229	7.4
20	1.96	3.11	5.71	260	7.3
22	1.94	3.09	5.82	274	7.4
24	1.94	3.06	5.82	274	7.4
26	1.95	3.06	6.82	274	7.3
28	1.95	3.05	6.85	273	7.4
30	1.95	3.02	6.82	272	7.4

Figs.IIIA4-7 clearly demonstrate the morphology of *C. rugosa* cells at different intervals of growth in UY-medium under optimised condition. Fig.IIIA4 describes the actively dividing *C. rugosa* cells which appeared to be swollen, curved or even saucer-shaved (24 h fermentation). Interestingly, Fig.IIIA5 shows slender and filamentous nature of cells at longer period of fermentation (48 h). Pseudo-filamentous appearance was developed due to the budding and subsequent non-separation of the daughter cells[114]. Same phenomenon was noticed in Figs.IIIA6&7. Pseudo-filamentous branched thallus formation is much prominent in

Figs.IIIA6&7. From these observations it could be concluded that during active growth phase (exponential growth phase), *C. rugosa* assumed swollen and spherical morphology, while at the later stages of growth, it existed in the pseudo-filamentous structure. Swollen morphology indicated active nucleic acid synthesis along with the construction of cellular components to beget daughter cells. Pseudo-filamentous structure was the sign of sluggish but industrially significant growth and during this phase active synthesis of extracellular substances (primary and secondary metabolites) occur[114]. These findings were confirmed by further studies, which appear in the subsequent chapters.

Interestingly, Figs.IIIA6&7 bear chains of conidia and chlamydospores along the pseudo-filamentous 'mycelium'. These photographs were taken after 60 h of fermentation. It shows that at the brink of death, *C. rugosa* escapes death by forming resistant spores such as chlamydospores or conida to cross over unfavourable season. Such spores were also noticed by Milikhina *et al.*[147].

This chapter, in general, gives a vivid idea about the fermentation behaviour, growth characteristics and the morphology of the organism used. All the materials and methods described in this section were invariably adopted in the succeeding chapters, unless otherwise specified.
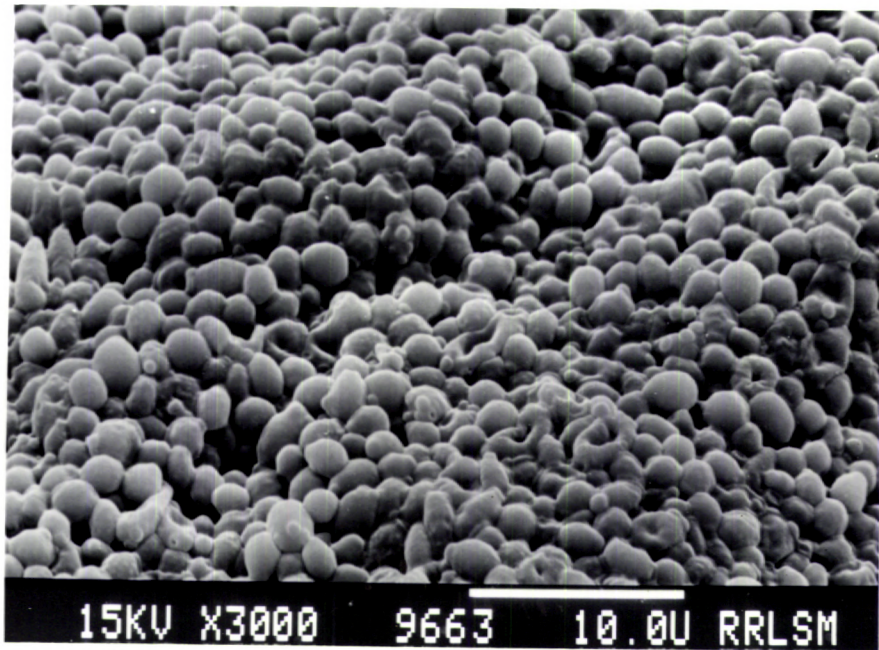
Fig.IIIA4. *C. rugosa* cells undergoing active division by fussion and budding in liquid medium at 24 h fermentation
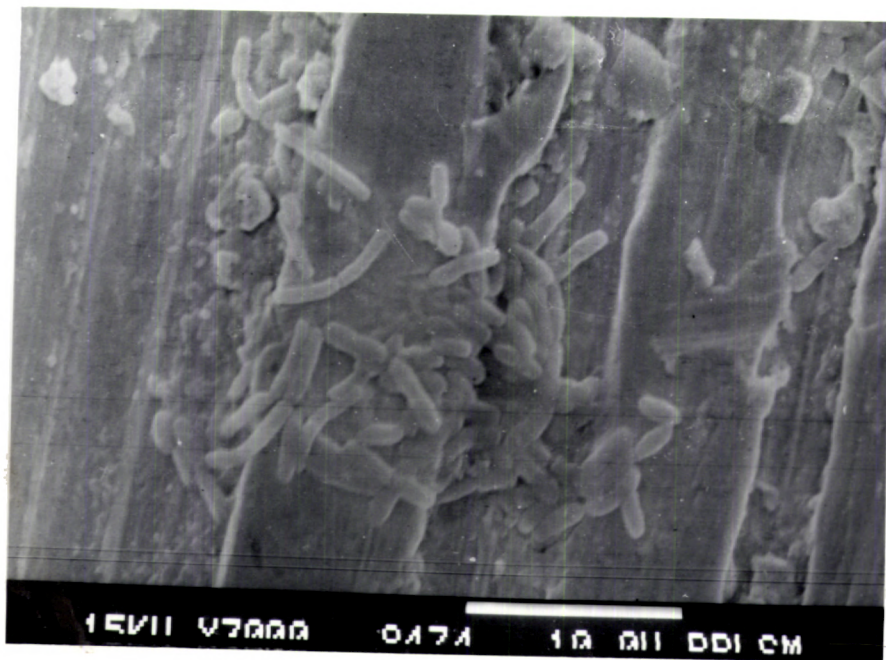

Fig.IIIA5.Pseudo-filamentous structure of *C. rugosa* in liquid medium at 48 h fermentation.
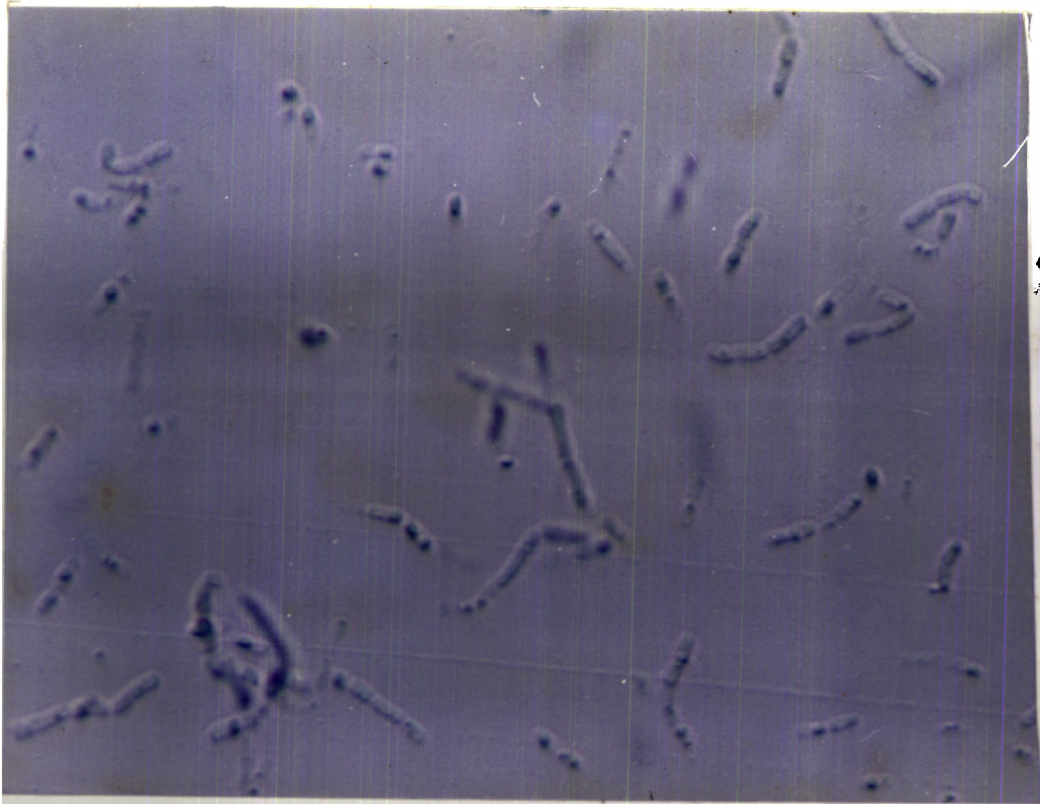
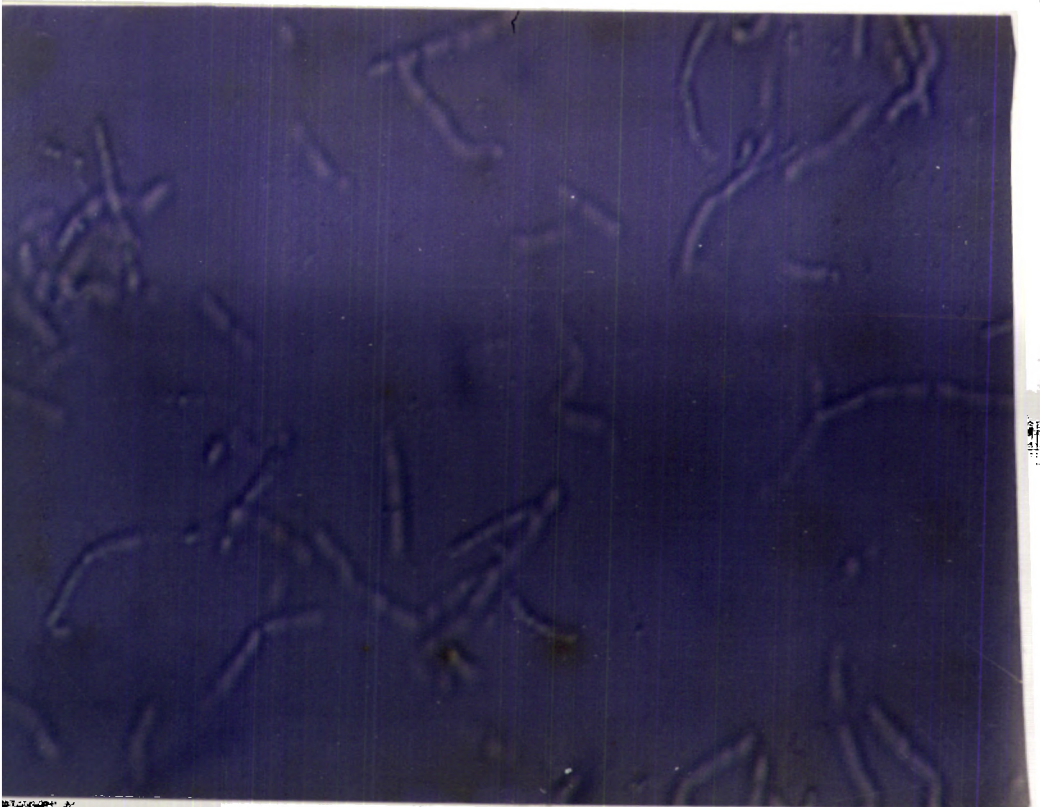Fig.IIIA6.Pseudo-filamentous structure with conidia and chlamydospores at 48 h of fermentation (X 400)

Fig.IIIA7.Pseudo-filamentous structure with chlamydospores (X 400)

CHAPTER - III B

SUBMERGED FERMENTATION

Introduction

Enzymes of industrial interest have traditionally been obtained from submerged cultures because of its control and handling advantages[17]. Upper hand of submerged fermentation (SmF) over solid-state fermentation (SSF) for the cultivation of *C. rugosa* has been described by many authors[199]. Fermentation conditions for the production of extracellular lipases by *C. rugosa* free cells in suspension cultures (SmF) have been investigated by Benjamin and Pandey[17]. It has been concluded that the presence of lipids and lipid-related substances induce secretion of copious amount of lipases along with other proteins in the culture medium. In addition, a significant influence of aeration with a minimum dissolved oxygen concentration has also been reported[62]. In this Chapter, optimization of universal yeast (UY) medium for lipase production by supplementing it with different elements, including nitrogenous salts, different carbon sources and the inducing role of olive oil is described.

Methods

Submerged Fermentation and Media

UY medium (without agar) was the basic medium used in all the experiments described in this chapter. Different media formulations were designated by letters from A to I (Table IIIB1). MA (M = medium) contained the constituents of UY-medium (without agar), which was used for inoculum preparation. MB contained the ingredients of MA and mineral nutrition as devised by Pandey[176]. MC was prepared by adding different concentrations (% w/v) of olive oil (2, 4, 6, 8, 10,

12 and 15) in MA. For preparing MD, some modifications were made to the medium formula devised by Ferrer and Sola[62]. ME contained constituents of UY-medium, inorganic nutrients and 10% olive oil. In MF, glucose was replaced by different carbon sources at 1% concentration. Slight modifications were made in ME to get MG, MH and MI. In MG, different ammonium salts and urea were tried at different concentrations. In MH, combined effects of urea and maltose were tested. MI was the enriched standard medium, which was used for the estimation of soluble proteins, carbohydrate and pH, apart from lipase activity and growth.

Table IIIB1. Media and their combinations

Media (pH 6.8)	Composition (liquid nutrients in v/v and solids in w/v basis)
A (UY-medium)	Glucose - 10; peptone - 5; yeast extract - 3 and malt extract - 3 (all g L^{-1})
MB	MA + mineral nutriton (ie., 3.4 mg Fe; 3.8 mg Zn; 0.43 mg Cu and 0.12 g N/100 ml basis)
MC	MA + different % of olive oil (2,4,6,10,12 and 15)
MD	Glucose 10; KH_2PO_4 2; urea 3; $MgSO_4.7H_2O$ 1; $CaCl_2.2H_2O$ 1 (all g L^{-1}) and $FeCl_3.6H_2O$ 10; thiamine-HCl - 0.2; biotin - 0.008 and inositol - 0.004 (all mg L^{-1})
ME	MA + $(NH_4)_2SO_4$ - 1.67; $CaCl_3.2H_2O$ - 0.5; KH_2PO_4 -0.4; K_2HPO_4 - 0.24, $MgSO_4.7H_2O$ - 0.2 (all g L^{-1}) and $FeCl_3.6H_2O$ - 4; thiamine-HCl - 0.2; biotin - 0.008; inositol - 0.004 (all mg L^{-1}) and 10% olive oil
MF	MA + (glucose was replaced by 1% starch, fructose, glucose, lactose, sucrose or maltose) 10% olive oil
MG	ME + (ammonium sulphate is replaced by different concentration of urea/other ammonium salts)
MH	ME + (ammonium sulphate is replaced by 400 mg L^{-1} urea and glucose is replaced by 1% maltose)
MI (enriched medium)	MH + 0.3% gum arabic

Inoculum Preparation

For preparing the inoculum, a loopful of cells from a freshly grown agar slant of *C. rugosa* culture was transferred to a 250 ml conical flask containing 50 ml of UY medium. The flask was incubated at 28°C on a rotary shaker at 250 rpm for 24 h and then the cells were harvested by centrifuging the broth (15 ml) at 4°C for 10 min at 8000 g in a sterile tube. The pellet was resuspended in 5 ml sterile distilled water (3 mg ml^{-1} biomass). One ml (2%) of this suspension was used for inoculating 50 ml medium.

Fermentation

Fermentation was carried out with 50 ml of the medium (Table IIIB1) in 250 ml conical flasks. All the media enlisted in Table IIIB1 were individually used for the cultivation of *C. rugosa*. After autoclaving, the flasks were inoculated with the cell suspension (2%) of *C. rugosa*, prepared as above. In all the experiments, the initial pH of the medium was maintained at 6.8. The flasks were incubated at 28°C on an incubator shaker (250 rpm) for 72 h. Sampling was done by removing the whole flasks in triplicate at every 12 h intervals. The results reported are the average values (standard deviation being ±0.22 to 1.3%) of three sets of the experiments.

Assay Methods

For different assays, an aliquot (20 ml) of the fermented broth was centrifuged at 8000 g for 10 min at 5°C. Supernatant so obtained was diluted (if necessary) to a suitable concentration. Lipase activity was determined by the modified method of Safarik[205]. In this spectrophotometric method, an aliquot of

triolein (100 mg) was transferred into a test tube containing 2 ml of 0.1 M Tris-HCl

(pH 7.0) and 1 ml of sample (after adequate dilution) was added to it. The mixture

was vortexed for 30 s and incubated at 37°C in a water bath under static condition

for 30 min, for the hydrolysis of triolein and subsequent release of free fatty acids

(oleic acid). Reaction was stopped by adding 1 ml conc.HCl. This mixture was

vortexed for 10 s on a vortex mixer. To all test tubes, 3 ml benzene was added and

the test tubes were vortexed for 90 s. Both aqueous and organic phases were

separated using a pasteur pipette. Benzene layer was withdrawn and transferred to a

tube containing 1 ml of aqueous cupric acetate solution (5% concentration, pH 6.2,

adjusted using pyridine) and the mixture (after vortexing for 90 s) was centrifuged at

5000 rpm for 10 min at 5°C to obtain a clear organic phase. The organic (benzene)

layer was transferred into a photometric cuvette and concentration of free FFA was

estimated at 715 nm against blank, which did not contain sample. One unit (U) of

lipase activity was expressed as μmoles of FFA (oleic acid) liberated/minute/ml

under the assay conditions, ie. U ml^{-1} (molecular weight of oleic acid = 282.5).

$$\text{Quantity of oleic acid liberated} = \frac{\text{concentration (for 1 ml) x dilution factor}}{282.5 \text{ x icubation time in minutes}} \times 1000$$

(lipase activity, U ml^{-1})

Soluble proteins and sugars were estimated out of the culture supernatant by

the spectrophotometric methods of Lowry et $al.$[133] and Miller[148], respectively as

described elsewhere. The dry cell weight (DCW) was determined by keeping the cell

pellet obtained after centrifugation of fermented broth in an oven overnight at 80°C.

OD was determined by estimating the turbidity at 618 *nm*.

Results and Discussion

Growth profile of *C. rugosa* in MA is shown in Fig.IIIB1. Maximum OD

(1.94) was observed at 20 h of

cultivation. But in the enriched

medium (MI) maximum OD

was 2.12 after 20 h of

cultivation (Fig.IIIB2). In both

the cases, OD did not show

much deviation from the peak

mentioned above, during the

subsequent growth. Cell count

experiments conducted in

Chapter IIIA revealed that by

20-24 h of growth, *C. rugosa*

Fig.IIIB1. Growth profile of *C. rugosa* in medium A

showed maximum OD. Viable

cell count remained more or

less constant up to 46-50 h of

growth. After this, the viable cell count showed a sharp decrease in number.

However, OD did not show much variation, which indicated simultaneous decay of

dead cells and compensatory genesis of new cells utilizing the dead organic
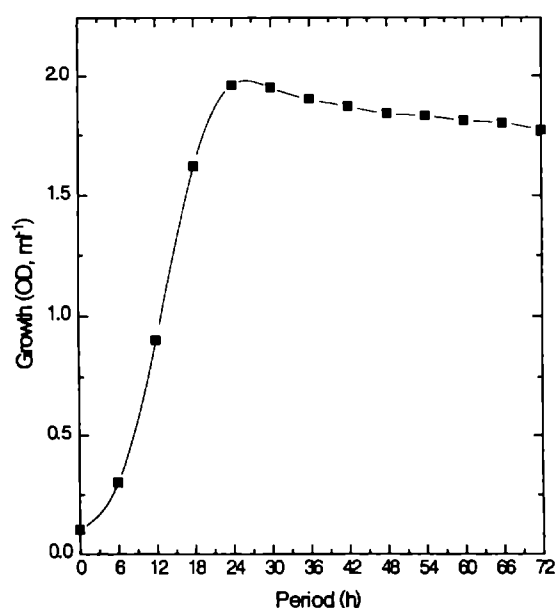
remains[25]. The same phenomenon can be viewed from growth profiles described above (Figs.IIIB1 & 2).

Dry cell weight (DCW) was also estimated in all the media (A to I) at 48 h of fermentation to assess the comparative growth profile in all the media (Fig.IIIB3). Surprisingly, in MD, which was purely synthetic, growth was very poor (3.08 mg ml^{-1}) in comparison to the growth in other media (Fig.IIIB3). Maximum DCW (36.65 mg ml^{-1}) was obtained in the enriched medium which contained 0.3 gum arabic (MI). In all other media, average DCW was 25 mg ml^{-1}. In fact, growth in media containing olive oil showed better results[17].

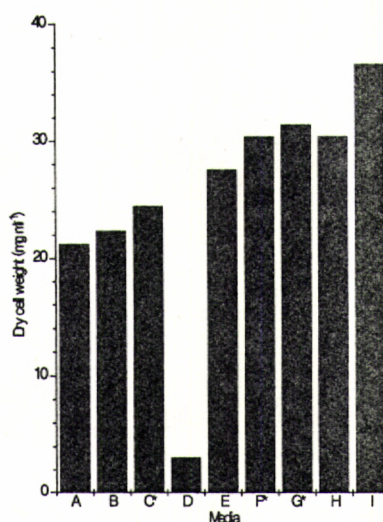Fig.IIIB2. Growth profile of C.rugosa in enriched medium (MI)

Fig.IIIB3. Comparative growth profile of C. rugosa in different media used. Dry cell weight at 48 h growth. C*: MC with 10% olive oil, F*: MF with 1% maltose and G*: MG with 400 mg L^{-1} urea

Though lipids are the most favourable substrates for lipase production, occurrence of limited quantity of lipids or lipid-related substances in the growth medium did favourably influence the quantity of lipase secreted[17,237]. This view was confirmed by the studies with olive oil, a widely used substrate for lipase assay (Table IIIB2). This table describes the inevitability of lipids (olive oil) in inducing lipase secretion, even below at 0.5% concentration. However, addition of mere 2% olive oil could enhance lipase secretion by five-fold. When the concentration of olive oil was increased upto 10%, it became a limiting factor but, by that time, the enzyme yield was increased to 8.36 U ml^{-1} at 48 h. Indeed, olive oil concentration beyond 10% was found to be unsuitable. Another striking feature was that upto 12 h of fermentation no lipase activity was noticed in any media studied. Yield from 12-24 h fermentation was also negligible, though exponential phase of cell growth was noticed during this period.

Table IIIB2. Lipase production profile in MC containing different concentrations of olive oil

Fermentation time (h)	Lipase activity* (U ml^{-1})							
	Concentration of olive oil (%)							
	0	2	4	6	8	10	12	15
24	0.18	0.86	0.96	1.35	1.61	2.38	2.24	2.18
36	0.25	1.30	1.82	2.48	3.13	4.99	4.70	4.58
48	0.33	2.58	3.15	5.81	7.38	8.36	8.18	8.00
60	0.29	2.55	3.98	5.09	7.49	8.28	8.17	7.98
72	0.25	2.32	3.85	5.67	7.47	8.03	7.95	7.97

*no lipase secretion upto 12 h samples

Maximum lipase secretion was noticed at 48 h of fermentation (Fig.IIIB4), a unique feature noticed in all the media studied. It was the senescence phase of growth. In the next 24 h, 90% of the cells were dead (Fig.IIIA3). Interestingly, *C. rugosa* cells mostly assumed pseudo-micelial or swollen morphology between 30-50h of fermentation (Figs.IIIA4-7) and also it was during this period maximum lipase.
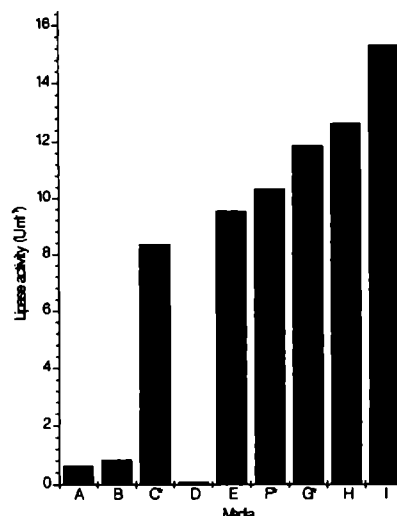

Fig.IIIB4. Comparative lipase production profile of *C. rugosa* in different media used, at 48 h of fermentation. C*: MC with 10% olive oil, P*: MF with 1% maltose, G*: MG with 400 mg L⁻¹ urea

Table BIII3. Lipase production profile in medium-F containing different carbon sources

Fermentation time (h)	Lipase activity* (U ml⁻¹)					
	Carbon source (1%)					
	Starch	Fructose	Glucose	Lactose	Sucrose	Maltose
24	0.13	1.83	1.75	2.14	2.18	2.47
36	0.35	4.87	4.92	5.16	5.19	5.40
48	0.75	8.29	8.30	8.74	9.76	10.34
60	0.75	8.27	8.28	8.74	9.74	10.20
72	0.74	8.20	8.26	8.74	8.73	9.18

*no lipase secretion upto 12 h

activity was noticed. As shown in Fig.IIIB4, lipase yield in media A, B and D was very low, even less than 1 U ml^{-1}. In all other media, 10% olive oil was added as an additional carbon source cum inducer, which caused significant enhancement in yield.

Table IIIB3 describes the individual role of each carbohydrate on lipase production (MF). Glucose is the usual carbon source, but in this experiment, disaccharides like lactose, sucrose and maltose favoured maximum yields than monosaccharides (glucose and fructose) used. In fact, yield (also growth) in the medium containing starch was significantly less. It also confirms that *C. rugosa* was not secreting the starch hydrolysis enzymes to assimilate it[43]. Among disaccharides, maltose was most preferred because its presence in the medium influenced to yield maximum among all the carbohydrates used[17].

