FERMENTATION PATTERN OF FUNGAL LIPASES AND THEIR APPLICATION FOR ESTERIFICATION IN NON-AQUEOUS MEDIA

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These studies were carried out in Biotechnology and Food Research Centre, Pakistan Council of Scientific and Industrial Research, PCSIR Laboratries, Lahore in collaboration with the Institute of Chemistry, University of the Punjab, Lahore. The author, hereby, declares that the work reported in this thesis has not been previously submitted to any University or Institute for the Degree of Doctor of Philosophy and shall not, in future, be submitted for obtaining similar degree from any other University.

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AUTHOR

SUMMARY

Lipolytic fungi were isolated from the soil and food samples using a selective isolation medium. The isolated fungal cultures were screened for maximum lipase production. The fungus *Rhizopus arrhizus* (isolate : 52) was found to produce maximum lipase. The fermentation medium M_5, consisting of (g/dm^3):- olive oil : 20, yeast extract : 1.0, ammonium sulphate : 5.0; dipotassium hydrogen phosphate : 0.5; magnesium sulphate heptahydrate : 0.25; calcium carbonate : 5.0 was found to be the best medium for lipase formation by *Rhizopus arrhizus*. The cultural conditions like medium composition, pH, acration rate and incubation temperature were optimized for the production of the intracellular lipase by *Rhizopus arrhizus* in shake flasks. The optimization of the fermentation conditions resulted in about three fold increase in the production of lipases. This optimized medium gave 13.5 g/dm^3

fungal biomass with 325 lipase units/g dry mycelium and about 2.4 units extracellular lipase per cm^3 of the broth. The fermentation was also carried out in 18 dm^3 stirred fermenter and 4 dm^3 airlift fermenter. The airlift fermenter was found to be especially suitable for the intracellular lipase production. In it, the fungus *Rhizopus arrhizus* produced 375 units lipase per gram mycelium and 14.2 g mycelium per dm^3 medium. In stirred fermenter, however, both fungal biomass and intracellular lipase activity were comparatively low.

Rhizopus arrhizus was also cultured in solid substrate for extracellular lipase production. Different agricultural by-products such as wheat bran, gram bran, rice husk, soybean meal and rice bran were evaluated for lipase production. A mixture of wheat bran and soybean meal, however, was found to be the best substrate. Additives like olive oil and the egg yolk emulsion increased lipase production.

The extracellular and intracellular lipases of the mould *Rhizopus arrhizus* were used for the biocatalysis of esterification reactions. Free exocellular lipase showed very low esterification activity (28 esterification unit/g). Whereas the celite immobilized lipase catalyzed synthesis of hexyl octanoate at the rate of 51 micromole/min./gram. Mycelial lipase of *Rhizopus arrhizus*, however, was very active in organic media and performed the synthetic work efficiently. In n-hexane, at 30°C the mycelial lipase of *Rhizopus arrhizus*

catalyzed the synthesis of hexyl octanoate at a rate of 92 micromoles/min./gram mycelium.

The mycelial lipase of *Rhizopus arrhizus* was also used for the production of fragrant esters from the fusel oil and terpene alcohols. Geranyl butyrate and butyrates of fusel oil were manufactured using a packed column reactor. In case of the fusel oil, ester yield of 87% was obtained by recycling the reaction mixture through the column for 3 times. In case of esterification of geraniol with butyric acid, the esterfication yield was 77%.

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Chapter 1

INTRODUCTION

Enzymes are biocatalysts produced by living cells and catalyze the specific reactions inside or outside the cells. For practical purpose all enzymes can be considered to be proteins, although recent work has shown that RNA, also can act as an enzyme[1]. Enzymes have been classified into six different groups by the Commission on Enzymes[2]. Hydrolases (E.C.3) constitute one of the six classes and include esterases as a subclass. Lipases are classified along with esterases as a sub-sub-class of hydrolases and International Union of Biochemistry (IUB) defined lipases as glycerol ester hydrolases (E.C. 3.11.3.) and further recommended that ester emulsion should be used as the substrate for lipases and water soluble esters for other esterases. After IUB had brought forward its recommendations many microbial enzymes, like crystalline lipase of *Aspergillus niger*, were found to be equally active against both water soluble and insoluble esters[3]. Therefore, it became difficult to differentiate between microbial lipases and esterases. However, due to the fact that long chain

triglycerides are the most common natural substrates of lipases, the most distinguishing feature of lipases is, that lipases attack water insoluble triglycerides at an oil-water or micelle-water interface, whereas esterases act on water soluble esters.

LIPASE PRODUCTION

Although lipases are produced in a wide range of organisms including plants and animals, but microbial sources are receiving increased attention and fermentation process has become the production technology of the day being cheaper and capable of fulfilling increased demands[4].

Fermentation is the biochemical activity of microorganisms in their growth, development and reproduction and constitutes the most fundamental and the most mature area of contemporary biotechnology[5]. During the last 20 years it has revolutionized the chemical and biochemical industry and has brought us valuable products such as antibiotics[6], amino acids, organic acids[7], vitamins and enzymes[8]. Microbial enzymes are produced by both submerged and solid state fermentations. Submerged fermentation technology employs a liquid medium in which the fermentation substrate is diluted in water. Whereas in solid state fermentation, microorganisms grow without free water, the moisture required by the organisms exists in an absorbed form in the solid matrix and often ranges between 10% and 80%. Although solid substrate fermentation involves less energy cost, simpler equipment and simpler growth medium and gives higher protein yield, it can be applied to a limited number of

microorganisms and processes[9]. In recent years solid substrate fermentation is getting increased attention as energy conserving fermentation technology. It is especially suitable for developing countries like Pakistan where electricity is costly and its supply is not continuous.

Although both bacteria and fungi can be exploited for lipase fermentation, fungal sources receive more attention because fungi are more potent lipase producers and dried mycelia of lipolytic fungi constitute naturally immobilized lipases which have high catalytic power and stability[10-11].

APPLICATIONS OF LIPASES

Lipases are extremely versatile enzymes. Their properties allow wide spread applications in various fields like chemical, food and pharmaceutical industries. Lipases from different sources can catalyze the hydrolysis or synthesis, depending upon the water activity of the reaction medium[12]. In the presence of excess water lipases catalyze the hydrolysis of ester bonds. This hydrolytic behaviour of lipases is used in oil and fat hydrolysis[13], fatty acid production from vegetable oils, cheese ripening, meat and fish processing, removal of fat from animal fur, detergents and paper industry[14-16].

In non-aqueous or microaqueous conditions, however, the direction of catalysis is reversed and lipases catalyze the synthesis of ester linkages, instead of the hydrolysis[17,18]. So, to employ the lipase on synthetic work, water activity of the reaction medium must be very low. Even when the reaction is performed in non-aqueous medium, water is produced during the course of

reaction which may drag the equilibrium of the reaction towards hydrolysis and synthetic activity of the enzyme may impair. For getting quantitative yield of esters, this formed water, somehow, must be removed from the reaction mixture and the enzyme[19]. However, complete removal of water from the system renders the enzyme inactive. Intramolecular forces in the enzyme protein such as salt bridges, hydrogen bonding and hydrophobic interactions which are responsible for conformational integrity of the protein molecules are dependent upon the presence of water[20]. Polar organic solvents, when used as reaction media, denature the enzyme reversibly or irreversibly by extracting the essential water. On the other hand non-polar solvents do not dehydrate the enzyme and prove to be good reaction media[21]. The denaturation is further prevented by enzyme immobilization. The immobilization increases the resistance of enzyme towards adverse conditions of pH, temperature and water scarcity[22]. Further the immobilized enzymes can be separated from the reaction mixture easily and used repeatedly or continuously[23].

IMPORTANCE OF ENZYMIC ESTERIFICATION

The lipase catalyzed esterification reactions have definite advantages over traditional chemical route of ester synthesis. It is gentle and efficient process giving reasonable esterification rates under mild conditions of temperature and pressure and hence involves much lower energy cost than the chemical synthesis. The process leads to selective esterification producing stereospecific esters. For example, if geraniol and nerol are both present in the reaction mixture, the mycelial lipase of *Rhizopus arrhizus* will esterify the

geraniol preferentially. Whereas, by chemical process both isomers are esterified. Moreover acid catalyzed esterification of pure geraniol gives a mixture of geranyl and neryl esters due to isomerization. On the other hand such undesirable reactions are avoided due to enzyme specificity. The enzyme based processes are environment friendly,cause little pollution and tend to have lower waste treatment costs[24].

Lipase mediated esterification reactions are exploited for the production of a variety of esters of immense importance. Some important products of enzymic esterification are discussed below.

1. Flavour Esters

Esters of short chain alcohols with short chain carboxylic acids are prepared using immobilized lipases in organic solvents and are important in food industry as flavour and aroma constituents[25].

The enzymatically synthesized stereospecific esters of terpene alcohols with short chain fatty acids e.g. geranyl acetate are fragrant in nature and are used in flavours and perfumes[24].

2. Wax Esters

These esters are formed by enzymic esterification of long chain alcohols and long chain fatty acids in organic solvents or solvent free system. These are of much commercial interest and have potential applications from lubricants to cosmetics[26, 27].

3. Medium Chain Glycerides

Medium chain glycerides are used as solvents for aromatics, steroids, lipophilic drugs, fat soluble vitamins, dyes, cosmetics, and in pharmaceutical field. A mixture of medium chain mono and diglycerides is an effective solvent for dissolving gallstones. Medium chain triglycerides are used as a nutritional supplement for patients suffering from malabsorption and as a component of infant feeding formula[28].

4. Sugar Esters

Carbohydrate esters of fatty acids are prepared by lipase catalyzed esterification of sugars or sugar derivatives[29,30]. These sugar esters are potentially important as emulsifiers in food, medicines and cosmetics. In addition these possess antitumor and plant growth inhibiting activities. Sucrose monostearate preparations are effective in inhibiting ascites sarcoma and leukemia[31,32].

5. Ester Oligomers

Aspergillus niger lipase was used for catalyzing the synthesis of ester oligomers from diols and dicarboxylic acids[33]. High molecular weight polyesters have also been prepared by lipase mediated esterification reactions[34].

6. Lactones

Another activity spectrum of lipases is stereospecific lactonization. Lactones are intramolecular esters formed from a compound with an alcohol

group at one end and a carboxylic acid group at the other end of the molecule. The lactones constitute key structures for many useful compounds such as macrolide antibiotics[35].

OBJECTIVE OF THE WORK

The objective of the present work is to develop a process for the enzymatic production of esters, especially those which find application in food industry as flavour constituents. The research project has two parts.

i) Production of Lipases

It involves isolation and screening of lipase producing fungi and optimization of the cultural conditions like medium composition, pH, aeration rate and incubation temperature for lipase production by the selected mould and yeast cultures.

ii) Application of Lipases in Ester Synthesis

It involves the selection of the most suitable form of the lipase (soluble, immobilized or cell bound) and its application in the catalysis of different esterification reactions. An important object of the work is to develop a process for the synthesis of flavouring esters such as short chain esters and terpene esters.

Chapter 2

LITERATURE REVIEW

2.1 ENZYME SOURCES

Lipases are produced by a wide variety of organisms including bacteria, yeasts, moulds, plants and animals[36]. These enzymes have traditionally been obtained from animal pancreases[37] . Lipases, as the fermentation products, receive much attention because of their increased use in industry and medicine[14]. Fungi are potent source of these enzymes[36]. There are about 50000-100,000 species of fungi, most of which are saprophytes[38]. These saprophytic fungi produce a wide range of hydrolytic enzymes to degrade the complex organic substrates on which they grow. The lipolytic fungi can grow on fat while the non-lipolytic fungi cannot. There are several fungal species belonging to different genera such as *Rhizopus, Mucor, Aspergillus, Fusarium,*

Geotrichum, Penicillium, Candida and *Torulopsis* that have both lipolytic and non-lipolytic strains[36-41].

2.2 ISOLATION & MAINTENANCE OF LIPOLYTIC MICRO ORGANISMS

Lipase producing bacteria and fungi have been isolated from different sources by different techniques. The principle behind all these techniques, is the growth of microorganisms on a selective medium having some lipid as the sole source of carbon and energy.

In 1967 Lawerence *et al*[42] reported the use of tributyrin agar for the detection of lipases in microorganisms. Fryer *et al*[43] developed a double layer technique for the screening of lipolytic organisms. The organisms were grown on nutrient agar overlying the tributyrin agar. Zones of clearance were formed in tributyrin agar layer around the lipase producing colonies which could be isolated. Kundu and Pal[44] reported a method for the isolation of lipolytic fungi from the soil on oil-mineral medium spread on silica gel plates. Ionota *et al*[45] isolated lipase producing fungi and yeasts using agar plate method. Many workers reported the presence of *Geotrichum candidum* in the sour cream and cheese[46]. Salleh *et al*[47] isolated a lipolytic strain of *Rhizopus oryzae* from effluent treatment pond of a palm oil mill. Omar and Jamil[48] isolated lipase producing organisms on palm oil medium.

In most of the cases isolated fungal cultures were maintained on the malt-agar and potato dextrose agar media[49]. However, some workers have chosen other media for culture maintenance. Pal *et al*[50] maintained *Aspergillus niger* on olive oil sucrose-agar medium. The lipase producing strain of *Aspergillus wentii* was isolated from the soil and maintained on yeast-dextrose-agar medium[51]. For the maintenance of cultures of *Mucor hiemalis* and *Mucor mucedo*, Sabouraud-dextrose-agar medium was used[52]. Valero *et al*[53], used malt extract peptone-agar medium for the maintenance of *Candida rugosa*.

Bacterial cultures were usually maintained on nutrient agar[54]. Breuil and Kushner[55] used semi-solid soil extract medium, containing soil, water, yeast extract, K_2HPO_4 and agar for the maintenance of *Acetobacter species*. Some bacterial cultures were also maintained on tripticase soy agar medium[56].

2.3 PRODUCTION OF MICROBIAL LIPASES

Microbial lipases have been produced by both solid substrate and submerged fermentations.

2.3.1 Submerged Fermentation

Cutchins and Coworkers[56] in 1951 and Nelsonand Nashif[57] in 1953 attempted to produce lipases on chemically defined media but low yields were obtained as compared to the complex media. Alford and Pierce[58] investigated a number of media for lipase production by Pseudomonas fragi and reported

that 1.0% peptone gave the highest lipase yield. A combination of amino acids arginine, aspartic acid, glutamic acid and lysine could produce considerable lipase activity. Breuil and Kushner[55] used different combinations of peptone and beef extract for lipase production by *Acinetobactor* species and found that a medium containing 2% peptone and 1.5% beef extract was the best.

Pal and coworkers[50] cultured *Aspegillus niger* in shake flasks on sucrose-oil-mineral medium whereas Omar and Jamil[48] used palm oil medium. Use of corn steep liquor, yeast extract and glucose was reported by Nadkarni[59]. *Penicillium cyclopium* produced two types of lipases. Maximum lipase production was achieved after six days cultivation at 27°C on a medium containing rice bran, corn steep liquor and dibasic ammonium phosphate[60].

Chander *et al*[61] cultivated Penicillium chrysogenm in shake flasks on glucose-yeast extract – polypeptone medium and found that lipase yield was increased by the addition of soybean oil to the fementation medium. Metwally *et al*[62] found that *P. citrinum* produced maximum lipase in palm oil containing growth medium on five days incubation at 30°C.

Geotrichum candidum produced an exocellular lipase when grown at 20°C in peptone broth or trypticase-glucose-mineral medium. The enzyme was reported to be a histidine-serine protein, active at pH 8.0-9.0[46]. Nadj and coworkers[63] reported a fast growing strain of *Geotrichum candidum* which produced maximum fungal biomass and lipase after 24 hours shaking at 30°C.

Blain *et al*[64] reported the production of mycelial lipases by *Rhizopus arrhizus* and *Mucor javanicus*. Fermentation was carried out in shake flasks for 5 days. Lipase production by ten species of *Mucor* and *Rhizopus* was reported by Mirza *et al*[65] and Akhtar *et al*[66]. The production involved five days fermentation in shake flasks containing peptone, glucose, salts and some oil. Benzoana[67] studied the fermentation pattern of *Rhizopus arrhizus* in a stirred fermenter on a medium containing casein hydrolyzate, corn flour and ammonium sulphate. The growth reached maximum after 20-25 hours but maximum lipase activity was obtained after 40 hours. Salleh *et al*[47] studied the production of extracellular and intracellular lipases by thermophilic strain of *Rhizopus oryzae* on peptone-salts medium. Miura[68] isolated fungi that had high lipolytic activities in biomass support particles. He found that *Rhizopus stolonifer* and *Rhizomucor miehi* had cell bound lipolytic activities. However, the production of extracellular lipases by these fungi was increased with increase in polypeptone concentration. Peter Rapp[69] studied lipase production during the cultivation of *Fusarium oxysporum* in shake flasks. He reported that the fungus required peptone, trimyristin, olive oil and oleic acid for significant production of the lipase. The lipase synthesis was repressed by glucose and glycerol.

Yoshida and coworkers[70] studied chemical and physical properties of lipase from *Torulopsis ernobii*. The yeast produced an acid stable extracellular lipase. The fermentation was carried out in 30 L jar fermenter with 15 L medium which was inoculated with two days old inoculum. The lipase

production was found to be related to cell growth and reached maximum in 25 hours. Nurmines and Suomalainen[71] reported the presence of lipases in cell envelopes of Baker's yeast but no esterase activity could be detected. In *Saccharomycopsis lipolytica* most of the lipase was cell bound, however, the extracellular activity was 125 folds increased when the yeast was cultivated in the presence of a protein like fraction of soybean[72]. The purified exocellular enzyme was found to require an activator for the hydrolysis of triglycerides[73]. The cell bound lipase did not require any activator and in fact was consisted of two proteins, with molecular weights 39000 and 44000, both being monomeric[74]. Novotny *et al*[40,75] studied the production of lipases and esterases by *Candida rugosa, Candida guilliermondii, Candida curvata, Candida deformens* and *Yarrowia lipolytica.* They provided evidence against linkage between high levels of lipase and mycelium formation in yeasts. Valero *et al*[53] reported that lipase fermentation of Candida rugosa involved two stages. In first part of the fermentation, ethanol was produced at the expense of glucose and lipase activity remained 1.0 unit/mL. When the glucose had been totally consumed, the ethanol was utilized promoting second stage of lipase formation (4.0 units/mL).

2.3.2 Solid State Fermentation

Fukumoto *et al*[4] isolated a strain of *Aspergillus niger* which showed very high lipase activitiy when grown on moist wheat bran buffered by $CaCO_3$. Fermentation was carried out at 30°C for three days. The enzyme was purified and finally obtained in crystalline form. Ohnishi *et al*[76] reported that

Aspergillus oryzae produced small amounts of lipase in solid culture in contrast to larger amounts in shake flask cultures. Kundu and pal[44] cultivated lipolytic fungi on moist wheat bran and extracted the lipase from fermented bran in phosphate buffer.

Ortiz-Vazquez *et al*[77] studied the lipase production by solid state fermentation of *Pnicillium candidum*. They exploited moist wheat bran as the fermentation substrate. The moisture content of 67.5% and temperature of 29°C were found to be the best for lipase production. The rice bran was used as solid substrate for lipase formation by Candida rugosa. Addition of oil, maltose and urea to the bran had positive effect on the production of lipase. The fermentation in tray fermenters indicated that 98% humidity was the most suitable for growth and lipase synthesis[78,79]. Benjamin and Pandey[80] used coconut oil cake as solid substract fermentation by *Candida rugosa* and obtained 87.76 lipase units/g of dry fermentation substrate.

2.3.3 Effect of Carbon Sources on Lipase Production

Nature of carbon sources and their proportion to nitrogen compounds, in the fermentation medium, exert important effects on microbial growth and enzyme yield. Pal *et al*[50] studied the relation of carbon sources to growth and lipase production by *Aspergillus niger*. Glucose, maltose, lactose, dextrins and starch supported good growth and moderate enzyme production. Sucrose induced appreciably good growth and very high enzyme formation. It was considered to be the best carbon source for *Aspergillus niger*. Similarly in case of *Aspergillus wentii* the best growth and lipase activity were noted with

glucose followed by mannitol. Other carbohydrates resulted in decreased growth and lipase activity. Oils have been reported to cause maximum inhibition of growth and lipase production in *Aspergillus wentii* and *Penicillium roqueforti*[81].

Chander *et al*[82] reported that glucose stimulated maximum lipase production whereas other monosaccharides and disaccharides caused low lipase production in *Rhizopus nigricans*. Salleh *et al*[47] reported that the level of intracellular lipase in *Rhizopus oryzae* was increased by the addition of sugars, particularly sorbitol. However, extracellular lipase was inhibited by including sugars into the basal medium containing peptone. For *Rhizopus delemar*, glucose was reported to be the best carbon source followed by galactose, sucrose, lactose, maltose, starch, dextrins and fructose in decreasing order[83]. Haas and Bailey[84] reported the ability of the fungus *Rhizopus delemar* to synthesize more lipase when grown on glycerol as the prime carbon source than glucose. A negative effect of glucose on lipase fermentation has been reported in case of *Mucor lipolyticus*, *Candida curvata* and *Candida deformans*. In these cases lipase synthesis was supposed to be subjected to catabolic repression by the glucose[85]. Sucrose was reported to be the most potent carbon compound for lipase biosynthesis by *Fusarium solani*[86].

The level of carbon source in the fermentation medium was very critical. The optimum carbon to nitrogen ratio for lipase and biomass production by *Candida rugosa* were 6.0:6.5 and 9.0:9.5, respectively[79]. Increased concentration of carbon source was found to lower both intracellular

and extracellular lipases in *Rhizopus chinesis*[10]. Higher oxidized carbon sources were found to have significant effect on the synthesis of esterases and lipases by yeasts, the most effective being citrate and succinate. However, in case of *Candida deformans*, lipase activity was decreased in the presence of higher oxidized carbon sources[40,86]. Benjamin and Pandey[87] reported that lipase of *Caudida rugosa* was inducible. Its production was increased when some lipid materials were present in the medium. Aabinose was reported to be the best carbon source for lipase production by thermophilic strain of *Rhizopus arrhizus*. In its presence the fungus produced 310u/ml lipase[88]. Tsujisaka *et al*[81] reported induced formation of lipase by *Geotrichum Candidum* in the presence of lipids. Freir *et al*[90] found that the lipase fermentation by Penicillium restrictum. He used meat peptone and ature oil in the growth medium with C/N ratio of 9.9. He found that higher C/N ratio forwarded cell growth and lipase production. It was also noted that the glucose was not a good source for lipase production. Peter Rapp[69] found that the fangus *Fusaricum oxysporum* required peptone, trimyristin, olive oil and oleic acid for significant production of lipase. Synthesis was reported to be repressed by glucose and glyurol.

2.3.4 Effect of Nitrogen Sources on Lipase production

Fujita *et al*[91] reported that the lipase production by *Mucor javanicus* and *Rhizopus delemar IFO 419* was enhanced using corn steep liquor in the fermentation medium. But in case of *Rhizopus delemar 1472*, corn steep liquor, rice bran and soybean extract proved to be poor sources of nitrogen. The

peptone was reported to be the best nitrogen source. The addition of inorganic nitrogen compounds did not cause any enzyme production[83].

Jensen[46] used peptone broth for *Geotrichum candidum* to get the best yield of the enzyme. Pal *et al*[50] studied different organic and inorganic nitrogen sources. Ammonium chloride, ammonium acetate, L-asparagine and peptone caused good growth but poor lipase yield. Addition of tryptone, aspartic acid or glutamic acid to the fermentation medium caused cellular growth and moderate lipase synthesis. Ammonium nitrate supported the best mycelial growth as well as lipase production. Kumar *et al*[88] cultured *Rhiropus arrhizus* on a medium containing soybean meal as a source of nitrogen. Chander *et al*[51,82] tried many organic nitrogen sources for the cultivation of *Aspergillus wentii* and *Rhizopus nigricans* and obtained highest lipase yield with peptone followed by wheat bran and soybean meal. Very low yields were obtained with corn steep liquor and ground nut proteins. Epsinosa *et al*[49] and Banerjee *et al*[92] have described organic nitrogen sources as superior to the inorganic nitogen sources supporting higher levels of growth and lipase production. Freir and coworkers[90] reported 2% meat peptone as the best nitrogen source for lipase production by *Penicillium restrictum*. For the lipase production by *Rhizopus Chinesis*, polypeptone and meat extract were used as nitrogen sources[93] Miura[68] studied the lipase production by *Rhizopus stolonifer* and *Rhizomucor miehei* and reported increased extracellular lipase production in the presence of polypeptone solution. Hamed[94] reported that aspartic acid was the best nitrogen source for *Aspergillas terreus*, *Fusarium*

solani and *Penicillium funiculosum*. While for *Fusarium maloniforme* and Mucor Circinelloides, L-glutamic acid was promotive for lipase production.

2.3.5 Lipase Induction

Akhtar *et al*[65] reported that significant amounts of intracellular and extracellular lipases were produced by *Mucor species* in the absence of triglycerides. The inclusion of various triglycerides, however, increased both intracellular and extracellular lipase activities to varying extents. So in *Mucor species*, lipase was supposed to be both inducible and constitutive. The lipase activities were increased with the increase in concentration of triglycerides upto 1.0%. Beyond this concentration lipase activities showed significant decrease. However, mycelial growth was increased with increase in the olive oil concentration upto 1.5%. Later it was found that lipase produced by *Mucor hiemalis* was especially active against triglycerides in the presence of which it was produced[95]. In *Rhizopus arrhizus*, *Rhizopus Japonicus* and *Rhizopus oryzae* there was no indication of lipase specificity for inducing glycerides, as in *Mucor javanicus*[66]. Addition of lipid materials to the fermentation medium increased lipase production by *Aspergillus niger*. Butter fat and olive oil supported the best growth and enzyme production but coconut oil did not[50].

Benzoana[67] reported that *Rhizopus arrhizus* did not require any inducer for lipase biosynthesis and secretion. In *Rhizopus delemar* the growth was significantly reduced by oils resulting in high specific lipase activity[96]. Lipid materials except glycerol were found to inhibit exocellular lipase production by

thermophilic *Rhizopus oryzae*. However, the intracellular lipase activity was increased when the fungus was cultivated in the presence of triolein, olive oil and oleic acid[47].

Like moulds, inducible synthesis of lipases in the yeasts, have also been reported[93,97]. Yoshida *et al*[70] reported two fold increase in lipase yield when 0.2% olive oil was added to the basal medium. The increase in lipase yield was related to increased cell growth. *Candida rugosa* produced more exocellular lipases when triglycerides and cholestrol were present simultaneously[74]. Induced production of lipases have also been reported in some *Candida* and *Yarrowia* yeasts[98].

Hamed[94] used corn oil for lipase induction and found that 0.3% concentration of corn oil was optimum for *Fusarium moloniforme* and *Penicillium funiculosum* and 0.4% for *Aspergillus terreus*, *Fusarium solani* and *Mucor Circinelloides*. de la Torre *et al*[99] studied lipase fermentation by *penicillium candidum*. They reported that among olive oil components only the oleate allowed significant lipase production whereas the other fatty acids did not cause derepression of lipase formation. Metwally and coworkers[62] found that the presence of lipids in the growth medium enhanced the production of lipases by *Penicilliam citrinum* and *Verticillium agaricinum*. However, addition of the lipids did not increase the biomass yield.

2.3.6 Lipase Inhibition

Pythium ultimum lipase was reported to be inhibited by Co^{++}, Fe^{++}, Sn^{++} and Mn^{++}[100]. *Candida rugosa* lipase was strongly deactivated by Cu^{++} and Zn^{++} and completely deactivated by detergents[101]. Lipase of *Pseudomonas fragi* was also inhibited by Fe^{++}, Fe^{+++} and Zn^{++}. EDTA was effective in removing toxicity of Fe^{+++} and Fe^{++}[102]. Hamed[103] studied many fungal strains and found that Cu^{++} inhibited lipase production by all strains. Low levels of Fe^{++}, Fe^{+++}, Mg^{++}, K^{+} and Zn^{++} were stimulatory whereas high levels were inhibitory. In case of Candida paralipolytica anionic surfactants acted as activator but cationic and nonionic surfactants were inhibitors[104].

Fatty acids and their salts have been frequently reported to inhibit microbial lipases. Yoshida *et al*[70] found that fatty acids lower than lauric acid, reduced lipase formation by *Torulopsis ernobii*. In *Pseudomonas fragi* and *Geotrichum candidum*, addition of sodium oleate and salts of other unsaturated fatty acids to the fermentation medium decreased lipase production without affecting cell growth. Some divalent cations, tweens, lecithin and bovine serum prevented oleate inhibition but did not reverse it[105]. Tetracyclin inhibited activity of Ca^{++} activated lipase of *Corynebacterium acnes*[106].

2.3.7 Effect of pH on Lipase Production

pH of the fermentation medium affects microbial growth and lipase production. Most of the fungi show optimum growth and maximum lipase production at slightly acidic pH[53,94,107]. For some yeasts optimum pH for

lipase production is as low as 3.0[70]. However, for some fungi optimum pH for lipase formation is 7.0 to 7.3[50,66]. The optimum pH for lipase action may or may not be the same as that for its formation or microbial growth[108,109].

2.3.8 Effect of Temperature

There are many reports which show that the highest lipase production is achieved when the organisms are grown at a temperature below the optimum growth temperature[47]. The optimum temperature for the lipase production varies from species to species. For some fungal strains, it is as low as 25°C[107] and for others it is as high as 45°C[47,110]. However for majority of fungi the optimum fermentation temperature ranges between 28-30°C[61,62,70,82].

2.3.9 Effect of Aeration

The decisive factor in aerobic fermentation is the rate of supplying and dissolving oxygen in the culture medium. If oxygen tension in the medium decreases below certain value, the respiratory activities of the cells decrease and metabolism changes. This critical value of dissolved oxygen (DO) is specific for each organism. For moulds like *Penicillium chrysogenum*, it is about 0.005 m.mole/L[111]. Iwai *et al*[60] found that *Penicillium cyclopium* produced maximum lipase when 500 mL flask, having 60 mL fermentation medium were shaken at 110rpm. Alford and Smith[112] have shown many fold increase in aerated cultures than in static cultures of *Geotrichum candidum*. In the case of *Aspergillus wentii*, *Rhizopus nigricans*, *Penicillium roqueforti* and *Penicillium chrysogenum*, aeration resulted in an increase in growth and lipase

synthesis. In all these cases shake flask cultures gave better lipase yield than the stationary cultures[51,82,61]. Similarly the enzyme yield was double in shake flasks as compared to stationary cultures of *Rhizopus delemar 1472*[83]. During shake flask studies on *Rhizopus oryzae*, the shaking speed of 100 rpm was the best for extracellular lipase production and 150 rpm for cell bound lipase activity. Increasing the speed to 200 rpm reduced both free and cell bound lipase activities[47].

2.4. LIPASE MEDIATED ESTER SYNTHESIS

2.4.1 Immobilization of Lipases

Enzyme immobilization is the imprisonment of an enzyme molecules in a distinct phase that allows exchange with, but is separated from the bulk phase in which substrate molecules are dispersed. The enzyme phase is usually insoluble in water and is often high molecular weight polymer. The imprisonment of the enzymes may be achieved by various means including adsorption, covalent binding, entrapment, crosslinking between enzyme molecules and immobilization within the microbial cells.

a) Adsorption

Many workers have reported the superiority of adsorption over other techniques of immobilization[113,114]. Brady and coworkers[115] studied many adsorbents for lipase immobilization. Hydrophobic microporous powders, membranes and fillers were found to give satisfactory performance, as no lipase activity was lost during immobilization. Many workers have reported that Celite was very effective adsorbent for the enzyme immobilization[25,116].

Wannerberger and Arnebrant[117] studied absorption of lipases to silica and methylated silica. They found that adsorption was increased in the presence of $CaCl_2$. *Mucor michei* lipase was immobilized by adsorption on Duolite ES 562 resin[118]. Ruckenstein and Wang[119] prepared microporous polymer support by polymerization of a mixture of styrene and divinyl benzene containing a suitable surfactant and an initiator. The lipase from *Candida rugosa* was immobilized on the internal surface of the microporous polystyrene divinyl benzene. Wang and Ruckenstein[120] also used polyurethane for the lipase immobilization. The lipase from *Candida rugosa* was immobilized by adsorption on porous polyurethane particles and crosslinked with glutaraldehyde to enhance the stability. Lipozyme is *Mucor miehei* lipase immobilized on microporous support and is used for ester synthesis[20]. Sato *et al*[121] immobilized the emyme on silicas of different pore size and utilized to catalyze ester synthesis. Gunnlaugsdottir *et al*[122] used glass beads for lipase adsorption. The enzyme immobilization was also achieved by adsorbing the enzyme on Alumina and silica gel based supports[123,124]. Ivanov and Schneider[125] have reported the immobilization of *Penicillium fluorescens* lipase on to different carriers. The celite adsorbed lipase was found to have maximum esterification activity and the lipase immobilized onto Eupergit C 250L exhibited the best operational stability. Basri *et al*[126] also found that Celite adsorbed lipase of *Candida rugasa* had very high esterification activity in all cases and broad specificity for fatty acids. Candida rugosa lipase was also immobilized by Jonzo and coworkers[127], using Duolite A 568 and Amberlite IRC 50. Duolite absorbed lipase was found to be more active then the

Amberlite immobilised lipase. The presence of sorbitol in the preparation enhanced the lipase activity by preserving the water shell around the catalyst. Gandhi *et al*[128] used ion exchange resin beads for lipase adsorption and reported that the thermal stability of the enzyme was enhanced by adsorption. de Lima *et al*[129] used chrysotile, a magnesium silicate, for the adsorption of lipases. Other inorganic supports have also been used for the adsorption of lipases[130].

b) Covalent Binding, Crosslinking and Entrapment

Covalent binding of enzymes on tosyl activated matrix (Corn cobs and agarose) have been reported by Arroyo *et al*[131] and Stevenson *et al*[132]. Ergan *et al*[133] prepared immobilized lipases by mixing the lipase with bovine albumen and glutaraldehyde in phosphate buffer. The mixture was allowed to stand and crosslinking to occur between the lipase and the albumin.

Murakata *et al*[134] entrapped lipase in poly - N - isoprylacrylamide gel beads which underwent thermally induced phase transition. Catalytic activity of immobilized enzyme in the gel beads (1EG) changed significantly before and after phase transition at which gel altered from the swollen to the shrunken state. The 1EG was the most active for ester synthesis, when it was shrunk and thus surrounded with a limited amount of water. Entrapment of the celite adsorbed enzyme with ENTP-2000 gave stable preparation[135].

c) *Immobilization within the Cells*

Intracellular fungal lipases have been immobilized simply by drying the mycelia[64]. The dead, dried and defatted mycelia constitute naturally immobilized lipase which is very efficient catalyst and shows good operational stability during the synthesis reactions in organic media[18]. Gancet *et al*[136] used dead mycelium of *Rhizopus arrhizus* as a lipase source for the synthesis of esters and glycerides. They have also reported the use of mycelial lipase of *Rhizopus arrhizus* for the natural flavour ester preparation, pharmaceutical drug synthesis and racemic mixture resolution[137]. Essamri and coworkers[138] optimized conditions for the production of mycelial lipase by *Rhizopus oryzae* and reported that it was stable in hydrophobic solvents. Valerie *et al*[139] used cell bound lipases of *Penicillium cyclopium* and *Rhizopus arrhizus* in a loop fixed bed reactor for continuous synthesis of esters.

d) *Enzyme Modification*

Lipases from various microorganisms were chemically modified with poly thylene glycol derivatives[140,141]. Kodera *et al*[142] used activated polymer PEG2 [2,4-Bis (O-methoxy polyethylene glycol)-6-chloro-s-triazine] and PEG1 [2-(O-methoxy polyethylene glycol)-4,6-dichloro-s-triazine] to immobilize the enzyme lipase. Such modified lipases were found to be soluble in organic solvents such as benzene, toluene and chlorinated hydrocarbons, as well as in water due to amphipathic nature of PEG. Basri *et al*[143] prepared activated poly ethylene glycol (PEG-nitrophexyl carbonate) using monomethoxy polyethylene glycol and p-nitrophenyl chloroformate. The

activated PEG was covalantly attached to *Candida rugosa* lipase. Lipase from *Psendommas fragi* was also modified with polyethylene glycol. The PEG modified lipase was used for esterification leading to resolution of recemic alcohols[144]. Modified lipases catalyzed ester synthesis at an increased rate as compared to the native lipases. Moreover, the specificity of the enzyme was altered by chemical derivatization and it became extremely thermostable. The PEG modified lipase was further endowed with magnetic properties by coupling it with magnetite. This magnetic lipase was dispersable both in aqueous and non-aqueous media. In organic solvents it catalyzed the ester synthesis and was completely recovered in 5 minutes in a magnetic field of 6000 Oe. It had half life of three months[145]. Mutation of the mould *Rhizopus delemar* led to the production of a lipase with modified specificity[146].

2.4.2 Effect of Water on Enzymic Ester Synthesis

In enzymic esterification water has been indentified as a crucial factor. Its activity in the reaction medium has a striking effect on rate and selectivity of the process[147-151]. Water has been reported as a competitive inhibitor of lipase catalyzed esterification in non-aqueous media. During glyceride and ester synthesis an equilibrium reaction mixture is obtained. One way to produce an excess esters is to remove the water produced during the synthesis and thus pull the equilibrium in forward direction[152]. Linfield *et al*[153] found it necessary, for esterification, to reduce water activity. Glycerides containing eicosapentanoic acid and docosanoic acid were synthesized only when water content in the reaction mixture was very low[154].

During esterification removal of water from the vicinity of the enzyme by lyophilization resulted, at first, in enhancement of reaction rate and then inactivation of the enzyme. This inactivation is reversible and the addition of water to the immobilized enzyme recovered the original activity[135]. Kim and Rhee[155] developed cold trap method for efficient removal of water during glyceride synthesis in solvent free system. Gubiza and Szakacs[156] found that the reaction rate as well as the enantioselectivity decreased with increase in water content. They also developed a technique for on line water removal from the reaction mixture. de Castro *et al*[157] investigated engineering strategies for removal and control of water generated during enzymic esterification of citronellol with butyric acid. Jeong and Lee[158] developed a technique for continuous stripping of water during the reaction. The technique of water stripping was also used by Dueret *et al*[159] during esterification of ibuprofen. Van der Padt et al[160] also developed pervaporation system for the removal of water. The enzyme was immobilized on the lumen side of a cellulose membrane where organic phase was present. Air was circulated at the shell side and the water activity was controlled with the use of condensers. Only at low water content an excess of triacyl glycerol was obtained. Lipozyme was found to be hydrated and inactivated during ester synthesis. Removal of water from the recovered enzyme restored the activity to the original levels. Regeneration of the enzyme was achieved by incubation with polar and nonpolar organic solvents especially acetone and n-hexane. This treatment regenerated about 70% of the original activity[161].

Complete removal of water from the system, however, was found to destroy the enzyme activity. The presence of some water was essential for the active conformation of the enzyme protein. Linko and Yu[162] studied the synthesis of butyl oleate using 25 different lipases. They concluded that the effect of added water varied with enzyme, some showed high activity with no water and some when a small amount of water was added. Takahashi *et al*[145], while working on PEG modified lipase, found that a trace amount of water was necessary for expression of enzyme activity. The ester synthesis activity of the celite immobilized lipase was maximum when water activity was 0.11 to 0.38[163]. Esterification activity of *Rhizopus niveas* lipase in n-hexane was maximum when water activity was 0.75[164]. Wehtje *et al*[165] developed a technique to adjust water activity in organic media during enzymic esterification. A saturated salt solution was circulated inside the silicone tube submerged into the reaction medium. The circulating solution could exchange water with the reaction medium thus adjusting the moisture level there.

2.4.3 Reaction Media

The polarity of the organic solvents used in the biocatalysis is found to affect the enzyme activity. Gorman *et al*[166] and Narayan *et al*[167] reported that high biocatalytic rates could be obtained when the polarity of the organic solvent was low. Cui *et al*[168] carried out enantioselective esterification of naproxen in isooctane. Laane et al[169] reached similar results showing that high enzyme activity could be obtained in solvents having log P> 4. The polar solvents were found to distort the essential water layer that stabilizes the

biocatalyst. Workers compared esterification activities of lipozyme in different organic solvents. The enzyme was active in water immiscible organic solvents while it was inactive or less active in water miscible hydrophilic solvents. Some water miscible solvents like tetrahydrofuran (THF) were reported to cause reversible while others like dimethyl formamide (DMF) irreversible loss to the enzyme protein[20,170,172]. Petroleum ether gave the best results in most of the cases of enzymic ester synthesis. Charton and Macrae[173] used ter.butyl alcohol containing less than 0.01% water as the reaction medium for lipase catalyzed synthesis of hexyl oleate. Gancet and coworkers[174,175] used a variety of solvents for various esterification reactions and reported that the tertiary alcohols, especially ter. amyl alcohol, were the best for glyceride synthesis. In its presence more monoglycerides (MAGs) were produced than diglycerides (DAGs) and triglycerides (TAGs). The fluorinated hydrocarbons were reported to be preferred media for the synthesis of short chain esters, thioesters and amides. For the esterification of primary terpene alcohols, non polar solvents with log P>3.5 where reported to be the best[176]. Supercritical CO_2 was also used as the reaction medium for esterification reactions[177,178,179].

Lipase catalyzed esterification in micro emulsions have been reported by many workers[180-181].

Stamatis and coworkers[182,183] studied the esterification between lauric acid and (-) menthol in microemulsion. Microemulsion was made in isooctane and was stabilized by bis (2-ethil, hexyl) sulphonate. Due to their low water content, microemulsions assist in reversing the direction of lipase activity,

favouring synthetic reactions. Paul et al[184] stabilized the emulsion by anionic surfactant sodium bis (2-ethyl hexyl) sulfonate. In another system water-in-oil microemulsion was stabilized by hexadecyl trimethyl ammonium bromide. *Candida cylinderancea* lipase in water-in-oil microemulsion, stabilized by dodecyl sulphonate, was used to catalyze the hydrolysis and synthesis of esters[185]. Papadimitriou *et al*[186] used reverse miscelles to cosolubilize hydrophilic and hydrophobic reactants for lipase catalyzed esterification.

The lipases have also been used in biphasic water/organic systems for esterification and transesterification reactions. The enzyme dissolves in aqueous phase while the hydrophobic substrates dissolves in organic phase. The reaction takes place at the boundary of two phases[164].

2.4.4 Glycerol Ester Synthesis

Enzymic synthesis of glycerol esters in non-aqueousor biphasic media have been reported by many workers[187,188]. Tsujisaka *et al*[189] compared activities of lipases from *Aspergillus niger, Rhizopus delemar, Penicillium cyclopium* and *Geotrichum candidum* for glyceride synthesis. The cystalline lipase of Aspergillus niger was used to synthesize glycerol esters of oleic acid. It was found that glyceride yield was increased by decreasing the water activity[153]. Akoh[190] used lipases from *Mucor miehei* and *Candida antarctica* for esterification between isopropylidene glycerol and fatty acids. Acid catalyzed cleavage of *isopropylidene* groups, resulted in the formation of monoglycerides and diglycerides. Both oleic and eicosapentaenoic acid were successfully incorporated into glycerides. Maximum acid consumption was

upto 96.9% with monoglyceride content upto 88.5%. Lortie *et al*[191] studied kinetics of triolein synthesis catalyzed by *Mucor miehei* lipase. They reported that this 1,3-specific lipase could catalyze the synthesis of triolein because of isomerization of 1,3-diolein which undergoes further acylation by enzymic action. Further studies showed that the isomerization was the rate limiting step of the reaction[192]. Mazur *et al*[193] developed a methodology for region and stereo-seletive esterification of glycerol and its derivatives. The regional and stereoselectivity in esterification was achieved using fatty acid anhydrides and 1,3-specific lipase. Li and Ward[154] reported the synthesis of glycerides of n-3 polyunsaturated fatty acids using *Pseudomonas* and *Mucor miehei* lipases in isooctane and n-hexane. He and Shahidi[194] reported the lipase cutalyzed synthesis of glycerides from ω-3 fatty acids and glycerol. Tanaka and coworkers[195] produced docosahexaenoic acid-rich triglycerides with immobilized lipase of *chromobacterium viscosum*. Okumura *et al*[196] used radiolabelled oleic acid and measured the extent of esterification during triglyceride hydrolysis and found that *Rhizopus delemar* lipase showed the strongest esterifying action. Some lipases for example those from *Rhizopus arrhizus*, *Mucor miehei* and some strains of *Aspergillus niger* were reported to show specificity for external hydroxyl groups of glycerol and some like those from *Candida rugosa*, *Aspergillus niger* and *Geotrichum candidum* were reported to be non-selective in nature[197]. The lipases from *Geotrichum candidum* and *Aspergillus niger* showed considerable strain variations[198] in specificity. Enzymic esterification was also used to reduce free fatty acid content of oils producing more triglycerides[199-201].

2.4.5 Esterification of Monohydric Alcohols

Many workers have reported the use of immobilized lipases for esterification of primary monohydric alcohols[202-205]. Gitlesen *et al* (2.06) prepared dodecyl dodecanoate and dodecyl decanoate by enzymic esterification and determined the rate constants. Miller and coworkers[20] found that the esterification activity of the Lipozyme was increased with increase in carboxylic acid chain length upto octanoic acid. It showed very low activity for branched chain fatty acids. The range of alcohols as substrates for Lipozyme was very broad. An initial esterification rate of 12.93mM/h/g was obtained with 3-phenyl propanol. It was used to synthesize wax esters in solvent free system by reacting stoichiometric amounts of fatty acids and stearyl alcohol. Hydroxylated fatty acids were also esterified[26]. Marty *et al*[207] optimized reaction conditions for Lipozyme catalyzed synthesis of ethyl oleate in n-hexane. Mycelial lipase of *Rhizopus niveus* was used to esterify long chain alcohols with oleic acids to form wax esters[208]. Garcia and coworkers[209] optimized conditions for enzymic synthesis of myristyle myristate. Isono *et al*[210] developed a membrane reactor system to synthesize wax esters using lipase surfactant complex. The preparation of uncommon wax esters was achieved by application of *Rhizomucor miehei* lipase (Lipozyme TM) and *Candida antarctica* lipase (S 435). The PEG lipase from *Candida cylindraceae* was able to synthesize esters from 2 and 3-substituted carboxylic acids in benzene. Methanol and benzoic acid were esterified to give 30% yield

of Methyl benzoate. The PEG modified lipase from *Pseudomonas fluorescens* did not catalyze ester synthesis from 2- or 3-substituted carboxylic acids[145].

Esters of short chain fatty acids with short chain alcohols are fragrant compounds and these are used as food flavours[116,211]. The immobilized lipase of Mucor michei was used to synthesize low molecular weight esters from C_3 to C_6 fatty acids and C_2 to C_6 alcohols in organic media[114]. Welsh and Williams[212] produced fragrance esters from fusel oil (an industrial by-product consisting of short chain alcohols) using crude lipase from Candida cylinderaceae, in n-hexane. Gancet[137] reported that dead mycelium of *Rhizopus arrhizus* constituted highly active catalyst for ester synthesis.

Aspergillus niger lipase was used to estrify terpene alcohols. Primary terpene alcohols were esterified but secondary and tertiary terpene alcohols could not be esterified. Although *Aspergillus niger* lipase was suitable for the synthesis of esters of lower fatty acids but acetate esters could not be prepared[213]. The preparation of Geranyl acetate was achieved using PEG-modified lipase of *Pseudomonas fragi* or immobilized lipase of *Candida antarctica*[145,214]. Enzymic synthesis of geranyl acetate have also been achieved by transesterification with acetic an hydride as acyl donar[215]. de Castro and coworkers[216] used immobilized lipase to catalyze the esterification of citronellol and butyric acid. The equilibrium between the forward and reverse reaction was controlled by the level of water in the reaction vessel. Under optimum conditions, 95% yield of cironellyl butyrate was obtained. Claon and Akoh[217] reported enzymic esterification of geraniol and citronellol

with short chain fatty acids giving upto 100% ester yield. Geranyl farnesyl acetate, an antipeptidic drug, was sythesized by lipase mediated esterification of geraniol and farnesyl acetic acid in 1,1,1-trichloroethane[142,218]. Resolution of racemic ibuprofen and naproxen have been achieved by lipase catalyzed selective esterification of the racemates[219,220,221].

Stereospecificity of lipases is well documented[222-224]. However, in most of the cases we deal with degrees of selection rather than absolutes. The PEG lipase of *Pseudomonas fragi* could recognize the chirality of alcohols. It was reported that the yeast's lipase did not show stereoselectivity on primary alcohols and did not exhibit any activity on tertiary alcohols. The enzyme showed stereoselectivity in case of secondary alcohols[225]. Pancreatic lipase gave different esterification rates with E & Z isomers of allylic alcohols[226]. Lipase of Mucor miehei was reported to have strong stereobias for R-enantiomer[227]. Enantioselective esterification of glycidol has been performed with lauric acid in organic media dosed with surfactant lipase complex as the catalyst[228]. The Phenolic acids from green coffee were esterified in solvent free system using immobulized lipase from *Candida antartica*[229]. Huang *et al*[230] have reported that modified lipase of *Candida rugosa* catalyzed enantioselective esterification of (S) – naproxen from racemic naproxen. The stereoselectivity of the enzyme have been reported to increase by increasing the degree of enzyme modification. Chen *et al*[231] showed that the active site of the *Candida cylinderaceae* lipase was quite flexible and its selectivities towards both racemic substrates were affected reciprocally.

Enzymic Synthesis of Polyesters

Okumura and coworkers[33] reported lipase catalyzed syntesis of ester oligomers. Lactones and biodegradable polyesters were prepared by lipase catalyzed esterification in organic solvents[35].

Linko et al[34] prepared high molecular weight polyesters (max. Mr=131,190) which were biodegradable. Lipase catalyzed polyester synthesis was also studied by Jaeskelaeinen *et al*[232]. Wu and coworkers[233] used *Rhizomucor miehie* lipase to catalyze the esterification reaction between sebacic acid and 1,4-butandiol. During the reaction, molar mass of polyester increased rapidly and reached upto 56,000 g $mole^{-1}$.