Table IIIB4. Lipase production profile in medium-G at 48 h of fermentation using different concentrations of nitrogen sources (mg L^{-1})

Nitrogen source	Lipase activity (U ml^{-1})						
	Concentration of nitrogen source used (mg)						
	100	200	300	400	500	1,000	2,000
Diammonium hydrogen phosphate	0.02	0.08	0.49	0.47	0.45	0.38	0.26
Ammonium chloride	0.06	0.06	0.55	0.59	0.49	0.41	0.32
Ammonium sulphate	0.53	1.09	3.55	4.58	3.54	3.32	3.18
Ammonium nitrate	1.31	2.75	7.13	8.66	8.61	7.95	7.84
Urea	8.81	7.93	8.48	11.86	11.41	10.53	9.45

In another study, the optimum concentration of nitrogenous salts in the medium was tested (Table IIIB4). Diammonium hydrogenphosphate, ammonium chloride, ammonium sulphate, ammonium nitrate and urea were used as nitrogen source at different concentrations (100, 200, 300, 400, 500, 1000 and 2000 mg L^{-1}). Among them, 400 mg L^{-1} of the above sources was found to be optimum. While ammonium nitrate and urea yielded positive results, the other three salts showed negative results. For further studies urea was used as the nitrogen source on the basis of its superiority, in comparison with other salts.

When olive oil was added in the medium, it remained on the surface as a layer[62], which hindered the diffusion of oxygen into the medium, thereby reducing both growth and activity of microbial cells. This barrier was overcome by adding different concentration of gum arabic in the medium. Gum arabic emulsified the oil, which facilitated free diffusion of oxygen into the medium and unnecessary gases like carbon dioxide out of the medium. Moreover, the emulsified form of oil functioned as the best
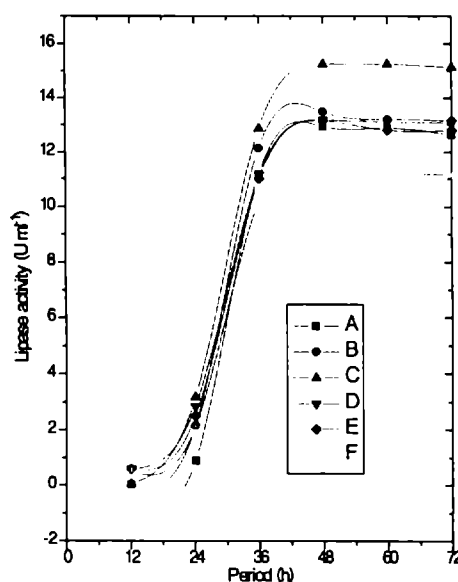

Fig.IIIB 5. Effect of different concentrations of gum arabic in lipase production on MH. A: 0.1%, B: 0.2%, C: 0.3%, D: 0.4%, E: 0.5% and F: 1.0%.

substrate for *C. rugosa*. As other additives, gum arabic also became a limiting factor, at its sub-optimal or supra-otpimal state. Its 0.3% concentration was found to be optimum (Fig.IIIB5). MH containing 0.3% gum arabic influenced both lipase production and growth to significantly high values (Fig.IIIB5 and Table IIIB5) by better substrate utilisation.

Table IIIB5. Fermentation medium of *C. rugosa* in enriched medium (MI)

Fermentation period (h)	Parameters analyzed				
	Lipase activity, (U ml^{-1})	Soluble sugars, (mg ml^{-1})	Soluble protein, (mg m^{-1})	Growth (OD, ml^{-1})	pH
0		14.26	4.01	0.10	6.8
12	0.05	14.01	3.71	1.27	6.7
24	3.18	5.31	3.75	2.12	6.9
36	12.86	3.82	5.86	2.06	7.1
48	14.31	2.02	6.98	2.01	7.4
60	15.26	1.98	6.94	1.96	7.4
72	15.14	1.96	6.93	1.93	7.4

Fermentation behaviour of *C. rugosa* has been depicted in Table IIIB5. While lipase yields (as already discussed), OD and pH show increasing trends, sugar concentration showed decreasing trend. By 48 h of growth, 86% of sugar was consumed, a sign of better utilization of the substrate, which also confirms the continuity of growth even after exponential growth phase (Figs.IIIA2&3). However, at 24 h where maximum OD was obtained, only 63% of sugar was consumed. Maximum lipase activity was noticed at 48 h. On the contrary, though protein concentration showed an initial decrease, it showed significant increase in the

succeeding hours, which was at par with increasing lipase activity. It clearly demonstrated the extracellular secretion of proteins including lipases, which also caused the increase in the pH of the medium[17].

This piece of work proved that in the absence of optimal level of lipids in the medium, C. rugosa could grow normally, but with a very low level of lipase secretion. Supplementation of the medium with lipids like olive oil, however, enhanced the lipase secretion. Del Rio et al.[54] described the mechanism behind the induction of lipase secretion by C. rugosa growing in olive oil. They proposed that the fatty acids released from the olive oil by the action of traces of lipase in the medium during the initial hours of fermentation, which might be contained in the inoculum were consumed by the organism and thus paved way for more lipase secretion. Consumption of fatty acid or lipids would activate the lipase gene, subsequently, they will be expressed as lipase protein. Action of gum arabic as surfactant in the medium further improved better substrate mixing and its accessibility to the organism. Wu et al.[250] found that presence of Tween-80 in the substrate substantially influenced the yield. As reported by Benjamin and Pandey[17], this study confirms the simultaneous growth and yield of C. rugosa under a healthy environment of balanced nutrients supplied and with optimum physical parameters like pH, rpm and temperature.

IRON REQUIREMENT

AND

SIDEROPHORE

Introduction

Iron has an invincible role in microbial physiology[160]. Pandey *et al.*[180] reported that for the balanced growth of microorganisms, adequate iron should be available because it acts as an important co-factor (either as activator or occupies in the prosthetic groups like haem) in many enzymes like cytochromes, hydroperoxidase, nitrogenase, etc. Active nitrogenase requires iron-protein complexes called molybdo-ferredoxin and azoferredoxin[144]. Ferredoxin is an important electron carrier to confer the reducing power in biological system, which strictly needs iron. In nature, however, limited amount of free iron is made available for microbial assimilation, owing to the insolubility of ferric iron at neutral or alkaline pH[91]. Bacteria and lower eukaryotes have evolved various powerful systems to overcome the low solubility of external iron. Because of the insolubility and toxicity of iron in the presence of oxygen, it is likely that all aerobic organisms have evolved a class of ubiquitous iron storage proteins, the ferritins which are able to sequester a few thousand iron atoms in their central cavity in a soluble, non-toxic bio-available form[7]. In the cell, metabolically inactive iron is generally transferred to the chelators - the storage compounds. The main iron-storage protein in animals and plants are ferritins and phytoferritins, respectively[34].

Under aerobic condition, when iron is present at its highly insoluble ferric (Fe^{3+}) from, bacteria and other microorganism fulfil their iron requirement through Fe^{3+} complexes of secondary metabolites termed siderophores, excreted usually in large amounts when cells are grown under iron deficient circumstances[7]. Membrane

carriers embedded in the plasmalemma specifically accept these Fe-chelates[110]. Most of the aerobic and facultatively anaerobic organisms secrete siderophores in response to iron-deprived environment[193]. The siderophores, the low molecular weight iron chelators, may also be synthesized by many microorganism in response to iron deprivation and are able to convert insoluble ferric hydroxide polymers into soluble chelates, which are substrates for high affinity transport mechanism[7]. Ismail et al.[91] suggested that the pathogenicity of virulent yeast, Candida albicans is dependent on its ability to acquire iron from its host. The same phenomenon was also reported in many bacteria, such as lactic acid bacteria, as reported by Pandey et al.[180].

Requirement of iron for the growth of C. rugosa has not yet been reported. However, conventionally, it is being added in slight quantities in the production media for lipase secretion[238]. This study envisages to unveil the metabolic role of iron in the growth of C. rugosa, and also its impact on lipase secretion. Secretion of siderophores in the deferrated medium is also highlighted.

Materials and Methods

Growth Medium

Two media (medium A and B) were employed in this study. Medium A (UY-medium) contained (g L^{-1}): glucose 10, peptone 5, yeast extract 3, and malt extract 3, which was used as the complex medium. However, synthetic medium (medium B) contained glucose: 20, KH_2PO_4 6, urea 1, $MgSO_4.7H_2O$, 1 (all g L^{-1}) and

biotin 0.008, inositol 0.004, $FeCl_3.6H_2O$ 0.04, Thiamine-HCl 0.2 (all mg L^{-1}). For

plate cultures, 15 g L^{-1} agar was added to these media as solidifying agent.

Culture Conditions

Initial pH, temperature and rpm were: 6.8, 28°C and 250, respectively

(Chapter IIIA). Same temperate and medium pH were also maintained in plate

cultures during incubation.

Detection of Iron Requirement

To assess iron requirement, C. rugosa was grown in both the media A, and B

as in their defined and also in deferrated forms. Medium A was deferrated by

8-hydroxy quinoline (5% in chloroform) treatment, by vigorous shaking in an

adequate borosil container. By autoclaving, it was ensured that the treated media did

not contain even traces of 8-hydroxyquinoline. Medium-B was used as such without

adding $FeCl_3$ to establish deferrated condition.

In another set of experiments, iron sufficient condition was established by

supplementing the sterilized deferrated medium with sterile ferric chloride (1 M) at

varying concentrations in $\mu l\ ml^{-1}$ (0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 5.0, 10.0 and 20.0) with

or without Ethylene Diamine Dihydroxyphenyl Acetic acid (EDDHA). EDDHA

was used as an iron chelator which was added in the medium on $\mu g\ ml^{-1}$ basis (25,

50, 100, 200, 500, 100). These studies were conducted in plate cultures using

8-hydroxyquinoline treated and untreated media. Roles of EDDHA and iron in

growth of C. rugosa were studied directly from colony formation.

Detection of Siderophores

Detection of siderophores production was carried out by growing *C. rugosa* as single cell colonies (after adequate dilution of inoculum) on the Chrome Azurole- S (CAS) added plates containing iron free UY-Agar medium. To prepare 1 litre CAS containing agar (blue) medium, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron(III) solution (1 mM $FeCl_3.6H_2O$ in 5 mM HCl). This mixture was slowly added to 72.9 mg Hexadecyl Trimethyl Ammonium bromide (HDTMA) dissolved in 40 ml distilled water, under stirring. The resultant dark blue liquid was autoclaved and was mixed with 900 ml of sterilized agar medium (50°C) and poured into the petri-plates in such a way that each plate received 30 ml of CAS-Agar medium. Final pH was 6.8-7.0. These plates were inoculated with dilute cell suspension before the medium was solidified. Streak cultures were also made after solidification. The behaviour of colonies formed was observed and scored.

CAS reagent was used to detect siderophores production in liquid cultures under iron-starved condition. For this, 6 ml of 100 mM HDTMA solution was taken in a 100 ml volumetric flask and diluted with water. A mixture of 1.5 ml iron(III) solution (1 mM $FeCl_3.6H_2O$ in 5 mM HCl) and 7.5 ml 2 mM aqueous CAS solution was slowly added to it under stirring. A weighed quantity (4.3) of anhydrous piperazine was dissolved in water and 6.25 ml of conc.HCl was also added to it. This buffer solution was rinsed into the volumetric flask containing above mixture and made up to 100 ml.

To detect siderophores, 0.5 ml of the above CAS reagent was mixed with 0.5 ml of culture supernatant in 4 ml test tube and change in colour of the mixture was observed. For obtaining clear supernatant, 1 ml of culture broth after stipulated period of fermentation was centrifuged in an Eppendorf centrifuge (5000 g). The colour change was compared with a reference sample which contained untreated supernatant

For estimating optimum iron requirement, different quantities of $FeCl_3.6H_2O$ salt was added in the iron-starved medium. Its effect was identified from corresponding OD and lipase production.

Results and Discussion

Growth of *C. rugosa* in both the media (untreated) is shown in Fig.IIIC1. Growth in Medium-B was very poor. Maximum OD in medium A was 1.96 at 20 h while in B, it was only 0.81. Studies with treated media showed the necessity of iron for *C. rugosa* metabolism (Fig.IIIC2). Treated media, however, showed comparatively less growth than in untreated media. On the basis of these
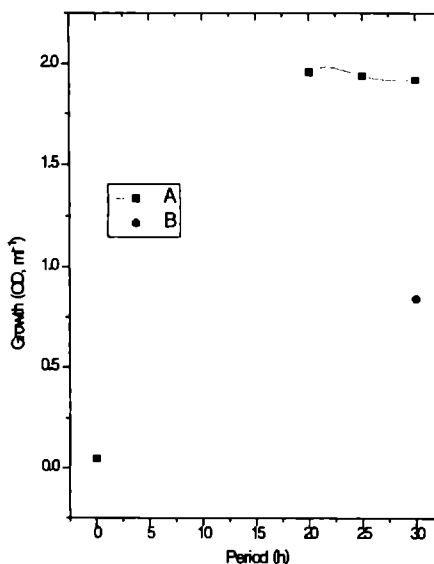

Fig.IIIC1. Comparative growth profile of *C. rugosa* in iron sufficient media, A and B. A: Medium A, B: Medium B.

preliminary studies, medium A was selected for further studies.

Table IIIC1 describes the effect of EDDHA (iron-chelating agent) on growth. Studies on the effect of EDDHA were carried out in untreated medium. Upto 100 µg ml^{-1} concentration EDDHA favoured the growth, but higher concentrations adversely affected the growth and even reduced to zero at 1000 µg ml^{-1}. Apart from drastic reduction in the number of colonies, the size of the colonies were also much affected by increasing

Fig. IIIC2. Comparative growth profile in deferrated media, A and B. A: Medium A, B: Medium B.

Table IIIC1. Influence of different concentration of EDDHA (µg ml^{-1}) on *C. rugosa* growth

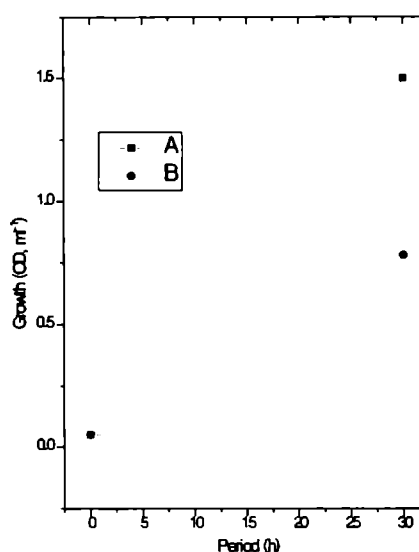|	Colonies	Size (mm)
Control (A)	Normal	3-4
A + 25 µg ml^{-1}	Moderate	2-3
A + 50 µg ml^{-1}	Scattered	2-2.5
A + 100 µg ml^{-1}	Scattered	1-1.5
A + 200 µg ml^{-1}	Moderate on heavy-streaked area	1-1.5
A + 500 µg ml^{-1}	Scattered on heavy-streaked area	1-0.5
A + 1000 µg ml^{-1}	Nil even at heavy streaked area	0

concentration. This adverse effect of EDDHA on growth was not its

iron-chelating effect alone, but its presence in the medium imparted toxic effect

or change in pH^{144}. Most probably, increasing pH was the reason for this toxic

effect. Further studies with liquid medium proved that presence of EDDHA in the

medium increased the pH, even to 10.0 which made unfit for the growth.

To investigate whether iron starvation caused the reduction in growth,

different concentrations of $FeCl_3$ were supplemented to the deferrated medium

in combination with EDDHA (Table IIIC2). Altogether, $FeCl_3$ at 1.0 μl ml^{-1}

concentration supported the growth even in the presence of low concentration of

EDDHA. However, higher concentration of EDDHA could not sustain growth even

if the concentration of iron was increased. Increased concentration of $FeCl_3$

beyond 1.0 μl ml^{-1} considerably reduced the growth. The reduction in growth was

due to the high acidic pH created by the increased concentration of $FeCl_3$ and was

not neutralised by $EDDHA^{144}$. At this stage, only 115 colonies were visible, but at

1.0 concentration, it was increased to 255. This showed C. rugosa required iron for

its better growth (Table IIIC2 and Fig.IIIC2). EDDHA, if present in the medium at

lower concentrations (upto 50 mg ml^{-1}) would show its pronounced effect on

iron-complex formation and subsequent retardation in growth. Both EDDHA and

$FeCl_3$ at higher concentration suppressed the growth by altering pH balance and, to a

certain extent, concentration-induced toxicity.

Like many bacteria and other microbes, C. rugosa also produces biological

iron chelating compound termed as the siderophores. Its presence in the iron-starved

Table IIIC2. Antigonistic effect of EDDHA and $FeCl_3$ in deferrated UY-medium

| Medium/EDDHA | 1M $FeCl_3$ (μl ml^{-1}) medium | | | | | | | |
	0.1	0.2	0.3	0.5	1.0	5.0	10.0	20.0
	Number of colonies							
Control (A)	115	115	201	215	255	16	4	0
A + 25 μg ml^{-1}	95	116	182	204	237	75	18	7
A + 50 μg ml^{-1}	80	123	148	161	173	84	16	2
A + 100 μg ml^{-1}	67	111	130	147	162	68	26	4
A + 200 μg ml^{-1}	34	28	167	152	195	70	32	3
A + 500 μg m^{l-1}	3	16	81	186	190	84	30	5
A + 1000 μg ml^{-1}	Nil	Nil	2	15	82	92	9	4

medium was confirmed by CAS assay. When it was added to the culture broth, blue colour of the mixture changed to orange, showing the presence of siderophores in the broth. A possible mechanism for this colour change was that iron complexed to the dye detached and joined with the siderophores, freeing the dye accompanied by colour change[214].

Secretion of siderophores by *C. rugosa* was more clearly revealed by the plate cultures (Fig.IIIC3). As shown in the Figure, a clear golden brown halo was developed around the colonies. These halos were formed by the above siderophores iron complex formation mechanism. Ismail *et al.*[91] observed the simultaneous secretion of hydroximate and phenolate-type of siderophores when *Candida albicans* was grown in a deferrated medium. Secretion of a siderophores by *C. rugosa* in the deferrated medium focuses the essentiality of iron for the metabolism

of *C. rugosa*. Since both *C. albicans* and *C. rugosa* are closely related members and also lipase producers, siderophores secreted by *C. rugosa* may have resemblance to those produced by *C. albicans*.

In another study, the exact requirement of iron for *C. rugosa* was investigated in the deferrated UY-medium under

Fig.IIIC4. Effect of different concentrations (μl ml^{-1}) of 1 MFeCl$_3$ in the growth of *C. rugosa*

agitated condition, by adding ferrous salt ($FeCl_3.6H_2O$). It was confirmed that *C. rugosa* required 1.0 mg ml^{-1} medium (Fig.IIIC4). Presence of more salt beyond this optimum concentration, did not show any betterment (Fig.IIIC5). Hence it can be concluded that, optimum requirement of iron is essential for *C. rugosa* growth, but it is not an essential factor for lipase
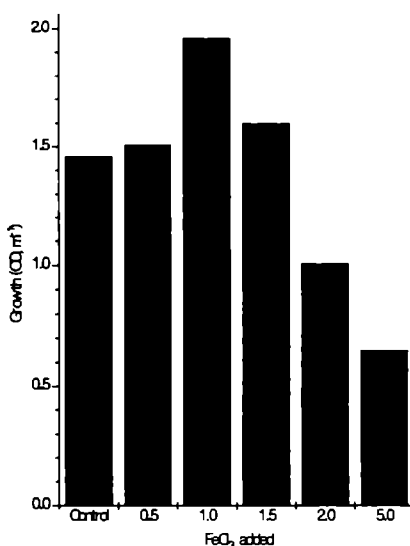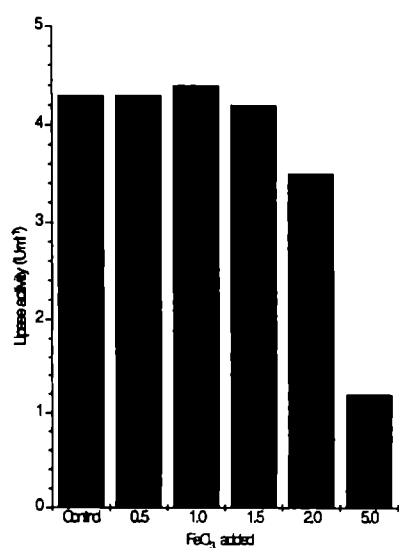

Fig.IIIC5. Effect of different concentrations of 1M iron (μl ml^{-1}) in lipase production at 48 h

secretion. However, retarded growth by the presence of more iron in the medium would also affect lipase secretion (Figs.IIIC4 and 5).

As a whole, this study revealed that *C. rugosa* required iron in microgram for optimum growth. If the availability of iron concentration is sub-optimum, it secretes siderophores to sequester scattered iron in the medium. However, iron is not an essential (directly) factor for lipase production. In other words, this study proved that iron is necessary for growth and growth indirectly involved in lipase production.
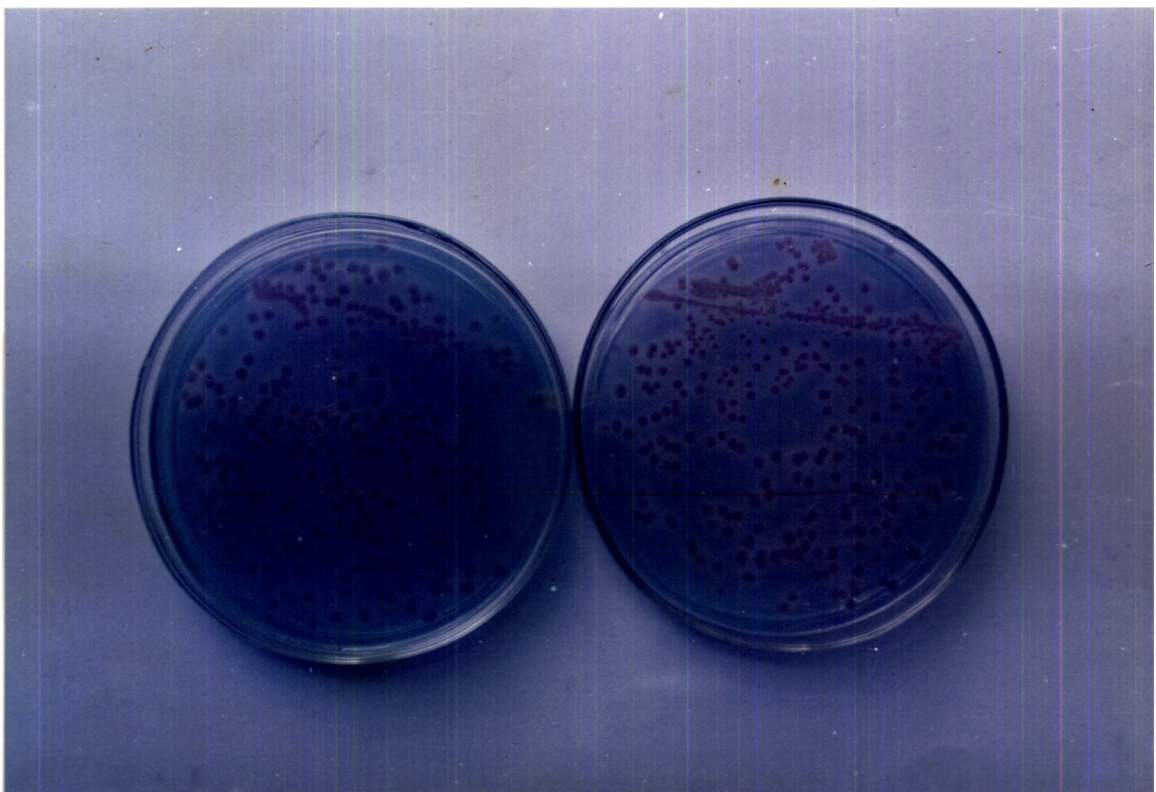
Fig.IIIC3. *C. rugosa* colonies in CAS-Agar
medium showing halos

IMMOBILIZATION

Introduction

Application of immobilized microbial cells for the production of extracellular enzymes is less well documented[220]. Their application for the bioconversion of materials hazardous to environment and for the production of useful low molecular mass components, which are unobtainable by the conventional chemical processes[19]. There is little literature published about immobilization of microbial cells for extracellular lipase production[159]

Natural and synthetic polymers are being used as efficient matrices for the immobilization or entrapment of microbial, plant and animal cells[249]. When an enzyme or cell is entrapped in these carriers, changed environment would considerably alter their chemical properties[225]. The most significant effect is the mass transfer of substrates and products into and out of the cells, respectively through the micropores of the carriers. Since the reaction in an immobilized system takes place in a heterogenous phase, the substrate has to diffuse into the immobilized cell and the product to diffuse back into the fluid phase, from which it absorbs nutrients. Porous particles are generally used for immobilization, because such materials can provide large surface area, along with proper means for diffusion[143].

Immobilized microbial cells are gradually attaining renewed interest for the large scale production of valuable primary and secondary metabolites[19]. A possible advantage of immobilized microbial cells relating to purified enzymes is its ability to catalyze a series of linked reactions, some of which require co-factors. It was

found that whole cells in gel matrix and collagen membranes could live for a month or even more under certain conditions[62]. Immobilized whole cells have a potential application to a new type of fermentation. For instance, production of biologically active substances can be performed with a column fermenter system. Significantly, downstream processing of the released products could be controlled in the fermenter itself[163].

Immobilization of *C. rugosa*[26] cells on a solid support for industrial production of lipase could offer several advantages. These are, improvement of microbial cell stability, higher dilution rates without culture washout, and facilitation of continuous operation in a bioreactor. Both the process control and downstream operation are significantly simplified, in comparison to the batch processing. However, diffusional limitations of both organic substrates and/or products have to be taken into consideration. In order to alleviate mass transfer resistance of the organic substrate, the immobilization of microbial cells on a support with an optimum hydrophobic/hydrophilic balance has been suggested[26].

Given the important advantages that any immobilized biocatalyst may offer a *priori,* the present work has focused its interest in a double objective - firstly, the selection and application of a suitable technology for *C. rugosa* cell immobilization, secondly, introduction of a new reactor design according to the physical and biological properties of the immobilized cell preparations, thus providing the optimum hydrodynamic and operational conditions[62] (O_2 requirement, medium mixing).

Materials and Methods

Feed Medium

Feed (production) medium contained: maltose (10), peptone (5), yeast extract (3), malt extract (3) KH_2PO_4 (0.4), K_2HPO_4 (0.24), $MgSO_4.7H_2O$ (0.2) (all g L^{-1}) and urea (40), $FeCl_3.6H_2O$ (0.4), thiamine-HCl (0.02), biotin (0.008), inositol (0.004) (all mg L^{-1}) and 10 (v/v) olive oil with 0.3% (w/v) gum arabic.