2.4.6 Esterification of Sugars and their Derivatives

Seino *et al*[29] made successful attempts to prepare carbohydrate esters of fatty acids using lipase of *Candida cylindereaceae*. These esters not only have surface activity but also antitumor and plant growth inhibiting activities. The workers made esters of glucose, fructose, sorbitol and sucrose in buffered solution of substrates. Lipases from *Pseudomonas* and *Enterobacter* species also catalyzed synthesis of carbohydrate esters but activities were less as compared to *Candida cylinderaceae* lipase. Oguntimein *et al*[234] reported lipase catalyzed ester synthesis in organic solvents. The immobilized fungal lipases were exploited for the synthesis of fructose and glucose esters of stearic acid in the presence of phenyl boronic acid or butyl boronic acid (solublizing agents). The only disaccharide they could esterify was platinose. The use of

phenyl boronic acid or butyl boronic acid as solulilizing agent for enzymic synthesis of suger esters was also reported by Wray and Wagner[235]. Regiospecific acetylation of pentoses and hexoses, after their preabsorption on silica gel have been reported[236]. The lipase catalyzed esterification of α-D-glucopyranoside by diacids was carried out in acetone using pig pancreatic lipase. The nature of the acylating agent influenced the regiospecificity of the reaction. Diacid esters were also obtained in solvent free system using the Lipozyme[237]. Fregpane et al[238] used sugar acetals and succeeded to produce their mono- and diesters in solvent free system. Working on the same lines Sarney et al[239] have reported the manufacture of lactose and maltose monoesters from corresponding disaccharide acetals. The final products were obtained by acid catalyzed cleavage of the acetal groups. The alkyl glucoside esters were prepared using *Candida antarctica* lipase in non-aqueous media[152]. The esterification of acetylated glucose using immobilized lipases of *Candida antarctica* and *Candida cylinderaceae* have been reported by Akoh[240].

Chopineau *et al* synthesired sugur alcohol monesters in non-aqueous media. Tsitsimpikou, *et al* used lipase of *Candida antarrtica* to catalyze the acylation of glucose in supercritical Carbon dioxide. Regioselective synthesis of 6-O-phenylbutyryl-1-n-butyl-β-D-glucopyranose was catalyzed by *Candida antratica* lipase and glucosidase[243] lipase catalyzed reqioselective acetylations and deacylation of nucleosides were reported by Ozaki etal[244] . They developed an efficient method for the selective protection of mucleosides.

Chapter 3

METHODOLOGY

3.1 LIPASE PRODUCTION

3.1.1 Isolation of Lipolytic Fungi

Lipase producing fungi were isolated from the soil samples by the method of kundu and Pal[44] The isolation medium (Table - A) was spread over TLC plates and was sterilized by exposure to U.V. radiations. Samples were spread over the plates and the plates were incubated at 30oC for four days. The microbial colonies were developed which were picked up and purified by streaking.

TABLE - A

Ingredients	g/dm^3
$(NH_4)_2SO_4$	1.0
KCl	0.7
K_2HPO_4	1.0
$FeSO_4.7H_2O$	0.01
Olive Oil	100
pH	6.5

3.1.2 Screening and Identification

The isolated cultures were screened for the production of lipases in 250 cm^3 shake flasks. The fermentation medium was consisted of (g/dm^3) peptone: 20; glucose: 10; Yeast extract: 5.0; NaCl: 5.0. The flasks were incubated at 30°C for 48 hours. Only that culture which gave maximum lipase activity, was selected for identification. The selected cultures were identified according to the scheme of Ainsworth[245].

3.1.3 Culture Maintenance

Following methods were adopted for the maintenance and storage of cultures.

a) Maintenance For The Routine Use

The isolated fungal cultures were maintained on potato-dextrose-agar slants (Table - B). The cultures were revived after every month.

TABLE – B

Potato-Dextrose Agar Medium

Ingredients	g/dm^3
Potato extract (200 g, potatoes, sliced, boiled and filtered through fine cloth)	1 dm^3
Glucose	20 g
Agar	15 g
pH	4 – 6

b) Maintenance For The Long Term Storage

For long term storage lipolytic fungi were maintained as soil cultures, silica gel cultures or freeze-dried cultures.

i) Soil Cultures

The spore suspension was added to sterilized loam and allowed to grow for about 10 days. The cultures were then stored in refrigerator at 5 - 10°C.

ii) Silica Gel Cultures

The spore suspension in 5% skimmed milk was cooled to 5°C and added to well cooled anhydrous and sterile silica gel in screw capped bottles. This was allowed to dry for two weeks. Then the caps were screwed down and the bottles were refrigerated.

iii) Freeze-Dried Cultures

In Lyophilization, the spore suspension was placed in small vials for freezing. The vials were then connected to a high vacuum line apparatus. The ice present in the suspension was sublimed under vacuum. The vials after sealing were stored in referigerator at 4°C.

3.1.4 Inoculum Preparation

The spore suspension of mould cultures was prepared by washing 72 hour old culture slants with 0.005% Monoxal O.T. The spores were centrifuged

at 2500 r.p.m. for 15 minutes in 20 cm^3 sterilized ceutrifuge tubes. The supernatant was discarded and pellet suspended in an adequate volume of sterilized distilled water. The number of spores per cm^3 were counted under the microscope using counting chamber. The suspension was diluted with sterilized distilled water to adjust the concentration of spores to 10 million spores per cm^3. Vegetative inoculum was developed by inoculating 50 cm^3 inoculum medium (Table - C) contained in 250 cm^3 conical flasks, with 1.0 cm^3 spore suspension. The flasks were incubated at 30°C with orbital shaking at 100 rpm.

TABLE - C

INOCULUM MEDIUM

Ingredients	g/dm^3
Peptone	10.0
Soluble starch	10.0
Ammonium Nitrate	2.0
Yeast Extract	2.0
Olive Oil	5.0
pH	5.0

3.1.5 Fermentation Techniques

Production of fungal lipases was studied by both by submerged and solid state substrate fermentations.

a) Submerged Fermentation

Both natural or synthetic fermentation media (M_1 — M_6) were screened for the maximum lipase production by the fungus *Rhizopus arrhizus*. The compositions the media of are given below.

M_1:-

Ingredient	g/dm^3
Peptone	20.0
Dextrose	10.0
Yeast Extract	5.0
NaCl	5.0

pH = 7.0

M_2:-

Ingredient	g/dm^3
Olive oil	10.0
Sucrose	10.0
NH_4NO_3	1.0
KH_2PO_4	2.0
$MgSO_4.7H_2O$	0.4
$FeSO_4.7H_2O$	1.0

pH = 7.0

M_3:-

Ingredients	g/dm^3
Corn steep liquor	50.0
Olive oil	20.0
KH_2PO_4	2.0
KCl	0.5
$NaNO_3$	0.5
$MgSO_4.7H_2O$	0.5

pH = 4.6

M_4:-

Ingredients	g/dm^3
Peptone	30.0
Glucose	10.0
K_2HPO_4	2.0
$NaNO_3$	0.5
KCl	0.5
$MgSO_4.7H_2O$	0.5

pH = 7.0

M_5:-

Ingredients	g/dm^3
Olive oil	20.0
Yeast extract	1.0
$(NH_4)_2SO_4$	5.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.25
$CaCO_3$	5.0

pH = 6.0

M_6:-

Ingredients	g/dm^3
Soyabean meal	35.0
K_2HPO_4	5.0
$(NH_4)_2SO_4$	1.0

pH = 6.0

The fermentation was carried out in :

i) Conical flasks (250 cm^3)

ii) Stirred fermenter (18 dm^3 – Glass Jar)

iii) Air lift fermenter (4 dm^3)

The fermentation media contained in the conical flasks or jar fermenter, were sterilized by autoclaving at 121°C (15 psi pressure) for 15 minutes. In case of $4dm^3$ airlift fermenter, the vessel was sterilized chemically by using sodium hypochlorite solution. Then the fermenter was aseptically flushed with sterile distilled water. The separately sterilized fermentation medium was, then, transferred aseptically to the airlift fermenter. The carbohydrates or $CaCo_3$ were added to the fermentation medium after separate sterilization. The medium was cooled in inoculated with fungal spores. The fermentation was carried out at 301°C for 48 hours. The agitation and aeration rate of the fermenter were 100 rpm and 1 $dm^3.dm^{-3}.min^{-1}$, respectively. Samples were taken out at regular intervals for the analysis of dry cell mass, pH, extracellular and intracellular lipase activities.

b) Solid State Fermentation

The solid state fermentation was carried out in 1L conical flasks. 30g of a substrate such as soybean meal, sunflower meal, cotton seed meal, rice bran, rice husk, gram bran or wheat bran was taken into the flasks. The substrate was wetted by 30 cm^3 diluent (water unless otherwise stated). The flasks were autoclaved at 121°C (15 psi) for 15 minutes. After cooling the flasks were inoculated with spores of *Rhizopus arrhizus* and incubated at 30± 1°C for 72 hours. The samples were drawn at regular intervals for the analysis. After termination of the fermentation, the fermented mash was soaked in distelled water for one hour. It was then centrifuged and the supernatant was analyzed for lipase activity.

3.1.6 Lipase Purification

The extracellular lipase of *Rhizopus arrhizus* was partially purified by employing ammonium sulfate fractionation and acetone precipitation.

The mycelia were separated from the fermented broth by centrifugaltion. Ammonium sulphate was added to the supermatant upto 35% saturation. After eight hours, the mixture was centrifuged to remove the precipitates. The precipitates formed at this stage were found to have negligible lipase activity. However, the supernatant was lipase active. To the supernatant more ammonium sulphate was added to 70% saturation. The mixture was kept at 4°C for 12 hours. The precipitates were collected by centrifugation and supernatant was tested for lipase activity which proved to be absent. Precipitates, thus obtained, were desalted by dialysis against distilled water.

From the dialyzed solution the lipase was precipitated by the addition of chilled acetone (55% v/v) and precipitates were freeze-dried.

3.1.7 Lipase Immobilization

Two methods were used for the immobilization of lipases.

a) Adsorption

Adsorption technique was used for the immobilization of extracellular lipase. Celite, cellulose, silica gel and alumina were investigated as adsorbents. To 10 mL buffered lipase solution 0.5g adsorbent was added and stirred for 30 minutes. After immobilization the support material was separated by vacuum filteration and washed with 100 mL buffer to remove any unadsorbed lipase. The immobilized material was dried under vacuum and stored over anhydrous $CaCl_2$ in desiccator.

b) Immobilization within the cells

The cell bound fungal lipases were immobilized within the cells. Lipase bearing mycelia were separated from the fermented broth. These were washed with water and squeezed between the folds of filter paper. Then these mycelia were dried and defatted by treating, first with chilled acetone (1 min.) then with acetone - ether mixture (1:1) for one minute and finally with pure diethyl ether for two minutes. The mycelia were ground to a fine powder and stored over anhydrous $CaCl_2$, in the desiccator.

3.2 ESTERIFICATION REACTION

The esterification between various alcohols and acids was carried out in stoppered flasks as well as by circulating the reaction mixture through the column packed with the enzyme.

3.2.1 Batch Reaction

Esterification reactions were carried out in 100 cm^3 stoppered conical flasks. A definite amount of lipase was added to 50 cm^3 reaction mixture containing 0.6M alcohol and 0.6M organic acid in a suitable organic solvent. The reaction mixture was incubated on orbital shaker with a speed of 100rpm at 30°C for 24 hours. After regular time intervals samples were drawn and analyzed.

a) Repeated Use of the Enzyme

The enzyme in the reaction medium was repeatedly used for further esterification. After the termination of the reaction, the mixture was filtered to separate the mycelium. The recovered mycelium was washed with chilled acetone, chilled acetone: diethyl ether mixture and then n-hexane. It was then reused for the catalysis of esterification reactions.

3.2.2 Packed Column Reactor

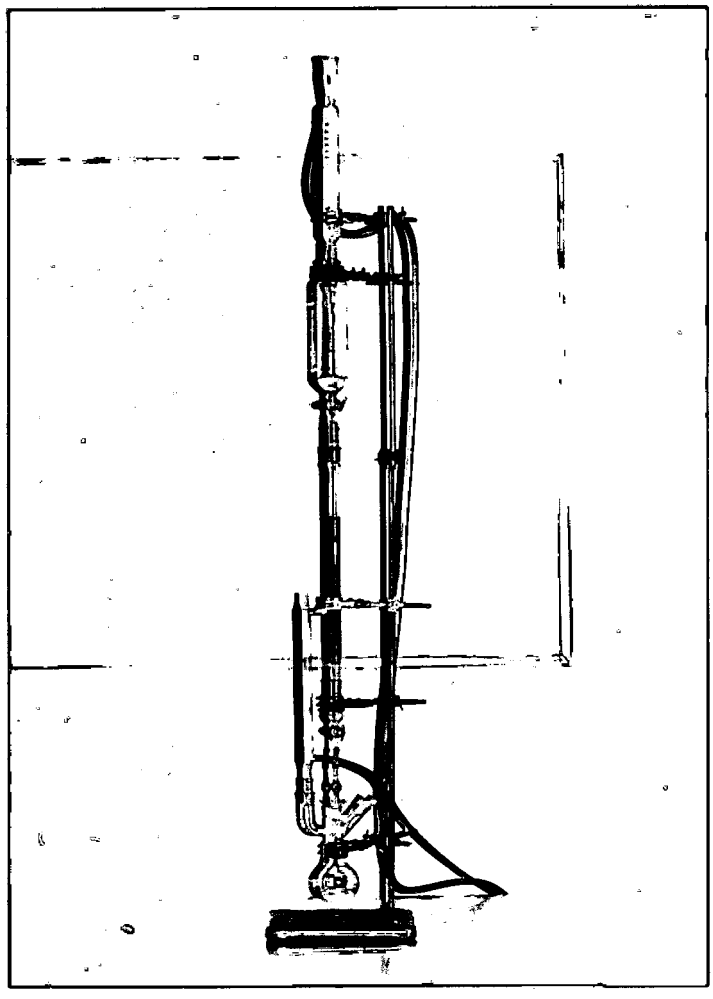
Lipase mediated ester synthesis was also studied by using a packed column reactor. Following are the characteristics of the column.

Length of the column	45 cm
Internal diameter of the column	18mm
Height of the packed material	30cm
Ratio of the enzyme to the filler	variable
Reservoir's volume	500Cm^3
Nature of flow through the column.	Down flow under gravity

The column was packed with the lipase bearing mycelial powder and neutralized sand. The reaction mixture was then passed through it at varying rates. Flow rate of the reaction mixture was controlled by two stoppers, one above and other below the column. To achieve maximum contact between reactants and the enzyme, the reaction mixture was recycled. For recycling, the reaction mixture was lifted from the collecting flask to the reservoir with the help of peristaltic pump.

a) Reactivation of the Enzyme

The enzyme absorbed water in the column and got inactivated after sometime. It was reactivated by passing chilled acetone, chilled acetone - diethyl ether mixture (1:1) and then n-hexane respectively through the column.



PHOTOGRAPH PACKED COLUMN REACTOR

3.3 ANALYSIS

3.3.1 Dry Cell Mass Determination

To determine the fungal biomass the mycelium was filtered through filter paper (wattman 40). It was washed with 0.1N HCl to remove $CaCO_3$ and then with distilled water. The washed mycelium was dried at 105+1°C to constant mass. It was placed in desiccator to bring it at room temperature and then mass was determined[246].

3.3.2 Spore Count

The fungal spores were counted by Thoma Counting Chamber[246].

3.3.3 Lipase Assay

Lipase activity in the fermentation broth or mycelia was determined titrimetrically on the basis of olive oil hydrolysis by the modified method of Samad *et al*[8].

One cm^3 culture supernatant or 10mg mycelial powder was added to the assay substrate containing $10cm^3$ 10% homogenized olive oil in 10% gum acacia, 2.0 cm^3 0.6% CaCl2 solution, and 5.0cm^3 0.2M tris maleate buffer. The enzyme substrate mixture was incubated on shaker with a speed of 100 rpm. At 37oC for 1 hour. 20cm^3 ethanol acetone mixture was added to the reaction mixture to stop the reaction. Liberated fatty acids were titrated with 0.05M alcoholic KOH using thymolphthalein indicator. A lipase unit was defined as

the amount of the enzyme which released one micromole fatty acids per minute at 37°C. The intracellular lipase activity was expressed as units per gram dry mycelium and extraellular lipase activity as units per cm^3. The term "Total lipase activity per dm^3" represents the sum of intracellular and extracellular lipases produced in 1 dm^3 medium. It is calculated as:

$$\text{Total lipase activity per dm}^3 = \left(\text{Intracellular lipase activity per gram mycellium} \times \text{Mycelial mass per dm}^3\right) + \text{Extracellular lipase activity per dm}^3$$

3.3.4 Determination of Lipids

Total lipids present in the fermentation broth, at any stage, were determined by Rose-Gottlieb method[247].

3.3.5 Determination of Moisture

The moisture content of the as the reaction media was determined by Karl Fisher method[248] and the moisture content of this mycellium or the solid substrate was determined by drying at 105±1°C to constant mass[246].

3.3.6 Determination of Proteins

The proteins present in the biocatalyst or the brotlr were determined by Lowery's method[249].

3.3.7. Analysis of Estirification Mixture

The course of esterfication reaction was monitored by free fatty acid titration. Thin layer chromatography, Gas chromatography, G.C Mass

spectroscopy and Infra red spectrometry were also used for the analysis and to monitor the purification of the product.

a) Free Fatty Acid Titration

The samples were drawn at regular intervals from the reaction flasks, and titrated for residual fatty acids, against 0.1M alcoholic KOH using thymolphthalein as an indicator. Blank samples (with inactivated enzyme) were titrated, similarly. The initial reaction rates (esterification activity) of the enzyme were calculated from the slope drawn at the beginning of the progress curve of residual fatty acid concentration. One esterification unit of the lipase was defined as the amount of the enzyme which catalyzes the synthesis of one micro mole of ester, per minute, under the specified conditions. The percentage esterification over certain period of time under certain conditions was calculated as follows:

$$\text{Percentage esterification} = \frac{V1 - V2}{V1} \times 100$$

V1 : Volume of 0.1M KOH (alc) used for 1 cm^3 blank.

V2 : Volume of 0.1M KOH (alc) used for 1 cm^3 sample.

b) Thin Layer Chromatography

TLC was carried out on silica gel (G-60 Merk) coated glass plates (20cm x 6cm x 0.25cm) using solvent system hexane/ether/acetic acid

(80:20:1). The spots were visualized by spraying with H2SO4/Methanol (5:95) and heating at temperature of 110oC until the spots appeared.

c) Infra Red Spectroscopy

Intra red spectra were recorded on Hitachi 270-30 Infra Red Spectrophotometer The product mixture was freed from solvent by evaporation and was pressed between NaCl plates and spectra were recorded (screened between 4000-400Cm-1).

d) Gas Chromatography

The sample of the fusel oil esters was also analyzed by Gas chromatography on Shamadzu G.C-14A apparatus equipped with FID and capillary column Se-30 (25m x 0.22mm i.d.). The temperature of the column was 170oC and Nitrogen was used as carrier gas. The peaks were recorded on Shamadzu C-R 4A Chromatopac and were identified by comparison of their retention time with those of standard samples.

e) GC Mass Spectrometry

Mass spectrometer JEOL - A x 505 combined with gas chromatograph (Hewlett Packard) 5890 and Data Acquisition and Reprocessing JEOL JMA-DA 5500 system was used. Sample was injected into BP-5 capillary column. Helium was used as carrier gas. The oven temperature was maintained at 170oC.

Chapter 4

RESULTS & DISCUSSIONS

4.1. ISOLATION , SCREENING AND SELECTION OF LIPOLYTIC FUNGI

4.1.1 Isolation of Liplytic Fungi

The data of the table-1 shows that about 88 different cultures of fungi were isolated from various sources i.e., 51 from the soil, 10 from milk, 10 from butter, 4 from putrefying fruits and 11 from putrefying vegetables. The fungal colonies were isolated on mineral medium containing olive oil as the sole source of carbon and energy (Table-A of Methodology).

4.1.2 Screening of Lipase Producing Fungi.

The isolated fungi were screened for lipase production in shake flasks. The fermentation medium used for the screening contained (g/dm^3): peptone, 20; glucose, 10; yeast extract, 5.0 and NaCl : 5.0. The flasks were incubated at 30°C for 48 hours on orbital shaker, rotating at 100rpm. After the termination

of fermentation, the extra and intracellular lipase activities of the broth and mycelium were determined. About 70% of the isolated cultureswere found to possess lipolytic activity. The isolation of non lipolytic fungi may be related to the presence of organic matter in the samples. By using same technique Kundu and Pal[44] isolated fungi, 95% of which were lipase positive. The difference in the efficiency seems due to the nature of the samples. Isolate no. 52 was found to produce maximum lipase under the given conditions. It was selected for further studies and identified as *Rhizopus arrhizus*. Each experiment was performed in triplicate and so the reported lipase activity in each case is the mean of three values.

TABLE-1

ISOLATION OF LIPASE PRODUCING FUNGI FROM DIFFERENT SOURCES

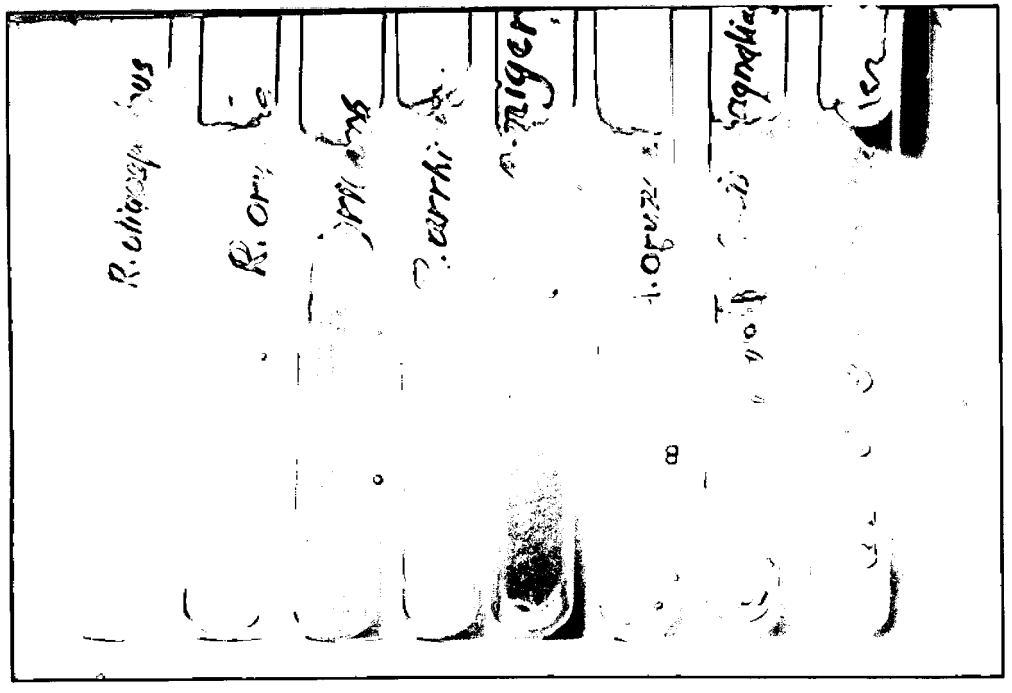
Source	No. Of Specimens Taken	No. Of Isolated Cultures	Assigned Culture No.
Soil	5	51	1-51
Milk	5	10	52-61
Butter	5	12	62-73
Putrefying Fruits	5	04	74-77
Putrefying Vegetables	5	11	78-88

TABLE-2

SCREENING OF ISOLATED FUNGAL CULTURES IN SHAKE FLASKS

Isolate No.	Lipase Activity		Isolate No.	Lipase Activity	
	Intracellular u/g mycelium	Extracellular u/Cm^3		Intracellular u/g mycelium	Extracellular U/Cm^3
1	13.0	0.66	43	13.0	0.98
2	14.6	0.70	44	27.0	0.48
3	38.6	0.8	47	0.0	0.22
4	0.0	0.39	48	14.0	0.76
5	0.0	0.18	49	0.0	0.11
7	8.3	0.42	50	14.0	0.60
8	35.0	0.8	51	8.2	0.40
9	35.0	0.8	52*	85.0	1.0
12	4.8	0.5	53	44.0	0.8
13	44.0	1.0	54	9.2	0.2
15	60.0	0.2	55	30.0	0.42
17	0.0	0.9	57	38.0	0.42
18	6.0	0.5	58	61.0	0.48
20	0.0	0.1	59	5.0	0.10
21	22.0	0.7	60	17.0	0.12
23	20.0	0.6	61	0.0	0.50
26	18.0	0.42	62	12.0	0.50
27	18.0	0.45	63	35.0	0.7
28	0.0	0.8	64	19.0	1.1
31	0.0	0.16	65	70.0	0.6
32	39.0	0.35	67	14.0	0.2
33	56.0	0.15	68	21.0	0.8
34	8.3	0.44	69	41.0	0.6
35	14.0	0.66	72	0.0	0.44
36	22.0	0.56	73	9.5	0.28
37	4.6	0.36	79	12.1	0.52
38	28.0	0.23	81	6.0	0.8
39	31.0	0.18	83	29.0	0.9
40	0.0	0.30	84	0.0	0.4
41	41.0	0.46	86	28.0	0.6
42	20.0	0.44	87	12.0	0.9

* *The Selected Culture*



LIPASE PRODUCING FUNGAL CULTURES MAINTAINED ON POTATO DEXTROSE AGAR MEDIUM.

4.1.3 Selection of Fermentation Medium for Lipase Production By *Rhizopus arrhizus*

The composition of the growth medium is an important factor for the lipase production by a microorganism. It is obvious that each type of mirco-organism requires a different medium to produce lipase at its maximum level.

The selected fungal strain of *Rhizopus arrhizus* was grown in different fermentation media (M_1-M_6) to observe the extracellular and intracellular lipolytic activities. The fermentation was carried out in 250cm^3 conical flasks at 30±1°C for 48 hours on a rotary shaker. The parameters studied, at the termination of fermentation were dry mycelial mass, intracellular lipase activity and extracellular lipase activity. All the experiments were performed in triplicate and the data of figures 1-3 represents means of three experiments.