Immobilization of C. rugosa on Different Gel Support

Immobilization of the free C. rugosa cells was accomplished with different entrapment materials viz., calcium alginate (CA), agar (A) and polyacrylamide (PA). Immobilization of yeast in CA was done according to the method of Tanaka et al[232]. To find out a suitable concentration, different percentages of sodium alginate (0.5, 1.0, 2.0, 3.0, 4.0) and calcium chloride (0.1, 0.2, 0.4, 0.5 and 1.0 M) were tested. To obtain CA beads with immobilized C. rugosa, the inoculum (5 mg wet cell mass g^{-1} gel) was mixed with 2% (pre-melted and cooled) sodium alginate solution and dropped in 0.5 M calcium chloride solution using a column of suitable sized nostril under sterile condition, so as to obtain beads of 3-5 mm diameter. The beads were kept for curing in the same $CaCl_2$ solution overnight at 28°C. The free Ca^{2+} ions were removed by repeated washing of the beads with sterile saline followed by sterile distilled water and then incubated in the feed medium.

Immobilization of C. rugosa in A was carried out following the method of Gogoi et al.[72]. As in CA different percentages of agar (0.5, 1.0, 2.0, 3.0 and 4.0) were tested. The inoculum (as above) was mixed with aqueous agar solution (2%) at

41°C. The mixture was quickly placed dropwise using the same column mentioned above into a hydrophobic liquid phase (olive oil) to get spherical beads of 3-5 mm size. The beads were thoroughly washed with normal saline followed by distilled water and incubated.

Polyacrylamide granules (cubes) containing the cells were prepared using the method of Woodward[249]. The gel was prepared by dissolving 1.98 g acrylamide and 0.1 g methylene bis-acrylamide in 11 ml distilled water followed by addition of ammonium persulphate (APS) (15 mg in 3 ml distilled water). To this, 0.1 ml Tetramethyl Ethylene Diamine (TEMED) was added as polymerising catalyst. This gel (14 ml) was mixed with 6 ml of sterile cell suspension (5 mg wet cell mass g^{-1} gel) (final volume = 20 ml). The mixture was suddenly transferred and spread in a petri-dish (3 mm thickness) and kept in the refrigerator for 6 h. After polymerisation, the beads were prepared (3 mm^3) by pressing the immobilized gel against a specially made Nylon net (3 mm^2 mesh size) to get 3 mm cubes. The beads were washed, cured and incubated as above.

The immobilized gel beads prepared so were incubated in the feed medium for 36 h in order to increase the cell concentration in the beads and also to sensitize the cells for lipase secretion. The beads were repeatedly washed and used for further studies. To study outward diffusion, the marker molecule added in the medium before preparing the beads/cubes, but for the studies of inward diffusion, the marker molecule was added in the distilled water, in which the beads/cubes were subjected to agitation.

For studying the diffusional characteristics of all the gelling agents (without

immobilised *C.rugosa*), glucose (280 Da), Bovine Serum Albumin BSA (66 kDa)

and Cyanocobalamine (Vitamin B12) (1355 Da) were used.

Release of Immobilized Cells

A 1.5% solution of sodium citrate was used to free yeast cells from Ca-alginate gel

beads (at 6 h intervals) without any loss of cell viability for counting the viable cells

using colony counting technique, as described in Chapter IIIA.

Operation of Bioreactor

The schematic set up of the bioreactor is shown in Fig.IIID1. It consisted of

an air pump, an air filter unit, reservoir containing feed medium (connected with

another flask containing water for air exit), a peristaltic pump and a column made of
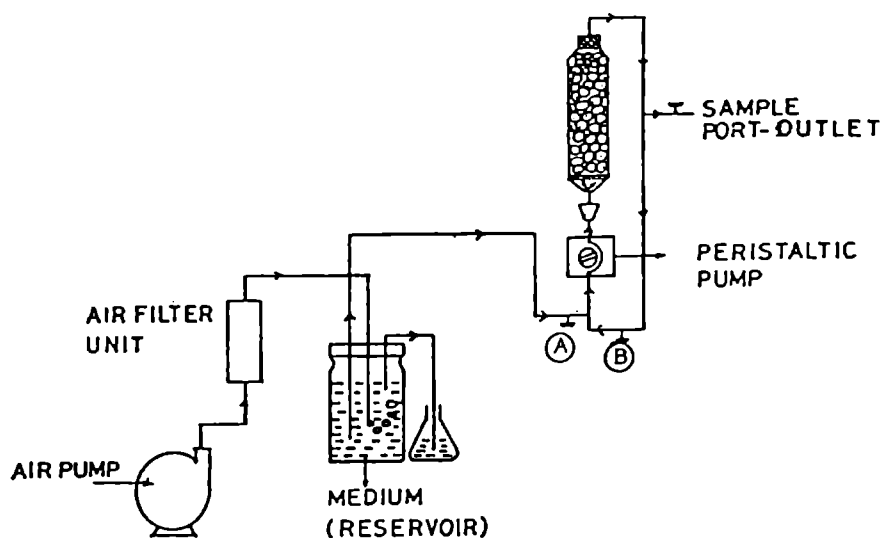

Fig.IIID1.Schematic set-up of packed-bed bioreactor containing
calcium alginate beads with immobilized *C. rugosa*.

glass (Vol.176 cm^3). The pre-incubated beads/cubes were packed in this column in different experiments. In the case of Packed-Bed Bioreactor (PBR) under batch mode, the feed medium was added to the column containing the immobilized beads/cubes and incubated under static condition (28°C) for required period. For PBR under fed-batch mode, the fresh medium was pumped from the reservoir into the column from the bottom in the reverse flow mode using a peristaltic pump at required flow rate. Once the column was full of fresh medium (pumped from reservoir) the tap A was closed and B opened to recycle the feed medium. The medium coming out of the column was recycled for required time and then taken out for assay. After each cycle, fresh medium was pumped into the column. the experiments were conducted for several hours. The entire process was maintained at aseptic condition throughout.

Assay Methods

Concentration of glucose and BSA were estimated by the methods of Miller[148] and Lowry et al.[133], respectively for studying diffusional characteristics. Rate of to and fro diffusion (%) of cyanocobalamine with respect to beads/cubes was found out by using the formula

$$D = \frac{OD \ (at \ 0 \ h) - OD \ (at \ given \ time)}{OD \ (at \ 0 \ h)} \times 100$$

where D = diffusion rate;   OD = at λ, 530

Results and Discussion

Duffussional characteristics in Ca-alginate gel beads were studied by
Thanaka et al.[231]. However, Benjamin and Pandey[19] employed three gelling agents
to choose the best among them. Diffusional characteristics of all the three gelling
agents were compared using glucose, BSA and cyanocobalamine as markers.
Different initial concentrations of sodium alginate were tested in bead formation
(Table IIID1). All the concentrations of calcium chloride used were equally good
for obtaining mildly hard beads, but except 0.5% of sodium alginate, all other
concentration could yield beads of size 3-5 mm in diameter with varying diffusional
properties. Low concentration of sodium alginate (0.5-1.0%) were not suitable
because such concentrations yielded deformed (wrinkled) beads, but 2.0%
concentration could yield mildly hard beads with medium diffusional characteristics.

Table IIID1. Studies on the diffusional properties of calcium alginate beads with
glucose as marker molecule by using (A) varying sodium alginate concentration
with 1.0 M $CaCl_2$ and (B) varying $CaCl_2$ concentration with 2% sodium alginate

Con. of sodium alginate (%)	A			B		
	Size of beads (mm)	Hardness	Diffusional property	Strength of $CaCl_2$ solution	Size of beads (mm)	Hardness	Diffusional property
0.5	wrinkled	very soft	00000	0.2 m	3-5	mildly hard	00000
1.0	3-5	soft	0000	0.4 M	3-5		0000
2.0	3-5	mildly hard	000	0.5 M	3-5		000
3.0	3-5	hard	00	1.0 M	3-5		00
4.0	3-5	very hard	0	2.0 M	3-5		0

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Thus for further studies, 2.0% sodium alginate in 0.5 M calcium chloride was used to obtain unique mildly hard calcium alginate beads with medium diffusional characteristics (Fig.IIID2).

Studies with agar concentration followed the characteristics observed in CA beads (Table IIID2). In comparison to lower concentration, 2.0% of agar resulted in obtaining mildly hard beads with medium diffusional properties with glucose. Diffusion of immobilized glucose and BSA in all the three gelling agents showed quite different diffusional pattern. For immobilization, 200 mg of glucose or BSA were used with calcium alginate, agar (both 2%) and polyacrylamide cubes (for 20 ml preparation). All the beads/cubes obtained so were agitated in distilled water in 250 ml flask on a rotary shaker (150 rpm) for 120 min (Tables IIID3 & 4). By 30 min 96.5% of glucose was diffused out of CA beads, while agar and polyacrylamide beads/cubes retained 25% of glucose at the same time (Table IIID3). Beyond 30 min, the differences were negligible.

Outward diffusion of BSA took more time, possible reason for this slow mobility is its high molecular weight[231]. After 60 min (under the same condition), only 58% of BSA diffused out of CA and maximum was 65% after 120 min agitation, as against 59% and 55% in A and PA, respectively at the same time. Initial concentrations of both glucose and BSA were comparatively higher than in CA and A beads. Even in CA, only 65% of BSA diffused out against 96.5% of glucose. It was due to the difference in molecular weight (MW) of these two

compounds[232], MW of glucose is 180 Da and that of BSA is 66 kDa. This effect could also be noticed in the case of cyanocabalamine[26] (1355 Da) .

Table IIID2. Studies on the diffusional properties of Agar beads with glucose as marker molecule by varying agar concentration

Concentration of agar used (%)	Size of beads (mm)	Hardness	Diffusional property
0.5	wrinkled	very soft	00000
1.0	3-5	soft	0000
2.0	3-5	mildly hard	000
3.0	3-5	hard	00
4.0	3-5	very hard	0

Table IIID3. Diffusion of glucose (mg/50 ml) out of the different gelling agents

Time (min)	Calcium alginate	Agar	Polyacrylamide
10	145.3	131.5	122.4
20	185.8	141.6	136.1
30	192.6	152.4	148.4
40	154.3	162.8	153.6
50	156.3	166.4	153.9
60	198.3	168.7	154.6
120	198.4	168.8	154.6

To and fro diffusional characteristics of cyanocobalamine showed vast differences. Upto 20 min, out ward diffusion was two-fold higher than inward diffusion in all the three gelling agents. Agitation was continued upto 180 min. Diffusional characteristics of CA was higher with 79% inward and 90% outward diffusion (Table IIID5).

Table IIID4. Diffusion of BSA (mg/50 ml) out of the different gelling agents

Time (min)	Calcium alginate	Agar	Polyacrylamide
10	15.6	14.3	24.8
20	42.3	45.6	41.1
30	62.5	64.4	60. 2
40	85.6	86.8	76.3
50	98.4	93.8	92.3
60	116.5	110.6	105.8
90	124.2	115.7	107.3
120	129.8	118.2	108.6
180	130.1	118.4	109.4

Table IIID5. Diffusion (D) of cyanocabalamine (100 mg) into and out of different gelling agents

Time (min)	% of cyanocobalamine					
	Calcium alginate		Agar		Polyacrylamide	
	into	out	into	out	into	out
10	38.1	67.5	26.5	48.1	21.4	27.5
20	46.5	82.7	31.5	65.3	26.5	34.6
30	59.3	86.5	50.4	73.2	31.2	38.1
40	68.6	88.7	61.5	73.6	33.6	38.6
50	78.4	90.1	64.8	73.6	33.6	38.7
60	78.6	90.1	64.8	73.7	33.7	38.7
90	78.6	90.2	64.9	73.7	33.7	38.7
120	78.9	90.2	64.9	73.7	33.7	38.7
180	78.9	90.2	64.9	73.7	33.7	38.7

Diffusional characteristics of the three gelling agents were studied to compare the mass transfer of feed into the beads (solid phase) and products towards the external aqueous phase. Since these two phases possess two distinct dynamism, diffusional characteristics was also greatly influenced by the phase change[143,231]. As a matter of fact, diffusion into aqueous phase would be more rapid from the solid

phase (Table IIID5). In the beads, polymerization of the gelling agent would cause cross-linking with micropores (Fig.IIID3). To and fro movement of molecules across these pores would not be hindered by internal factors, when the beads are free of cells[143].

Most significant outcome of the diffusional studies was that CA was the best gelling agent among all the three agents studied and hence it was selected for further studies (Figs.IIID2&3). Diffusion of molecules through the beads is dependent on the molecular size of the diffusing molecule[232]. Molecules with less MW diffused faster than those with high MW, and also if the diffusing molecules are ionic in nature, the influence of that molecules would be reflected during fermentation[19].

Fig.IIID4 shows multiplication profile of *C. rugosa* cells inside the CA beads. At zero h its concentration was only 1×10^8 cells. After 36 h of incubation the
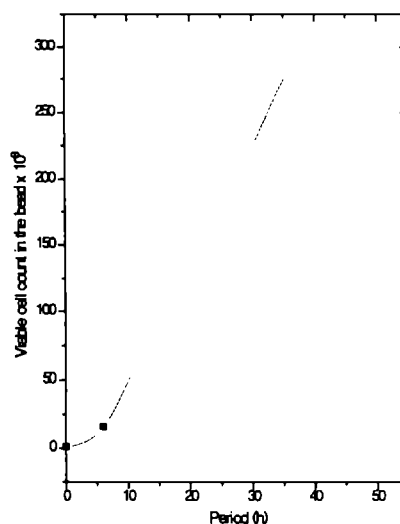

Fig.IIID4. Cell growth in 1g calcium alginate bead during incubation

cell concentration was increased to 281×10^8 cells per gram gel. However the cell concentration is mainly confined to the periphery of the beads (Fig.IIID5). It indicates that along the periphery, the availability of dissolved oxygen and feed was

high. This profuse growth along the interior crust of the beads would chock the micropores in the core of the beads and thus reduce the growth at the centre[19].
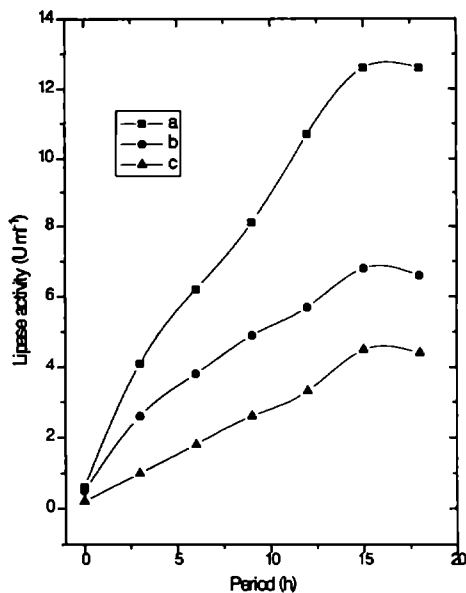
Fig.IIID6 shows results of comparative lipase yield obtained by 3 gelling agents used in batch experiments, using

Fig.IIID6. Comparison of lipase yield in CA, A and PA, PBR under batch mode.
a: CA, b: A and c: PA

the same feed media. Best results were obtained with CA. Yield with PA was very

poor. It could be attributed to the poisoning nature of chemicals used in preparing the gel[232]. Benjamin and Pandey[19] found that lipase yield by immobilized cells in agar was also not encouraging, due to the large pore size of the beads in retaining the substrate for a minimum period inside the beads and most cell leakage[72]. Maximum
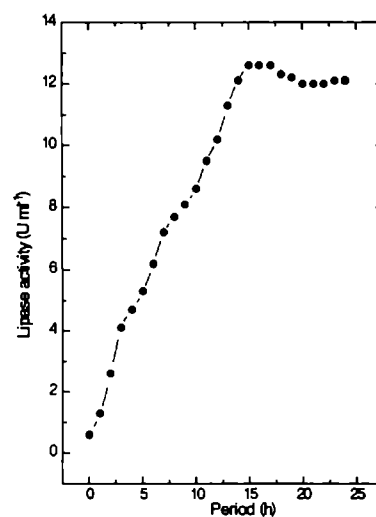

Fig.IIID7. Lipase production in PBR under batch mode without recycling the substrate using immobilised CA beads.

production of lipase by *C. rugosa*

immobilized in CA was noticed at

15 h (Fig.IIID7). Beyond 15 h, no

further yield was obtained.

Repeated batch experiments

were conducted only with CA

packed in PBR, owing to the

superiority in results (Fig.IIID8).

PBR under reverse flow mode

(repeated fed batch) with different

flow rate showed significant

Fig.IIID9. Lipase production in PBR under
repeated batch mode using different flow
rate. A: 0.2, B: 0.4, C: 0.6, D: 0.8 and E: 1.0.

improvements (Fig.IIID9). Different flow rates (0.2, 0.4, 0.6, 0.8 and 1.0 ml min^{-1})

were tested and 0.6 ml min^{-1}

with a residence (retention)

time of 110 min could yield

maximum lipase (21.6 U ml^{-1})

at 12 h. Thus several cycles of

feed medium (each cycle was

persisted for 12 h) were fed

into the column under repeated

batch mode to study the life

expectancy (half life period) of
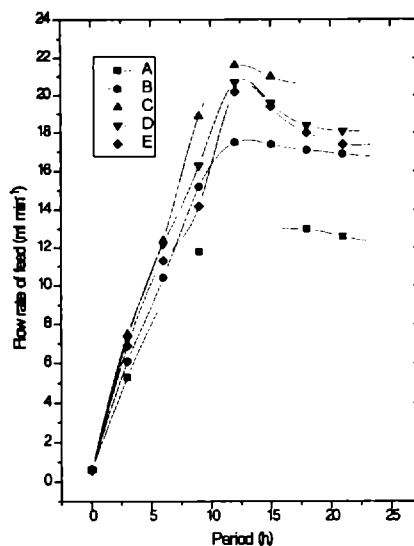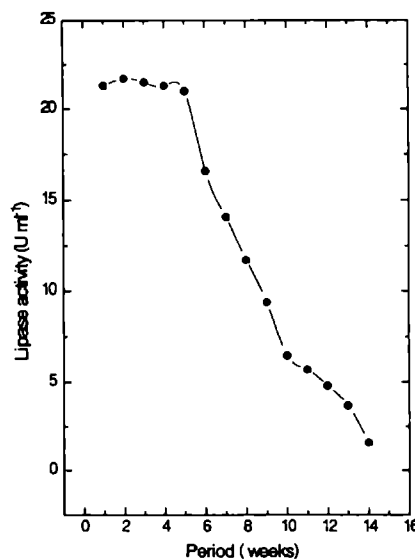
the immobilized *C. rugosa*

Fig.IIID10. Half life period of *C. rugosa*
under immobilised state in CA in PBR.

(Fig.IIID10). Maximum activity of *C. rugosa* was persisted for 1 month in beads packed in the column, and half life was 2 months

Compared to the results obtained in SmF, production by immobilized *C.rugosa* under batch experiments in reverse flow mode of feed medium showed significant enhancement. Maximum production in SmF was only 15.14 U ml^{-1} (Chapter IIIB), which obtained at 48 h[17]. However, under immobilized conditions, increased yield (by 30%) was obtained at 12 h of running the reactor (1 cycle)[19]. Same yield was retained upto one month with a half life period of two months (Fig.IIID10). The results obtained in the present study undoubtedly declare the efficiency of immobilised system as stated in the introductory part of this Chapter. This longevity and efficiency at low cost passively attracted new entrepreneurs and technocrats towards this potential area for harvesting maximum profit[71].
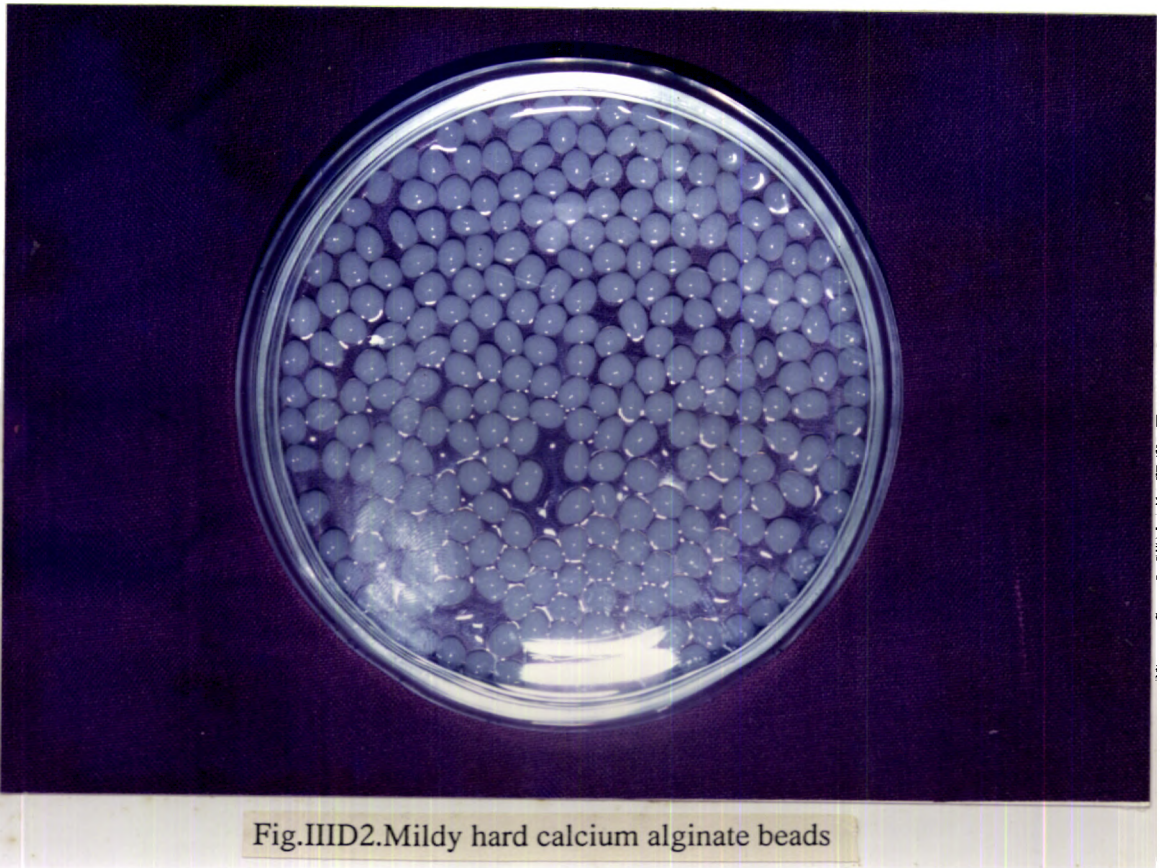
Fig.IIID2.Mildy hard calcium alginate beads
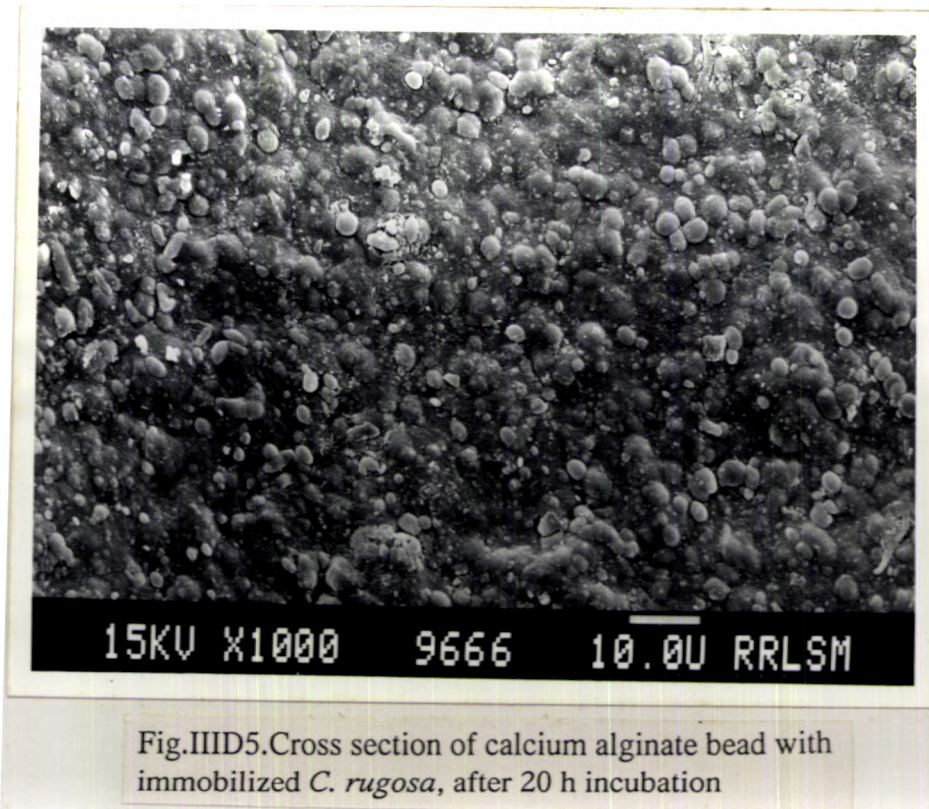

15KV X2000 9654 10.0U RRLSM
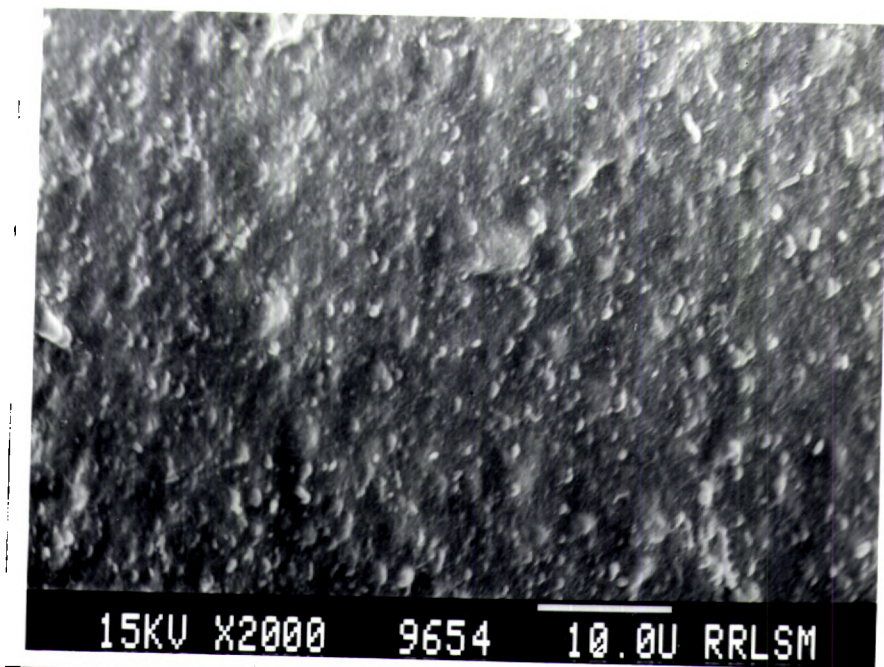
Fig.IIID3. Cross section of calcium alginate
bead showing cross-linkings