Medium M_5 was found to be the best for lipase production. It contained (g/dm^3), olive oil, 20.0; yeast extract, 1.0; $(NH_4)_2SO_4$, 5.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25 and $CaCO_3$, 5.0. Fig.1-3 show that when the fungus *Rhizopus arrhizus* was cultivated in the medium M_5, it produced 8.1 g/dm^3 fungal biomass, 150 units intracellular lipase per gram mycelium and 0.95 units/cm^3 extracellular lipase. Although, in the fermentation media M_1 and M_3, comparable amounts of extracellular lipase were produced but intracellular lipase and fungal biomass remained low. In case of the medium M_6, the fungus grew around the solid particles of the substrate. Thus the apparent biomass (as reported in figure-1) was the sum of biomass supporting particles and the actual mycelial mass.

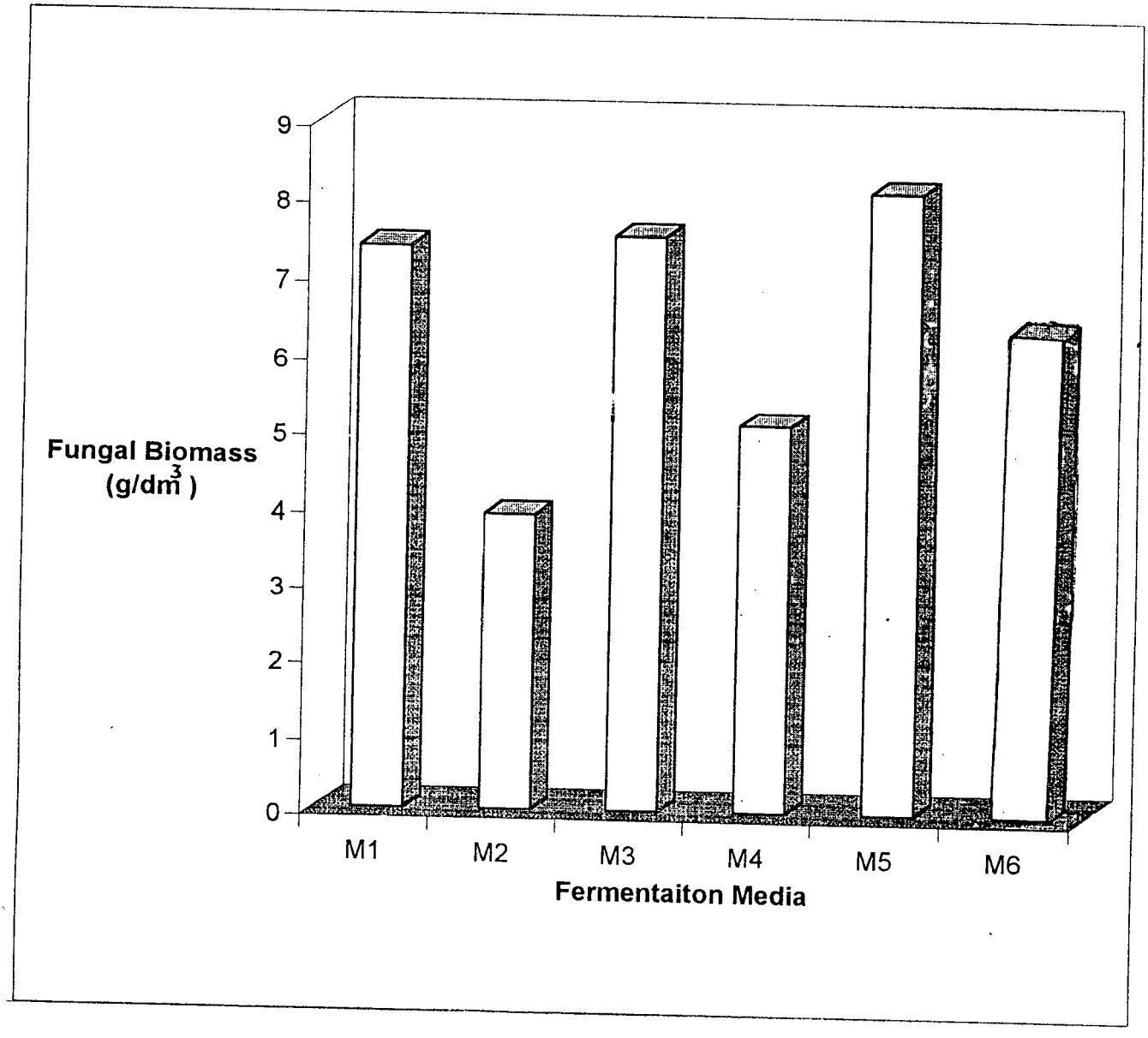


FIG. 1 SCREENING OF FERMENTATION MEDIA FOR MAXIMUM BIOMASS PRODUCTION BY *RHIZOPUS ARRHIZUS.*

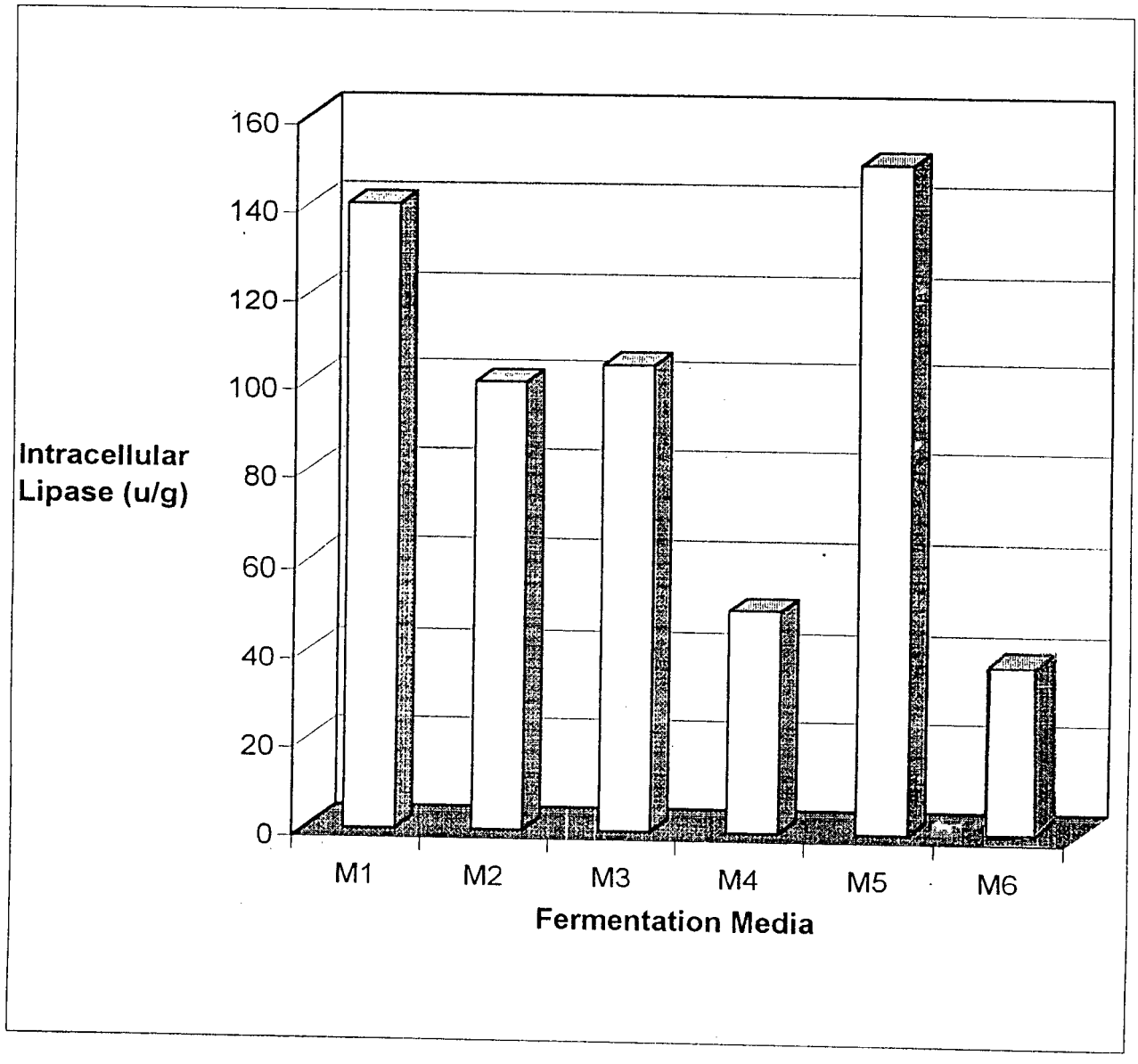


FIG. 2. SCREENING OF FERMENTATION MEDIA FOR MAXIMUM INTRACELLULAR LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS*.

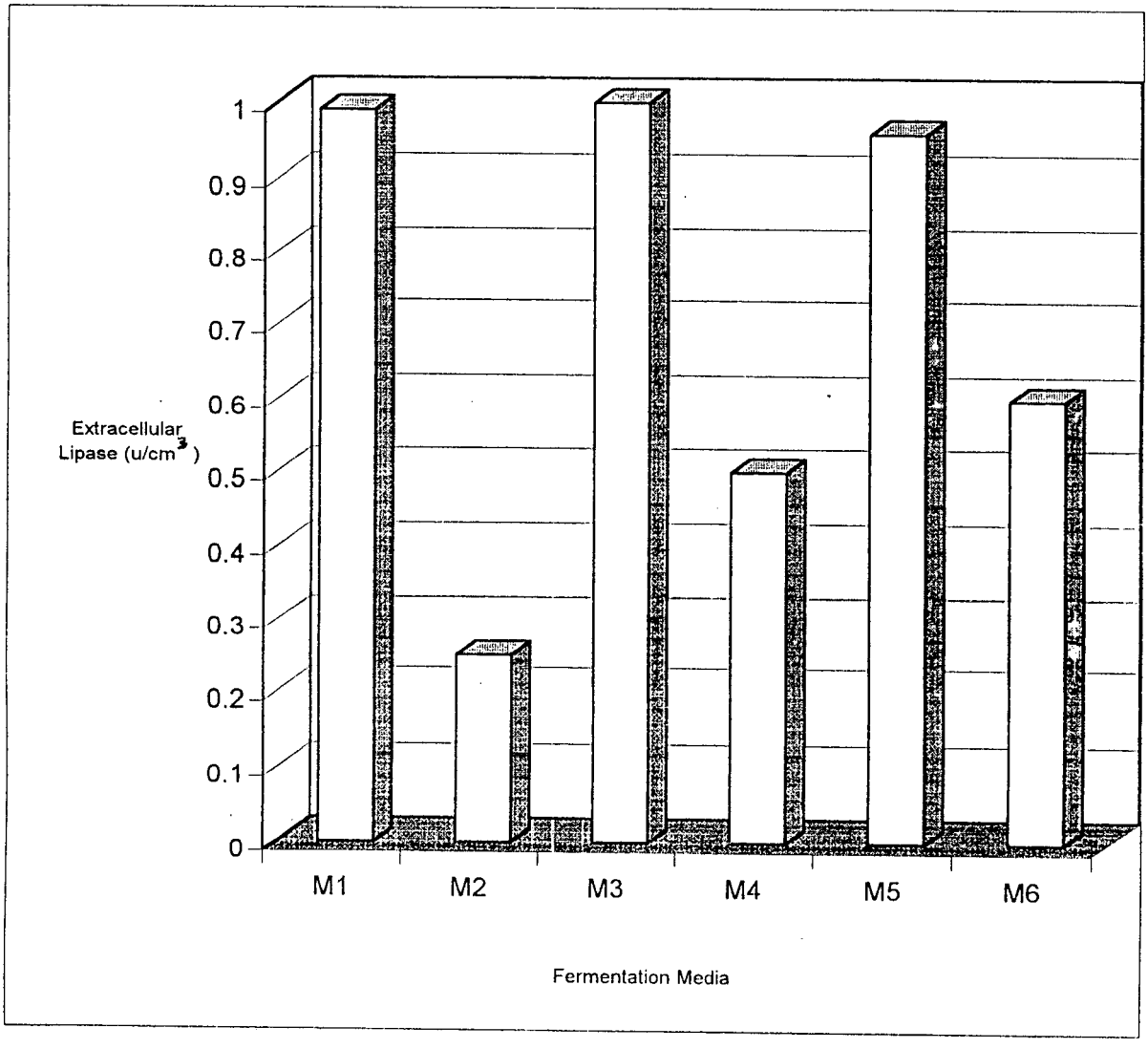


FIG.3. SCREENING OF FERMENTAITON MEDIA FOR EXTRACELLULAR LIPASE PRODUCTION.

4.2 LIPASE PRODUCTION IN SUBMERGED FERMENTATION BY *RHIZOPUS ARRHIZUS*

Rhizopus arrhizus, the mould selected for the lipase production belongs to the class zygomycetes and order mucorales. The strain was isolated from a sample of raw milk and was found to produce intracellular as well as extracellular lipase. Fermentation conditions were optimized for the maximum production of lipases in shake flasks, stirred fermenter and the airlift fermenter.

A. Optimization of Cultural Conditions for Lipase Production By *Rhizopus arrhizus* in Shake Flasks.

The fermentation was carried out at 30°C in 250cm^3 shake flasks, having 50cm^3 fermentation medium. It was found that the strain was predominantly intracellular lipase producer when cultivated in the fermentation medium 'M_5' consisting of olive oil, 20; yeast extract, 1.0; $(NH_4)_2SO_4$, 5.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$,0.25 and $CaCO_3$,5.0. The medium composition and the physcial parameters were optimized. All experiments were performed in triplicate and the data presented in tables and graphs are means of triplicate experiments.

4.2.1. Effect of Organic Nitrogen Sources on Lipase Production by *Rhizopus arrhizus* in Shake Flasks.

The effect of different organic nitrogen sources on the production of lipase by *Rhizopus arrhizus* in shake flasks was studied. For this, the fermentation medium M_5 without ammonium sulphate and yeast extract was used. Different nitrogenous compounds were added to the basal medium keeping the initial concentration of nitrogen at constant level of 1.3 g/dm^3. The fermentation flasks were inoculated using 6% (v/v) vegetative inoculum. The flasks were incubated at $30\pm1^{\circ}C$ for 48 hours, with shaking at 100 rpm.

The nature of the organic nitrogen sources remarkably influenced the fungal growth and production of the enzyme. Fig. 4 shows that the yeast extract was the best nitrogen source. In its presence the fungus produced $10.5g/dm^3$ biomass that had lipase activity of 168 units / g. The culture, also produced 1.6 $units/cm^3$ extracellular lipase. These results are in accordance with the work of Epsinosa *et al* [96] who reported the yeast extract to be the most effective organic nitrogen source for the lipase production by *Rhizopus delemar*. The yeast extract is very important constituent of the medium. In addition to its action as a source of nitrogen it provided a range of micronutrients and minerals required for the fungal growth. The cell bound lipase activity of the mycelium was maximum in the presence of yeast extract followed by the tryptone and the caseitone. The clarified corn steep liquor and the peptone were found to be suitable organic nitrogen sources for extracellular lipase production but intracellular lipase activity per gram mycelium was lower. Similar results have

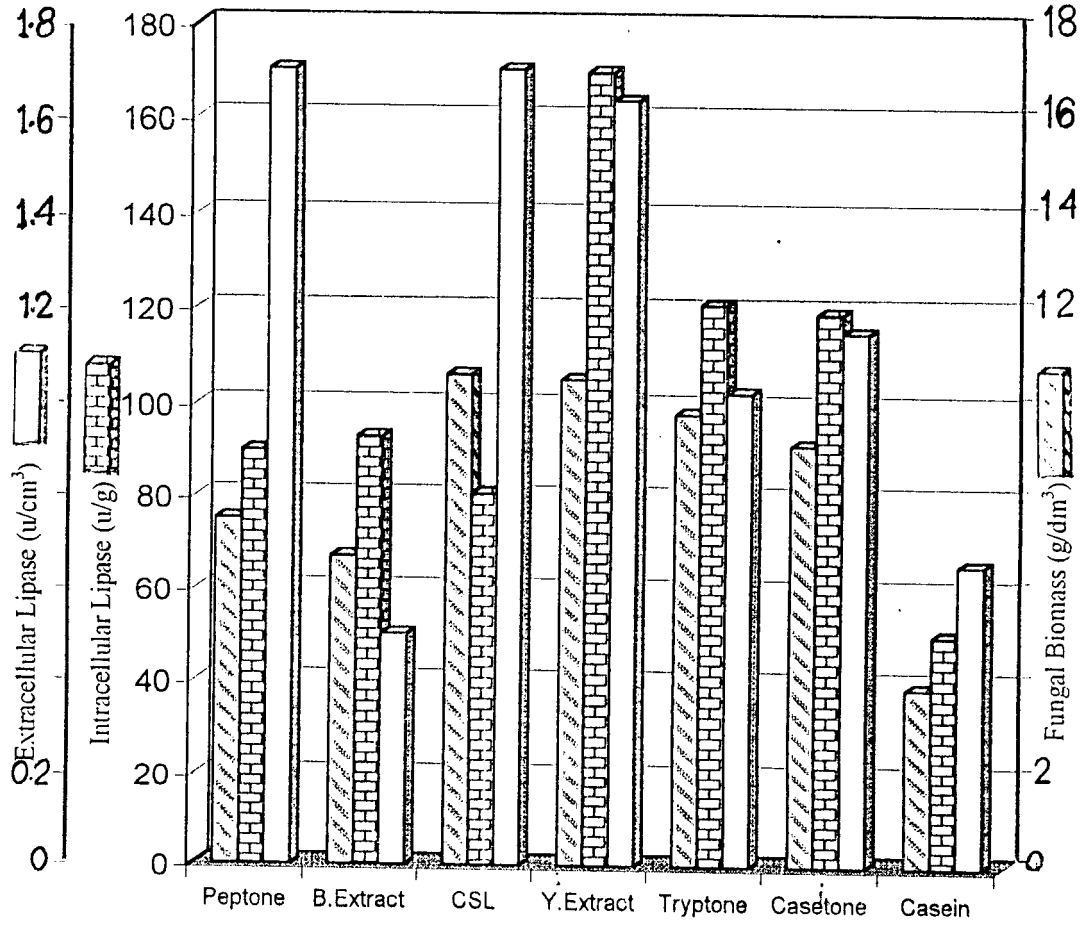


FIG. 4. EFFECT OF ORGANIC NITROGEN SOURCES ON LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS.*

been reported by salleh *et al* [47] in case of thermophilic *Rhizopus oryzae* which produced maximum extracellular liapse in the presence of peptone.

4.2.2 Effect of Inorganic Nitrogen Sources on Lipase Production by *Rhizopus arrhizus* in Shake Flasks.

Figure-5 shows the effect of the addition of different inorganic nitrogen sources such as sodium nitrate, potassium nitrate, ammonium citrate, ammonium nitrate, ammonium sulphate, diammonium hydrogen phosphate, ammonium dihydrogen phosphate and ammonium chloride. It was observed that the ammonium salts were more effective as compared to the nitrate salts. The fungus fulfilled its nitrogen demand from inorganic salts showing that the strain was capable of synthesizing all the amino acids and other nitrogen containing biomolecules from simple nitrogenous salts. Figure-5 shows that $NH_4H_2PO_4$ was rapidly metabolized giving maximum fungal biomass but lipase production was maximum in the presence of NH_4Cl rather than $NH_4H_2PO_4$. So NH_4Cl was selected as a best source of inorganic nitrogen in contrast to Benzoana's report [67] $(NH_4)_2SO_4$ was less effective than NH_4Cl or $NH_4H_2PO_4$. These differences in results might be due to strain differences and synergistic effects with other factors present in the medium.

4.2.3 Combined Effect of Organic and Inorganic Nitrogen Sources.

To find out a more effective nitrogen source for lipase fermentation by *Rhizopus arrhizus*, different combinations of the best organic nitrogen source (yeast extract) and the best inorganic nitrogen source (NH_4Cl) were

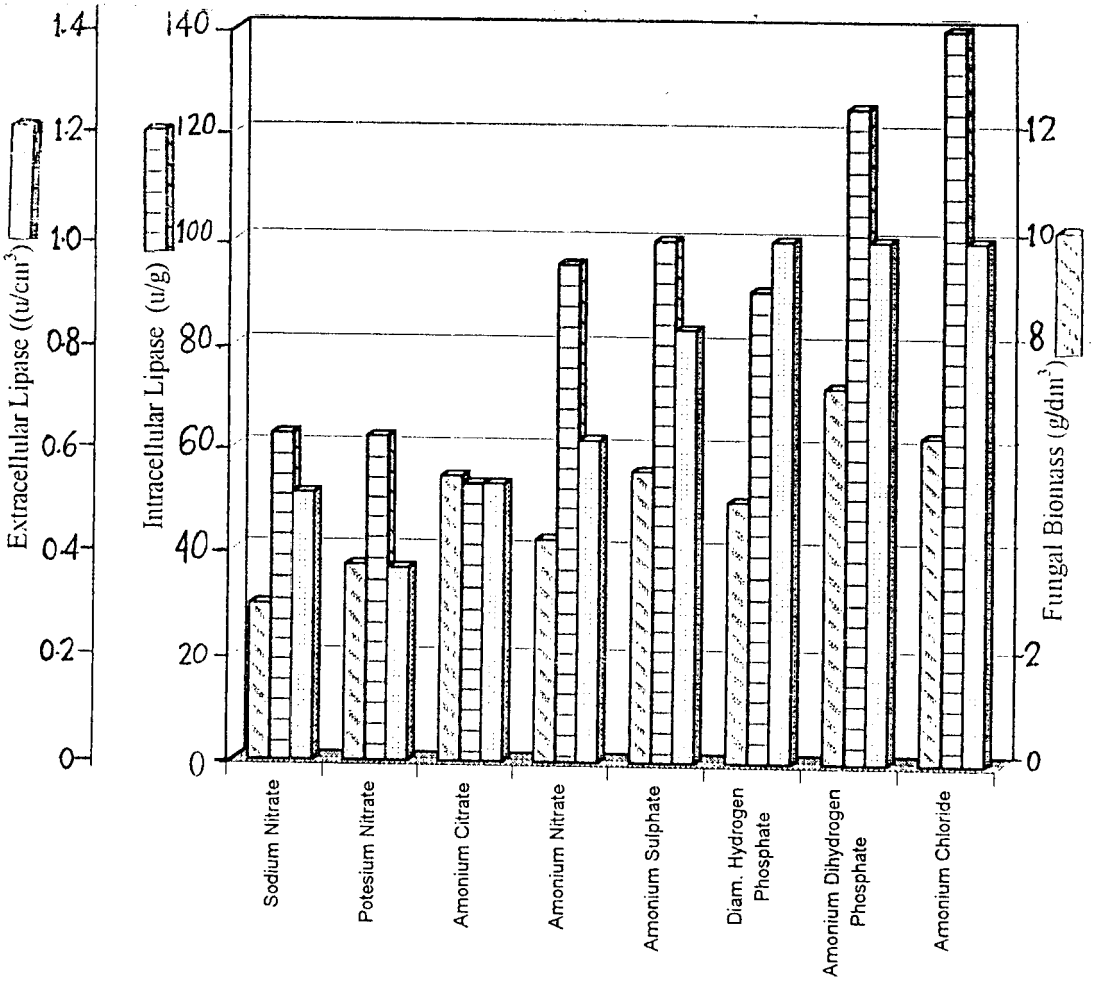


FIG. 5. EFFECT OF INORGANIC NITROGEN SOURCES ON LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS.*

TABLE-3

PARTIAL REPLACEMENT OF AMMONIUM CHLORIDE BY YEAST EXTRACT

NH4Cl (g/dm^3)	Yeast ext. (g/dm^3)	Biomass (g/dm^3)	Intracellular liapse activity (unit/g)	Extracellular lipase activity units/cm^3
5.0	0.0	6.2	140	1.0
4.5	1.0	8.6	166	1.5
4.0	2.0	9.4	175	1.7
3.5	3.0	9.6	175	1.7
3.0	4.0	9.9	175	1.7
2.5	5.0	10.0	170	1.7
2.0	6.0	10.2	170	1.7
1.5	7.0	10.2	170-	1.7
1.0	8.0	10.5	168	1.7
0.5	9.0	10.5	168	1.6
0.0	10.0	10.5	168	1.6

investigated. For this purpose, in a series of experiments NH_4Cl was partially replaced by the yeast extract without changing the total nitrongen content of the medium. Table-3 shows that the replacement of 0.26 g/dm^3 inorganic nitrogen (1.0g/dm^3 NH_4Cl) by same amount of organic nitrogen (2.0 g/dm^3 yeast extract) resulted in 52% increase in the fungal biomass, 70% increase in the extracellular lipase activity and 25% increase in cell bound lipase activity. Further increase in the concentration of yeast extract caused very small increase in the biomass but no increase in liapse activities. Hence combination of yeast extract and NH_4Cl (2g/dm^3 and 4.0 g/dm^3) was selected to enhance the production of lipases by *Rhizopus arrhizus*.

4.2.4 Effect of Nitrogen Level of the Medium on Lipase Fermentation.

To study the effect of nitrogen level different concentrations of the optimized combination of yeast extract and NH_4Cl (1:2 w/w) were used.the data of the table-4 shows that there was little growth in the absence of nitrogen compounds. The fungal growth and the lipase formation increased with the increase in nitrogen content of the medium upto 1.3 g/dm^3 nitrogen (2 g/dm^3 yeast extract and 4.0 g/dm^3 NH_4Cl). Further increase in the concentration of nitrogen sources could not enhance the growth and lipase formation. This may be due to increase in N/C ratio in the medium. Adverse effect of high N/C ratio on lipase synthesis have already been reported by Freir *et al*[90].