Fig.IIID5.Cross section of calcium alginate bead with immobilized *C. rugosa*, after 20 h incubation

Fig.IIID8. Packed-bed bioreactor used for fermentation

SEMI - SOLID FERMENTATION

ON
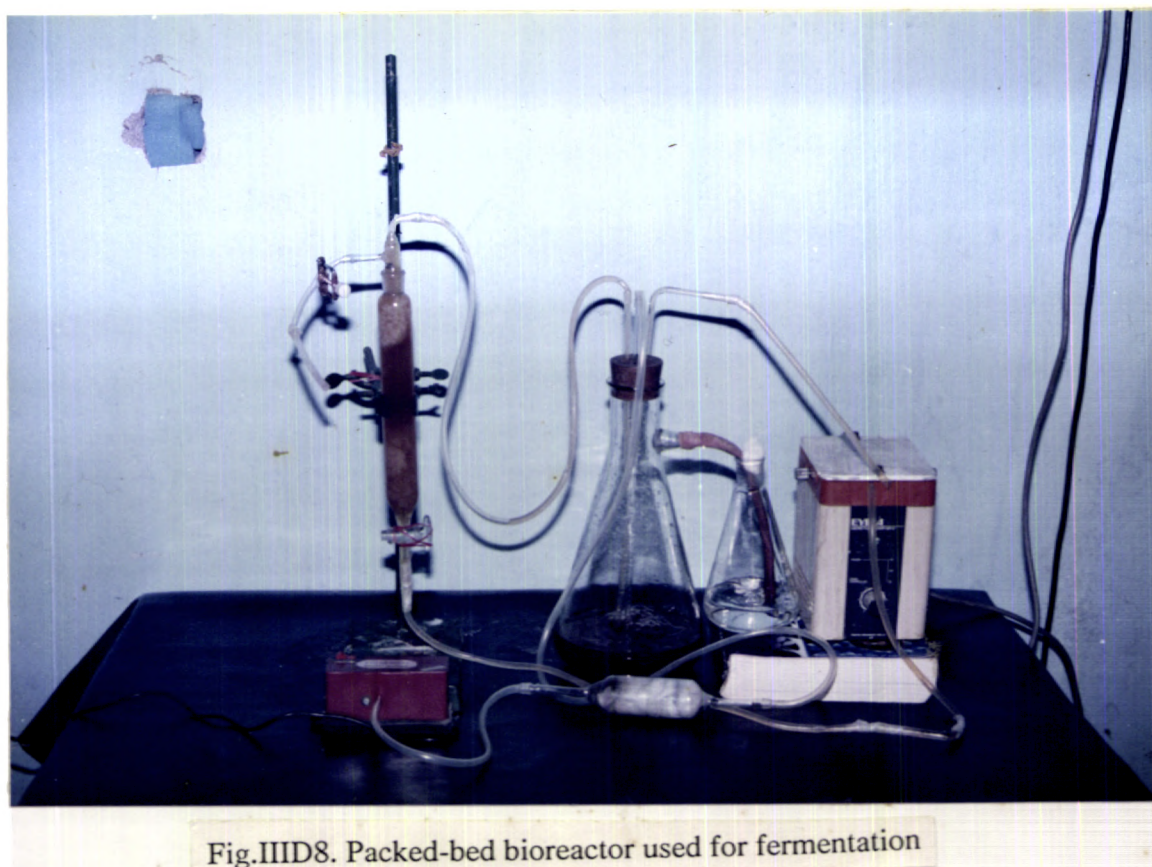
COCONUT CAKE EXTRACT

Introduction

Bioconversion of cheap agro-industrial waste has gained significant momentum in the present decade, mainly in view of its great economic viability[43]. Its prime objective is to recycle the value-less (or less valued) wastes or by-products into value-added products. In this context, new possibilities of coconut oil cake (copra waste a by-product obtained after oil extraction from dried copra) to be used as a substrate for lipase production were investigated[18]. Present section describes the utilization of coconut oil cake extract (COCE) for the cultivation of *C. rugosa* and optimization of fermentation parameters at semi-solid state.

Materials and Methods

Inoculum Preparation

Inoculum was prepared as described under Materials and Methods in Chapter IIIB. For each 50 ml production medium, 1 ml (2%) of inoculum was used.

Chemical Analysis of COC

Coconut cake was subjected to chemical analysis. Soluble proteins in the extract were estimated by the method of Lowry *et al.*[133]. DNS method was employed to analyse soluble sugars[148]. Total insoluble carbohydrates (starch and cellulose) were also determined by analysing sugars obtained after acid hydrolysis. It was done by reflexing 0.5 g COC in 100 ml 10% HCl for 150 min, using a heating mantle. After cooling, the hydrolysate was filtered and the filtrate was neutralised with 1N NaOH. The solution so obtained was used for the estimation of sugar by DNS method[148]. Nitrogen in the COC was estimated by the Micro-Kjeldahl[198] method. Two grams of COC was boiled in a Kjeldahl's digestion chamber containing

20 ml conc.H_2SO_4. To avoid charring, few drops of H_2O_2 were added at final stage in the mixture after cooling. Digestion was continued till a clear solution was obtained. The digested mixture was diluted to 50 ml by adding distilled water and poured into the Kjeldahl's distillation apparatus and added 8 ml 40% NaOH to neutralise it. Ammonia liberated was collected in 0.2% boric acid containing the indicator, bromocresol blue. It was titrated against 0.01 N H_2SO_4 to find out total nitrogen in the sample. The lipid content in the substrate was estimated by extracting it with petroleum ether. For extraction, 5.0 g COC was reflexed with 100 ml petroleum ether in a reflex condenser for 6 h (60-70°C). After digestion, the hydrolysate was cooled and filtered. The residue was washed repeatedly with the same solvent. All the filtrate were pooled and the solvent was evaporated off to get the lipid dissolved in it in the concentrated form. Dry weight and moisture contents were estimated by using an oven and moisture balance, respectively. Ash content in the cake was determined by heating it at 600°C for 12 in a furnace. The details of COC analysis obtained by various means as described above are enumerated in Table IIIE1.

Table IIIE1. Chemical composition of coconut oil cake (COC)

Chief Constituents	Quantity (%)
Starch	18.82
Soluble sugars	6.31
Total protein	14.34
Soluble proteins	4.81
Total nitrogen	0.46
Total lipids	6.68
Total moisture	8.41
Ash content	5.63

Production Media

Coconut oil cake (copra waste) obtained as a gift from Kannan Oil

Industries, Karamana, Trivandrum was used to prepare the substrate. A weighed

quantity of coconut oil cake (COC) was soaked with 70% distilled water and

autoclaved for 1 h. After cooling, the liquid content in the cake was squeezed out

using a muslin cloth by a hand mill. This extract (COCE) was used as the raw

substrate (control) and designated as Medium A (MA). A number of additional

nutrients were added in COCE to constitute different production media. All the

media and their ingredients used in this study are shown in Table IIIE2. Additional

nutrients (w/w or w/v basis with respect to COC) were added to the substrate prior to

autoclaving. Initial pH of all the media were adjusted as 6.8.

Table IIIE2. Enzyme production media and their composition

Media (pH 6.8)	Composition
MA	raw COCE (dry weight = 16.41%)
MB	COCE + mineral solution (%): 0.5, 1.0, 1.5, 2.0 and 2.5
MC	COCE + corn steep liquor (%): 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 and 2.5
MD	COCE + organic nitrogen sources (%): 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0
MH	COCE + Carbon source (%):0.1, 0.2, 0.5, 1.0, 2.0 and 5.0
MF	COCE + free fatty acids (%):0.05, 0.1, 0.15, 0.2 and 0.25
MG	COCE + 10% olive oil + gum arabic (%): 0.2, 0.4, 0.6, 0.8 and 1.0
MH	COCE + 0.2% peptone + 0.1% glucose + 0.15% free fatty acids + 10% olive oil + 0.4% gum arabic

Fermentation

For fermentation, 50 ml of the production medium (MA-MH) was taken in

250 ml flasks, autoclaved cooled and inoculated with cell suspension of *C. rugosa*.

After thorough mixing, the flasks were incubated at 28°C under static condition and under agitation (150 rpm) on an incubator rotary shaker.

Assay Methods

Soluble proteins, sugars and lipase activity were estimated as described in Chapters IIIA and IIIB. The results so obtained were expressed in per gram dry substrate (gds) basis, in the case of sugars and proteins. But lipase activity was expressed as U g^{-1} ds (µmoles of free fatty acids liberated per minute per gram dry fermented substrate). Samples were withdrawn at 12/24 h intervals. From each flask, 20 ml fermented matter was centrifuged at 8000 g for 10 min at 5°C. Supernatant so obtained was diluted, if necessary, to a suitable concentration and used for assays. The residue so obtained was used for the estimation of glucosamine content in the cell wall of yeast, after hydrolysis, as described by Sakurai et al.[206]. Total solid contents in the COCE (before and after fermentation) were determined by keeping the samples (5g extract) in an oven at 80°C. For the estimation of glucosamine, 0.5 gm of pellet obtained from the fermented broth was digested with 2 ml conc.H_2SO_4 for 24 h at room temperature, as described by Benjamin and Pandey[18]. To this hydrolysate 36 ml H_2O was added and autoclaved for 1 h. After cooling and filtration, the filtrate was neutralized with 1N NaOH. The volume (n) was noted. In order to estimate glucosamine content 0.5 ml of the above sample was mixed with equal volume of acetyl acetone reagent, sealed and heated for 20 min in a boiling water bath. After cooling to room temperature, 3 ml absolute alcohol was added followed by 0.5 ml Ehrlich's reagent. The mixture was incubated at 65°C for 10 min, cooled to room temperature and concentration was read at 530 *nm* against a

reagent blank and glucosamine as standard. Acetyl acetone reagent contained 1 ml

acetyl acetone and 50 ml 0.5N sodium carbonate. Ehrlich's reagent contained 2.67

gm of p-dimethyl amino benzaldehyde in 1:1 mixture of absolute alcohol and con.

HCl and made upto 100 ml in a volumetric flask. Glucosamine content was

calculated using the formula:

$$\frac{X \text{ (adjusted to 1 ml) x n}}{0.5 \text{ g x dry wt. for 1 g residue}} = mg \ g^{-1} \ ds \text{ of glucosamine}$$

where X = concentration at = 530 nm

n = volume obtained after neutralization

Results and Discussion

Chemical composition of COC and different production media are recorded

in Tables IIIE1 and IIIE2, respectively. Soluble sugars (6.3%) and soluble proteins

(4.8%) make the COCE as a potent substrate for lipase production by $C.$ $rugosa.$

Apart from this, Kjeldahl's experiment with the COC showed that, COC contains

comparatively a very good quantity of nitrogen (0.46%) and the ash content in the

substrate was 5.63%[20]. However, raw COCE was not so good enough to support

good growth and lipase yield[18] (Table IIIE3). In this table, data on sugar

consumption, protein depletion and lipase yield are presented which describes two

different sets of experiments, using MA (control). Data 'A' obtained under static

culture and 'B' by agitated culture. Compared to agitated culture, assimilation of

sugar and protein under static culture was very poor. In agitated culture, though

protein concentration showed an initial depletion, after 36 h of fermentation, its

Table IIIE3. Fermentation behaviour of *C. rugosa* on raw coconut oil cake extract (COCE)

Fermentation time (h)	Soluble sugars mg g⁻¹ ds		Soluble protein mg g⁻¹ ds		Lipase production U g⁻¹ ds		pH	
	A	B	A	B	A	B	A	B
0	63.1	63.1	48.1	48.1			6.8	6.8
12	58.1	48.2	37.5	35.4			6.9	6.9
24	44.8	35.6	35.7	31.4	0.56	1.31	7.2	7.3
36	39.7	28.6	36.1	36.5	2.81	6.37	7.3	7.5
48	35.1	16.4	37.2	41.5	3.56	9.81	7.3	7.6
60	34.5	15.3	47.8	54.5	4.48	11.45	7.3	7.5
72	33.1	14.9	47.7	54.3	4.43	11.35	7.3	7.5
84	32.8	14.6	47.5	54.3	4.38	11.35	7.3	7.5

A: under static condition (28°C), B: under agitated condition (150 rpm, 28°C)

concentration increased to 54.5 mg g⁻¹ds at 60 h, but such influencing result was not noticed in static culture. Simultaneously, lipase production was also increased, and the maximum was 11.45 U at 60 h as against 4.48 U in static culture. pH also showed slight increase with the advancement of protein concentration. Maximum activity was noticed at 60 h. After this period, yield and sugar consumption did not show much change. Influence of agitation
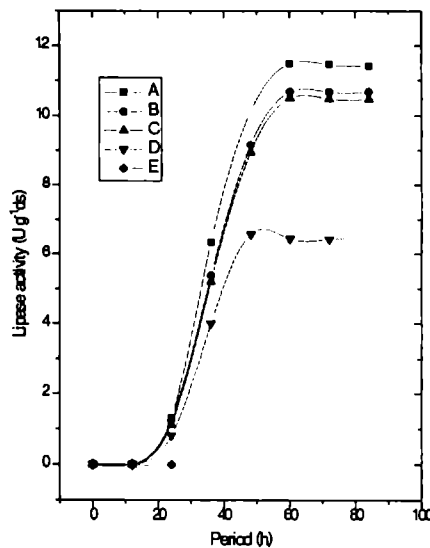

Fig.IIIE1. Effect of mineral (%) solution on lipase production in MH. A: 0.5, B: 1.0, C: 1.5, D: 2.0 and E: 5.0.

obtained in this experiment clearly explains the importance of O_2 requirement as reported by Valero *et al.*[238] Hence in the succeeding experiments, agitated culture was preferred to static culture.

For additional nutrient supplementation, mineral nutrition $(MB)^{176}$ and corn steep liquor (MC) were found to be not promising. At higher concentrations, both these nutrients even adversely affected the enzyme yields (Figs.IIIE1 and IIIE2). Mineral nutrition at 0.5% concentration has no effect on lipase yield in the agitated culture with medium MA (Table IIIE3). At 5% level, it was found to be highly toxic (Fig.IIIE1) with marked decrease in yield (1.85 U g^{-1} ds). Probably, the minerals

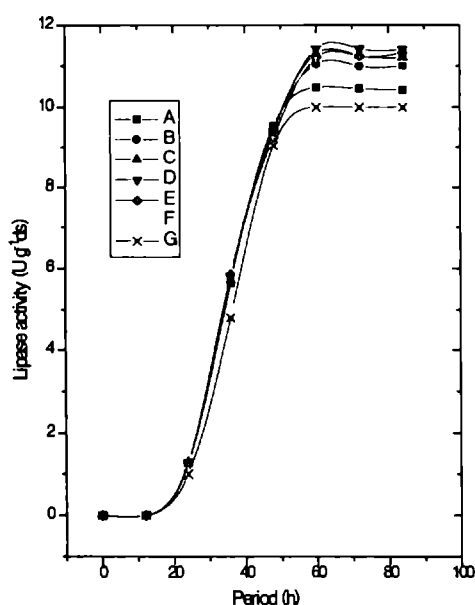Fig.IIIE2. Effect of corn-steep liquor (%) on lipase production in MH. A: 0.1, B:0.2, C: 0.3, D: 0.4, E: 0.5, F: 1.0 and G: 2.5.

(Cu, Zn, Fe) in the mineral nutrition could have altered the osmoregulatory mechanism of *C. rugosa*, thereby altering the yield[207]. Cornsteep liquor was also found to be of any utility on lipase production[18]. Hence, both these nutrients were not added in the media in succeeding studies.
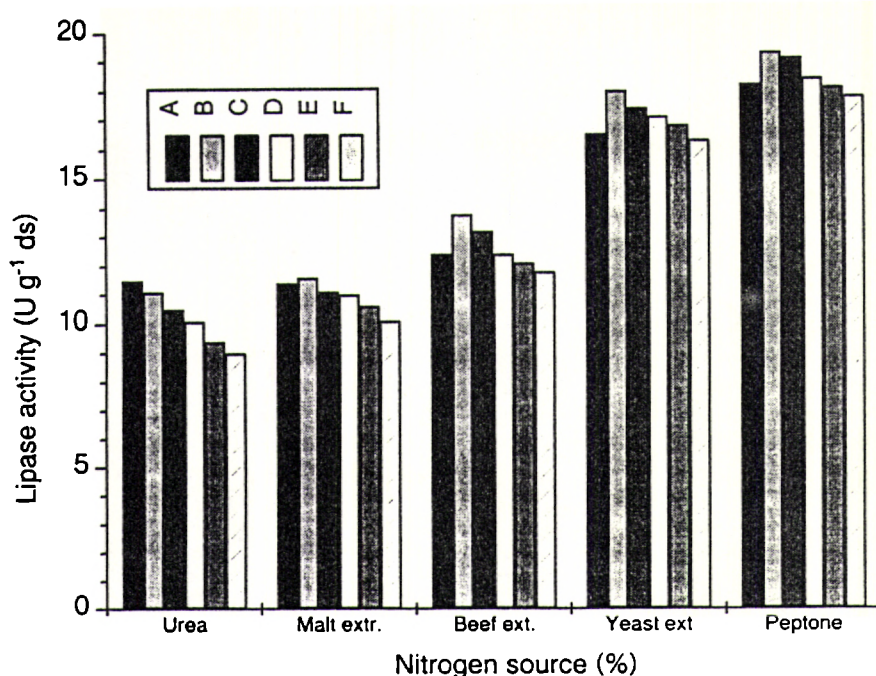
Fig.IIIE3. Influence of different organic nitrogen (%) in lipase production on MD at 60 h of fermentation.A: 0.1, B: 0.2, C: 0.3, D: 1.0, E: 2.0 and F: 5.0.

Addition of organic nitrogen source to the COCE (MD) was highly encouraging. Of the different organic nitrogen sources tested, urea and malt extract were found to be insignificant as the yields of enzyme were marginally increased or comparable to that of control experiment (Table IIIE3 & Fig.IIIE3). Beef extract, another complex nitrogen source, showed slight increase in the enzyme yield. Influence of yeast extract and peptone were worth mentioning, compared to other sources. As a whole, peptone was the most efficient organic nitrogen source. All these nutrients were found to have positive effect upto 0.2% concentration. Increase in concentration beyond this level was not effective. At 0.2% level, peptone yielded 19.3 U g^{-1} ds lipase, a significant increase over the previous studies, in the control experiments (Table IIIE3).
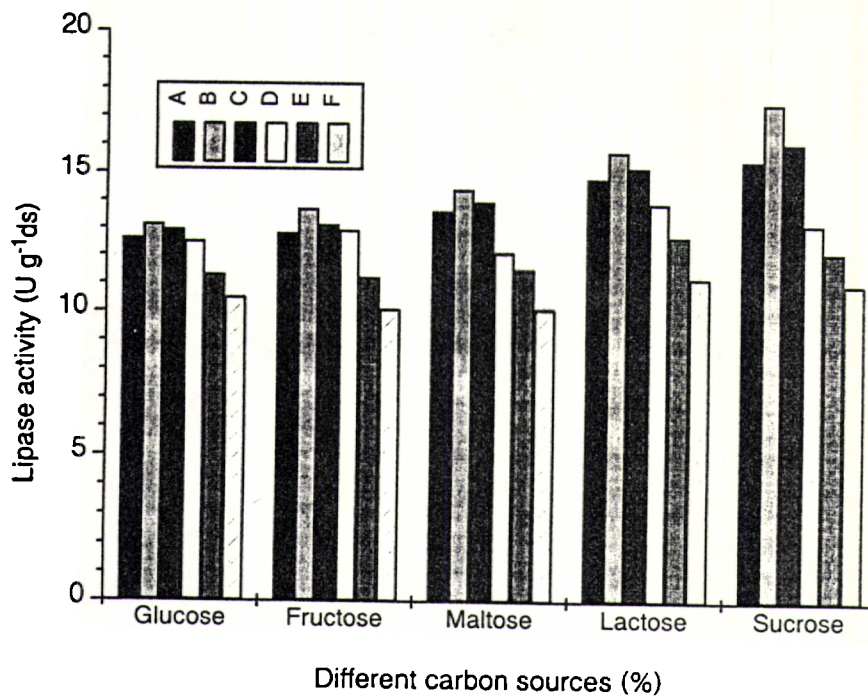
Fig.IIIE4. Influence of different carbon sources (%)in lipase production on ME at 60 h of fermentation.A: 0.1, B: 0.2, C: 0.3, D: 1.0, E: 2.0 and F: 5.0.

Results of addition of additional carbon source in the medium are shown in Fig.IIIE4. Apparently, monosaccharides like glucose and fructose were not having an effect on enhancing enzyme yields. Disaccharides (Maltose, lactose and sucrose) were exhibited to be superior in comparison to monosaccharides. Among the disaccharides, sucrose at 0.2% concentration, yielded 17.5 U lipase. In fact, it was a significant increase compared to the efficacy of MA, MB and MC.

Fig.IIIE5 shows the impact of addition of free fatty acids (FFA) in lipase production by *C. rugosa*. FFA induced lipase secretion to surprisingly higher titres (MF). Butyric acid and caproic acid were much toxic even at low concentration (0.05%). Capric acid and caprylic acids were much effective. They were optimally
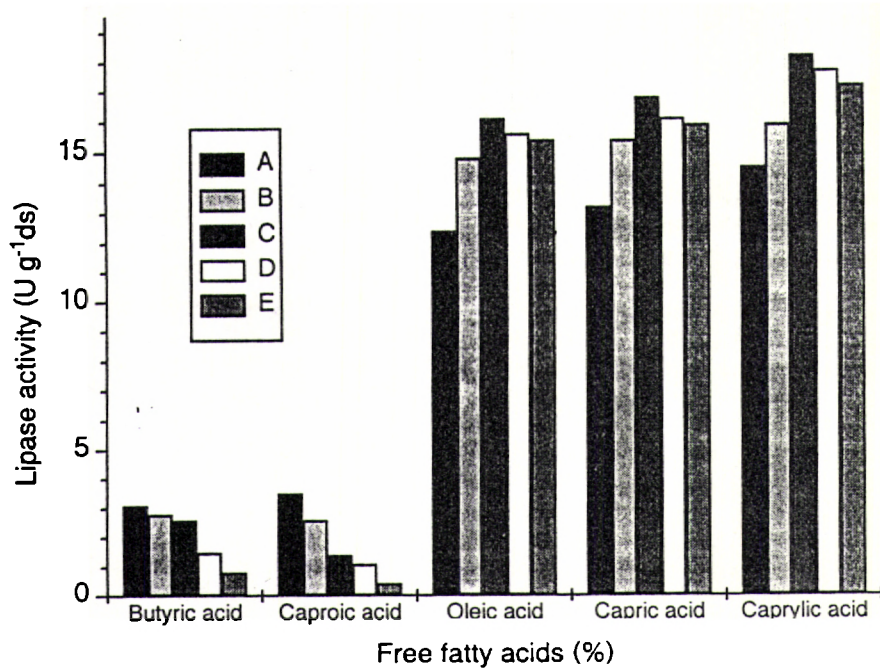
Fig.IIIE5. Influence of different free fatty acids (%)in lipase production (MF) at 60 h of fermentation.A: 0.01, B: 0.1, C: 0.15, D: 0.2 and E: 0.25.

active at 0.15% concentration. At this concentration, caprylic acid yielded 18.2 U g^{-1} ds lipase in COCE, which marks 37% increase in comparison to control (Table IIIE3). The inducing role of lipids like oils, fatty acids, sterols, etc was well documented by Obradors et al.[167] Benjamin and Pandey[18] with their studies on COCE have concluded that presence of traces of lipids in the medium would switch on the

Fig.IIIE6. Influence of gum arabic (%) in lipase production (MG) containing 10% olive oil at 60 h fermentation.

lipase gene and thereby increase in secretion of lipases. Same effect was noticed when olive oil was added in the medium (Fig.IIIE6).

Presence of 10% olive oil in combination with 0.4% gum arabic in MG showed significant impact. Emulsifying property of gum arabic[62] could serve olive oil as a better additional substrate for *C. rugosa* (Fig.IIIE6). Otherwise, olive oil would remain on the surface of the fermentin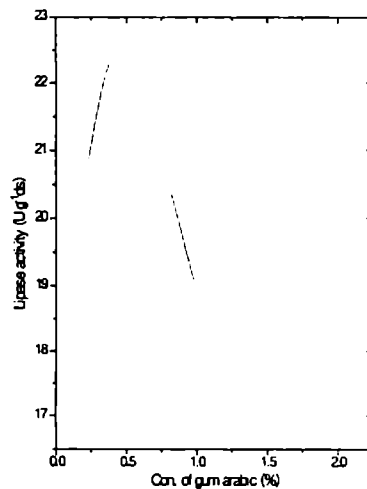g medium, in the inaccessible form[18]. Higher concentration of gum arabic (0.6, 0.8, 1.0, 2.0%) showed retarding effect. It indicates the difficulties in the diffusional properties of *C. rugosa* membrane. A detailed profile of fermentation characteristics of *C. rugosa* in MH is tabulated in Table IIIE4.

Table IIIE4. Fermentation behaviour of *C. rugosa* in enriched medium (MH)

Fermentation time (h)	Soluble sugars (mg g^{-1} ds)	Soluble protein (mg g^{-1} ds)	Glucosamine (mg g^{-1} ds)	Lipase activity (U g^{-1} ds)	pH
0	72.4	58.2			6.8
12	65.3	46.4	0.8		6.9
24	41.6	31.8	3.1	6.4	7.1
48	10.6	59.5	16.6	23.2	7.4
60	8.4	66.4	16.5	31.4	7.5
72	8.1	66.5	16.4	31.3	7.5
84	8.0	66.4	16.4	30.8	7.4

Fermentation behaviour of *C. rugosa* in enriched COCE was obeyed more or less the same pattern observed with liquid fermentation. In general, maximum cell growth in all the media studied was obtained at around 48 h of fermentation, while activity (enzyme production) was at around 62 h. Yield in Semi-solid Fermentation

described here shows very good increment over the previous studies. Around 90% sugar consumption was achieved, which was a very good sign for utilisation of the substrate. As a common factor, pH variation with increasing fermentation time was dependent on the protein secretion. These results show that COCE can be employed in a much profitable form for the effective utilisation of cake for lipase production by cultivating *C. rugosa* on it

CHAPTER - III F

SOLID - STATE FERMENTATION
ON
COCONUT CAKE

Introduction

It is economically significant to note that solid-state fermentation (SSF) or surface culture technology offers numerous well recognized advantages over its counterpart, the submerged fermentation (SmF) or suspension culture[1,169]. The upperhand of SSF could be attributed to: (a) less space requirement, because of the limited quantity of water used (absence of any free water) to moisture the substrate; (b) no need of inoculation tank, instead spores or concentrated cell suspensions are directly used to inoculate; (c) low moisture (only in bound form) excludes bacterial contamination; (d) less substrate is used to produce more product, and also less solvent is required to extract the product; (e) the micropores embedded among the substrate particles provide enough space for maintaining better aeration; (f) comparatively higher yield over SmF; (g) the products obtained are much concentrated. Concentrated products in solid substrates may be dried and incorporated directly into the animal feed at less cost, and finally lower capital input and recurring expenditure, higher concentration of the product at less energy input, substantial saving in downstream processing, negligible effluent output and less environmental problems[85,177].

Growth of microbes on solid-substrates approximates its growth that occurs on the natural moist solid substrates and is well proved to be economically significant even at lower scale of production in contrast to SmF[178]. Compared to occidental developed countries, oriental developing countries give much attention for SSF[181]. SSF gained great developments especially in the former USSR and

Japan. Until the beginning of 1980's, SmF was in vogue in terms of microbial lipase production by dint of its so-called handling and control advantages[20]. However, the dawn of this decade witnessed an unprecedented resurgence in the SSF. The forehand of this juvenile technology over suspension culture has rightly been pointed out by many authors. In the present study, a maiden and comprehensive attempt was accomplished to cultivate *C. rugosa* on coconut oil cake by SSF technology.

Materials and Methods

Medium Preparation for SSF

Coconut oil cake (COC) was the basic substrate used in the present study. Various modification were made in COC so as to boost the enzyme yield[20], and different modified media were designated as MA, MB, MC, MD, ME, MF, MG and MH (M = medium) (Table IIIF1). In all the cases, the final moisture content was 65%. MA contained only the raw COC. Medium B constituted by adding 5% (v/v) mineral solution in MA. MC had the composition of MA and 1.0 (w/w) nitrogenous salt [$(NH_4)_2HPO_4$, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3 or urea]. Urea was sterilized by filtration. In MD, nitrogenous salt in MC were replaced by 3% organic nitrogen source (beef extract, malt extract, yeast extract or peptone) and in ME by 2.5% carbon source (starch, glucose, fructose, sucrose, lactose, maltose or sucrose), respectively. To prepare MF, 2.5% sucrose and 3% peptone were added to MA. The percentage of the ingredients mentioned above were fixed after repeated trials with different concentrations. MG was prepared by adding varying quantities (%) of

olive oil (5, 10, 15 or 20) in MF. MH was the finally standardized (enriched) medium comprising MF and 10% olive oil. In another experiment, influence of different concentrations of mineral solution was studied using MH. Initial pH of all the formulae was set at 6.8 before autoclaving.

Table IIIF1. Media composition for 10 g wet substrate (initial pH 6.8, before autoclaving)

Component(g)	Media							
	MA	MB	MC	MD	ME	MF	MG	MH
COC	3.5	3.5	3.465	3.395	3.412	3.307	3.307	3.307
Dist.H_2O	6.5	6.0	6.5	6.5	6.5	6.5	5.2	5.85
Mineral solution*		0.5						
Inorganic N source			0.035					
Organic N source				0.105		0.105	0.105	0.105
C. source					0.088	0.088	0.088	0.088
Olive oil							0 to 1.3	0.65
Total	10	10	10	10	10	10	10	10

*1.0 g contains (in mg): $(NH_4)_2HPO_4$ - 34.189; $ZnSO_4.7H_2O$- 1.079; $FeSO_4.7H_2O$ 0.959 and $CuSO_4.5H_2O$ -0.122

Water Activity (a_w)

COC and glycerol were mixed in different proportion on percentage basis to find out standard a_w values against different concentrations of glycerol used[179]. The respective COC: glycerol (w/v) ratios used were; 60:40, 55:45, 50:50, 45:55, 40:60, 35:65, 30:70 and 25:75. Before estimating a_w, the mixture was autoclaved for 30 min. In further experiments, glycerol was replaced by distilled water and

autoclaved. The results were compared with standard values obtained with glycerol

trial. Novasina water activity meter was used for the estimation of a_w.

Fermentation

For fermentation, 25 g moist substrate was taken in each 250 ml flasks,

autoclaved cooled and inoculated with 1 ml cell suspension (4%) of *C. rugosa*.

After thorough mixing, all the flasks were incubated at 28°C under static condition,

and cultivated for 144 h. After stipulated period, the samples (as whole flasks in

triplicate) were withdrawn and the fermented matter was homogenized manually and

5 g of such sample from each flask was taken for extraction and analysis[20]. In all the

experiments, moistened sterile air (1 vvm) was passed through the fermenting matter

under aseptic condition using an aerator. The schematic version of the set-up used
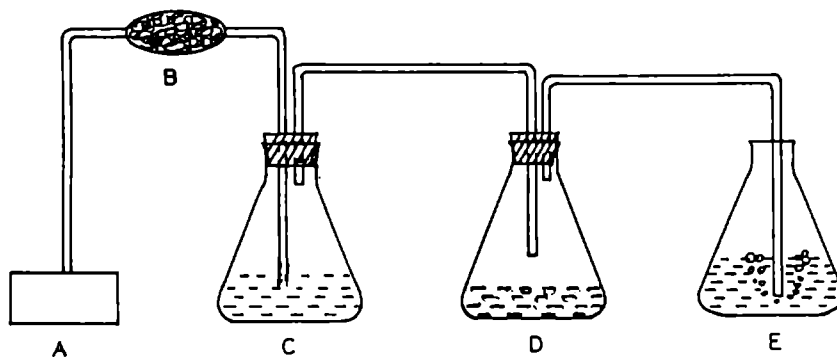
for aeration is shown in Fig. IIIF1.

Fig.IIIF1.Schematic set-up of aerating system used for fermentation in flask. A: aerator; B: air filter unit; C: moisturing unit; D: fermenting substrate and E: air exit unit.

Assay Methods

For quantitating various constituents such as lipases, soluble proteins and soluble sugars from the solid fermented matter, 5 g of the homogenised fermented matter was extracted with 45 ml distilled water for 15 min on a magnetic stirrer and the whole content was vacuum filtered. Extracellular lipase was estimated out of this aqueous extract with olive oil as described elsewhere. One unit (U) of lipase activity was defined as μmol of FFA liberated per minute per gram dry fermented substrate (gds), under the assay conditions[20]. Soluble proteins and sugars were also estimated using the aqueous extract. Concentrations of soluble sugars and proteins were expressed in mg g^{-1} ds. The change in a_w throughout the experiment was estimated using the fermented substrate. Growth was also estimated (by glucosamine method) using the fermented matter as described in Chapter IIIE.

Results and Discussion

The ultimate goal of the present study was to enrich COC for better growth and yield of *C.rugosa* using SSF technology. In the present study, optimization of a_w is an important parameter required for SSF. Optimum a_w was determined using MA[179]. As described in materials and methods of this Chapter, various initial a_w (0.954, 0.936, 0.915, 0.885, 0.857, 0.832, 0.795, 0.742) were established using MA to determine the best initial a_w for the growth and activity of *C. rugosa* (Fig.IIIF2). As this figure exemplifies an initial a_w of 0.915 (which corresponds to 65% moisture) was the suitable for maximum cellular growth. At 25 h of incubation the glucosamine content in the fermented medium was 1.63 mg g^{-1} ds. It increased to

11.6 mg g^{-1} ds at 72 h. Beyond

that no increase was observed,

indicating maximum growth by

72 h. This observation finds

uniqueness in all the

experiments. Whatsoever, initial

a$_w$ above 0.915 resulted in a

different pattern of initial

growth. At this condition, initial

growth (upto 48 h) was faster

than the latter stages, for

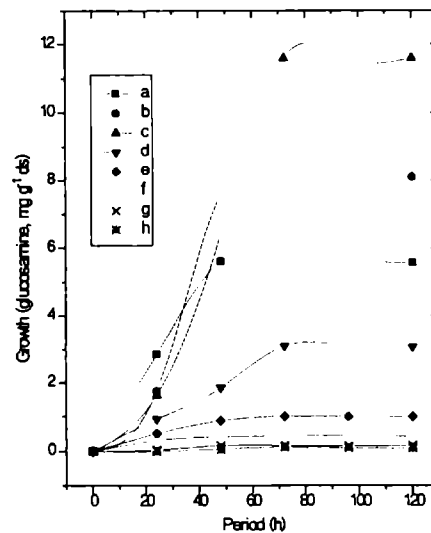instance, at initial a$_w$ 0.954, 2.85

Fig.IIIF2. Effect of initial a$_w$ on the growth of *C. rugosa*. a-h are growth at initial a$_w$ values: 0.954, 0.936, 0.915, 0.885, 0.857, 0.832, 0.795 and 0.742, respectively.

mg g^{-1} ds glucosamine was obtained after mere 24 h. This was two times greater

than that obtained with a$_w$ 0.915 within the same period. Surprisingly, after 50 h of

growth no much increase was obtained in substrates with a$_w$ above 0.915. This

interesting finding was mainly due to the less availability of micropores among the

substrate particles, because at higher a$_w$ the substrate would be coagulated, by

clogging aeration[20]. Valero *et al.*[238] investigated oxygen requirements for better

growth of *C. rugosa*. The dissolved oxygen in the substrate caused enhanced initial

growth. By faster growth, *C. rugosa* attained swollen morphology and such cells

impregnated into the available microspaces, thereby blocked further growth

(Fig.IIIF3). This view was further supported by the luxuriant growth on the free

surface of substrate facing the aerating mouth of the flask. Cells on this portion got

enough fresh air, supplied by the aerator.

Contrastingly, initial a_w below 0.915 was also not suitable for growth. It was

principally due to the inadequacy of bound water in the substrate, as evidenced by

drastic reduction in growth with reduction in moisture content[169]. At 0.742 level

(40% moisture) no growth was observed upto 40 h of growth. All these findings

project a vivid idea that optimum aeration and moisture are two important

preliminary parameters for SSF, both being co-existing independent factors[181]. In

these studies, initial a_w 0.915 (65% moisture) satisfactorily proved to be the ideal

initial a_w, and thus the same was fixed as the invariable parameter in all subsequent

studies on SSF.

Solid-state Fermentation

Studies on the influence of supplementation of mineral (as described by Pandey[176]), on the cultivation of *C. rugosa* in SSF showed that among different concentrations of mineral solution tested, 0.5% was found suitable (Fig.IIIF4). It did not
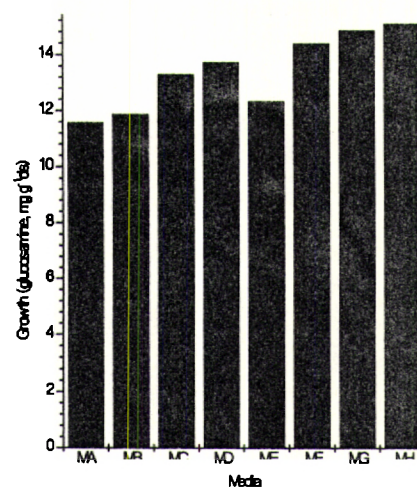

Fig.IIIF4. Comarison of growth profile on all 8 media studied. MC with 1% urea, ME with 2.5% sucrose, MF with 3% peptone + 2.5% sucrose, MG with 0.65% olive oil and enriched MH

enhance growth over that obtained

with MA, but enzyme yield was

three fold higher (47 U) than that

found in MA (16 U) (Fig.IIIF5).

This significant increase in yield

focuses on the sub-optimum

nutrition in the raw cake[20]. If an

organism gets only minimal supply

of nutrients, that would be utilized

for sustaining its life. Enough

nutrients would switch on different
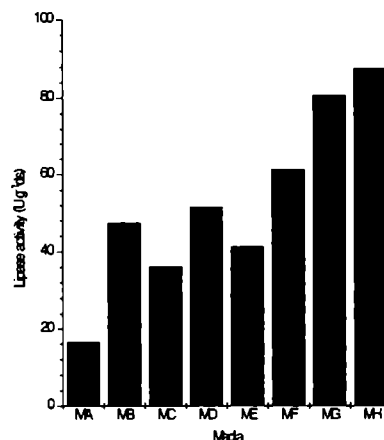
Fig.IIIF5. Comparison of lipase production in all 8 media studied at 96 h fermentation (MC with 1% urea, MD with 3% peptone, ME with 2.5% sucrose, MF with 3% peptone and 2.5% sucrose, MG with 0.65% olive oil and enriched MH).

Table IIIF2. Effect of different inorganic nitrogen sources (1.0%) on the fermentation behaviour of *C. rugosa* at 28°C. Growth and lipase activity are shown after different time intervals

Duration (h)	Diammonium hydrogen phosphate	Ammonium chloride	Ammonium sulphate	Ammonium nitrate	Urea
24	*1.71	1.82	1.64	2.01	3.24
	**1.75	1.75	1.85	2.62	2.70
48	10.32	10.32	10.32	10.32	15.49
	3.80	3.80	3.82	6.75	8.55
72	11.45	10.32	20.65	22.65	25.81
	8.66	9.49	10.06	12.48	13.3
96	15.17	17.10	20.65	30.97	36.14
	8.75	9.0	9.45	10.94	10.62
120	14.36	16.33	15.49	30.97	30.97
	7.88	8.77	8.08	9.19	9.63
144	10.32	10.32	10.32	20.65	25.81
	8.33	8.10	7.65	9.25	8.75

*lipase activity, U g⁻¹ ds; **glucosamine, mg g⁻¹ ds

biosynthetic pathways and cause the secretion of different primary as well as secondary metabolites for more active life and better substrate utilization[1]. In fact, occurrence of lipids in the medium would induce more lipase secretion[54]. COC contained 6.68% lipids. It was responsible for the secretion of lipase in MA, even if it contained only minimum nutrients, but in MB, sufficient feeding could elevate the organism to the second stage of production and active life, thus enhanced the lipase producing activity.

Table IIIF2 describes the individual effect of different ammonium salts in COC (MC). In comparison to control, MA (Fig.IIIF5) diammonium hydrogen

Table IIIF3. Effect of different organic nitrogen sources (3%) on the fermentation behaviour of *C. rugosa* at 28°C. Growth and lipase activity are shown after different time intervals

Duration	Beef extract	Malt extract	Yeast extract	Peptone
24	*2.32	2.77	3.51	3.94
	**1.75	2.25	3.06	4.68
48	10.24	10.24	15.49	20.65
	9.71	9.90	11.25	11.48
72	25.81	20.65	30.93	36.14
	11.38	12.60	13.13	13.75
96	36.14	26.14	41.30	51.62
	11.56	12.15	12.06	13.41
120	30.27	24.97	36.14	41.30
	10.94	11.70	11.56	11.25
144	30.97	24.07	30.97	36.14
	10.80	11.25	10.80	10.94

*lipase activity, U g^{-1} ds; **glucosamine, mg g^{-1} ds

phosphate caused the least enzyme yield (15.17 U g^{-1} ds) and urea emerged as the best enhancer of both growth and yield (13.3 mg g^{-1} ds glucosamine, 36.14 U g^{-1} ds, respectively) (Figs. IIIF4&5). Ammonium nitrate stood next to urea followed by ammonium sulphate, but diammonium hydrogen phosphate, ammonium chloride and ammonium sulphate were not significant nutrients (Table IIIF2). Though no much difference from MA was observed in lipase production by these nutrients, they repressed the biomass production slightly. This retarding effect discussed above could be mainly due to the free and unnecessary ions released by these salts in the medium, which disturbed nutrient uptake mechanism of the organism[20]. Thus this piece of work explains a significant behaviour that C. rugosa requires additional nitrogen source, which should be free of unnecessary ions.

Complex organic nitrogen sources offer yet another important nutrient supply. In using complex nutrients, they have an added effect, ie. most of the required trace elements would contain in such media, which entered as impurities. Table IIIF3 depicts the results obtained by four complex organic nitrogen sources, which are frequently used in most of the microbiological experiments. Effect of beef extract was more or less similar to that obtained with urea (Table IIIF2). Malt extract was used as a dual substance, which acted as both carbon and nitrogen source. Its performance on lipase production was very poor, an evidence for poor nitrogen content in it[25], but growth was slightly higher than as obtained with beef extract. Yeast extract not only supported better growth (13.13 mg glucosamine) but lipase yield was also high (41.30 U). While effect of peptone equalled with yeast extract in growth, its influence on lipase producing activity was much promising

(51.62 U). Increased biomass production by peptone and yeast extract tokens the

role of amino acids and its derivatives present in them, which acted as prominent

precursors for lipase production, through biosynthesis of proteins[20].

Table IIIF4. Effect of different additional carbon sources (2.5%) on the fermentation behaviour of *C. rugosa* at 28°C. Growth and lipase activity are shown after different time intervals

Duration (h)	Starch	Glucose	Fructose	Lactose	Maltose	Sucrose
24	*0.21	0.34	0.37	3.21	3.27	4.52
	**1.75	2.19	2.25	2.62	2.70	3.24
48	6.33	10.32	11.49	20.65	20.65	25.87
	8.10	10.80	11.49	9.90	10.06	11.56
72	11.79	25.81	27.32	28.90	30.65	41.29
	11.60	11.25	11.69	10.05	11.13	12.34
96	16.97	26.35	28.51	30.51	31.55	41.32
	11.15	11.38	11.15	10.25	11.69	12.16
120	16.37	25.29	28.17	30.10	28.72	46.46
	11.70	10.63	11.70	9.74	11.56	11.96
144	15.41	22.97	27.71	27.33	28.02	41.29
	10.93	10.06	11.25	9.35	10.20	11.86

*lipase activity, U g⁻¹ ds; **glucosamine, mg g⁻¹ ds

Preferential requirement of additional carbon source when added in COC

was confirmed by the studies with medium ME (Table IIIF4). *C. rugosa* preferred

disaccharides to monosaccharides and polysaccharides. Effect of starch signified

nothing because, results obtained with starch were at par with the results with MA[43].

It was an indication for its inertness as a carbon source in this study. As for the effect of the monosaccharides were concerned, effect of glucose and fructose were similar. In both these cases, however, though the growth was not significant, lipase secretion was better over MA (Figs.IIIF4&5). Among the disaccharides (sucrose, maltose and lactose), sucrose was the most favourable choice.

In medium F, combined effect of peptone and sucrose was studied and the results were plotted on Figs. IIIF4&5. Maximum lipase at 96 h of fermentation was 61.34 U, the best ever result obtained in the present study. Accordingly, at 72 h maximum growth was 14.40 mg. Both the nutrients enriched the substrate, which provided optimum conditions for the active growth of *C. rugosa*. These better results are direct evidence to state that raw COC was short of soluble sugars and proteins.

Effect of olive oil in lipase production has been reported by many authors in submerged fermentation. However studies on its influence on solid substrate is new[20]. As described in Table IIIF5, four concentrations (%) (5, 10, 15, 20) of olive oil were added to MF to get MG. In fact, in all the cases further increase in growth was not observed, but enzyme yield was increased by 43% (87.7 U) in the presence of 10% olive oil at 96 h (over 61.31 U, that obtained in the same medium without olive oil) (Table IIIF5). Concentration of olive oil above 10% was not so suitable. It was proved that lipid substances are essential for higher titres of lipase production. Reaction scheme of lipase production by *C. rugosa* growing on olive oil was described by Del Rio *et al.*[54] According to them, traces of lipases which initially

secreted would split olive oil into glycerol and oleic acid (FFA) and the production

of lipase at higher levels would be associated with the consumption of fatty acids.

Thereby, induction of lipase gene in the presence of lipids in the medium leads to the

overproduction of lipases.

Table IIIF5. Effect of different quantities of olive oil on the fermentation behaviour
of *C. rugosa* at 28°C. Lipase activity and glucosamine are tabled after different time
intervals

Duration (h)	0%	5%	10%	15%	20%
24	*1.31	2.72	3.65	3.1	2.81
	**5.10	5.55	5.85	0.45	0.45
48	20.65	25.81	41.30	30.97	30.97
	9.35	9.45	9.90	9.90	9.19
72	41.30	41.30	72.77	61.95	61.60
	14.40	14.44	14.85	14.44	14.40
96	61.31	77.27	87.70	77.43	72.27
	14.40	14.72	13.50	14.40	14.36
120	60.18	61.95	72.27	72.27	72.27
	13.05	13.30	12.75	13.05	13.30
144	51.62	51.62	61.95	56.78	51.62
	12.15	12.25	12.68	11.40	11.4

*lipase activity, U g⁻¹ ds; **glucosamine, mg g⁻¹ ds

Complete fermentation profile of *C. rugosa* on Medium H is enumerated in

Fig. IIIF6. As in the previous studies, maximum growth (15.1 mg glucosamine) and

activity (87.76 U) were achieved at 72 and 96 h of fermentation, respectively. After

72 h of fermentation a_w declined from the initial value 0.91 to 0.82. It shows that
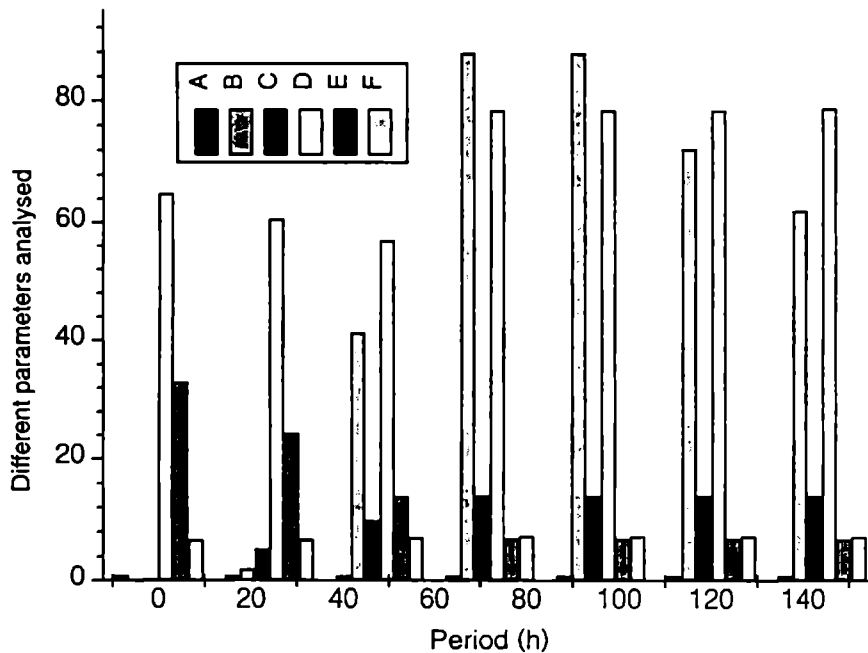
Fig.IIIF6. Fermentation behaviour of *C. rugosa* on standardised
medium (MH) at different time intervals. A: a_w, B: lipase activity
(U g^{-1} ds), C: glucosamine (mg g^{-1} ds), D: soluble protein
(mg g^{-1}ds), E: soluble sugar (mg g^{-1}ds) and F: pH.

this final value is roughly equivalent to an initial a_w for substrate containing 50%

moisture , *ie*. 30% of the bound water in the substrate was utilized for the active life

of *C. rugosa* (Fig.IIIF2). Beyond 72 h of fermentation, further fluctuation in a_w was

not prominent. Fig.IIIF3 describes the morphology of *C. rugosa* growing on

coconut cake at 72 h of fermentation. Here the cells are oval and so swollen, ready to

secrete more by-products. Depletion in soluble sugar concentration shows the extent

of substrate utilization. Maximum consumption was 75%. As a common factor, in

this experiment also soluble protein concentration showed sudden increase after 50 h

of fermentation. Extracellular protein in the medium was responsible for this

fluctuation[20]. Final pH of the medium after 96 h of fermentation was 7.5. This

alkaline pH was due to the alkaline protein in medium which is accompanied by
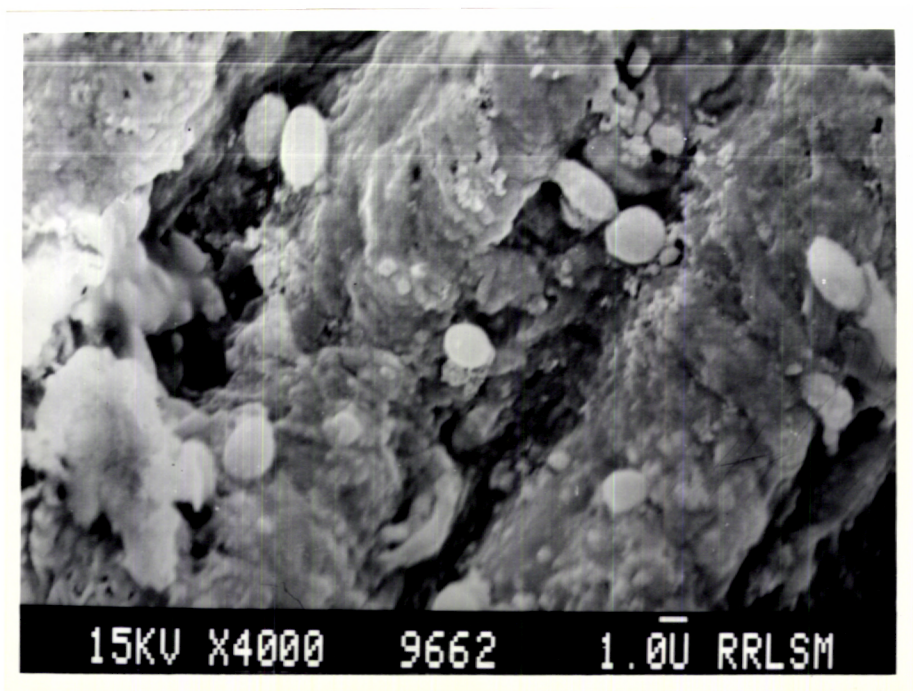
progressively increasing lipase activity[84].