TABLE-4

EFFECT OF NITROGEN CONCENTRATION ON LIPASE FERMENTATION OF *RHIZOPUS ARRHIZUS*

Total amount of nitrogen source (g/dm^3)	Amounts of components		Nitrogen level (g/dm^3)	Fungal biomass (u/g)	Intracellular lipase activity (u/g)	Extracellular lipase activity (u/cm^3)
	NH4Cl (g/dm^3)	Yeast Ext. (g/dm^3)				
0.0	0.0	0.0	0.0	0.8	70	0.0
2.0	0.33	0.66	0.433	4.6	127	0.6
4.0	0.66	1.33	0.866	7.4	148	1.0
6.0	4.0	2.0	1.3	9.4	175	1.7
8.0	5.33	2.66	1.73	9.1	175	1.5
10.0	6.66	3.33	2.16	7.6	175	1.1

4.2.5 Effect of Carbon Sources on Lipase Production by *Rhizopus arrhizus* in Shake Flasks.

The composition of the fermentation medium is an important factor in the rate of lipase production by microorganisms. Each microorganism requires a different carbon source to produce lipase at its maximum level. To select the most potent carbon source for lipase production by *Rhizopus arrhizus*, a variety of carbohydrates and lipid substances were used. These substances were added to the basal medium containing (g/dm^3) NH_4Cl, 4.0; yeast extract, 2.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25 and $CaCO_3$, 5.0. The effect of carbon sources on the fungal growth and lipase formation is presented in fig-6. Amongst all carbohydrates tested, the maximum biomass was obtained in the presence of glucose. However, the lipase activity was poor. On the other hand, in the presence of dextrin a good fungal biomass together with high lipase activity was obtained. These results are in good agreement with Iwai *et al*[60] and Espinosa *et al*[96] who reported that starch and dextrin were most potent carbohydreates for maximum lipase formation.

In case of oils their emulsification was found to be very important for their utilization by the fungus. It increased the availability of the lipid substrates in the medium. Fig 6 shows that the replacement of carbohydrates by emulsified oils, in general resulted in an increase in fungal biomass as well as intracellular lipase activities. Extracellular lipase activities, however, were lower than those in the presence of dextrin or glucose. So it is concluded that oils enhance the fungal growth and lipase formation but inhibit the secretion of

the enzyme into the medium. These findings about the influence of trighlycerides on lipase formation and secretion are similar to those of Akhtar *et al*[66] but more experiments are needed to confirm the mechanism of inhibition of the secretion of lipases and their location within the cell in the presence of glycerides. The corn oil was proved to be the best source for the growth and lipase production. When it was present in the medium, the fungus produced 11.4 g/dm^3 biomass, 222 u/g intracellular lipase and 1.4 units /cm^3 extracellular lipase.

4.2.6. Effect of Concentration of Corn Oil on Lipase Fermentation by *Rhizopus arrhizus*

The data of the table - 5 shows that both fungal biomass and lipase yield were increased with the increase in corn oil concentration. The biomass and the lipase activities reached the maximum when the concentration of the oil was 20g/dm^3. Further increase in the concentration of oil did not increase the production of the enzyme. Nakashima *et al*[10] and Epsinosa *et al*[96] also studied the effect of carbon sources on the production of lipases by *Rhizopus chinesis* and *Rhizopus delemar*, respectively. Both of them reported that the formation of lipasees was rapidly decreased when the concentration of carbon source was increased beyonds its optimum level. However, we found that in case of *Rhizopus arrhizus*, negative effect of increased levele of corn oil was negligible. This may be due to water insoluble nature of the oil as compared to soluble carbohydrates used by Nakashima *et al*[10] and Epsinosa *et al*[96], as carbon sources.

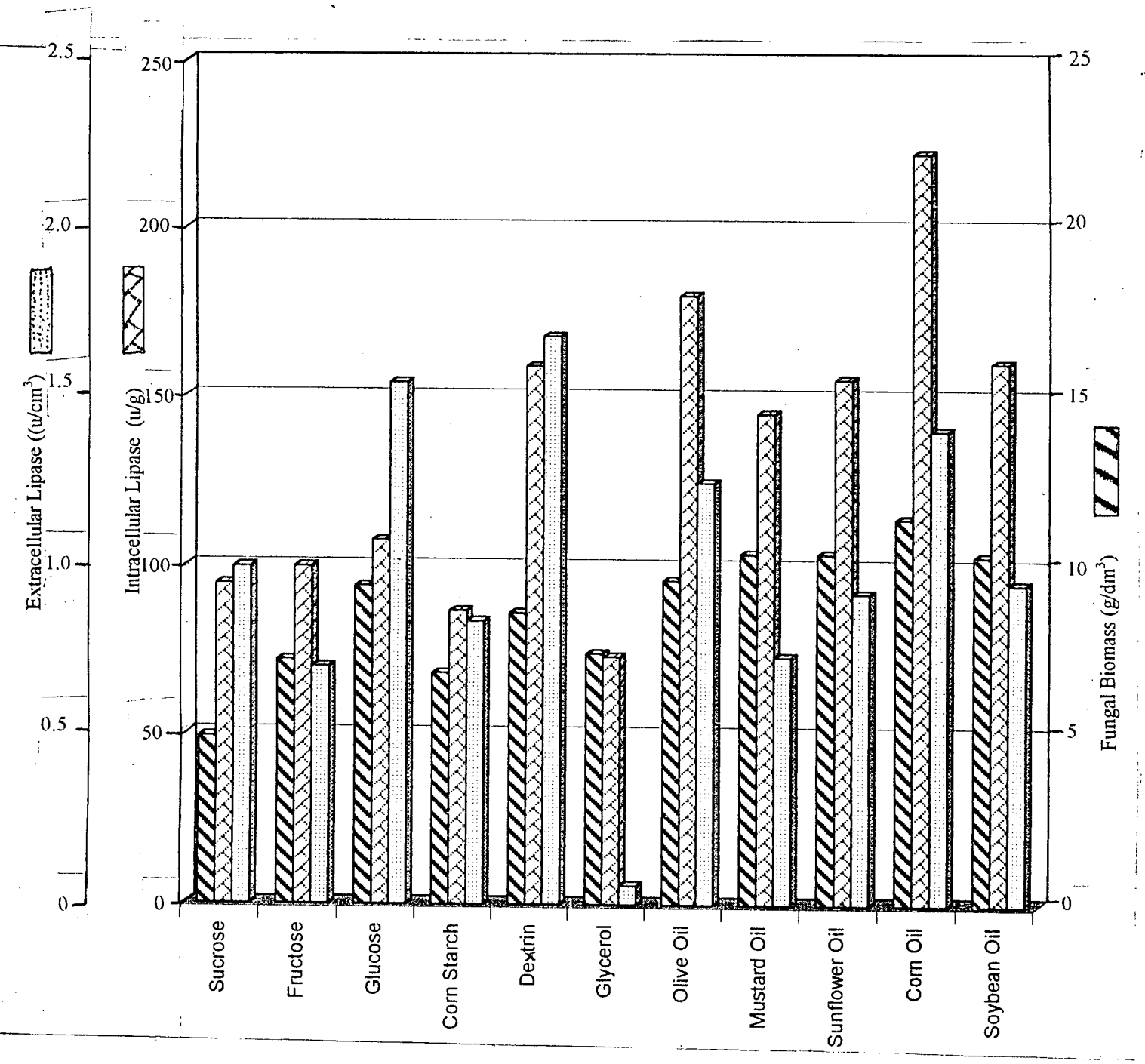


FIG. 6. EFFECT OF CARBON SOURCES ON LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS*

TABLE - 5

EFFECT OF CONCENTRATION OF CORN OIL ON LIPASE FERMENTATION BY *RHIZOPUS ARRHIZUS*

Concentration of Corn Oil (g/dm^3)	Fungal Biomass (g/dm^3)	Intracellular Lipase Activity (u/g)	Extracellular Lipase Activity (u/cm^3)
0.0	2.0	69	0.4
5.0	5.6	125	0.8
10.0	8.4	162	1.0
15.0	10.2	196	1.2
20.0	11.4	222	1.4
25.0	11.4	206	1.1

4.2.7 Effect of Concentration of Inorganic Phosphate on Lipase Fermentation by *Rhizupus arrhizus*

Data of the Fig. 7 shows the effect of different concentrations of dipotassium hydrogen phosphate on the fungal biomass and the lipase yield. When the mould was cultivated in the absence of inorganic phosphate,only 4.2 g/dm^3 fungal biomass was obtained with 96 lipase units per gram mycelium,and the extracellular lipase activity was also very low (0.2 u/cm^3). In this case, phophate required for the synthesis of new DNA, RNA, nucleotides, phospholipids and energy transfer reactions was provided by the yeast extract. However, the phosphate remained the limiting nutrient. It was observed that the fungal growth and enzyme activity per unit weight of mycelia were increased by the addition of $K_2H\ PO_4$ upto 0.5 g/dm^3. Further increase in the concentration of inorganic phosphate was ineffective.

4.2.8. Effect of the Addition of Egg Yolk to the Fermentation Medium.

One of the many substances, which were investigated for increasing lipase production by *Rhizopus arrhizus*, was the Broyler's egg yolk. The yolk was transferred, aseptically, from the egg to the sterilized water and the emulsion thus formed, was added to the sterilized fermentation medium. The addition of the yolk to the fermentation medium was found to enhance both the fungal biomass and lipase production. The data of the table-6 shows that an addition of 10 g/dm^3 yolk to the fermentation medium resulted in 22% increase

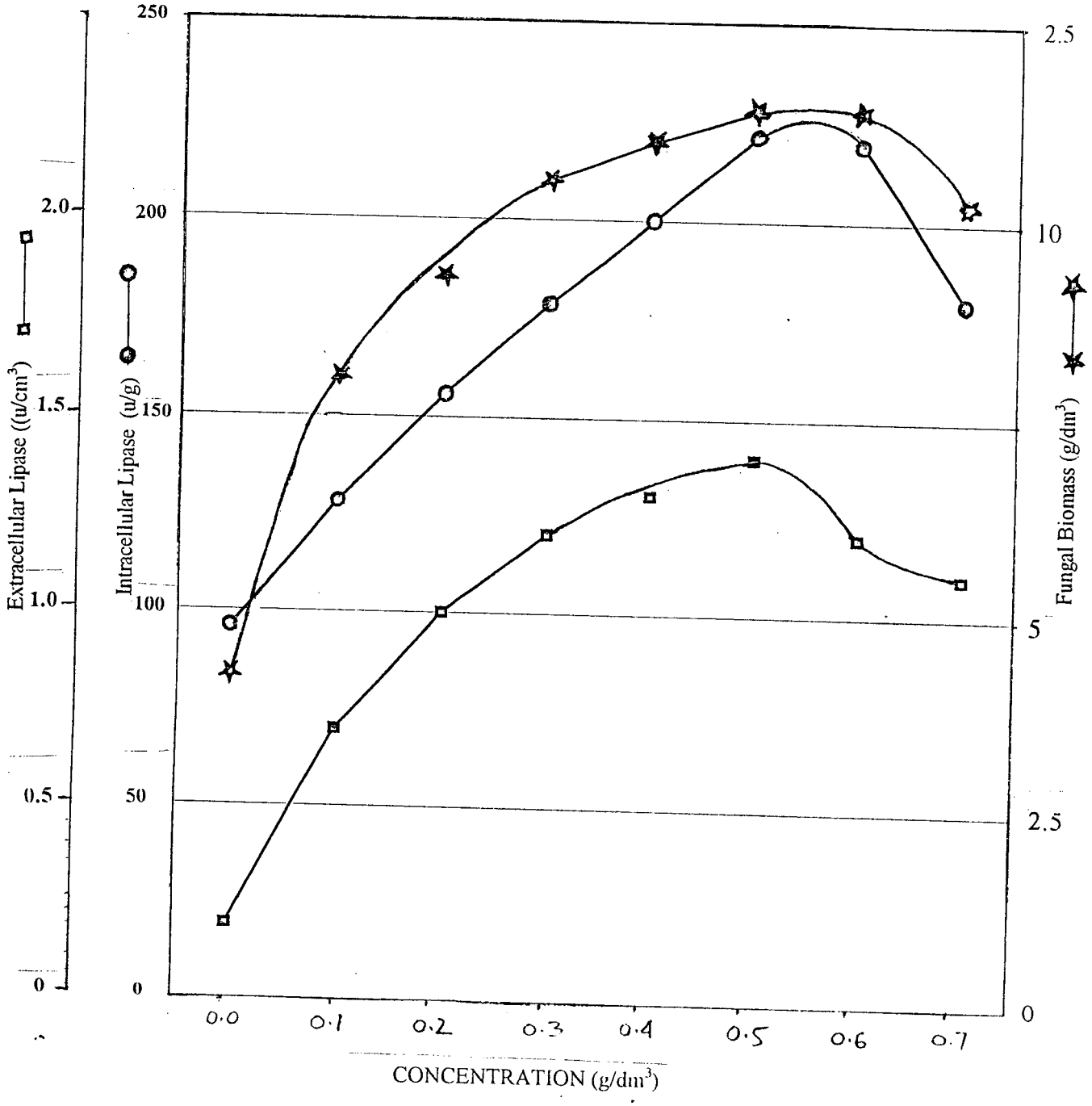


FIG. 7. EFFECT OF CONCENTRATION OF PHOSPHATE ON LIPASE FERMENTATION OF *RHIZOPUS ARRHIZUS.*

in dry mycelial wt, 37% increase in the intracellular lipase activity per gram mycelium and 50% increase in the extracellular lipase activity. Further increase in the level of egg yolk did not cause a significant change in growth and lipase yield. The increase in growth and lipase formation is related to the presence of nutrients especially lipids as shown in a table D.

Lipids of the egg yolk include cholesterol and triglycerides[250]. Simultaneous presence of these two substances in the fermentation medium have stimulatory effect on the fungal growth and lipase synthesis as reported by valero *et al*[53]. This increase in the intracellular lipase activity per gram mycelium may also be related to the retention of the lipase inside the mycelia due to the formation of mycelial aggregates in the presence of egg yolk. A decrease in lipase formation by increasing egg yolk beyond 15% may be related to impaired oxygen supply in the fermentation medium because its higher viscosity.

TABLE-D

COMPOSITION OF EGG YOLK [250]

Water	48.0%
Proteins	17.5%
Lipids	32.5%
Carbohydrates	1.0%
Ash	1.0%

TABLE-6

EFFECT OF ADDITION OF EGG YOLK ON LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS*

Conc. of Egg Yolk (g/dm^3)	Fungal Biomass (g/dm^3)	Intracellular Lipase Acitivity (u/g)	Extracellular Lipase Activity (u/cm^3)
0.0	11.4	222	1.4
5.0	12.8	288	1.6
10.0	13.9	305	2.1
15.0	14.0	292	2.7
20.0	13.5	286	2.0
250	12.0	245	1.0

4.2.9 Effect of $CaCO_3$ on Lipase Production by *Rhizopus arrhizus* in Shake Flasks.

Fig-8 shows the fermentation pattern of *Rhizopus arrhizus* for the production of enzyme lipase in the presence & absence of $CaCO_3$. The parameters studied were pH changes & enzyme formation at different intervals. 0.5% $CaCO_3$ was added in the fermentation medium before inocculation. Initial pH of the fermentation medium was kept at 7.0. It was observed that the $CaCO_3$ did not allow the pH to fall below 6.0 which favoured good mycelial growth as well as good enzyme production. On the other hand, in its absence, a sharp fall in pH was noted which retarded the growth and the lipase formation. The low pH also enhanced inactivation of the preformed lipase. A pH about 2.0 was noted in 48 hours old culture flasks. At such a low pH formation of the lipase was inhibited and inactivation of lipase was quite rapid.

4.2.10. Effect of Temperature on Lipase Production by *Rhizopus arrhizus*

To study the effect of temperature on the production of lipase by *Rhizopus arrhizus*, the fermentation was carried out in shake flasks at different temepratures such as 20°C, 25°C, 28°C, 30°C, 35°C and 40°C.

It is evident from the Fig-9 that the maximum growth of the fungus was observed at 35°C (14.9 g/dm^3) but the mycelium, thus obtained, had only 126 lipase units per gram dry mass. At this temperature, the extracellular lipase activity was only 1.3 units /cm^3. Maximum intracellular lipase activity per unit

mass of dry mycelium (363 u/g) was obtained at 25±1°C. However, an increase in the temperature upto 28°C resulted in a small decrease in the intracellular lipase activity along with considerable increase in the extracellular lipase activity and fungal biomass. The decrease in the intracellular lipase activity by increasing temperature from 25°C to 28°C seems to be related to increased secretion from the mycelium to the borth rather than the decreased formation as indicated by corresponding increase in the extracellular lipase activity.

Although an increase in temperature beyond 28°C, enhanced the fungal growth, but both intracellular and extracellular lipase activities were lowered. Thus, the locally isolated strain of *Rhizopus arrhizus* had different optimum temperatures for the growth and lipase formation. Different optimum temperatures for the growth and lipase production by *Rhizopus oryzae,* have also been reported by Salleh *et al* [47].

The total lipase activity per dm^3 medium, was calculated as discussed in the Methodology. It was found that the total lipase activity per dm^3 of the fermentation medium was the highest at 28±1°C (6787u/dm^3) followed by 6339u/dm^3 at 30±1°C. So 28±1°C was selected as the optimum temperature for lipase fermentation by *Rhizopus arrhizus*.

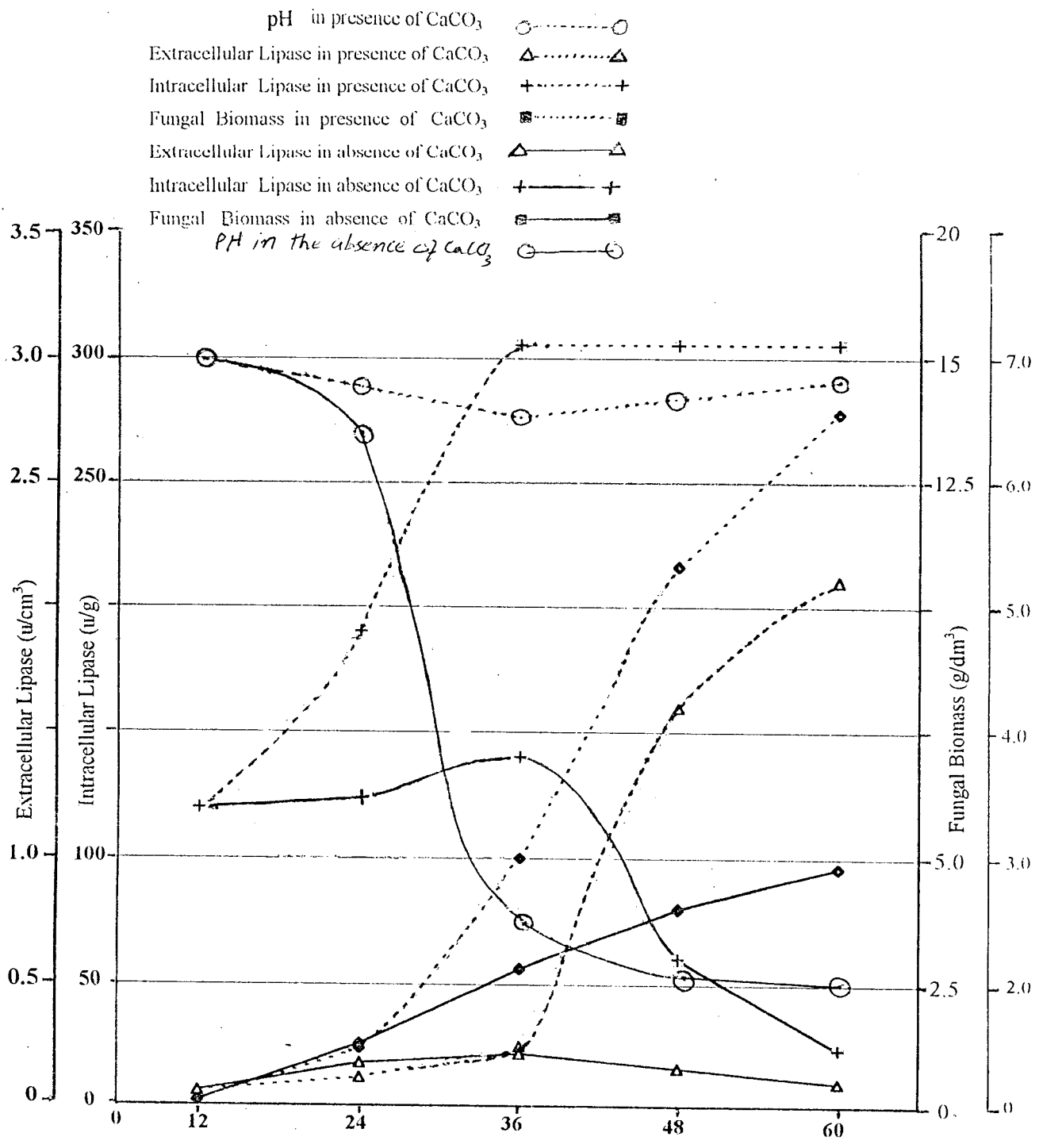


FIG. 8. EFFECT OF CALCIUM CARBONATE ON LIPASE FERMENTATION OF *RHIZOPUS ARRHIZUS.*

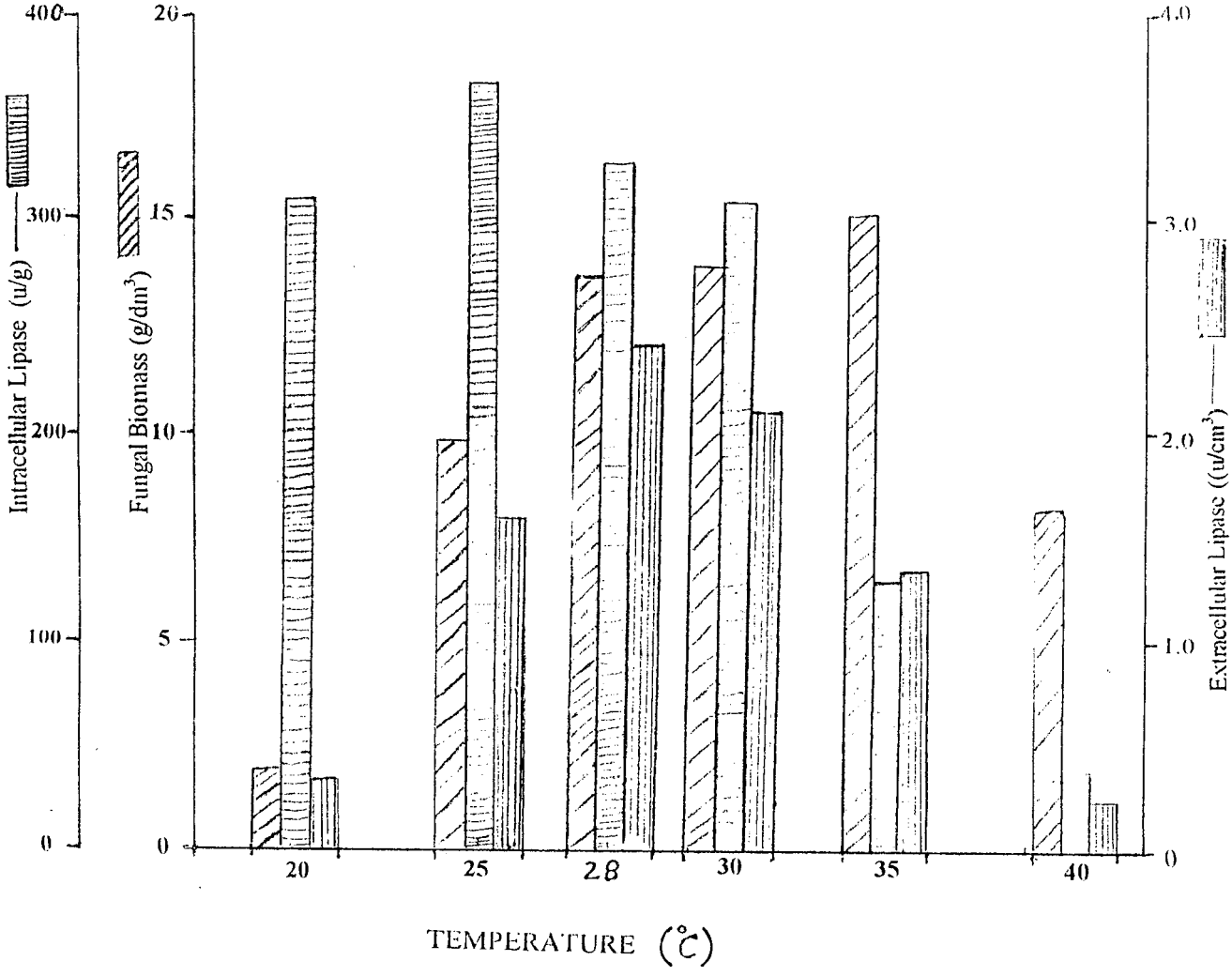


FIG. 9. EFFECT OF TEMPERATURE ON LIPASE FERMENTATION OF *RHIZOPUS ARRHIZUS.*

4.2.11. Effect of Size and Age of Inoculum on Lipase Fermentation by *Rhizopus arrhizus*.

Fig-10 shows the effect of different concentrations of spore suspension or vegetative inoculum. The spore suspension was prepared from 72 hours old culture slants as described in Methodology and the spore count was brought to 10 million spore /cm^3 using sterile distilled water. It was used as inoculum over a range of 1.0% to 5.0% v/v. The fermentation was completed in 72 hours (rather than 48 hours as in the case of vegetative inoculum). The fungal biomass was found to increase with the increase in the level of spore inoculum and reached the maximum value of 10.4 g/dm^3 when the size of the inoculum was 4.0% (4 x 10^4 spore /cm^3 in the fermentation medium). Further increase in the size of the inoculum did not affect the fungal biomass.

Extracellular lipase activity followed the same pattern as the fungal mass. Intracellular lipase activity, however, was least affected by the variations in the size of inoculum and remained at same level of 280u/g dry mycelium.

To investigate the effect of size of the vegetative inoculum (18 hours old) it was used at the rate of 1.0% (v/v) to 7.0% (v/v). The fermentation was carried out at 28±1°C for 48 hours. The fungal biomass and the extracellular lipase activity were increased with increase in the level of vegetaise inoculum upto 6.0%. Intracellular lipase activity, however, remained unaffected. Further increase in the size of inoculum resulted in a negligible increase in the fungal biomass and considerable decrease in intra and extracellular lipase activities. It

is due to depletion of nutrients in the late growth phase of the culture. So lesser enzyme was formed in the late logarthmic phase which is the major stage for the formation of enzymes.

Effect of the age of vegetative inoculum on lipase fermentation by *Rhizopus arrhizus* was also investigated. The age of vegetative inoculum was (12, 18, 24, 30, 36 hrs). The data of the table 7 shows that 18 hrs old inoculum was the most effective. Use of such a juvenile inoculum, has not been reported by any otherworker. It was noted that 18 hours old inoculum became highly viscons. Later palletes were formed. Less lipase formation with 24, 30 or 36 hours older inoculum might be due to the presence of pellets compared to homogeneous mycelial growth observed in 18 hours old inoculum.

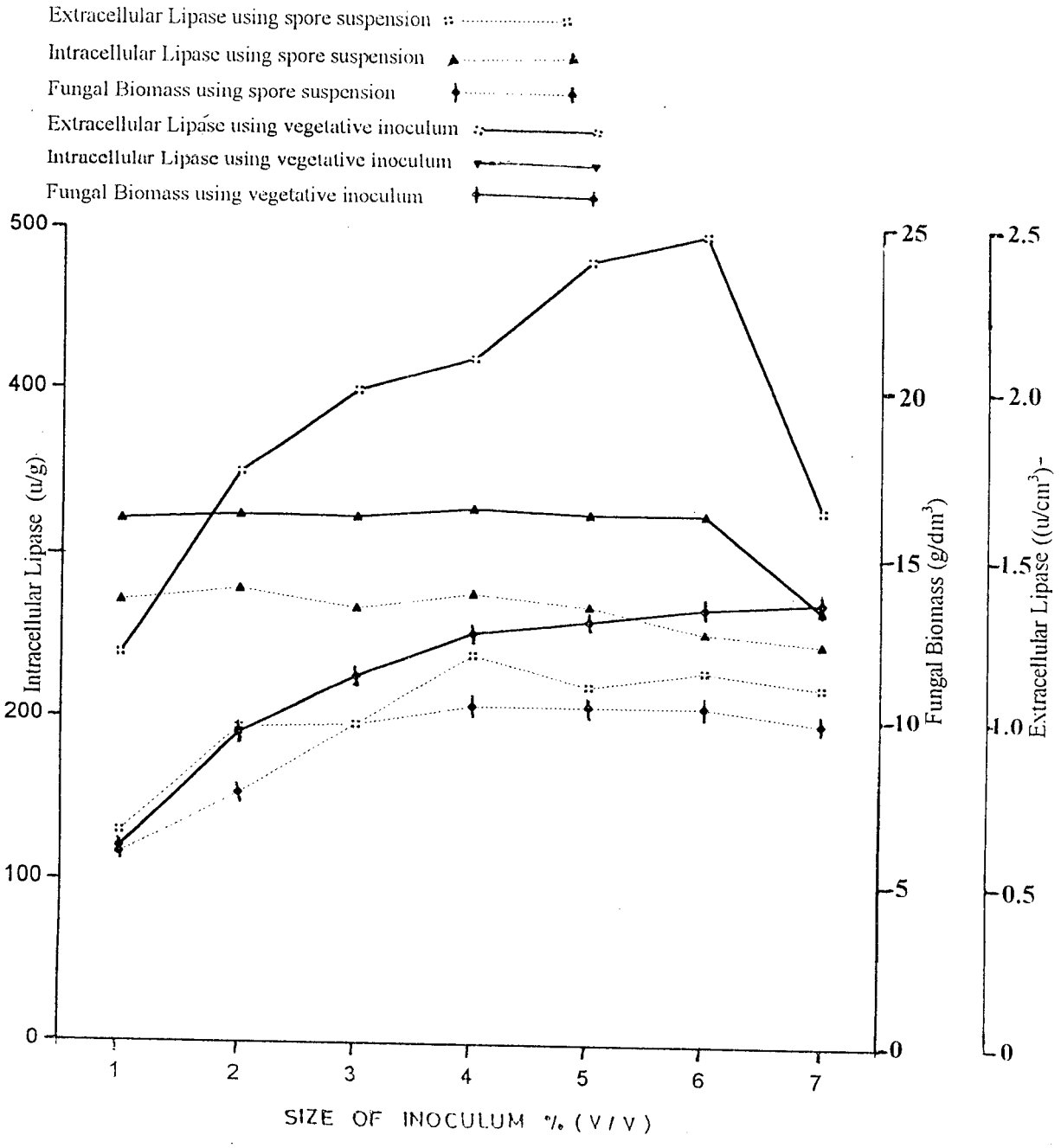


FIG. 10. EFFECT OF SIZE OF INOCULLUM ON LIPASE FERMENTATION OF *RHIZOPUS ARRHIZUS* IN SHAKE FLASKS.

TABLE-7

EFFECT OF THE AGE OF VEGETATIVE INOCCULUM ON LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS*

Age of inoculum (h)	Fungal biomass (g/dm^3)	Intracellular lipase activity (u/g)	Extracellular lipase activity u/ cm^3
12	8.4	292	2.00
18	13.5	32 5	2.48
24	11.7	309	2.3
30	9.4	270	1.6
36	9.0	189	1.6

4.2.12. **Effect of Aeration on Lipase Production in Shake Flasks**

Effect of aeration rate on lipase production by *Rhizopus arrhizus* in shake flasks was studied by changing the speed of shaker as well as the volume of the fermentation medium in the shake flasks. Different volumes of the fermentation medium in 250 cm^3 conical flasks such as 20, 30, 40, 50, 60, 70 or 80 cm^3 and different speeds of the shaker such as 50, 100 or 150 rpm were used (Table-8). The maximum amount of lipase was produced in the shake flasks which contained 50 cm^3 of the fermentatin medium and were shaken at 100 rpm. At lower speeds of the shaker or more medium per flask, the fungal growth and lipase formation were reduced. The fungal growth and the lipase foramtion were reduced in such cases, because dissolved Oxygen became the limiting nutrient[251]. Chander *et al* [51, 61] obtained similar results after working on *Penicillum chrysogenum* and *Aspergillus wentii*. He found that a shaking speed of 200 rpm was the best for lipase production in 250cm^3 Erlenmeyer flasks containing 50cm^3 fermentation medium.

TABLE - 8

EFFECT OF AERATION ON LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS* IN SHAKE FLASKS

Volume Per Flask (Cm^3)	Shaker's Speed					
	50 rpm		100 rmp		150 rpm	
	Fungal Biomass (g/dm^3)	Total Lipase Activity (u/dm^3)	Fungal Biomass (g/dm^3)	Total Lipase Activity (u/dm^3)	Fungal Biomas (g/dm^3)	Total Lipase Activity (u/dm^3)
20	11.2	4880	12.9	6450	9.0	4132
30	10.9	4900	13.0	6500	10.2	4908
40	9.8	4170	13.5	6867	11.5	5244
50	8.6	3691	13.5	6867	12.3	5800
60	7.0	2866	12.8	6295	12.4	5478
70	5.8	1950	11.0	5760	12.0	5800
80	4.8	900	7.0	4800	12.0	4900

4.2.13. Time Course of Lipase Fermentation by *Rhizopus arrhizus* in Shake Flasks

The rate of growth and enzyme production by microorganisms is quite important to understand their fermentation pattern. Figure-11 shows the rates of production of fungal biomass, extracellular lipase activity and intracellular lipase activity during lipase fermentation by *Rhizopus arrhizus* at 28±1°C for 96 hours. It was found, that the culture entered into the logarithmic growth phase after 12 hourse of incubation. Maximum growth rate was observed between 12 and 48 hourse of fermentation. After 60 hours, there was no increase in the mycelial dry mass. The intracellular lipase activity after 12 hours of incubation was 150 u/g. It started increasing and reached upto 318 u/g after 36 hours fermentation. It remained about the same (i.e. 325 u/g at 48 hours) till 48 hours and then started decreasing and reached to 105 u/g at 96 hours. The rate of lipase secretion into fermentation broth was maximum between 24 and 48 hours. However, maximum accumulation of extracellular lipase in the broth was observed after 60 hours fermentation, which was decreased later. For intracellular lipase production by *Rhizopus arrhizus* the optimum fermentation duration was 48 hours and for extracellular lipase production it was 60 hours. Benzoana[67] found that the maximum extracellular lipase by *Rhizopus arrhizus* was produced after 40 hours of inoculation whereas as the fungal biomass was reported to reach the maximum between 20 and 25 hours of inoculation.

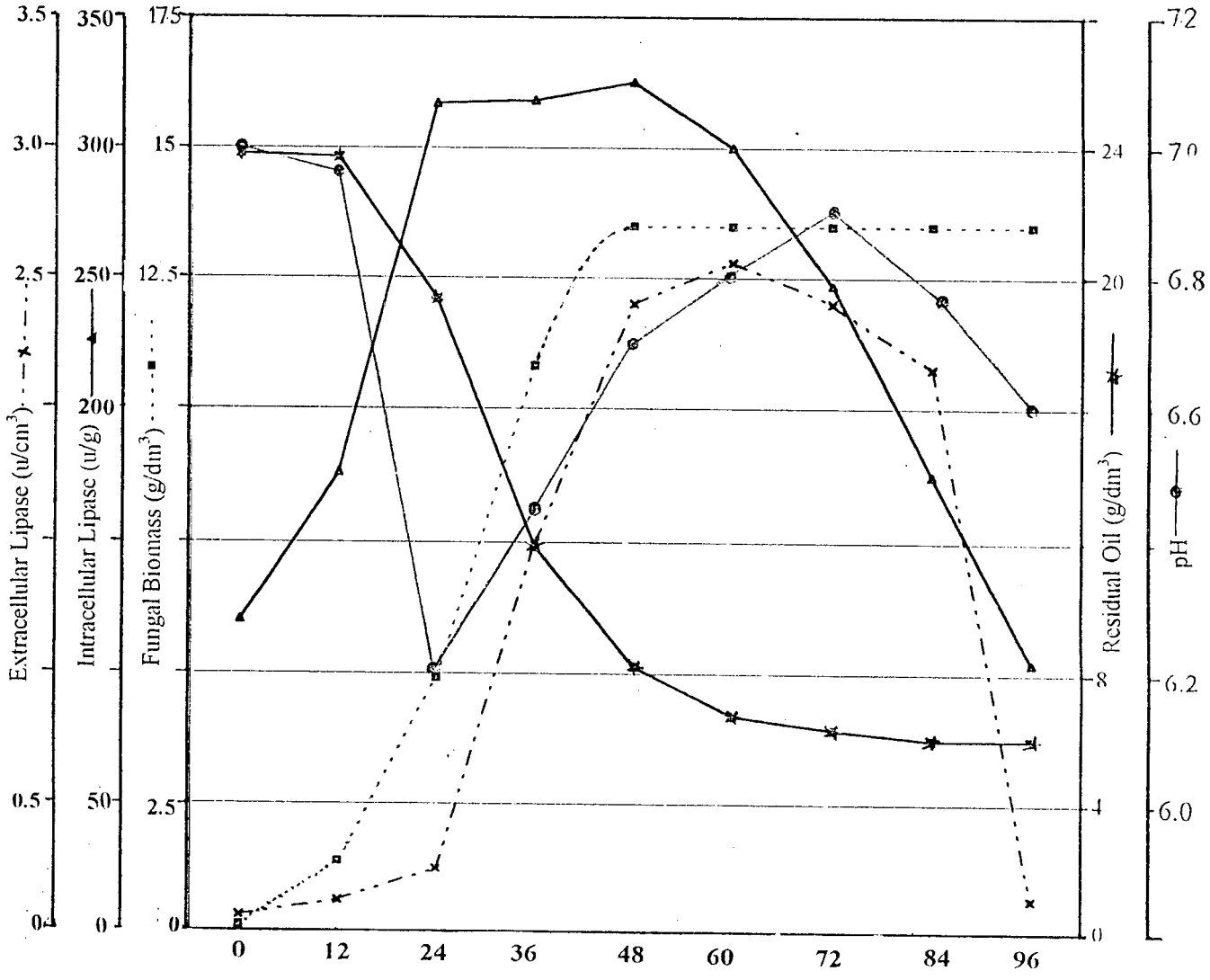


FIG. 11. TIME COURSE OF LIPASE FERMENTATION BY *RHIZOPUS ARRHIZUS* IN SHAKE FLASKS.

B. Lipase Production by *Rhizopus arrhizus in* Stirred Fermenter

Lipase fermentation was also carried out in 18dm^3 glass jar fermenter of following dimensions.

CONFIGURATION OF GLASS JAR FERMENTER

(VirTis USA Model 43-100)

Fermenter diameter	220 mm
Fermenter height	480 mm
Impeller diameter	18 mm
Impeller diameter / fermenter diameter	0.08 mm
Number of impellers	3.0
Type of impellers	Open turbine
Number of blades	6.0
Blade width	14 mm
Type of sparger	L-shape (sintered stainless steel)
Number of baffles	4.0
Baffle width	20 mm
Impeller maximum speed	500 rpm

4.2.14.Effect of Agitation on Lipase Fermentation by *Rhizopus arrhizus* in 18dm^3 Glass Jar for Fermenter.

Agitation is very important for the distribution of nutrients and oxygen in the fermenter. To study the effect of stirring speed on lipase fermentation in 18dm^3 jar fermenter, the fermentation was carried out at different speeds of 20, 50, 100 150 and 200, rpm while keeping all other parameters constant. The fermentation medium was consisting of (g/dm^3) corn oil, 20; egg yolk, 10; yeast extract, 2.0; NH_4Cl, 4.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25 and $CaCO_3$, 5.0. The medium was aerated at rate of 1dm^3.dm^{-3}.min^{-1} throughout the fermentation. The data of the table-9 shows that at 100 rpm the fungal biomass and intracellular lipase activity were maximum (13.0 g/dm^3 and 228 u/g, respectively) at 100 rpm. Further increase in stirring speed resulted in a decrease in fungal biomass and intracellular lipase activity along with a small increase in the extracellular lipase activity (from 2.7 units/cm^3 to 2.9units/cm^3). The decrease in the intracellular lipase per gram mycelium at 150 rpm and higher speeds, may be related to the release of the enzyme from the broken mycelium into the medium due to the higher shear rate. However increase in extracellular lipase activity was not as much as the decrease in intracellular lipase indicating the denaturation of some of the released enzyme.

TABLE-9

EFFECT OF AGITATION RATE ON LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS* IN 18-dm^3 GLASS JAR FERMENTER

Speed (rpm)	Fungal biomass (g/dm^3)	Intracellular lipase activity (u/g mycelium)	Extracellular lipase activity (u/cm^3)
20	5.2	122	0.49
50	9.8	190	1.45
100	13.0	228	2.7
150	11.0	149	2.9
200	8.3	78	1.6

4.1.15. Effect of Aeration on Lipase Production by *Rhizopus arrhizus* in Stirred Fermenter

Moulds require large quantities of oxygen for their growth, multiplication and enzyme production. So, the effect of aeration rate on lipase fermentation in 18-dm^3 glass jar fermenter was studied using optimized medium consisting of (g/dm^3) corn oil, 20; egg yolk, 10; yeast extract, 2.0; NH_4Cl, 4.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25 and $CaCO_3$, 5.0. The fermentation was carried out at 28±1°C with a stirring speed of 100 rpm. The aeration rate was changed from 0.2 dm^3 dm^{-3}. min^{-1} to 2.0dm^3 dm^{-3}. min^{-1}. The data of the table-10 shows that the fungal biomass and lipase activities were increased with the increase in the aeration rate and reached maximum when the aeration rate was kept at 1.0 dm^3 dm^{-3}. min^{-1}. Further increase in the aeration rate upto 2.0 dm^3 dm^{-3} min^{-1} adversely affected the biomass and lipase activities. The extracellular lipase activity, however, was affected more. This may be due to the direct effect of air on the dissolved enzyme. The cell bound enzyme was resistant to the oxidative degradation to such an extent.

TABLE-10

EFFECT OF AERATION ON LIPASE FERMENTATION

BY *RHIZOPUS ARRHIZUS* IN 18dm^3 GLASS JAR FERMENTOR

Aeration Rate ($dm^3\ dm^{-3}\ min.^{-1}$)	Fungal Biomass (g/dm^3)	Intracellular Lipase activity (u/g mycelium)	Extracellular lipase activity (u/cm^3)
0.2	5.8	96	6.3
0.5	8.4	179	1.9
1.0	13.0	228	2.7
1.5	13.0	222	2.0
2.0	11.2	189	0.9

4.2.16. Effect of pH on Lipase Fermentation by *Rhizopus arrtrizus* in Stirred Fermenter.

Fig.12 shows the effect of pH on the biomass yield and lipase production by *Rhizopus arrhizus* in stirred fermenter. The fermentation medium contained $CaCl_2$ instead of $CaCO_3$ and was consisting of (g/dm^3):corn oil, 20.0; egg yolk, 10; yeast extract; 2.0; NH_4Cl, 4.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25 and $CaCl_2.2H_2O$, 5.0. The medium was stirred at 100 rpm and aerated at the rate of $1dm^3dm^{-3}$ min^{-1}. The initial pH of the medium was set with 0.1M NaOH or 0.1M HCl and during fermentation the pH of the medium was monitored and controlled by automatic pH controller uisng of 0.1M NaOH/HCl. There was moderate growth but insignificant lipase production at pH:5.0. An increase in pH upto 7.0 enhanced mycelial growth and lipase formation. At pH 7.0, maximum mycelial mass (12.0 g/dm^3) and lipase production (218 units/g intracellular and 2.7 units/cm^3 extracellular lipase) were observed. Further increase in pH of the fermentation medium lowered both the growth and lipase formation. No growth took place at pH 9.0 and above.

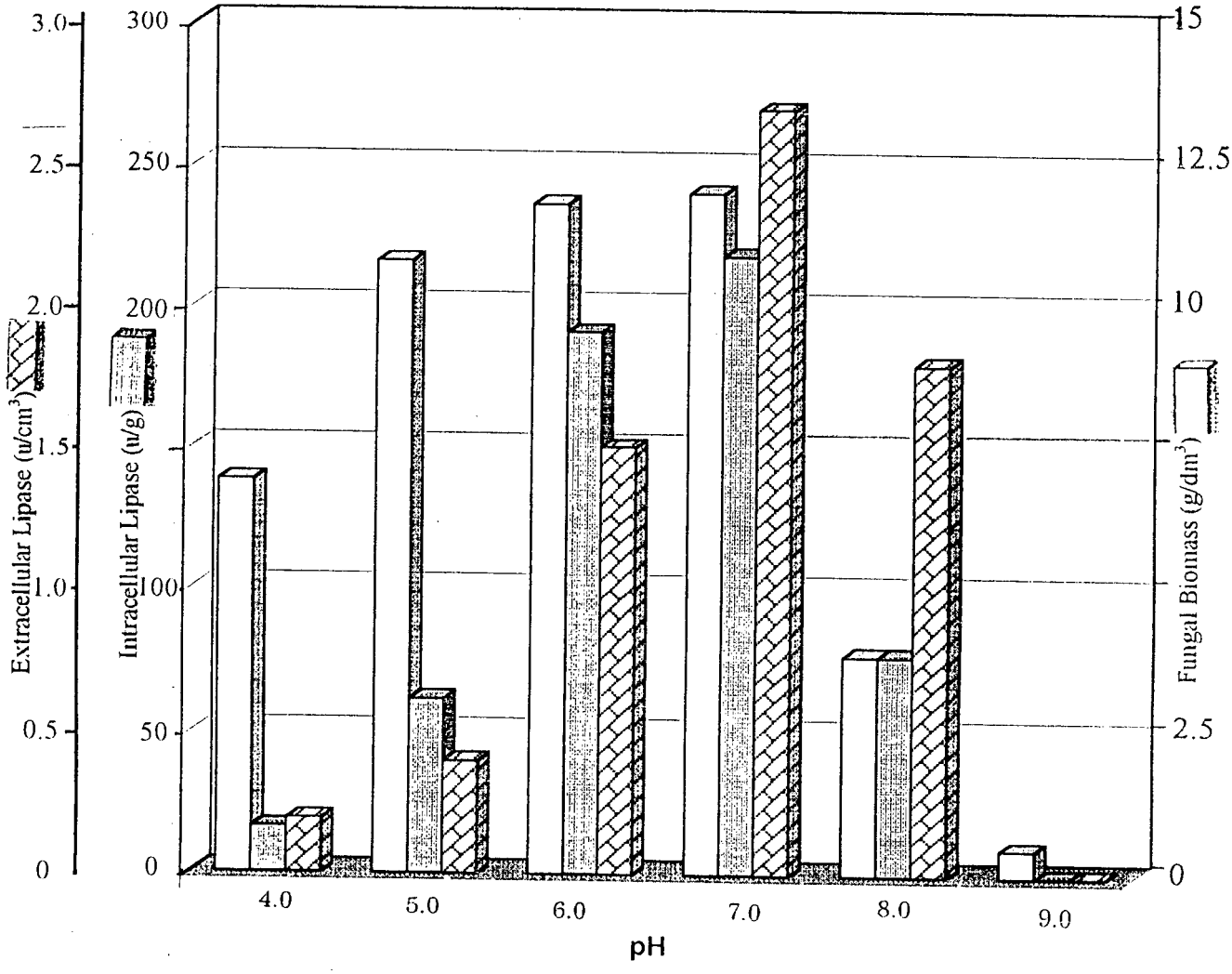


FIG. 12. EFFECT OF pH ON LIPASE FERMENTATION BY *RHIZOPUS ARRHIZUS* IN STIRRED FERMENTER.

4.2.17.Fermentation Profile of *Rhizopus arrhizus* in 18dm^3 Glass Jar Fermenter.

Fig.13 shows the fermentation profile of lipase production by locally isolated strain of *Rhizopus arrhizus* in 18dm^3 glass jar fermenter. The fermentation medium was consisting of (g/dm^3) corn oil, 20.0; egg yolk, 10; yeast extract, 2.0; NH_4Cl, 4.0; K_2HPO_4, 0.5; $MgSO_4$ $7H_2O$, 0.25 and CaCO3, 5.0. The sterilized fermentation medium was inoculated with 6.0% vegetative inoculum. Vegetative cells of the mould took very small time for adaptation and soon entered into logarithmic growth phase. The rates of fungal biomass production and corn oil utilization were the highest between 12 and 36 hours. Maximum fungal biomass (13 g/dm^3) was present after 48 hours fermentation.

The intracellular lipase activity of the inoculum was 157 units /g dry mycelium. After 12 hours fermentation, the intracellular lipase activity was 121 units / gram. However, 12 hour results of four independent experiements showed large variations in intracellular lipase activities ranging from 62 to 143 units / g dry mycelium. It appears that during the adaptation phase, preformed mycelia from inoculum secreted the lipase and little new enzyme was formed. After 12 hours the intracellular lipase activity per gram mycelium began to increase and reached its maximum level (237 units/g dry mass) at 36h and thereafter it remained almost constant till 48h (228 units /g). The intracellular lipase activity per gram mycelium decreased due to inactivation of the enzyme with the time and also its secretion into the medium.

Although the strain of *Rhizopus arrhizus* under the present grwoth conditions was predominontly intracellular lipase producer, some free lipase was always secreted into the borth. The extracellular lipase was gradually accumulated into the broth and attained a value of 2.70 u/cm^3 after 48 h of fermentation. This is in contrast to the work of Benzoana[67] and Akhtar *et al*[66]. According to Akhtar *et al*[66], lipase production reached maximum after 96 hours. Benzoana[67] reported that *Rhizopus arrhizus* secreted most of the lipase into the broth and extracellular lipase activity reached the maximum in 40 hours. These differences in nature and rate of fermentation may be duc to different strains and growth conditions.

Initial pH of the medium was 7.0. It dropped to a value of 6.1 due to acid formation. Further decrease in pH was prevented by the buffering action of $CaCO_3$ present in the medium. After 24 hrs. of incubation, pH increased gradually and reached to 6.7 on the second day of fermentation. This may be due to utilization of organic acids produced in the first 24 hours and anabolic conversin of acidic NH_4Cl into organic nitrogenous compounds. A fall in pH was again noted on the 4th day which might be due to lysis of the cells.