Fig.IIIF3. Growth of *C. rugosa* on coconut cake
at 60 h fermentation

MIXED - SOLID
SUBSTRATE FERMENTATION

Introduction

Potential role of coconut oil cake (COC) to be used as a substrate has already been established by Benjamin and Pandey[20]. Studies with COC extract showed significant results for the overproduction of lipases. COC in its enriched solid form was very much attractive for *Candida rugosa* growth and yield. Rao *et al*[196] also employed SSF technology for the cultivation of *C. rugosa* on rice bran. However, no literature is available on the utilisation of mixed-solid substrate for lipase production by growing *C. rugosa* on it under restricted water environment nor even with other microorganisms[182]. Nevertheless, success with COC influenced to examine the efficacy of mixed-solid substrate containing COC and wheat bran as co-substrate. In the present investigation, this juvenile technology has been employed for monitoring the growth and yield of *C. rugosa* and much promising results so obtained are discussed here.

Mateials and Methods

Media preparation

Cheaply available coconut oil cake (COC) and wheat bran (WB) (both fine and coarse) were procured from the local market. In all the studies (media A to J), COC, and fine and coarse WB were used in 1:1:1 ratio (Table IIIG1) and invariably, in all the experiments, final moisture content in the substrate (65%) and solid substrate (35%) were kept as constant parameters. Different ingredients were tested for substrate enrichment. Modified media were designated as medium A to J and only optimum concentration (obtained after repeated trials) of ingredients for the

growth and activity were reported in Table IIIG1. Initial pH of all the media was set

at 6.8.

Table IIIG1. Media composition used in the experiments

Medium (pH 6.8)	Composition
MA	Coconut Oil Cake (COC) + Fine Wheat Bran (FWB) + Coarse Wheat Bran (CWB) (1:1:1)
MB	MA + 5% mineral nutrition (Pandey,1991)
MC	MA + 6% corn steep liquor
MD	MA + 2% maltose
ME	MA + 3% maltose
MF	MA + 10% olive oil + 0.4% gum arabic
MG	MF + 2% maltose + 3% peptone
MH	MG +0.3% different fatty acids
MI	MG + 0.5% different Tweens
MJ	MG + 0.3% caprylic acid + 0.5% Tween-20

Fermentation

For fermentation, 25 g moist substrate was taken in each 250 ml flask,

autoclaved, cooled and inoculated. After thorough mixing all the flasks were

incubated at 28°C under static condition, and cultivated for 120 h. Initial

experiments were conducted in flasks. As a final step in this study, a column

fermenter model was devised to monitor the influence of aeration during

fermentation. Schematic set-up of the column fermenter is shown in Fig.IIIG1. It

contained an aerator (A), which infused air into a moisturing unit (C) through an air

filter (B). Sterile and moistened air released from C entered into a distribution unit

(D). From D air entered into the column' (E) (30x5 cm) containing fermenting

substrate on reverse flow mode.

In case of flask experiments, after distributing fresh and moist air to the

fermenting substrate, the exhaust air went out through the exit unit (Fig.IIIF1).

Aseptic condition was maintained throughout the experiments.   After stipulated

period, the samples (as whole flasks in triplicate) were withdrawn (12/24 h

intervals). The fermented matter was homogenized manually and 5 g sample from

each flask was taken for extraction and subsequent assays.   In case of column

experiments, fermentation was stopped after 24 h intervals and whole contents were

homogenized and a weighed quantity (5 g) was processed.
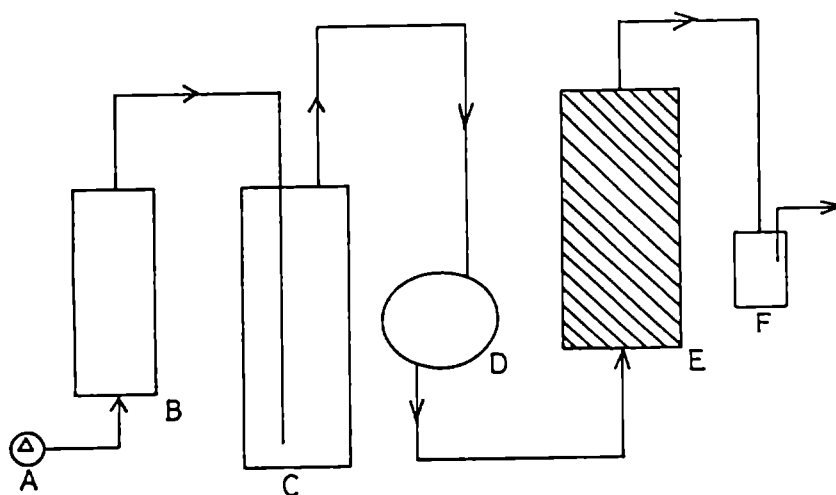
Fig.IIIG1.Schematic set-up of column bioreactor for lipase
production in SSF. A: aerator; B: air filter unit; C: air
saturation unit; D: air distributor; E: column bioreactor
and F: air exit unit.

Assay methods

Soluble sugars, proteins, lipase activity, a_w and glucosamine content were

assayed as described in previous chapters.   Growth was also measured indirectly

from the evolved CO_2. Wheat bran was also subjected to chemical analysis and the methods employed were similar to that of COC.

Results and Discussion

A comparative study of the results obtained by chemical analysis of the substrate shows that COC is superior to wheat bran (WB) in possessing higher amount of total protein and lipid contents (Table IIIG2). However, soluble sugar content was more in WB. Soluble sugar content in the fine wheat bran showed 27% increment as against coarse WB. Total lipid and protein contents in both fine and coarse wheat bran were less in comparison to COC.

Table IIIG2. A comparative study of chemical composition (%) of COC and WB

Chief constituents	Quantity (%)		
	COC	Coarse WB	Fine WB
Starch	18.82	24.21	26.82
Soluble sugars	6.31	8.34	11.37
Total protein	14.31	3.65	4.65
Soluble protein	4.81	0.27	0.75
Total lipid	6.68	0.36	0.73
Total moisture	8.94	6.31	7.12
Ash content	5.63	6.62	5.84

Pandey et al.[179] found that maintenance of optimum a_w in the solid substrate is an indispensable prerequisite for SSF. With this view, optimum initial a_w requirement was analysed in four substrates: COC, coarse WB, fine WB and in mixed substrate which contained all the substrates mentioned above in 1:1:1 ratio (MA) with 65% initial moisture. Maximum lipase activity was obtained at 96 h used

Table IIIG3. Comparison of growth (glucosamine content, mg g⁻¹ds) and yield (lipase U g⁻¹ds) of *C. rugosa* in different substrates by SSF

Duration of fermentation (h)	[a]Coconut cake		[b]Coarse wheat bran		[c]fine wheat bran		a,b & c (medium A) in 1:1:1 ratio	
	Glucosamine	Lipase	Glucosamine	Lipase	Glucosamine	Lipase	Glucosamine	Lipase
24	2.1	3.2	1.3	0.6	1.8	1.2	2.5	5.1
48	7.5	12.1	2.6	1.5	4.5	6.1	7.6	16.6
72	11.6	22.4	7.8	5.9	10.3	10.0	10.8	26.3
96	11.2	26.0	7.4	6.2	9.7	17.0	10.2	34
120	11.1	25.2	7.4	6.0	9.5	16.4	10	33.8

cultivation as against 72 h for growth. Lipase yield was less when the substrates were used individually. Mixed substrate showed improved results, however, growth did not follow this pattern (Table IIIG3). Growth was slightly better in COC (11.6 mg g⁻¹ ds) than in mixed substrates (10.8 mg g⁻¹ ds). It indicated that though growth and enzyme yields were two independent factors,
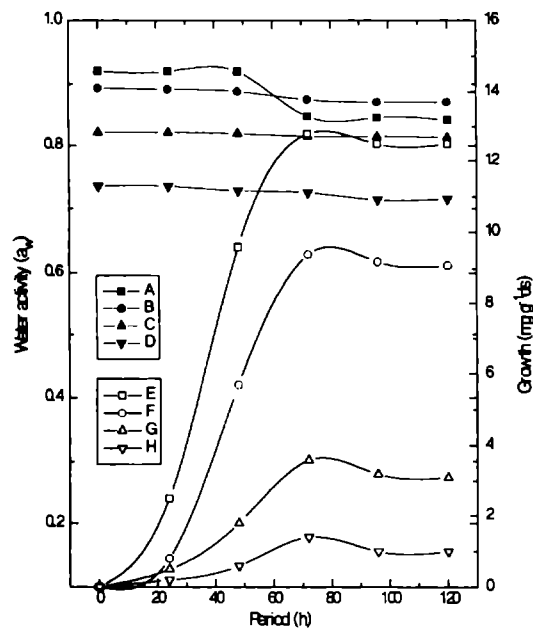

Fig.IIIG2. Influence of different initial a_w on the growth of *C. rugosa* in the mixed substrate (medium A) A, B, C & D: initial a_w set at 0.921, 0.894, 0.822 & 0.735 respectively. E,F,G&H are corresponding growth of A, B, C & D

maximum lipase yield was noticed at the senescence phase of growth itself. Higher

enzyme yields in mixed substrate indicated that the trace elements in different

substrates under mixed state enabled the yeast to yield more, by influencing its

biochemical pathways for lipase production[207]

A clear picture on the influence of a_w in the growth of *C. rugosa* is shown in

Fig.IIIG2. Optimum moisture (65%) in the substrate recorded an a_w 0.921. It was

found to decrease progressively with advancing growth. Lower a_w values, *viz.* 0.894

(60% moisture), 0.822 (50% moisture) and 0.745 (40% moisture) adversely affected

the growth. Optimum

moisture is essential for better

release of nutrients, which are

substrate-bound and their

release occurs during

autoclaving[18]. If initial

moisture is supra-optimum

that would chock the

inter-particular air spaces in

the substrate and eventually

retard the growth of the

organism, hence growth was

not promising at a_w above

Fig.IIIG4. Enrichment effect of raw mixed substrate with different nutrient sources in lipase production at 96 h A: raw maixed substrate, B: mineral nutrition (5%), C: corn steep liquor (6%), D: maltose (2%), E: peptone (3 F: olive oil (10%) + gum arabic (0.4%), G: F + peptone (3%) + maltose (2%)

0.92[179]. Observations through scanning electron microscope (SEM) showed that at

the optimum a_w (0.92), *C. rugosa* appeared as actively dividing sac-like cells on the
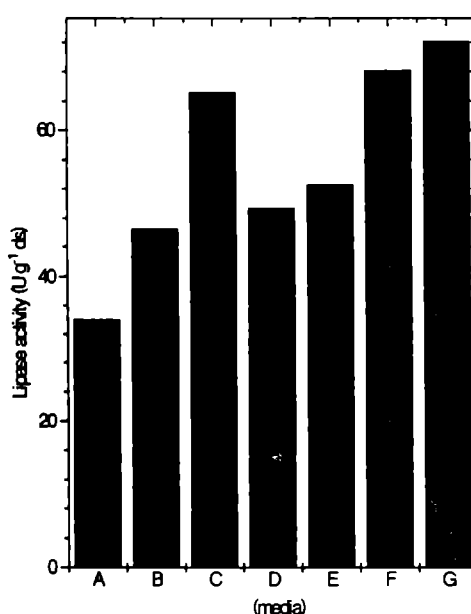
mixed substrate (Fig.IIIG3). It was a clear sign for the better growth and yield, as discussed in succeeding paragraphs.

Mixed substrate (MA) was enriched in various ways as depicted in Table IIIG1. The potential role of different nutrients, which were used as efficient source of macro and micro elements for lipase production is shown in Fig.IIIG4. Effects of additional nutrients added in media B to F were tested in different combinations, but the combinations mentioned in medium G resulted in comparatively high lipase yields (72.2 U). However, this yield was not significantly higher than that obtained in the MF (68.2 U), which contained olive oil. It is a direct evidence for the inducing role of lipid substances for favourably altering lipase secretion.

Table IIIG4. Comparative study on the individual effects of caprylic acid (Medium H) and Tween-20 (Medium I) and their antagonistic effect (Medium J) in lipase production at different time intervals

Medium	Period (h)				
	24	48	72	96	120
G (partially enriched)	5.4	24.6	54.8	72.2	71.3
H (containing caprylic acid)	3.4	16.4	76.8	84.4	83.2
I (containing Tween-20)	12.0	34.8	79.1	96.7	96.5
J (enriched, containing caprylic acid and Tween-20	5.5	15.4	50.2	55.4	52.3

Figures IIIG5 and IIIG6 describe the inducing role of different free fatty acids (FFA) and Tweens, respectively in lipase production. These inducers were tried individually in MG for lipase production. From Fig.IIIG5, butyric and caproic acids were found to be much toxic and adversely affected the enzyme yield[167]. Caprylic acid, however, enhanced the lipase yield (84.4 U). The inducing role of
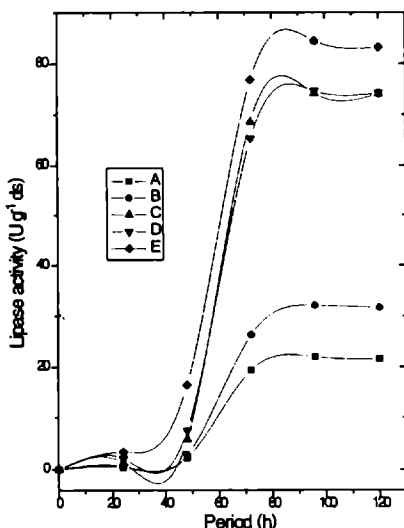
Fig.IIIG5. Influence of different fatty acids (0.3%) in the partially enriched substrate for lipase production (medium H) A: butyric acid, B: caproic acid, C: oleic acid, D: capric acid, E: caprylic acid

caprylic acid in SmF was already confirmed by Benjamin and Pandey[17]. Induction of lipase production by FFA was reported by many authors[54]. Of four Tweens tested, Tween 20 was found to be much effective (Fig.IIIG6) which, probably, was due to

the occurrence of short chain (12 C) dodecanoic acid in it. However, the combined action of Tween 20 and caprylic acid (MJ) revealed the antagonistic effect of these two individual inducers, possibly a mutual competition suppression in the act of feeding the cells[23] (Table IIIG 4).
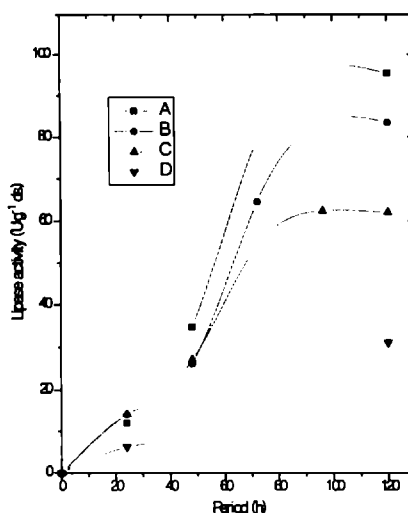

Fig.IIIG6. Influence of different Tweens (0.5%) in partially enriched substrate for lipase production (medium I) A: Tween-20, B: Tween-40, C: Tween-D: Tween-80
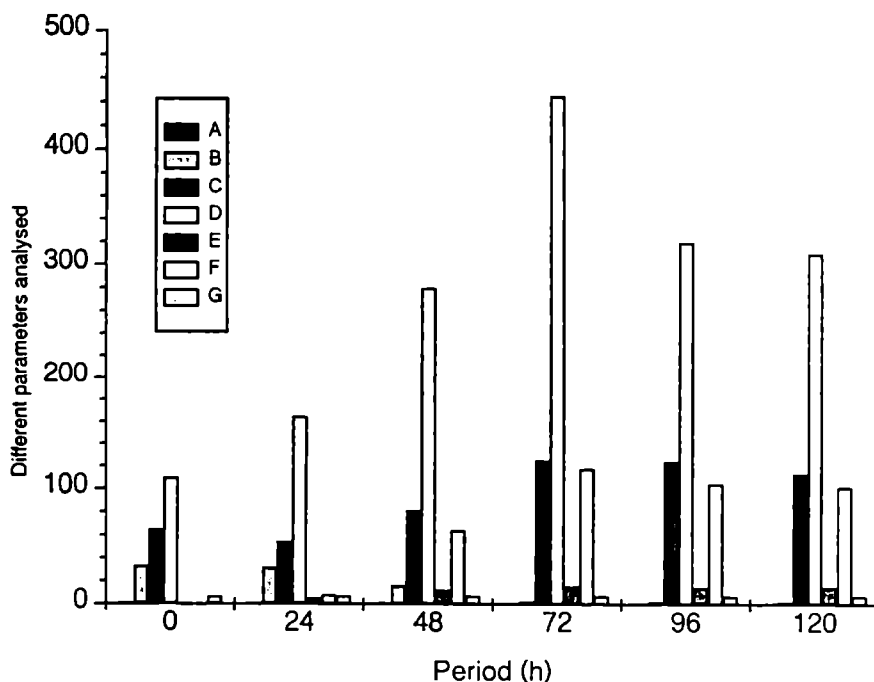
Fig.IIIG7. Different parameters studied in the fermentation behaviour of *C. rugosa* in the final enriched medium after aeration (1 vvm), in column fermenter. A: a_w, B: soluble sugars (mg g^{-1}ds) C: soluble proteins (mg g^{-1}ds), D: evolution of CO_2 (ppm), E: glucosamine (mg g^{-1}ds), F: lipase (U g^{-1}ds) and G: pH

Supply of sterile air (1 vvm) through the column fermenter model (Fig.IIIG1) greatly altered the fermentation behaviour of *C. rugosa* (Fig.IIIG7). In the column fermenter, the most significant influence was that both growth (15.8 mg g^{-1}ds) and yield (118.2 Ug^{-1}ds) were synchronized at 72 h with a slight increase in pH[23]. In contrast, in flask experiments, maximum growth was at 72 h and yield at 96 h. Maximum carbohydrate consumption was also noticed (90%) at this stage. However, protein concentration in the fermented matter gradually increased. Apart from glucosamine estimation, evolved CO_2 also gave an indirect evidence for growth, which was at par with glucosamine content.

This study proved that strain improvement for better performance is one of the prime objectives of industrial microbiology for the large scale production of valuable metabolites, which can be achieved by balanced nutrient supply and optimization of external physical factors required for SSF. The present study gives a vivid picture on the influence of solid substrate and additional nutrients coupled with aeration for extracellular lipase production. In comparison to SmF, SSF can be preferred for lipase production by *C. rugosa* owing to its upperhand as picturised by the present study.
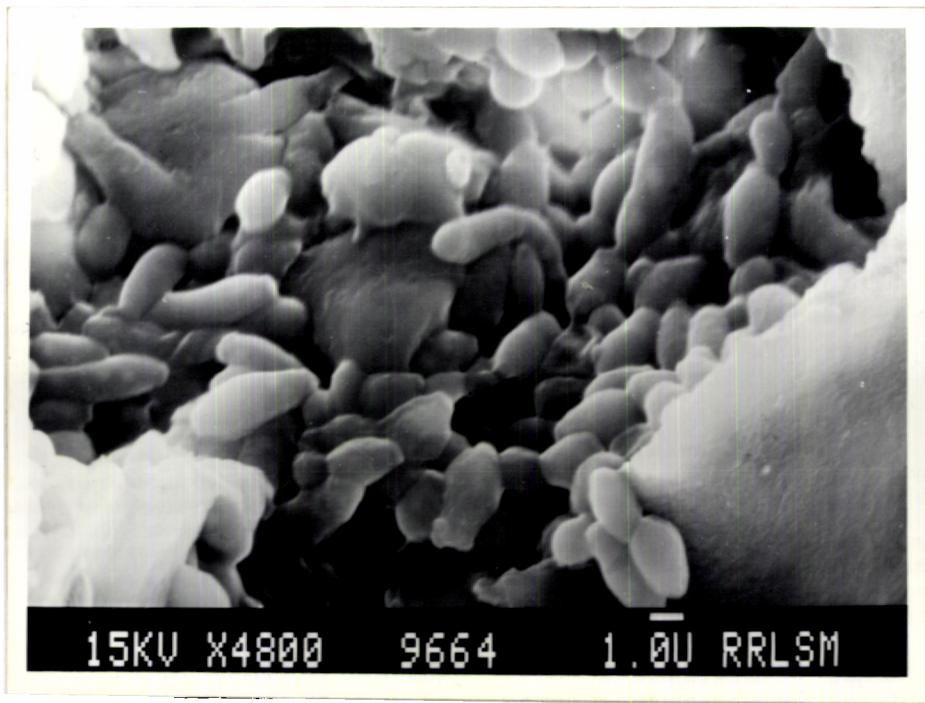
Fig.IIIG3. Growth of *C. rugosa* on mixed-solid
substrate at 60 h fermentation

PURIFICATION, ISOLATION

AND

CHARACTERIZATION OF LIPASES

Introduction

Existence of *Candida rugosa* lipase isoforms has been reported by eminent groups working in various parts of the world. Occurrence of isolipases is a common feature in many yeasts (*Geotrichum candidum, Candida antartica, C. rugosa*), mold (*Aspergillus niger, A. oryzae, Pencillium cyclopium, Rhizopus miehei*) and bacteria (*Chromobacterium viscosum*)[22]. However, the authors are not having a consensus in ascertaining the exact number of isolipases encoded in the lipase 'minigene family' of *C. rugosa* genome Rua and Ballesteros[202] isolated and characterised two main lipase isoforms (Lipases A and B) which had similar amino acid content, N-terminal sequence and molecular weight, but differ on neutral sugar content, hydrophobicity, stability and substrate specificity. Linko and Wu[129] also claimed two distinct lipases (L1 and L2) from *C. rugosa*, isolated by hydrophobic interaction chromatography on phenyl sepharose, gel filtration on Sephadex G-25, ion-exchange chromatography on DEAE-Sepharose Fast Flow and ultrafiltration. Interestingly, Shaw and Chang[217] succeeded in identifying three distinct forms from the commercially available crude *C. rugosa* lipase preparation by the technique of activity staining on a gradient polyacrylamide gel. Contrary to these reports, Lotti *et al*[132]. of Universita degli Studi di Milano, Italy reported five isolipases from *C. rugosa*. A unique feature of all these reports was that, in all the studies, commercial lipase preparation was used[24].

In the present study, attempts were made to purify, isolate and characterize three distinct lipase isoforms directly from the culture broth, obtained by solid-state fermentation.

Materials and Methods

Production Medium and Fermentation

Enriched mixed-solid substrate was used as the production medium (initial pH 6.8). The mixed-solid substrate contained coconut oil cake (COC), fine and coarse wheat bran in 1:1:1 ratio, which was enriched by supplementing 10% olive oil, 2% maltose, 3% peptone, 0.5% Tween-20 and 0.4% gum arabic, as described in Chapter IIIG. Quantity of total moisture and solid substrate were invariably maintained as 65% and 35%, respectively throughout the fermentation. Initial a_w was set at 0.92. Fermentation was carried out in a column fermenter (Fig.IIIG1) under aseptic condition at 28°C. Sterile and moist air was passed through the column (1 vvm) under reverse flow mode.

Extraction of Proteins from the Fermented Matter

Fermented matter (72 h) was homogenized manually and 5 g was extracted with 20 ml distilled water on a magnetic stirrer for 15 min. The extract so obtained was centrifuged at 6000 g for 10 min at -5°C to get a debris free supernatant. It was the crude extract (Fraction I).

Fractionation with Ammonium Sulphate

Solid ammonium sulphate was added to the crude extract (Fraction I) at 20% saturation and allowed to stand for 30 min. The precipitate (Fraction II) was obtained by centrifugation (16000 g, 20 min at -5°C) and the resulting supernatant was further treated with solid ammonium sulphate at 40% saturation. The precipitate (Fraction III) was collected by centrifugation (as above) and the

supernatant was further treated with ammonium sulphate at 60% saturation. The precipitate fraction (IV) was again obtained by centrifugation and the supernatant was finally treated with 100% saturation (Fraction V). All the precipitates (II-V) were resuspended in a minimal amount of buffer solution (0.1 M L^{-1} Tris-HCl, pH 7.0) and dialysed against the same buffer by using successive large volumes of the same buffer. The process was continued till the last trace of ammonium sulphate was removed. The protein fractions were concentrated by ultrafiltration using an ultrafiltration unit with a cut off range of 10 kDa (Millipore, USA). All the fractions (II-V) were subjected to protein measurement and lipase assay to choose the fraction containing maximum lipase activity[24].

Assays

Protein and lipase assays were performed as described in the previous chapters (IIIA and IIIB), respectively.

Sephadex G-200 Gel Filtration Chromatography

Concentrated fractions obtained at 40-60% saturation (Fraction-IV) was applied on a column (30x1.5 cm) packed with Sephadex G-200 (Sigma, USA) which was previously equilibrated with 0.1M L^{-1} Tris-HCl (pH 7.0)[191]. The column was eluted with the same buffer with a flow rate of 60 ml h^{-1} using a peristaltic pump. Fractions of 3 ml were collected by fraction collector and each fraction was assayed for lipase activity and the amount of protein in each fraction was estimated by measuring the absorbance at 280 nm using spectrophotometer. Fractions showing only lipase activity were pooled and subjected to the next step of purification, $ie.$

sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and also for PAGE under non-denaturing condition. In another set of experiments, specific peaks obtained by the lipase active fractions with gel permeation chromatography were also separately collected for loading on the PAGE, after concentrating by ultrafiltration.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (in the presence of SDS) was performed as described by Laemmli[116], in 10% polyacrylamide. Samples and control were boiled for 3 min in the sample buffer, which contained 5% 2-mercapto ethanol and 1% bromophenol blue. Coomassie brilliant blue was used to stain the protein band. Electrophoresis in the absence of SDS (non-denaturing or native PAGE) was carried out on 8% polyacrylamide gels in Tris-glycine buffer (pH 8.8)[32].

Electrophoresis was performed on a vertical slab mini-gel apparatus (Biotech, India) at 100 V for 5-6 h. Active lipase bands were detected in the non-denaturing gel after its extraction from the untreated gel slices. The gel slices were homogenised and kept in 0.1 M L^{-1} Tris-HCl buffer (pH 7.0) for 1 h for passive diffusion of the lipase into the buffer. Lipase containing extract was obtained by eliminating the gel debrises on centrifugation in an Eppendorf Centrifuge (5000 g for 10 min)[32]. Molecular weight markers contained myosin (205 kDa), β-galactosidase (116 kDa), Phosphorylase-B (97 kDa), Bovine Serum Albumin (66 kDa) and Ovalbumin (45 kDa) (Sigma, USA)[24].

Electrophoretically separated lipase fractions (as described above) were used for further studies and the characterization of lipase isoenzymes.