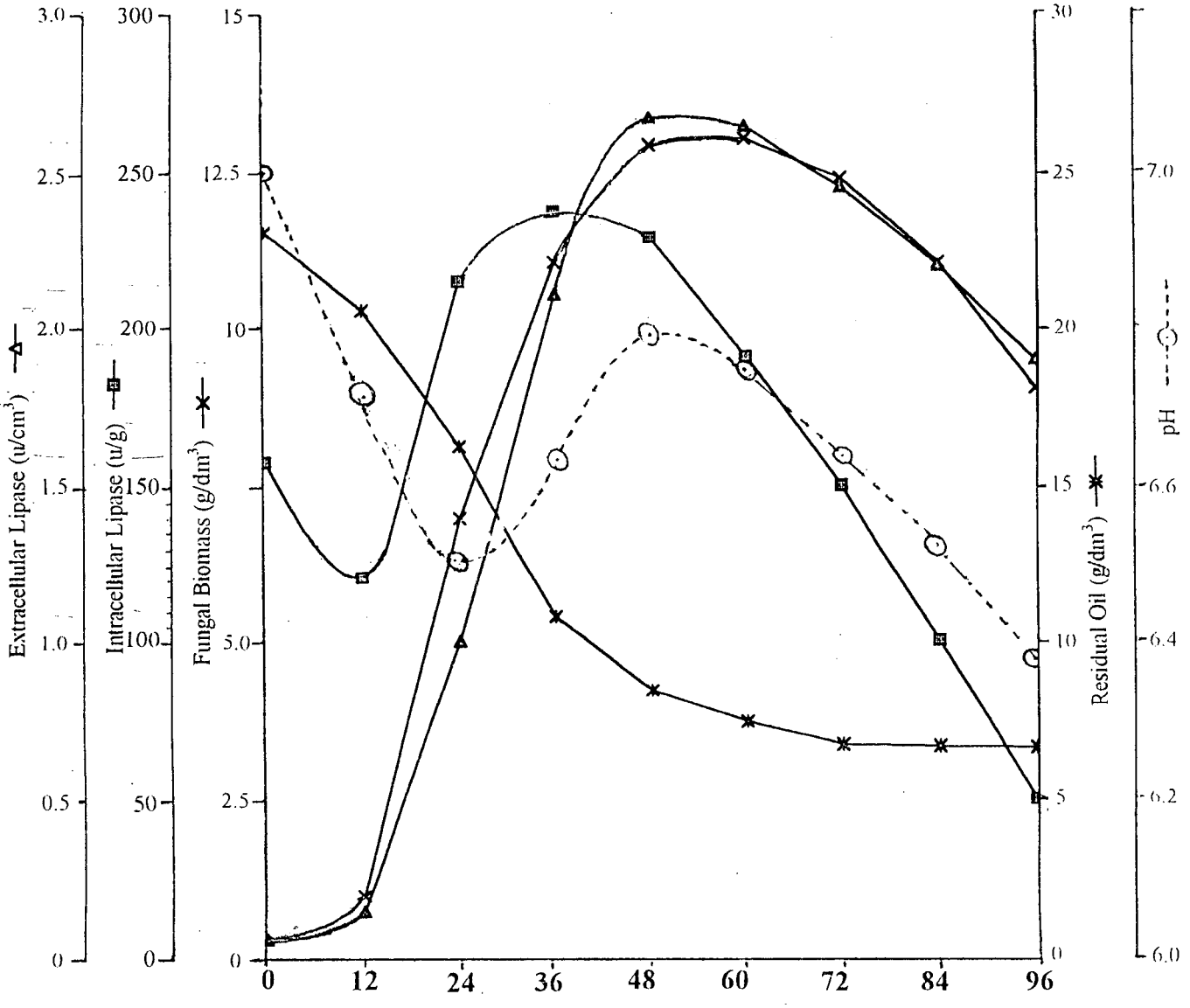
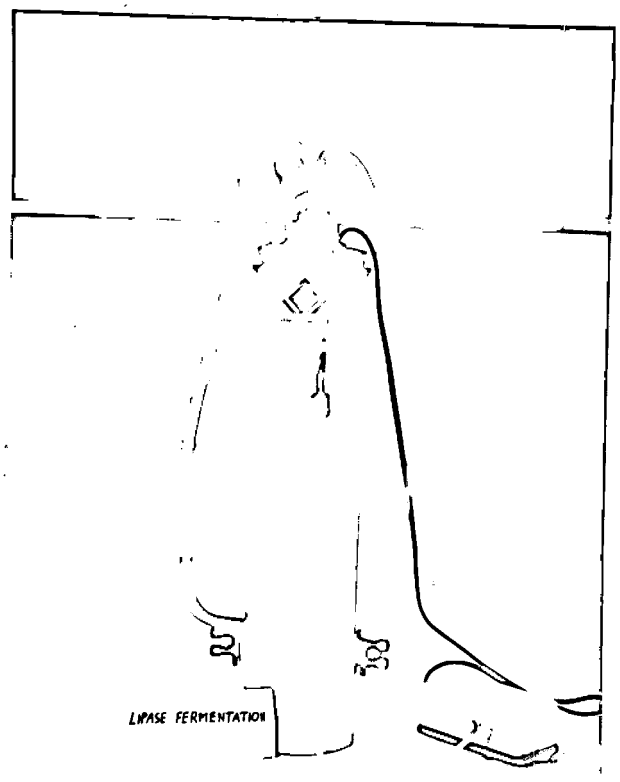


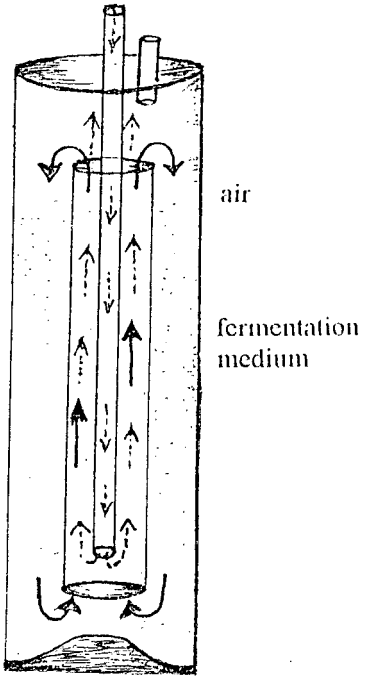
FIG. 13. FERMENTATION PROFILE OF *RHIZOPUS ARRHIZUS* IN 18-DM^3 GLASS JAR FERMENTER.

C. Lipase Fermentation by *Rihizopus arrhizus* *in* 4dm^3 Airlift Fermenter

Airlift fermenters are loop fermenters, which are especially suitable for those fermentations in which low shear rate is required. These fermenters do not have stirring mechanism, instead a circulating flow of the liquid is responsible for mixing. An airlift fermenter consists of two parts, a riser and a downcomer separated by a vertical wall. It appears as a column like vessel inside which a concentric draft tube has been fixed. Aerating the riser produces a circulating flow of the liquid. The presnet study involves 4 dm^3 airlift fermenter (working volume 3dm^3) having a height of 50cm and diameter of 11 cm with a concentric riser tube of 6 cm diameter. The air was sparged 4 cm above the lower end of the riser tube (6 cm above the base of the fermenter).



Airlift Fermenter



Circulation of the medium in air lift fermenter

4.2.18. Effect of Aeration Rate on Lipase Fermentation by *Rhizopus arrhizus* in 4dm^3 Airlift Fermenter

As efficiency of an airlift fermenter is very much dependent on aeration rate, the effect of aeration rate on lipase and biomass production by *Rhizopus arrhizus* was studied. The fermentation medium consisted of (g/dm^3) corn oil, 20.0; egg yolk, 10.0; yeast extract, 20; NH_4Cl, 4.6; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25 and $CaCO_3$, 5. The initial pH of the medium was adjusted to 7.0 and it was inoculated using 18h old vegative inoculum at the rate of 6.0%. Differnet aeration rates, which were investigated for maximum production of the enzyme, were 0.1, 0.5, 1.0, 1.5 and 2.0dm^{3}dm^{-3} min^{-1}. The data of the table 11 shows that both biomass and extracellular lipase were increased with increase in aeration rate upto 1.0 dm^{3}dm^{-3}min^{-1} (14.2g/dm^3 and 2.6 u/cm^3 respectively). Intracellular lipase activity, on the other hand, remained fairly constant (about 370 u/g dry mass) till the aeration rate was upto 1.0 dm^{3}dm^{-3} min^{-1}. A 32% decrease was noted when the aeration rate increased from 1.0 to 2.0 dm dm^{-1}min^{-1}. However corresponding decrease in extracellular lipase activity was much higher i.e. 58%. This may be due to excessive oxidation of the soluble lipase at elevated aeration rates.

TABLE - 11

EFFECT OF AERATION RATE ON THE LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS* IN AIRLIFT FERMENTER

Aeration Rate ($dm^3 \cdot dm^{-3} min^{-1}$.)	**Fungal Biomass** (g/dm^3)	**Intracellular Lipase Acitvity** (u/g)	Extracellular **Lipase Activity** (u/cm^3)
0.1	8.0	362	.20
0.5	11.9	376	1.4
1.0	14.2	370	2.6
1.5	13.9	288	1.6
2.0	13.0	250	1.1

4.2.19.Fermentation Pattern Of *Rhizopus arrhizus* In Airlift Fermenter

Figure-14 shows the fermentation pattern of *Rhizopus arrhizus* in the airlift fermenter. The fermentation medium was consisted of (g/dm3) corn oil, 20.0; egg yolk, 10; yeast extract, 2.0; NH_4Cl, 4.0; K_2HPO_4,0.5; $MgSO_4.7H_2O$, 0.25 and $CaCO_3$, 5.0. The initial pH of the medium was adjusted to 7.0 after sterilization and the medium was inoculated at the rate of 6.0% using 18 hours old vegetative inoculum. The temperature and the rate of aeration were kept constant throughout the fermentation i.e. $28\pm1^{\circ}C$ and 1.0 dm^3 dm^{-3} min^{-1}, respectively. The parameters studied during the course of fermentation were pH changes, dry mycelial weight, residual oil and intracellular and extracellular lipase activities. The inoculum used for the fermentation was 18 hours old consisting of loose mycelia. Ten hours after inoculation the microorganisms in the fermenter turned into fine pellets. The growth reached a maximum value of 14.2 gdm^{-3}, 48 hours after inoculation. A small decrease in the fungal biomass was observed after 60 hours due to cell lysis and decaying.

Initially 23.4 g/dm^3 lipids were present in the medium. These were utilized at the highest rate between 12h and 36h. At the end of 72 h fermentation, 5.0 g/dm^3 extractable lipids were present in the broth.

The initial pH of the medium was 7.0. First it decreased to 6.0 (after 24 hours) and then started increasing till 48 hours. After 60 hours, pH of the fermentation medium again started to decrease and reached 6.3 at the end of

fermentation (96 hours). In first 24 hours the fungus produced acids. The pH could not fall below 6.0 due to the presence of $CaCO_3$ in the medium. The pH rose again due to the utilization of fatty acids between 24-48h. The lysis of the cells started between 60 and 72 hours which decrease in pH so at the termination of fermentation at 96 hours pH of the broth was 6.38 ± 1.

Intracellular lipase activity of 375 u/g was observed after 36 hours of fermentation. It remained at the same level till 48h. Then it gradually decreased and reached 140 u/g after 96 hours. The decrease in lipase activity per gram mycelium might be due to decrease in the synthesis and increase in the secretion with the passage of time. A sharp increase in the extracellular lipase acitivity was observed between 24h and 36h. It reached maximum value of ($2.6u/cm^3$) after 48h of fermentation and after 60h it decreased rapidly probably due to deterioration of the dissolved enzyme.

4.2.20. Comparison Between Stirred Fermenter and Airlift Fermenter

In the airlift fermenter the mycellium grew as small pallets (2-3mm diameter), whereas, a viscous pulpy mass was obtained in case of stirred fermenter.

Th intracellular lipase activity per gram mycelium obtained in case of airlift fermenter was 375 u/g: i.e. 51.8% higher than that in case of stirred

fermenter. The extracellular lipase activity, however, was in the same range (27u/cm^3 and 26 u/cm^3) in stirred and airlift fermenters.

Fermentation in the airlift fermenter required much less energy than the energy needed for fermentation in the stirred fermenter.

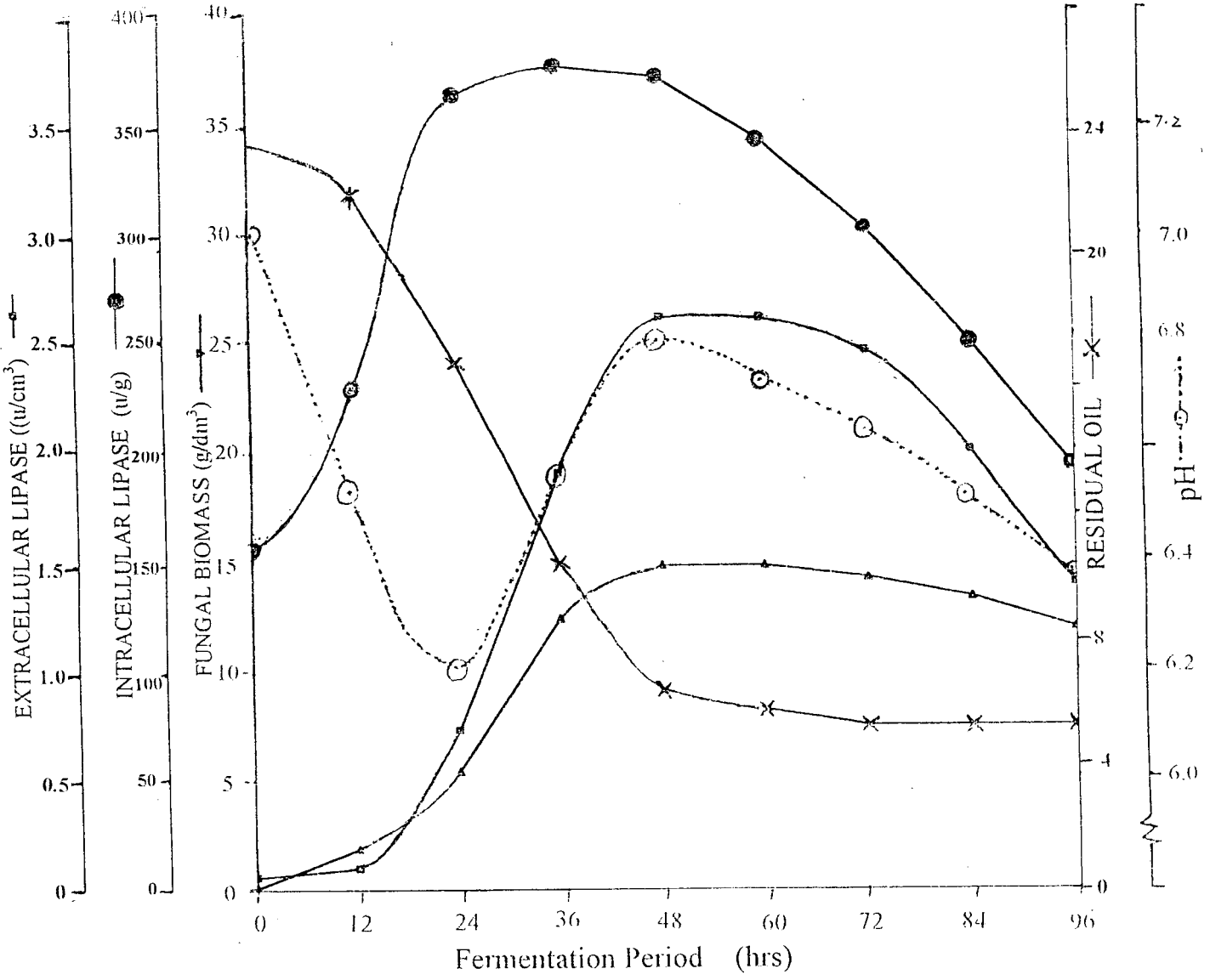


FIG. 14. FERMENTATION PROFILE OF *RHIZOPUS ARRHIZUS* IN 4-DM^3 AIRLIFT FERMENTER.

4.3 SOLID SUBSTRATE FERMENTATION

In solid substrate fermentaiton (SSF) the microorganisms grow on moist solid substrate. The water required by the organisim, exists in an absorbed form in the solid matrix and often ranges between 10% to 80%. Although the SSF is applicable to a limited number of microorganisms and processes, the cultivation of a wide group of moulds on solid media to produce fungal enzymes has opened a new era in the field of industrial biotechnology. It is especially suitable for developing countries like Pakistan as it involves less energy cost, simple equipment and cheaper growth media consisting of agro/industrial by-products.

4.3.1 Evaluation of Different Substrates for the Production of Lipase by *Rhizopus arrhizus* in the Solid State Fermentation

Different agricultural by-products like soybean meal, cotton seed meal, sunflower meal, wheat bran, gram bran, rice bran and rice husk were evaluated as substrates for lipase production. In each flask, 30g substrate was added along with $30cm^3$ distilled water as the diluent. The flasks were autocalved at 121°C for 15 minutes and after inoculation using 4% spore inoculum, were incubated at 28±1°C for 72 hours with occasional shaking. After termination of the fermentation, the fermented mash was soaked in distilled water for one hour. It was then centrifuged and the supernatant was analyzed for lipase activity. Wheat bran was found to be the best substrate for lipase production, giving 6.0 units per gram bran (dry wt. basis). The wheat bran is cheap and abundantly available in Pakistan. Moreover, it is an ideal substrate for

fermentation being rich in nutrients. The soybean meal was the next most effective substrate for lipase production by *Rhizopus arrhizus* giving 4.8 units lipase per gram. The lipase activities were lower with the rice bran, cotton seed meal, sunflower meal, gram bran or rice husks.

4.3.2 Effect of the Depth of Substrate on Solid State Fermentation by *Rhizopus arrhizus.*

Availability of oxygen to microorganisms plays an important role on the production of enzymes by solid substrate fermentation. To study these effects differnet surface/volume ratios of the substrate were used for the production of lipases by *Rhizopus arrhizus*. Different amounts of wheat bran such as 10, 20, 30, 40, 50, 60 or 80 g were taken into $1dm^3$ conical flasks. Distilled water was added according to the weight. of the bran. The depths of the wheat bran were 6, 9, 13, 17, 21, 26, 32 and 42 mm, respectively, in flasks. The fermentation was carried out for 60 hours at 30°C. At the termination of the fermentation it was observed (Table-12) that maximum enzyme production was achieved in the flask containing 20 or 30 g of wheat bran i.e. 6.0 u/g. Further increase in the quantity of wheat bran did not show any increase in the enzyme formation because greater thickness reduced the oxygen supply and caused poor production of the enzyme. Decrease in the production of lipases under reduced air supply have also been reported by other workers[61, 83]. The flask containing 10 gm of wheat bran also gave slightly lower production of the enzyme. It might be due to excessive evaporation of the moisture from thin layers of the bran. So, the 13mm depth of the wheat bran in 250 cm^3 conical flasks was preferred for the maximum production of enzymes.

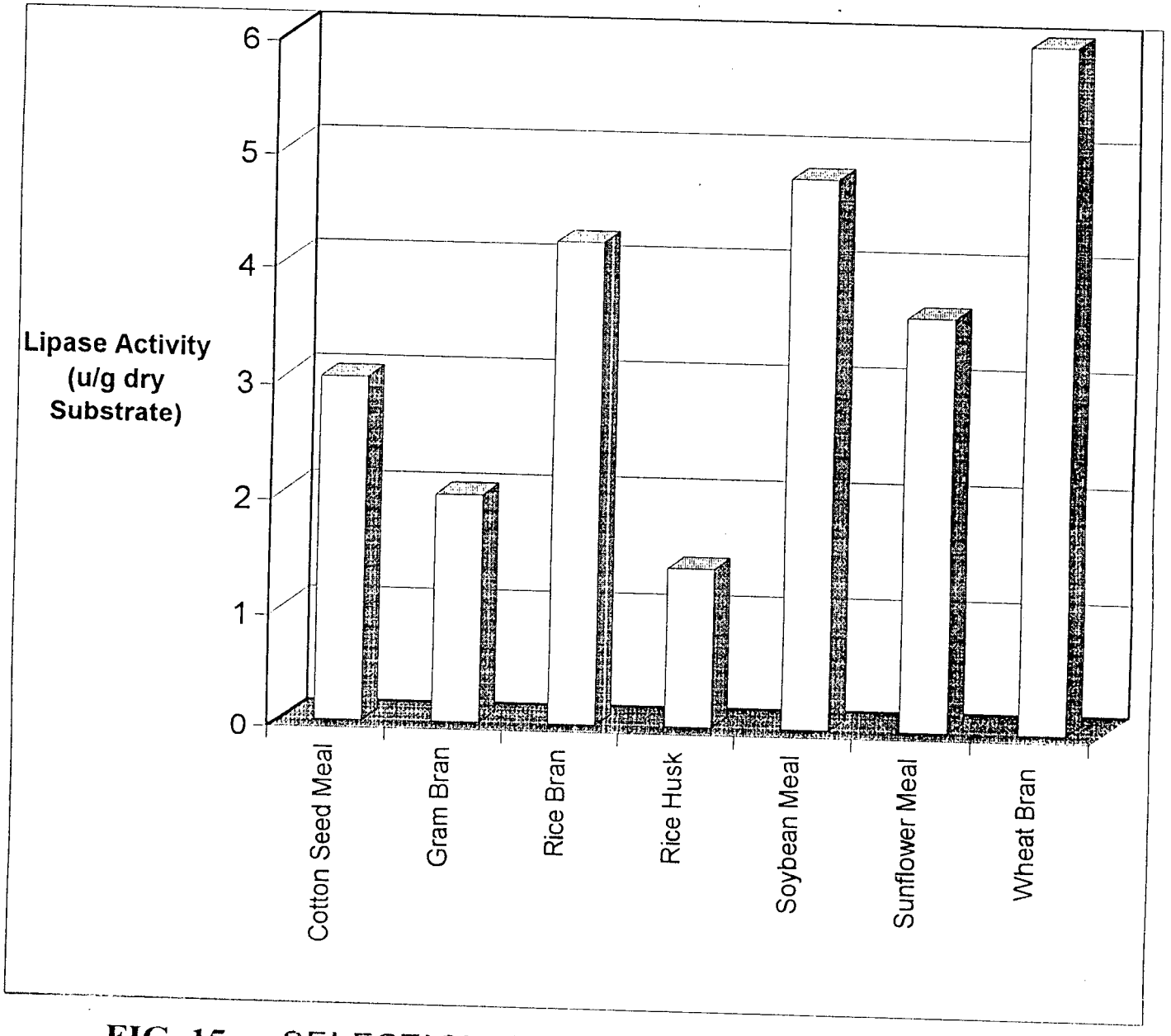


FIG. 15. SELECTION OF SUBSTRATES FOR SOLID STATE LIPASE FERMENTATION BY RHIZOPUS ARRHIZUS

TABLE - 12

EFFECT OF DEPTH OF THE SUBSTRATE ON SOLID STATE FERMENTATION BY *RHIZOPUS ARRHIZUS*

Wheat bran per flask (g)	Depth of the bran layer (mm)	Extracellular lipase activty (u/g. dry Substrate)
10	6.0	5.4
20	9.0	6.0
30	13.0	6.0
40	17.0	4.8
50	21.0	3.9
60	26.0	3.1
70	32.0	2.4
80	40.0	1.8

4.3.3 Optimization of the Moisture Level in the Substrate for Lipase Production by *Rhizopus arrhizus*

In addition to playing the key role in the fungal metabolism water also affects the texture and porosity of the substrate. The wheat bran used was found to contain 10-14% moisture in it. Table-13 shows the effect of addition of water to the substrate on lipase formation. It was observed that the addition of 30g water to 30g wheat bran in flask (1:1 ratio) resulted in maximum enzyme production. It was also noted that at lower level of the moisture, the bran was not properly softened as required for the fungal growth and lipase formation. On the other hand addition of more water caused the bran to clump together and oxygen penetration was impaired which resulted in decreased lipase formaton. The effect of moisture level on solid substrate fermentation has also been studied by Ortiz - Vazquez *et al*[77]. They reported that *Penicillium candidum* produced maximum lipase at the moisture content of 67.5% saturation.

TABLE - 13

OPTIMIZATION OF MOISTURE LEVEL IN THE SUBSTRATE FOR SOLID STATE LIPASE FERMENTATION BY *RHIZOPUS ARRHIZUS*

Ratio of Wheat bran : Water	Lipase Activity (u/g dry substrate)
1.0 : 0.0	--
0.9 : 0.1	0.0
0/8 : 0.2	0.8
0.7 : 0.3	3.2
0.6 : 0.4	5.6
0.5 : 0.5	6.0
0.4 : 0.6	4.5
0.3 : 0.7	1.8
0.2 : 0.8	~0.0
0.1 : 0.9	~0.0
0.0 : 1.0	0.0

4.3.4 Effect of Partial Replacement of Wheat Bran by Soybean Meal

Effect of partial replacement of the wheat bran by soybean meal on biosynthesis of lipase was studied. The table-14 shows that the lipase production was increased by 51% on the replacement of 20% wheat bran (w/w) by soybean meal. Further increase in the concentration of SBM lowered the lipase formation.

4.3.5 Selection of Diluent for Solid Substrate Lipase Fermentation by *Rhizopus arrhizus.*

Fig.-16 shows the effect of addition of different diluents to the solid substrate on lipase production by *Rhizopus arrhizus*. In each case 30g solid substrate (24g wheat bran and 6.0g soybean meal) was wetted by an equal amount of diluent ($30cm^3$) before sterilization. However, in case of egg yolk the substrate was wetted by $20cm^3$ distilled water and sterilized, cooled and then $10cm^3$ egg yolk emulsion of varying centractions, prepared in sterlized water, was added aseptically to get the desired concentration of the yolk in the medium. The contents of flasks were analyzed after 60h fermentation for lipase activity. The fig-16 shows that the replacement of water by phosphate buffer or oils resulted in an increase in lipase biosynthesis but the best diluent was 2% egg yolk emulsion. In its presence 17.5units lipase per gram dry substrate were obtained. The increase in lipase formation in the presence of lipids has been reported by many workers. The egg yolk contains glycerides and cholestrol. It has already been reported by Valero *et al*[53] that the simultaneous presence of glycerides and cholestrol has stimulating effect on lipase synthesis.