Characterization of Lipase Isoenzymes

In order to distinguish distinct characteristics of isolated isoenzymes, substrate specificity, thermosensitivity, pH tolerance and effect of ions and inhibitors on their activity were investigated.

Acyl Glycerol Specificity

The enzyme fractions obtained from native PAGE were incubated with triolein, tripalmitin, trilaurin, tricaprin and tributyrin as test substrate to a final concentration of 20 mM. The reaction mixture contained the ingredients used for lipase assay in 0.1M Tris-HCl buffer (pH 7.0) and at 37°C. Sequential dilutions of the substrates were done to determine Km values.

Thermostability

To investigate lipase stability, the enzyme mixtures were preincubated at various temperature (25 to 65°C at pH 7.0) in Tris-HCl buffer, before lipase assay. Incubation persisted for 0-300 min under the assay conditions. Samples were withdrawn after 5-30 min intervals.

pH Tolerance

To investigate pH tolerance, enzyme mixtures were preincubated at different pH values (5.5 to 9.5 at 37°C) for 30 min in Tris-HCl buffer as above.

Effect of Ions and Inhibitors

Lipase fractions were preincubated for 1 h at pH 7.0 and 37°C in 0.1M

Tris-HCl buffer with various ions or inhibitors. Assay was performed with the

mixture which did not contain $CaCl_2$ (except in test sample). One dozen metal ions

were tested at varying concentrations (10^{-2} - 10^{-4} M). Varying molar concentration

of chelator or inhibitors tested were: Ethylene Diamine Tetra Acetic acid (EDTA),

anionic detergent, Sodium Dodecyl Sulphate (SDS), thiol-binding Parachloro-

mercuric Benzoate (PCMB) and serine protease inhibitors such as Phenyl Methane

Sulphonyl Fluoride (PMSF), 3,4-Dichloroisocoumarin (3-4 DCl) and Pefabloc[24].

Results and Discussion

Purification and Isolation of Lipases

A representative purification profile is summarised in Table IIIH1. *Candida*

rugosa lipases (CRL) were

purified approximately to

43-fold over the culture

supernatant with 22.5%

activity recovery, employing

native PAGE[24]. As shown in

Fig.IIIH1, lipase active

fractions contained in

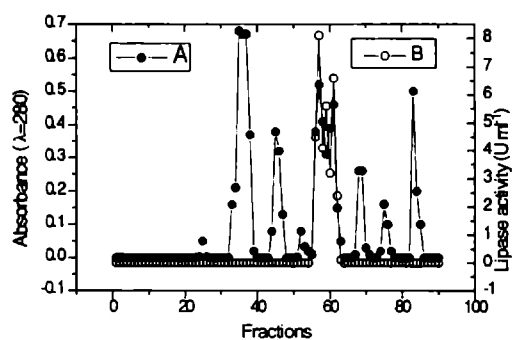between 55 and 63. A

magnified version of

Fig.IIIH1. Elution profile of lipases from
Sephadex G-200 column. A: absorbance
at 280 nm and B: lipase activity (U ml⁻¹)

Sephadex G-200 elution is presented in Fig.IIIH2. Three distinct peaks are visible in the active fractions. It indicates that these fractions contained different lipase isoforms with varying activity characteristics. These findings are also confirmed by the results obtained by SDS-PAGE electrophoresis[24] (Fig.IIIH3).
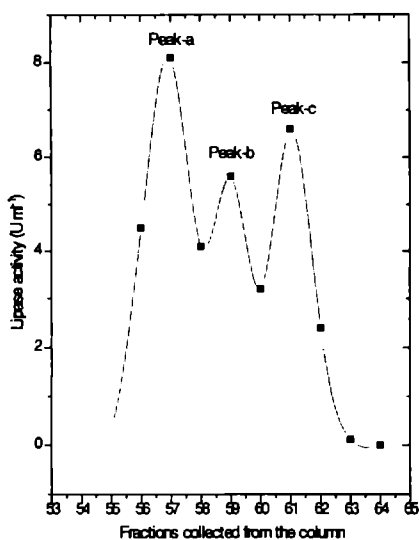
Fig.IIIH2.Elution profile of ammonium sulphate precipitated (40-60% saturation). Lipase active fractions showing 3 distinct peaks.

Table IIIH1. Flow sheet for purification of *C.rugosa* extracellular lipases

Purification	Total protein (mg ml⁻¹)	Specific activity (U ml⁻¹)	Total activity (U ml⁻¹)	Activity recovery (%)	Purification (fold)
Culture supernatant	32.77	1.48	48.61	100	1
Ammonium sulphate precipitate	4.68	3.88	18.14	37.32	2.62
Sephadex G-200	0.46	28.96	13.32	27.40	19.56
PAGE (native) Fraction	0.17	64.35	10.94	22.51	43.48
Lip A	0.06	75.89	4.25	11.62	51.27
Lip B	0.021	26.67	0.56	1.15	18.02
Lip C	0.018	97.22	1.75	3.64	65.69

In Fig.IIIH3, pattern on lane D clearly shows three distinct bands contained in the pooled lipase active fractions (Sephadex G-200 elute between 55 and 63).

Bands in lanes E (peak-a), F (peak-b) and G (peak-c) represent 3 distinct forms of lipases, which are the representatives of the peaks in order of their elution from the column (Fig.IIIH2). To get these bands, fractions corresponding to each peak were separately pooled and loaded on lanes E, F and G, respectively.

For convenient interpretation, bands on lanes E, F and G were named as Lip A, Lip B and Lip C, respectively (Fig.IIIH3). Lane E shows a major and a minor bands. The major band was considered as the representative of Lip A and minor one should be of Lip B. Bands on lanes F and G are very clear. The location of these 3 individual bands coincides with 3 bands on lane D. Though apparent molecular weight (MW) of these lipases is around 60 kDa, as reported by Lotti *et al.*[132] and Rua and Ballesteros[202], they show a slight variation on a range ~ 2- 4 kDa. This variation can be attributed to the partial purity and also difference in amounts of glycosylation[202]. However, apparent MW of Lip C (60 kDa) is in concordance with the observations of Lotti *et al.*[132]. It could be purified to 66-fold (maximum purity obtained in the present investigations), with 97 U ml^{-1} specific activity (Table IIIH1). Comparatively, purification fold of Lip B was very low, *ie*, only 18-fold with least specific activity (27 U ml^{-1}). However, performance of Lip A is worth mentioning in terms of purification, activity recovery, specific activity, etc. (Table IIIH1).

Characterization studies on these lipases, obtained after native PAGE elution also confirms the individuality of 3 distinct lipase isoforms of *C rugosa*.

Characterization Studies

(a) Substrate Specificity

Lip A prefers short chain saturated triglycerides, and is exemplified by its affinity (Km = 0.4 mM L^{-1}) for tributyrin (C6:0) (Fig.IIIH4). Its affinity for fatty acids was found to decrease with increasing chain length. However, it showed same preference for both saturated (Tripalmitin, C18:0) and unsaturated triolein (C18:1, 9) with same chain length . Lip B showed strong discrimination towards unsaturated triglyceride (triolein), but its kinetic behaviour shows that it has much preference for medium chain triacyl glycerols

Fig.IIIH4. Reaction kinetics of *C. rugosa* lipases on different substrates [triolein (C$_{18}$: 1,9), tripalmitin (C$_{18}$: 0), trilaurin (C$_{14}$: 0), tricaprin (C$_{12}$: 0) and tributyrin (C$_{6}$: 0). a = Lip A, b = Lip B and c = Lip C.

(Lip C also showed strong affinity towards medium chain saturated triglyceride rather than one with short chain. In general, this piece of work shows that *C. rugosa* lipases prefer short chain or medium chain saturated fatty acids for their hydrolysis from glycerol moiety, non-selectively with respect to ester bond. Langrand *et al.*[120] found that *C. rugosa* lipase was very effective in synthesizing short chain flavour esters for valuable dairy products.
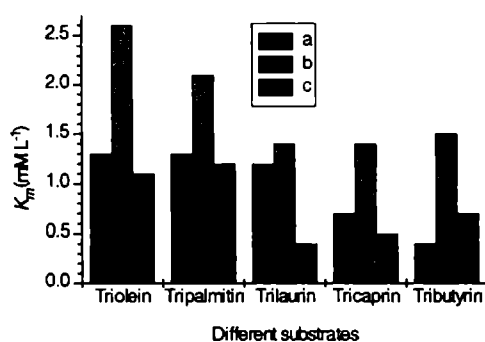
With regard to substrate specificity, CRL exhibited specific individuality in choosing suitable substrate. As for the physiological relevance of lipase isoforms for the producing organism, one could speculate that the availability of related but non-identical enzymes would improve the adaptation to different nutrients[132]. This view is further supported by the recent description on lipase isoenzymes of *G. candidum*, a close relative of *C. rugosa* with different substrate specificities[29]. Two distinct lipases of *C. rugosa* characterised by Rua and Ballesteros[202] were also in concordance to this view.

(b) Effect of Temperature

Generally, all the 3 lipases manifested that prolonged incubation would adversely affect their activity (Fig.IIIH5-7). However unlike Lip A and C, Lip B could tolerate a higher temperature (45-50°C) for 2 h without much loss in activity (Fig.IIIH6). On the contrary, both Lip A and C showed optimum activity at 35-45°C range (Figs.IIIH5 and IIIH7, respectively). During the first 10min of incubation at 60°C, over 85% of the activity of Lip A was lost, while
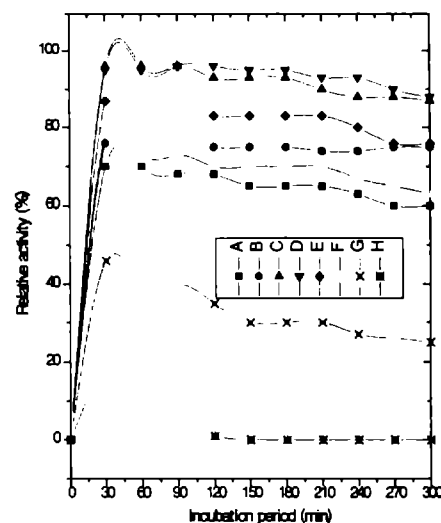
Fig.IIIH 5. Effect of temperature and incubation time on the relative activity (%)and stability of Lip A: A: 25°C, B: 30°C,C: 35°C, D: 40°C, E: 45°C, F: 50°C, G: 55°C and H: 60°C.

Lip B and C showed slightly more tolerance. Even then, beyond 60°C, no CRL could remain in their active form. From this, it is evident that Lip A is more

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Fig.IIIH6. Effect of temperature and incubation
time on the relative activity (%) and stability
of Lip B. A: 25, B: 30, C: 35, D: 40, E: 45, F: 50,
G: 55, H: 60 and I: 65 (all °C).

thermolabile than Lip B
and C. However, all the 3
lipases showed that
optimum incubation time
is 30 min at a temperature
of around 40°C. At these
conditions, maximum
relative activity (~ 90%)
obtained with all the 3
lipases (Figs.IIIH5-7).

As for the temperature

sensitivity is concerned,
behavioural features.
One of the striking
aspects is their
thermostability for
prolonged incubation
with a retention of ~ 75%
activity upto 55°C. The
basis for this stability at
relatively high tem-
perature is still unclear,
but it might be linked to
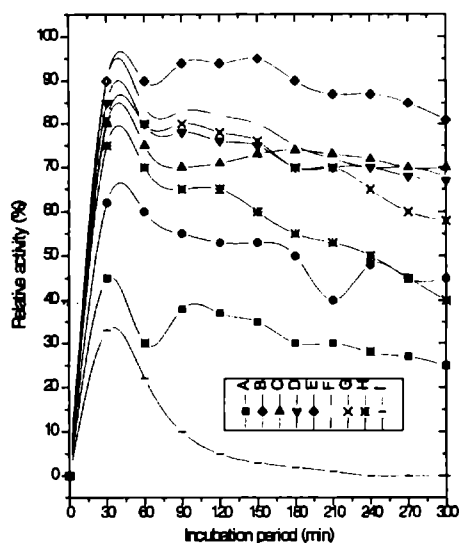
C. rugosa lipases showed common but distinct
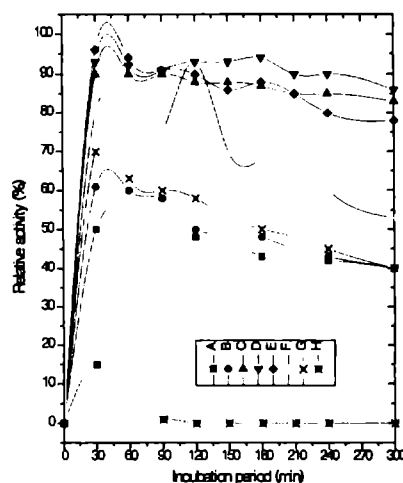
Fig.IIIH7. Effect of temperature and incubation
time on the relative activity (%) and stability of
Lip C. A: 25, B: 30, C: 35, D: 40, E: 45, F: 50,
G: 55 and H: 60 (all °C)

the fact that the lipases are highly hydrophobic[73]. The hydrophobic amino acids

must be concentrated near the catalytic centre. Hydrophobic amino acids presented

on the side of the lid that overarching the catalytic site become completely exposed,

greatly expanding the non-polar surface around the active site and may explain the

stability of lipase at relatively high temperatures[77].

(c) pH Sensitivity

All the 3 lipases

followed more or less the

same pattern towards pH

strength. All of them

showed equal agreement

towards the neutral pH

(Fig.IIIH8). In fact, while

Lip A was found to be

highly sensitive to pH

change, Lip B preferred

slightly alkaline pH

(7.0-8.0 range). Lip C

also showed slight
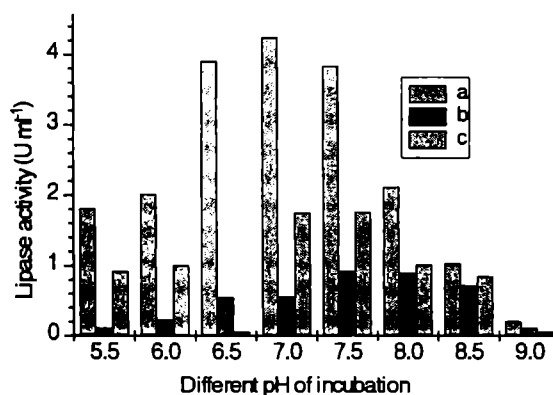
Fig.IIIH8. Effect of different pH on lipase activity at 37°C with 0.1 M Tris-HCl buffer. a = Lip A, b = Lip B and c = Lip C.

tolerance towards alkaline pH. In general, none of these lipases showed preference

for acidic or slightly acidic pH. Exact mechanism behind pH sensitivity is not clear.

However, the stability studies of C. rugosa lipase conducted by Hernaiz et al.[84] focus

on the conformational changes on the lipase's topology caused by pH changes. It

might reduce or impose strain on the lid overarching the active centre, thereby open

or shut down the catalytic centre for substrate binding[77,138].

(d) Effect of Ions and Inhibitors

Table IIIH2 describes the effect of various metal ions on the activity of the

lipase isoforms. Even at 10^{-4} M concentrations, Ag, As and Hg ions highly retarded

activity of all the lipase fractions. While Mn behaved as neutral, Ca and Mg ions

highly favoured the activity at a final concentration of 10^{-2} M. All other ions studied

showed slight inhibition.

Table IIIH2. Effect of metal ions on lipase activity

Metal ion	Final concentration (Molar)	Relative activity (%)		
		Lip A	Lip B	Lip C
Control	Nil	100	100	100
$AgCl_2$	10^{-4} M	57	50	48
$AsCl_2$	10^{-4} M	73	71	76
$CaCl_2$	10^{-2} M	127	131	125
$CoCl_2$	10^{-3} M	85	84	81
$CuCl_2$	10^{-3} M	83	80	81
$FeSO_4$	10^{-3} M	87	75	77
$HgCl_2$	10^{-4} M	46	54	49
KCl	10^{-3} M	84	84	49
$MgCl_2$	10^{-2} M	118	118	117
$MnCl_2$	10^{-2} M	103	100	104
NaCl	10^{-3} M	86	73	71
$ZnSO_4$	10^{-3} M	78	80	80

Effect of different inhibitors is shown in Fig.IIIH9. Activity of all the 3

lipases was completely inhibited by the protease inhibitors such as 3,4-DCI and

Pefabloc. PMSF, another serine protease inhibitor, however, retained around 25% of activity. Influence of thiol-binding reagent such as PCMB showed only 25% inhibition. The potent chelating agent, EDTA highly retarded activity, while SDS, the anionic detergent inhibited the activity by 70%. Significantly, effect of metal ions and inhibitors created same pattern of influence on the 3 lipases[24].
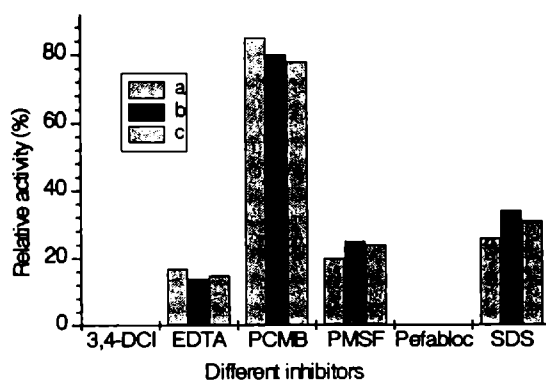
Fig.III-9. Effect of different inhibitors (10^{-5} M) on lipase activity. a = Lip A, b = Lip B and c= Lip C

Poisonous nature of Ag, As and Hg has already been confirmed by many authors[47]. However, nobody has investigated the biochemical mechanism involved in the inhibition. One possible mechanism to interpret is that these ions strongly bind to the sulphur moiety of amino acids and impart steric hindrances to the lipases. This would cause conformational changes to the lipase protein, which eventually culminates at the retardation of enzyme activity by making catalytic site alterations. However, the positive roles of Ca and Mg were interpreted in a different way by Hernaiz et al[84]. They opined that ions interact with the glycopeptides that are placed out side the enzyme, and make confirmational changes to the protein (lipase).

These glycopeptides interact with the interface of the hydrophobic substrate and water, and with the ions, owing to the presence of sugars, which makes them very much hydrophilic. The presence of ions alters the turn over number of the enzyme activity.

As in the case of ions, the activity of inhibitors is not well established. However, the actions of serine protease inhibitors can be attributed to the inhibition of serine in the catalytic triad (Ser-His-Glu) situated in the active site[132,138]. This active site is located within a cavity closed by a flap and therefore not accessible to the substrate, unless activation occurs[132]. Presence of lipids in reaction mixture will strongly activate the catalytic centre at the water-lipid interface, a process requiring a conformational rearrangement of surface loops named the 'flap' [132,138]. Presence of serine protease inhibitors (10^{-5} M) in the reaction mixture would totally deactivate the enzyme, even if all other factors are optimum. It confirms that such inhibitors (3,4-DCI and Pefabloc) strongly bound to serine located in the catalytic triad. However, PMSF, another serine protease inhibitor inhibits the action only by 75%. PCMB, a thiol-bonding inhibitor caused only 25% inhibition. The ion chelator EDTA and anionic detergent SDS were also inhibited the activity. Inhibitory action of the former was stronger than that of the latter. Action of these two chemicals can be attributed to the imbalance of ions in the reaction mixture by absorption or release, respectively. Altogether, action of ions and inhibitors showed same effect on all the 3 lipase fractions.

In conclusion, the basic aspect that can be deduced from the characterization studies is that C. *rugosa* is a highly evolved protistan to adapt various physical and

chemical environments. Secretion of all the lipase isoforms should not be equitable, but the environment in which it lives would influence the secretion of a particular isoform in abundance. It would afford specific specificity either to substrate, pH, temperature or chemical environment and make the organism fit for adjusting the available but diverse environments. Thus, in evolutionary point of view, *C.rugosa* can be considered as a highly evolved eukaryotic unicellular organism with magnificent adaptive values.
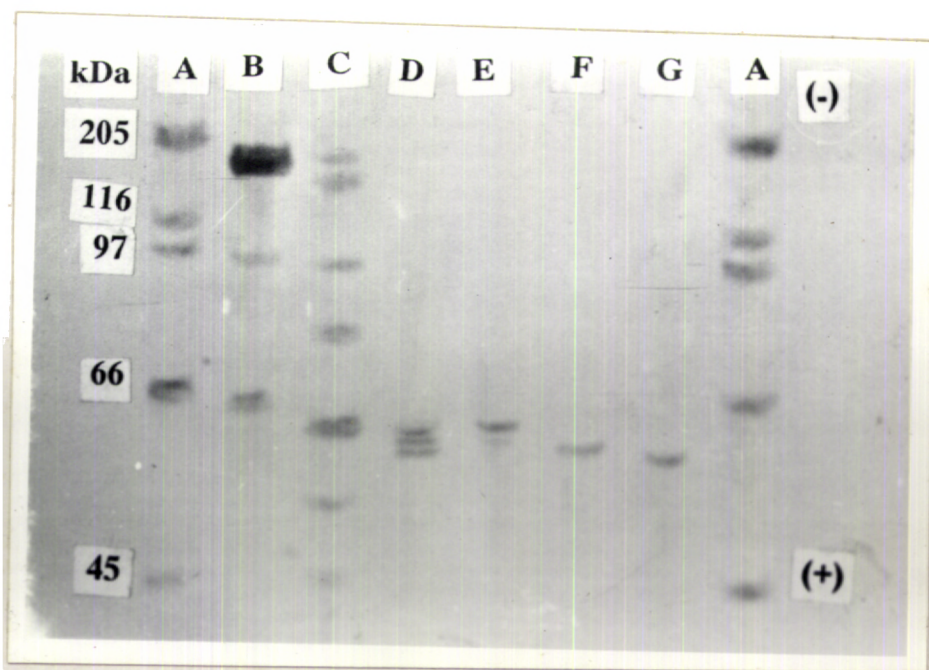
Fig.IIIH3. Electrophorogram of *C. rugosa* lipases on SDS-PAGE. Bands on lanes A: molecular markers; B: concentrated crude extract; C: 40-60% ammonium sulphate precipitate fraction; D: three distinct bands obtained by pooled lipase active fractions (53-63) from Sephadex G-200 column; E: bands obtained by fractions by Peak-a of Sephadex G-200 column; F: band corresponding to Peak-b and G: band corresponding to Peak-c.

CHAPTER - III I

GENERAL DISCUSSION

On the basis of the major findings described in the preceding chapters and current literature, a compendious discussion is accomplished in this chapter , so as to fetch deep insight into *Candida rugosa* and its lipase secretion. For convenience, prominent observations are odded out and projected up with scientific background.

(a) Morphology of *Candida rugosa*

Kreger van Rig[114] described *Candida* genus as the budding yeast having pseudo-mycelium and reproduced by arthrospores. However, van Uden and Buckley[114] observed that *C. rugosa* would become ovoid to elongate, sometimes sausage-shaped or like a pellicle. In fact, photographs obtained in the present study through Scanning Electron Microscope and Polarised Optical Microscope showed slight variations from the description given above. It was found that morphology of *C. rugosa* was dependent on the duration of growth and type of substrate in which it grew. After 24 h growth in liquid medium, the cells appeared as ovoid, kidney-shaped, or even sausage-shaped due to active fission and budding, as described by van Uden and Buckley[114] (Fig.IIIA4). However, on solid substrate, this type of morphology was attained only at 48-60 h fermentation under optimum a_w (Figs.IIIF3 and IIIG3). In addition, *C. rugosa* on solid substrate showed swollen morphology, instead of pseudo-filamentous form at later stages of fermentation.

Pseudo-filamentous phenotype is a common feature of *C. rugosa* in liquid media at the senescence phase of growth (Fig.IIIA5). At the same time, towards decay phase, *C. rugosa* reproduced actually either by producing chains of conidia, arthrospores or chlamydospore (Figs.IIIA6&7). These findings have parallelism to

the reports of Kreger van Rig[114] and Milikhina *et al.*[147]. Fig.IIID5 gives another feature of *C. rugosa* under immobilized state. Within the bead, *C. rugosa* tried to occupy the whole available space by beaded filament formation in achieving active fission and budding.

Morphology of *C.rugosa* is a significant clue to demonstrate the copious secretion of lipases[25]. In the present study [17-26] , it was found that lipase secretion of lipase was 5-12 fold higher in solid culture than that obtained in liquid culture. Hence, it could be concluded that sac-like morphology in solid culture was associated with more lipase secretion than filamentous forms. The mechanism behind this behaviour is not known. One possible mechanism might be the active expression of lipase genes, when the nucleus is in the interphase, which in turn controlled by the physical and chemical environments of growth.

(b) Growth and Enzyme Yield Profile

In liquid medium *C. rugosa* attained maximum growth (OD) by 24 h of fermentation (Chapters IIIA & B). In the next 24 h, OD and viable cell count maintained a stagnant pattern. However, after 48 h, viable cell count showed sharp decrease and by 72 h total death occurred. Still longer time was taken for maximum growth under semi-solid culture (Chapter IIIE). But under immobilized state, 100% viability of cells, which entrapped in calcium alginate retained upto two months (Chapter IIID). Growth on solid substrates (Chapters IIIF&G) followed a different pattern. On these substrates, maximum growth was attained by 72 h of fermentation.

As far lipase secretion is concerned, in liquid media (Chapters IIIB&C), maximum activity was noticed by 48 h of fermentation, but in semi-solid conditions (Chapter IIIE), around 60 h was taken for obtaining maximum activity. On solid substrates, maximum activity was resulted at a longer period. SSF in flasks took 96 h to secrete maximum lipase (Chapter IIIF) but in the column fermenter, this long period could be reduced to 72 h for maximum activity (Chapter IIIG). In all these studies lipase secretion upto initial 24 h was negligible. Lipase secretion under immobilized state did not follow any of these pattern (Chapter IIID).

Growth and lipase yields were found to be two independent but co-existing factors[20]. During the active growth phase, lipase secretion was comparatively less. Maximum lipase secretion was noticed only after attaining maximum growth. As Benjamin and Pandey[20] have reported, when growth was stagnant, all the pooled energy could have diverted for activating the biosynthetic pathway involved in lipase biosynthesis. Moreover, the peculiar morphology of the organism at this stage induce less energy consumption for mobility and it might be the reason for enhanced synthesis on solid substrates, and it can be considered as a best example for energy management.

(c) Lipase Production - A Comparison

Screening of literature on lipase production using *C. rugosa* employing different fermentation techniques showed that in SmF[238] it was 12 U ml^{-1}, 36 U g^{-1} rice bran in SSF[196] and 9 U ml^{-1} under immobilized state[62]. However, the present investigations let to the enhanced yield (118.2 U g^{-1} ds) using a mixed-solid

substrate containing coconut oil cake and wheat bran in a column fermenter[23]. In enriched coconut oil cake, enzyme yield was 87.8 U g^{-1} ds^{20}, but in semi-solid fermentation (which employed coconut oil cake extract as the substrate), the yield was 31.4 U g^{-1} ds^{18}. Only 15.14 U ml^{-1} was obtained in SmF^{17}, which showed slight improvement over the reported value (12 U ml^{-1}) by Valero et al.[238]. Yield of lipase at immobilized state showed much improvement (21.6 U ml^{-1}) with a half-life period of 2 months[19]. These results clearly upheld the upperhand of solid-state or semi-solid fermentation technology over the conventional SmF^{20}.

Until 1993, C. rugosa was being cultivated by SmF technology. Cultivation of C. rugosa on rice bran by Rao et al[196] was a break through to this monopoly. For the last 30 years, since the discovery of C. rugosa, SmF technology has been in vogue but the production was surprisingly low. As a brief estimate, 98% of publications on C. rugosa have been focused on the biotechnological spectrum of its lipases. Hence a healthy comparison between these two fermentation aspects becomes difficult. Advantages of both the systems were reviewed by many authors[1,85]. However, reports of Benjamin and Pandey[17-26] showed that SSF is highly promising than SmF, and semi-solid fermentation[18] offers still more potentialities. Mixed-solid substrate fermentation is yet another novel process devised by them[23].

Most of the scientists who worked on SSF opined that the main reason for the better performance of C. rugosa on solid substrate was the simulus nature of solid-state fermentation conditions created in the laboratories to that occur in the

natural condition[1,177,183]. In fact, it is an added support for the broadening eco-friendly approach and bioremediation of agricultural as well industrial wastes.

(d) Immobilization of *C. rugosa*

Major significance of immobilization was reflected by the longevity of *C. rugosa* in the gel beads. In liquid culture, activity of *C. rugosa* expired by 72 h. But in the Ca-alginate beads, activity was retained upto three months, when used under repeated batch culture in a Packed-Bed Bioreactor[19] Lipase production retained a unique range for one month with a half life period of two months (Fig.IIID10). Reason for longevity is not clear. However, Suzuki *et al*[225] reported that *Pseudomonas fluorescence* remained in the immobilized active stage for a month. Indirect contact to the substrate and immobility under aseptic condition were the reasons attributed to the active life of the organism[225]. Norton and Amor[163] reported the physiological effects of yeast cells during immobilization. Their findings focus that the lack of energy loss (which might be needed for mobility) by immobilization of the cells might have enabled the yeast for prolonged life.

(e) Iron Requirement

Studies described in Chapter IIIC confirmed that for the luxuriant growth of *C. rugosa*, iron is required in microquantities. Perhaps, the micronutrient status of iron may be the reason for the lack of any report on iron requirement of *C. rugosa*. As described in Chapter IIIC, in the absence of iron, *C. rugosa* secreted siderophores. Secretion of siderophore is a clear evidence to show that *C. rugosa* needs iron. Ismail *et al*.[91] found that *Candida albicans*, a pathogenic yeast, secreted

hydroximate and phenolate-type of siderophores. Usually, in most of the synthetic media, where iron is not added directly, it would enter into the system as impurity. Hence, deficiency symptoms of iron would be least pronounced. In short, C. rugosa required iron and its essentiality for metabolism has been confirmed by CAS-Assay (Chapter IIIC).

(f) Effect of Carbon Source

Both liquid and solid culture experiments proved that C. rugosa preferred disaccharides in comparison to monosaccharides. Among disaccharides, maltose and sucrose highly influenced both growth and enzyme yield. At the same time, monosaccharides such as glucose and fructose were also found to influence growth and enzyme yield, but with a less effectiveness. As discussed in Chapter IIIH, C.rugosa secretes a mixture of isolipases to accommodate a variety of substrates[24]. Similarly, it should have secreted various enzymes to assimilate different carbohydrates, as described by Johri et al.[100] with the yeast, Sporotrichum thermophila. Most likely preference to certain disaccharides was another evidence for its struggle to cope up with diverse environments of nutrition. However, C. rugosa was found to be not secreting any hydrolytic enzyme for the hydrolysis of starch[43]. This view is supported by the findings described in Table IIIB3.

Generally, 80-90% soluble sugars supplemented or contained in the medium was consumed during the active growth of C. rugosa. Hence soluble carbohydrate consumption could be taken as a better standard for assessing the substrate

utilization profile. In a nutshell, carbohydrate assimilation was directly proportional to the growth of *C. rugosa*[17].

(g) Effect of Nitrogen Source

Being an essential element, presence of nitrogen as a nutrient source in the medium is a major prerequisite for better growth and yield of *C. rugosa*. It can either be in the inorganic salt form or in complex organic forms. Organic nitrogen sources like yeast extract and peptone were much attractive. The simplest organic nitrogen salt, urea was also much effective. However, nitrogenous salts in the medium created retarding effect on *C. rugosa* (Chapter IIIF)[20] Komatsuzaki[111] observed on *C. albicans* that dissolution of salts, would release unnecessary ions in the medium, which would harmfully affect the growth of organism and thus retard yield also. Possibly pH fluctuation may be the main reason for the negative effect of some nitrogen salts[124].

Superior effects of yeast extract and peptone were not only due to the occurrence of finished substrates in such compounds but also the micro-nutrients present in them can afford optimum growth conditions[30]. Significance of trace and ultratrace elements has been studied by many authors[207]. Yeast extract and peptone are the best sources of amino acids[5]. Their occurrence in the nutrient mixture can cause their passive absorption into the cytoplasm with subsequent lipase secretion[20].

(h) Effect of mineral nutrition

Supply of mineral nutrition elicited mixed responses (Chapters IIIB, E, F & G). It can be concluded that under nutrient sufficient condition, supplementation of

mineral solution is unnecessary and if the medium is short of trace elements, mineral nutrition act as a necessary factor, as in liquid culture[17] (Chapter IIIB). Another aspect to be focused is that to a certain extent, mineral solution favours growth but that much influence is not found in lipase secretion. However, its addition in the enriched substrate (Chapter IIIF) resulted in significant reduction of lipase production[20].

(i) Effect of Lipids

Olive oil, free fatty acids and Tweens were tested as inducers for lipase secretion. Many authors found that presence of lipid in the medium was a paramount factor for lipase secretion[17,54,174,237]. Del Rio *et al*[54] described the mechanism involved in lipase secretion in the presence of olive oil. They concluded that consumption of free fatty acids was accompanied by lipase secretion. Findings of Obradors *et al*.[167] also confirms this view. Ota *et al*.[174] reported that steroids could enhance lipase secretion. Valero *et al*.[237] found that olive oil as an efficient inducer. Lipids are the favourite substrate for lipases. As a general fact, lipase needs to be secreted only if its substrate is available. But, the mechanism involved in the consumption of free fatty acids in association with lipase secretion still remains as a puzzle. Benjamin and Pandey[20] attributed this phenomenon to the excitation of lipase 'mini-gene family'. They concluded that the entry of traces of lipid in the cytoplasm would alter the electronic environment and subsequent expression of gene by stripping off of the inhibitory proteins, such as histones, associated with the DNA segment, which codes for lipases[20].

(j) Effect of Gum Arabic

When lipid substances added in the liquid media, they remained as a layer on the medium. It became a hindrance for gaseous exchange. But, addition of small quantities of gum arabic in the medium emulsified the lipid for an equitable distribution throughout the medium. Ferrer and Sola[62] found that *C. rugosa* was inefficient to assimilate olive oil as a substrate in the unemulsified form, but when gum arabic was added in the medium, growth and yield showed significant improvement. Wu *et al*[250] noticed the same phenomenon with Tween 80 as the emulsifier cum inducer. In the present study also, it was found that presence of gum arabic (below 1%) could be effective for better yield (Fig.IIIB5).

Gum arabic behaved as the best emulsifier not only in SSF (Chapter IIIG) but SmF (Chapter IIIE) as well. Its emulsifying property should have eased the membrane carriers on the plasmalemma to transport lipid substances into the cell, thereby enabling to secrete more lipases[23]. The inducing role of gum arabic in lipase production occurred indirectly through its emulsifying property.

(k) Effect of Physical Parameters

pH, temperature and aeration significantly influenced the growth and enzyme yield of *C. rugosa*. Initial pH (6.8) and temperature (28°C) could be maintained in all the experiments. But, maintenance of proper aeration was found to be difficult in SSF. In order to over come this barrier, two fermenters were designed (Figs.IIIF1 and IIIG1) to monitor aeration. Valero *et al*[238] found that optimum air supply is essential for the active life of *C. rugosa*. It further confirmed that *C. rugosa* was strictly an aerobic microorganism. However, in the absence of O_2, it could lead

facultatively anaerobic life to a certain extent. But in such a condition, secretion of metabolites was significantly less or even reduced to zero[196].

Careful study of *C. rugosa* morphology in the fermenter models (as mentioned above) showed its profuse growth on the areas where supply of fresh air was more[23]. Description in liquid and semi-solid culture[18] also confirm this report. Positive impact of aeration is correlated with the active metabolism of the organism[238].

(l) Protein Secretion

During the initial phase of growth, soluble protein concentration showed a sharp depletion (Chapters IIIA-G) followed by gradual hike. It shows that *C. rugosa* consumed soluble proteins contained in the medium, which caused initial depletion. Increasing protein concentration with advancing growth designates large amount of protein secretion including lipases. Due to this extracellular protein the exact quantity of protein consumption was not easy to measure[20]. Impact of protein secretion was directly visible in lipase activity and pH fluctuations.

Fusetti *et al*[68] reported that *C. rugosa* lipases as alkaline proteins. Most of proteins (enzymes and non-enzymes) secreted by *C. rugosa* were also alkaline in nature[123]. These reports reaffirms the findings of Benjamin and Pandey[17] that pH change towards basicity (with advancing fermentation) should be attributed to the protein secretion[17].

(m) *Candida rugosa* Lipases

Chapter IIIH embodies a detailed account on a mixture of three distinct lipases secreted by *C. rugosa* on mixed-solid substrate[24]. It is the first ever account

that claims three lipase isoforms of *C. rugosa* from crude culture broth. Lotti *et al.*[132] sequenced the CRL proteins and concluded that all the lipase isoforms have 534 amino acids with 66% total amino acid identify and 84% amino acid similarity with an apparent MW of 60 kDa and certain degree of glycosylation. Extend of glycosylation makes slight difference in MW[41]. Three lipases, as obtained in the present studies[24] showed an average MW of 60 kDa, as reported by Lotti *et al.*[132]. Existence of different lipases makes *C. rugosa* as a much evolved organism to thrive on various substrates[129,132]. Characterization studies of the isolated lipases also confirmed this view[24] (Chapter IIIH).

Conclusion

In brief, the present study is a comprehensive treatise on the fermentation behaviour of *C. rugosa* with a view to maximize lipase production by opitimizing fermentation conditions. Conventional Submerged culture techniques appeared as attractive, in comparison to cheap and economic solid-state or mixed-solid substrate fermentation, in a suitable fermenter. As far as the economy was concerned, immobilization was equally important and lower enzyme yields in comparison to SSF would be nullified by the longevity of the yeast in the gelling agent with stable viability and long half-life period in yield. In general, industrial feasibility of SSF should be preferred to SmF for the overproduction of lipases at low cost. Similarly, barring from huge initial investment, immobilization can also be employed for the continuous production of lipases in a reactor under repeated batch mode.

SUMMARY, CONCLUSION

AND

PROSPECTS

SUMMARY

Industrial curiosity on *Candida rugosa* (formerly *Candida cylindracea*) relies upon its lipase secreting capacity, which was recognised nearly 30 years ago. Extracellular lipase (EC.3.1.1.3) of *C.rugosa* has been proved as a wonderful biocatalyst, capable of non-specific hydrolysis of all the ester bonds in the triglycerides. In addition, its proven GRAS (generally regarded as safe) status has attracted the food technologists to exploit its boundless potentialities for lipid-based food processing. Initially, though *Candida rugosa* lipase (CRL) was popular as a fat-splitting biocatalyst, newer applications to its credit have been reported over the successive years.

A brief spectrum of CRL's recent applications include: invincible role in food and flavour industry, biocatalytic resolution of life-saving pharmaceuticals, synthesis of carbohydrate esters and amino acid derivatives unobtainable by conventional chemical synthesis, potent biocide making, wonderful biosensor modulations, advancing bioremediation by eco-friendly approach, manufacture of biosurfactants for efficient detergent making, production of delicious ice cream and costly single cell protein (SCP), biocatalysis of novel esters and secondary alcohols for organic synthesis, manufacture of efficient lubricants, emerging roles in pulping and paper industry, production of attractive plastic goods, and of late, cosmetics and perfumery.

With the advancement in molecular biology, focus is being given to purification, isolation and characterization of *C. rugosa* lipases. It was confirmed that *C. rugosa* secretes a mixture of lipase isoforms with diverse characteristics.

Such lipases are encoded in a **lipase mini-gene family**. Existence of lipase isoforms has again pushed CRL towards more specific biotechnological processes and the production of robust industrial products.

The aforesaid facts on the pluripotency of *C.rugosa* lipases are the reflection of over 98% literature available on *C. rugosa*. Amazingly, only the remaining 2% of literature gives isolated and inconclusive ideas about its production aspects. When this study was initiated, the status of CRL production in the world was incomparably low, though industrial production conditions were not known. Hence, elaborate attempts were made to maximise lipase production by adopting conventional submerged, coupled with emerging solid-state and immobilized fermentation strategies employing the non-pathogenic yeast, *C. rugosa*. Robust strategies such as semi-solid and mixed-solid substrate fermentation techniques were successfully accomplished to achieve the objective. Another challenging output of this study is the partial purification, isolation and characterization of three distinct lipase isoforms of *C. rugosa*.

In order to make the construction of this thesis simple and unconventional, lucid and much legible language accompanied by prompt illustrations have been adopted, especially in Part three, which comprehends all the investigations and their outcome carried out in the present study. For convenience, Part three which is designated as Materials, Methods, Results and Discussion is subdivided into nine chapters. Chapters one to eight (IIIA to H) describe various aspects studied for lipase

production. Chapter nine (III I) gives a general discussion on all these eight chapters. The format of this thesis is described in the prelude.

Chapter one (III A) describes the standardization of the growth profile of *C. rugosa* in liquid medium along with the study of its morphology. Major parameters discussed under this chapter include: media selection, coupled with optimization of physical parameters such as pH, temperature and agitation. It has been concluded that Universal Yeast medium supported maximum growth among five liquid media studied. Likewise pH, temperature and agitation (revolutions for minute, rpm) optima were 6.8, 28 and 250, respectively. Another significant observation was that *C. rugosa* required 20-24 h of cultivation for the acquisition of exponential growth. Beyond this period, though growth was paralysed, turbidity did not show much change. However, between 24 to 48 h of fermentation, the viable cell count in the medium retained the same number, and by 72 h, total extinction occurred. As far as the morphology is concerned, *C. rugosa* showed pseudo-mycelial growth at the senescence phase of growth with chains of chlamydospore and conidia throughout the 'filament', though active fission and budding were preferred during the early stages. Advancing growth was accompanied by soluble sugar depletion and slight increase in pH and much hike in protein concentration.

Chapter two (III B) describes the enrichment of liquid medium for lipase production. Universal Yeast (UY) medium was the basic medium used in this study. Many trials were made with different additional nutrient sources to enrich it. It was found that modified medium containing olive oil, gum arabic and urea along with

vitamins and some salts serve as the best medium to support maximum growth and enzyme yield. Maximum growth and yield were obtained at 24 and 48 h, respectively. By 72 h viability of the cells in the medium was completely lost. This piece of work proved that growth and enzyme yield can be maximized by providing a competent environment of balanced nutrients along with optimum physical parameters.

Chapter three (III C) describes the iron requirement and siderophore production by *C.rugosa*. To find out whether *C. rugosa* requires iron as a nutrient, experiments were conducted in media both under iron sufficient and deferrated conditions. It was found that iron is essential for *C. rugosa* metabolism. In the deferrated medium, *C. rugosa* secreted siderophores. It further confirmed that iron is an inevitable micronutrient for *C. rugosa*. Chrome Azurole-S (CAS) assay was conducted to test siderophore production. It was concluded that iron is not directly involved in lipase secretion, but directly involved in growth, and growth is related to lipase secretion.

Chapter four (III D) gives a detailed picture on lipase production by *C. rugosa* under immobilized state and also growth profile in a bioreactor. Calcium alginate, agar and polyacrylamide gel supports were tested as immobilizing agents. Calcium alginate emerged as the best gelling agent with optimum diffusional characteristics. By 36 h of incubation, the cell concentration per gram calcium alginate gel was increased to 281×10^8 cells. Half life period for lipase production

was two months in calcium alginate beads packed in Packed-Bed Bioreactor (PBR) under reverse flow of the feed medium with each cycle persisting for 12 h.

Chapter five (III E) describes lipase production profile of *C. rugosa* in coconut cake extract (COCE) under semi-solid fermentation. The extract was enriched by different ways. Effect of different carbon and nitrogen sources, free fatty acids, olive oil, gum arabic, mineral solution, corn steep liquor were also tested. Final standardized medium contained COCE, 0.2%, peptone, 0.1%, glucose, 0.15%, caprylic acids, 10%, olive oil and 0.4% gum arabic. Maximum growth was noticed at around 48 h fermentation, but enzyme yield was maximum at 60 h. Best results were obtained under agitated culture at 28°C with an initial pH 6.8.

Chapter six (III F) describes the cultivation of *C. rugosa* on a solid support, the enriched coconut cake by solid-state fermentation. Eight modified media (65% moisture) were formulated to select the best one among them. It was found that an initial water activity (a_w) set at 0.915 could support maximum growth and activity. Sterile and moist air was also supplied through the fermenting matter. It was found that *C. rugosa* took 72h for attaining maximum growth on solid substrate, however, maximum lipase yield occurred after 24 h, compared to maximum growth. Growth was expressed by glucosamine content in the cell wall of the yeast, after hydrolysis.

Chapter seven (III G) describes the pattern of growth and yield of *C. rugosa* on mixed-solid substate. Coconut oil cake, fine and coarse wheat bran were mixed in 1:1:1 ratio to get 35% solid support (along with additional solid nutrients). In this experiment also, the ratio (%) of solid matter and liquid in the medium was

maintained as 35:65, respectively. During the culture in flasks culture maximum growth and enzyme yields were obtained at 72 and 96 h, respectively. However, in a column fermenter, both growth and activity were synchronized at 72 h of fermentation with significant improvement in yield.

Chapter eight (III H) describes partial purification, isolation and characterization of three distinct lipases of *C. rugosa* from the culture broth (the first ever report of such kind), obtained from mixed-solid fermented matter used for extracting the lipases. The extract was precipitated with ammonium sulphate to 40-60% saturation. After dialysis and ultrafiltration, the concentrated sample containing lipase isoforms was applied on a Sephadex G-200 column and eluted with 0.1 M Tris-HCl buffer. Flow rate was maintained as 60 ml h^{-1}. Fractions between 55 and 63 showed lipase activity. Such fractions were used for further analysis by PAGE under both denaturing and non-denaturing conditions. All the three isolipases which were isolated had an average molecular weight of 60 kDa, and showed diverse substrate, temperature and pH specificities. However, they showed mutual agreement towards the action of ions and inhibitors.

Chapter nine (III I) was added as a special chapter to give a coordinated picture on the fermentation aspects studied. It gives a general discussion morphology of *C. rugosa,* growth and yield profile, a comparative lipase production profile in all the media studied. Submerged *versus* solid-state fermentations, immobilization of *C. rugosa,* iron requirement, effects of carbon and nitrogen

sources and of lipids, gum arabic, physical parameters, protein secretion, followed by *C. rugosa* lipases and a brief conclusion.

When this study was initiated maximum production of lipase by *C.rugosa*, as reported in the literature, was only 12 h in submerged fermentation, and 36 U in solid-state fermentation and 9 U ml^{-1} under immobilized state. In fact, judicious management of *C. rugosa* by altering its fermentation parameters, this low production could be enhanced to 118.2 U g^{-1} by SSF and 15.3 U by SmF. However, lipase production employing semi-solid fermentation (a new strategy adopted) also offered much prospects (31.4 U), as obtained by immobilized *C. rugosa* under immobilized condition (21.6 U) in calcium alginate beads.

SUMMARY

Lipase producing capacity of *Candida rugosa* was achieved by employing judicious management of the fermentation techniques and parameters. This coordinated approach enabled to enhance lipase yield from 36 U to 118 U in solid-state fermentation along with significant improvement in lipase yield under immobilized state. Present investigations give a vivid picture on the upperhand of solid-state fermentation over conventional submerged fermentation in supporting lipase secretion by *C. rugosa*. Semi-solid fermentation on coconut cake extract also proved as a promising area. However, for the long term continuous production of lipases, immobilization of *C. rugosa* on a suitable support may offer an economic advantage. Coupled with overproduction of lipases, isolation and characterisation of distinct lipase isoforms offer vast avenues for the utilization of such lipases for ctalyzation of specific chemically and industrially significant reactions under mild conditions, and promise unseen new areas for its deployment. Each of this lipase forms has its own specificities towards various substrates, pH, temperature, etc. This diversity would further broden the scope of *C. rugosa* lipases in industry.

Proven GRAS status has lifted *C. rugosa* and its lipases to an elevated position amidst their counterparts, especially in the kingdom Monera, headed by bacteria, most of them being known pathogens. No adverse effect on human or on other form of life has been reported as a result of traditional or even open fermentation practices using both *in situ* *C. rugosa* or *ex situ* purified lipases, *eg.* for the production of food and flavours and SCP as well. A fascinating field, not yet exploited, is the meat technology, especially for delicious sausages, and also pulping, plastic and lubricant industries can be modernised by the introduction of

lipases. Infusion of lipase into living cells would make a radical change, if it could be applied *in vivo* on patients suffering from chronic cardio-vascular diseases.

The concept of weightless fats made of fatty acids brought from outer space by exploration would impart a fascinating imprint in the energy-rich food-making industry for the generations to come. Multifacetedness of CRL is the simple reason which elicited its unprecedented demand in the world market. It urges the scientists to improve both quality and quantity as maximum as possible to bridge the gulf between demand and production. Quantitative enhancement can be achieved by strain improvement and site-specific mutagenesis or by cloning and expression in a surrogate organism for overproduction. Optimization of reaction parameters with molecular restructuring of the enzyme by genetic adjustments would yield functionally stabilized CRL with maximum quality. Finally, it is hoped that this scientific account on *C. rugosa* would scatter ample light into the ongoing projects and great ventures to come.

CONCLUSION

Versatility of *Candida rugosa* lipases undoubtedly proclaims its bright future. Further, exploitation of site-directed mutagenesis in *C. rugosa* lipase gene for the overproduction of lipases and functionally stabilized engineered lipases with novel characteristics is one of the challenging aspects to be investigated. Unfortunately, so far, only lipase producing capacity of *C. rugosa* has been investigated. Other enzymes produced by this organism also warrants quick and detailed investigation.

Unorganized deployment of *C. rugosa* on oil-tainted waste reveals that oil splitting ability of CRL can be exploited for the production of single cell protein (SCP) and waste disposal, as *Candida utilis*, the fodder yeast being used. Prospects of SCP by growing *C. rugosa* on agricultural wastes such as coconut cake, which can be transformed into a value-added cattle feed offer yet another area of active research. Preliminary works are being done towards this direction.

Recombinant-DNA technology can be employed to express lipase gene of *C. rugosa* in industrially significant organisms for its large scale production, especially in single cell protein production. A genetically engineered organism, which performs multifunctions by secreting many enzymes simultaneously, would be much attractive for waste disposal, especially in the factory waste management. In such a way, bioconversion of wastes into valuable products can be obtained at low cost with the refreshment of the environment.

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(a) Reviews

1. **Sailas Benjamin** and Ashok Pandey (1996). The realm of microbial lipases in biotechnology. **Enzyme and Microbial Technology** (communicated).

2. **Sailas Benjamin** and Ashok Pandey (1997). *Candida rugosa* lipases: Molecular biology and its versatility in biotechnology. **Yeast** (communicated).

(b) Original Papers

3. **Sailas Benjamin** and Stephen, J. (1996). Mitoclastic and Clastogenic effects of Aspirin on mouse bone-marrow cells *in vivo*. **Cytologia, 61,** 27-32.

4. **Sailas Benjamin** and Ashok Pandey (1996). Optimization of liquid media for lipase production of *Candida rugosa*. **Bioresource Technology, 55,** 167-170.

5. **Sailas Benjamin** and Ashok Pandey (1996). Lipase production by *Candida rugosa* on copra waste extract. **Indian Journal of Microbiology, 36,** 201-204.

6. **Sailas Benjamin** and Ashok Pandey (1997). Enhancement of lipase production during repeated batch culture using immobilized *Candida rugosa*. **Process Biochemistry, 32,** 257-260.

7. **Sailas Benjamin** and Ashok Pandey (1997). Coconut cake - a potent substrate for the production of lipase by *Candida rugosa* in solid-state fermentation. **Acta Biotechnologica** (accepted).

8. **Sailas Benhamin** and Ashok Pandey (1997). Mixed solid substrate -a novel process for the enhanced lipase production by *Candida rugosa*. **Biotechnology Techniques** (communicated).

9. **Sailas Benjamin** and Ashok Pandey (1997). Production, partial purification, isolation and characterization of three distinct lipase isoforms of *Candida rugosa*. **Journal of Fermentation and Bioengineering** (communicated).

(c) Conference/Symposia

10. Stephen, J., Mohan Kumar, M., Ravindran Ankathil, Mercykutty, V.C., Shakkela, N., Mary Chacko, Babu, N., Meenakumari, T., Pradeep Kumar, V., Annie Roy and **Sailas Benjamin** (1994). Genetic toxicology of some therapeutic drugs. **Ann. Conf. Int. Med. Sci. Acad.** (12-15 March, 1994), Trivandrum, India.

11. **Sailas Benjamin** and Ashok Pandey (1995). Lipase production by *Candida rugosa* in both solid and liquid fermentation   a comparative study. **Louis Pasteur's Cent. Memr. Sym.** (Nov. 20)Trivandrum, India, p.11.

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