TABLE - 14

EFFECT OF PARTIAL REPLACEMENT OF WHEAT BRAN BY SOYBEAN MEAL, ON LIPASE PRODUCTION BY SOLID STATE FERMENTATION BY *RHIZOPUS ARRHIZUS*

Ratio Wheat Bran :Soybean Meal	Lipase Activity (units/g . dry substrate)
10 : 0	6.0
9 : 1	7.2
8 : 2	9.1
7 : 3	8.1
6 : 4	7.4
5 : 5	7.0
4 : 6	6.8
3 : 7	6.6
2 : 8	6.3
1 : 9	5.7
0 : 10	4.8

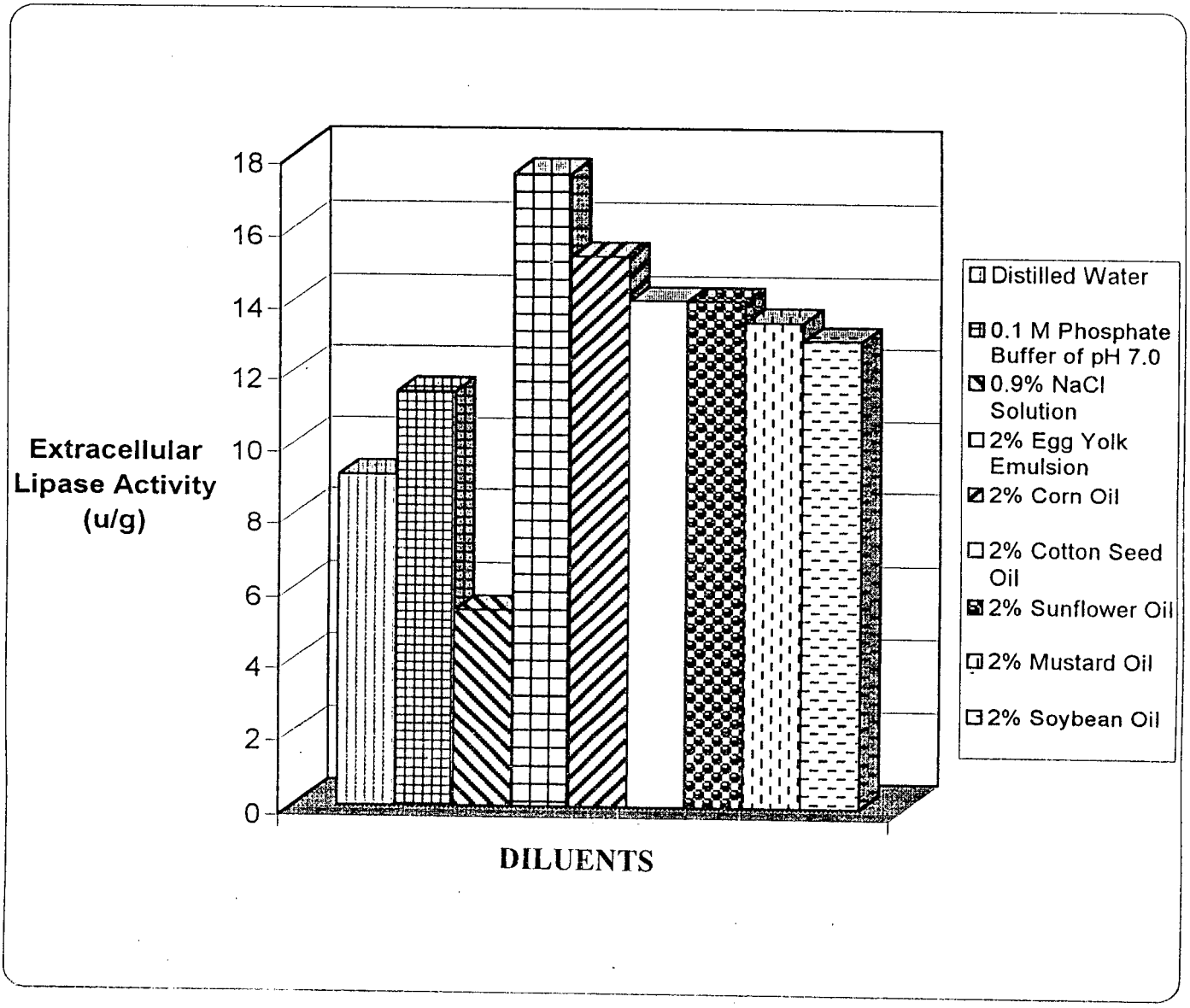


FIG. 16. SELECTION OF THE DILUMENT FOR SOLID SUBSTRATE FERMENTATION.

4.3.6. Effect of Addition of $CaCO_3$ on Solid State Lipase Fermentation by *Rhizopus arrhizus*

To study the effect of the addition of $CaCO_3$ on lipase production, different amounts of $CaCO_3$ were added to the fermentation flasks, each containing 24g wheat bran 6.0g soybean meal and $30cm^3$ 2% egg yolk emulsion. The fermentation was carried out for 72 hours at $28\pm1^{\circ}C$. At the termination of fermentation, the fermented mash was soaked in water for one hour, centrituged and the supenatant was analyzed for the extracellular lipase activity and pH. The control flasks were treated similarly except the addition of $CaCO_3$.

The lipase activity was increased with the increase in the concentration of $CaCO_3$ upto 4%. Without the addition of $CaCO_3$, the pH was dropped from 7.0 to 4.0 due to acid formation during fermentation. The addition of $CaCO_3$ resulted in the neutralization of acids and the final pH was found to be 6.2. The buffering action of $CaCO_3$ rerulted in the neutral pH. This neutral pH permitted the formation of more enzyme and prevented the inactivation of the formed enzyme. Calcium ions derived from $CaCO_3$ might also contribute to the "lipase enhancing effect" of $CaCO_3$ as reported by Tsujisaka *et al*[189]. Hence 40.5% increase in lipase activity in the presence of $CaCO_3$ is due to increase in lipase synthesis and decrease in the inactiviation because of buffering action of $CaCO_3$ as well as the effect of soluble Ca^{2+} salts derived from $CaCO_3$.

TABLE 15

EFFECT OF $CaCO_3$ ON LIPASE PRODUCTION BY SOLID STATE FERMENTATION BY *RHIZOPUS ARRHIZUS*

$CaCO_3$ % (on dry wt. basis)	Lipase activity (Units/g substrate)	Final pH
0	17.5	4.0
1	21.0	4.8
2	23.0	5.5
3	24.0	6.0
4	24.6	6.2
5	24.6	6.2
6	24.0	6.2

4.3.7 Effect of Incubation Temperature on Solid State Fermentation of Lipases by *Rhizopus arrhizus*

The effect of incubation temperature on lipase production by microorganisms is very important. To study this effect, the fermentation was carried out at different temperatures. It was found that optimum termperature for lipase production by solid state fermentation was the same as for submerged fermentation i.e. 28± 1°C (Figure-20). Below 28°C the rate of lipase formation was less due to slow metabolic activities. At temperatures above 30°C, low lipase activity was obtained which might be due to both lower production and rapid inactivation of the enzyme. Similar results have been obtained by Benzoana[67] who found that optimum temperature for the production of lipases by *Rhizopus arrhizus* was 29°C.

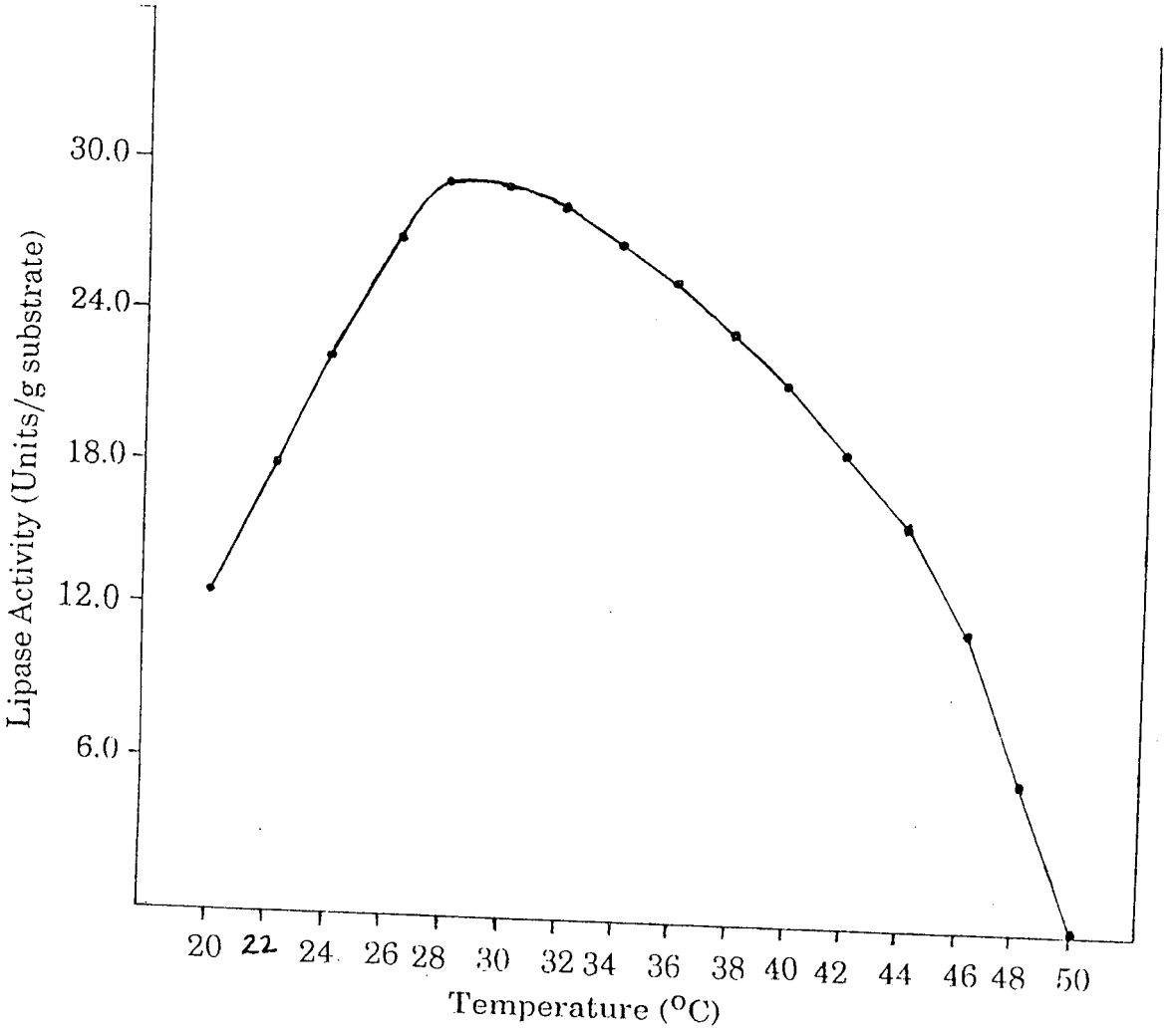


FIG. 17. EFFECT OF TEMPERATURE ON SOLID STATE LIPASE FERMENTATION BY *RHIZOPUS ARRHIZUS.*

4.3.8 Time Course of Lipase Production by Solid Substrate Fermentation by *Rhizopus arrhizus*

Time course for the production of lipases by *Rhizopus arrhizus* was studied in $1dm^3$ flasks, each containing 24 g wheat bran, 6.0 g soybean meal, $30cm^3$ 2.0% egg yolk emulsion and 1.2 g $CaCO_3$. After incoculation with 4% spore inoculum, the solid substrate was incubated at 28 $\pm$1°C for 96 hours. The flasks were analyzed after every 12 hours for extracellular lipase activity. From the fig-18 it is evident that the maximum rate of the lipase biosynthesis was achieved between 24 and 48h. However, the lipase was accumulated in the medium and reached maximum level of 24.6 units/g substrate after 72 hours and then it started decreasing due to reduction in the synthesis rate and increase in the denaturation of the enzyme.

The pH of the solid substrate was found to decrease from 7.0 to 6.2 within 48 hours due to the acid formation. Further decrease in pH was prevented by the buffering action of $CaCO_3$.

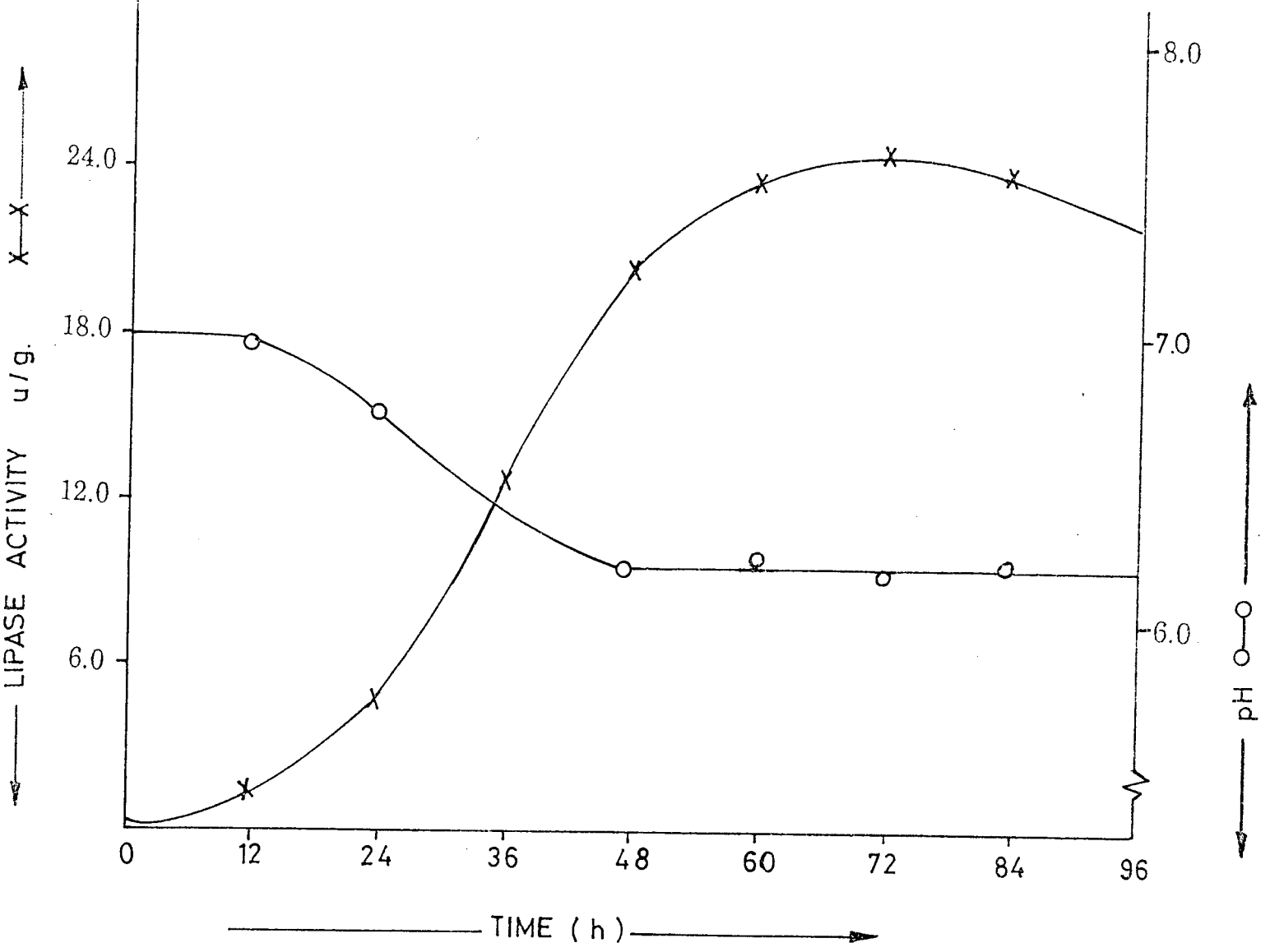


FIG. 18. FERMENTATION PROFILE OF *R. ARRHIZUS* IN SOLID SUBSTRATE FERMENTATION.

4.4 PARTIAL PURFICATION AND IMMOBILIZATION OF THE EXTRACELLULAR LIPASE OF *RHIZOPUS ARRHIZUS*

The mould *Rhizopus arrhizus* used in the study was predominantly intracellular lipase producer. However, some extracellular lipase activity was always found in the fermented broth. The mycelium was separated from the broth by centritugation and the extracellular lipase was salted out from the supernatant by ammonium sulphate fractionation. The salted out enzyme, after dialysis, was precipitated with chilled acetone. The partially purified extracellular lipase as such and after immobilization onto different supports was evaluated for lipase activities.

4.4.1 Partial Purification of the Extracellular Lipase of *Rhizopus arrhizus*

The fermented broth was centrifuged and the supernatant was found to have 4.9 mg/cm^3 proteins and 2.60 u/cm^3 lipase activity. To 1dm^3 filtrate having 2600 units lipase and 4.9 g proteins, solid ammonium sulphate was added to bring the saturation to 35% and after standing for 8 hours at 4°C the mixture was centrifuged at 8000 rpm to remove the precipitates. To the supernatant additional ammonium sulphate was added slowly to bring the saturation to 70% and the mixture was left standing overnight at 4°C. The precipitates were collected by centrifugation and dissolved in 100cm^3 distilled water. The solution was dialyzed against water for 60 hours using cellophane membrane. The dialyzed solution was found to have 2100 units lipase and 490

mg proteins. In this step purification was increased by 7.7 with 78% recovery of the original lipase activity.

To the dialyzed solution (below 4°C), chilled acetone was added with stirring to the concentration of 35% by volume. The precipitates were immediately removed by centrifugation. To the supernatant more acetone was added to bring its concentration to 55% and the precipitates were collected by centrifugation and dried under vacuum. In this step purification was increased to 14.37 with 61% recovery of the original enzyme (Table -16). The dried precipitates were stored in the desiccator over anhydrous $CaCl_2$.

4.4.2 Enzyme Immobilization

The etracellular lipase of *Rhizopus arrhizus* was obtained from various batches. It was partially purified and vacuum dried and stored at 4°C. It was analyzed before immobilization and was found to have 6000 units/g. Different supports such as celite-535, cellulose, silica gel, clay and alumina were evaluated as adsorbents for lipase immobilization. To $10cm^3$ solutation containing 0.5 g free lipase, 1g support was added for adsorption. The mixture was stirred slowly with a glass rod for 30 minutes and filtered and the liplolytic activity of the immobilized catalysts was determined. The data of the table 17 shows the activities of free and immobilized lipases. The celite-535 was found to adsorb maximum amount of lipase as compared to other supports. After washing it retained 928 units lipase per gram (dry weight basis). So the hydrolytic activity of celite immobilized lipase was about 15.1% of free lipase (600 u/g). The results resemble qualitatively to those of Tanaka and

Sonomoto[135] who reported the celite to be the best support for lipase adsorption. However, these results differ from those of Brady *et al*[115] who found that the cellulose and silica gel were better adsorbent as compared to the celite.

TABLE 16

PARTIAL PURFICATION OF EXTRACELLULAR LIPASE OF *RHIZOPUS ARRHIZS*

	Total lipase activity (Units)	Total proteins (mgs)	Specific lipase activity (u/mg)	Yield (%age of the original activity)	Purification
Culture filtrate ($1dm^3$)	2600	4900	0.53	100	1.0
Lipase active fraction salted out by $(NH_4)_2SO_4$ 35-70% saturtation and dialyzed.	2100	490	4.28	80	8.0
Precipitates obtained on addition of chilled acetone (35-55%v/v)	1600	210	7.6	61.5	14.3

TABLE 17

EVALUATION OF DIFFERENT IMMOBILIZATION SUPPORTS FOR THE ADSORPTION OF LIPASE OF *RHIZOPUS ARRHIZUS*

Support	Lipolytic Activity of Immobilized Enzyme (u/g)
Free	6000
Celite-535	918
Cellulose	760
Silica gel	744
Clay	421
Alumina	98

4.5 LIPASE CATALYZED ESTER SYNTHESIS

Lipases can catalyze hydrolysis, synthesis and interesterification of esters. For lipase mediated ester synthesis, it is essential to carry out the reaction in non-aqueous media to avoid hydrolytic reactions. Lipase catalyzed esterification reactions constitute an economical, efficient and evironment friendly route for the synthesis of a variety of esters.

4.5.1 Evaluation of Free and Immobilized Lipases of *Rhizopus arrhizus* For Esterification of Hexanol and Octanoic Acid

Different forms of lipase of *Rhizopus arrhizus* such as free (6000 u/g), celite adsorbed (928 u/g) and mycelial (300 u/g) were used to catalyze esterification of 1 - hexanol and octanoic acid. 0.025 g of the free lipase, 0.16g of the celite adsorbed lipase or 0.5g of mycelial lipase, each containing 150 units lipase, were added to 50cm^3 reaction mixture containing 0.6 M octanoic acid and 0.6 M n-hexanol in the petroleum ether. The stoppered flasks containing the reaction mixture were incubated at 30±1°C with shaking at 100 rpm. The samples were drawn periodically and analyzed to determine the esterification activities and percent yield of the ester.

The data of the Table 18 shows that the soluble lipase had very low esterification activity and negligible yield (2.0%) of the ester in 24h reaction. Singnificance of direct application of the soluble lipase in ester synthesis is further reduced when the ratio of its synthetic activity to hydrolytic activity (28/6000) is compared to those of immobilized lipases such as 72/300 for

mycelial lipase and 51/920 for celite adsorbed lipase. Low synthetic activity of soluble lipases in non-aqueous media is well documented and is due to direct exposure of the enzyme to unnatural organic environment and reactants, instead of natural aqueous envionment of the living system. The enzyme immobilization prevents the direct exposure and makes the enzyme protein resistant towards adverse conditions of pH water scarcity and unnatural organic environment. So immobilized lipases are preferred for ester synthesis in organic solvents.

The comparision of the two immobilized lipases indicates that the celite-adsorbed lipase, inspite of having higher hydrolytic activity in the aqueous system, had lower esterification activity in non aqueous solvents. Mycelial lipase, on the other hand was very active in the organic solvent and had an esterification activity of 72 micro moles per minute per gram dry mycelium. The yield of the ester obtained in 24 hours was 24% in case of celite adsorbed lipase as compared to 69% with mycelial lipase. Lower yield with the celite adsorbed lipase may be due to the enzyme inactivation during the reaction in organic solvent,in addition to lower initial rate of esterification. The mycelial lipase of *Rhizopus arrhizus* on the other hand, was found to have high efficiency and good operational stability during the esterfication in organic medium.

TABLE 18

COMPARISION OF SOLUBLE AND IMMOBILIZED LIPASES OF *RHIZOPUS ARRHIZUS* FOR THE SYNTHESIS OF HEXYL OCTANOATE

Enzyme	Hydrolytic activity in aqueous system (u/g)	Esterification activity in petroleum ether (μmol/min/g)	Yield of ester in 24 hrs. (%)
Soluble lipase	6000	28	2
Celite-Immobilized lipase	928	51	24
Mycelial lipase	300	72	69

4.5.2 Effect of Solvent Nature on Esterification Activity of Mycelial Lipase of *Rhizopus arrhizus*

It is known that the nature of the reaction medium greatly influences the activity of the biocatalysts. Differnet organic solvents such as n-hexane, n-octane, petroleum ether, diethyl ether, benzene, toluene, carbon tetrachloride, chloroform, acetone, tetrahydrofuran or dimethyl formamide were used as non-aqueous reaction media. The mycelial lipase of *Rhizopus arrhizus* was used at the rate of 1.0% (w/v) for the biocatalysis of the esterfication. Other reaction conditions such as temperatue, shaking speed and initital concentrations of octanoic acid and n-hexanol were same as given in section 4.5.1.

Figure 19 shows that the enzyme exhibited maximum esterification activity in n-hexane as the reaction medium. In it, the initial estrification rate was 86μ mole per minute per g mycelium. Other non-polar hydrocrbon solvents such as n-octane and petroleum ether were also quite suitable for the lipase catalyzed esterification. Polar and hydrophilic solvents such as acetone, Tetrahydrofuran (THF) and Dimethyl Formamide (DMF) were found to inhibit the enzymic ester synthesis. It may be explained that the hydrophilic solvents extract that water from the enzyme protein which is essential for conformational integrity of the protein. A structural change in the protein results in an inactive or less active conformation. In contrast, hydrophobic solvents such as n-hexane do not disturb the aqueous micro-environment of the enzyme protein. This behaviour of mycelial lipase of *Rhizopus arrhizus* in different solvents is similar to that of lipozyme (immobilized lipase of *Mucor*

miehei) as reported by Miller *et al*[20]. However, our results are somewhat different from those reported by Manjon *et al*[114] for immobilized lipase of *Mucor miehei* which was found to retain some acitvity even in THF, and DMF like solvents.

For further studies on enzymic esterification, n-hexane, was selected as the reaction medium.

4.5.3 Effect of Substrate Concentrations on Lipase Catalyzed Synthesis of Hexyl Octanoate

In enzymic esterification, the concentrations of the acid and the alcohol effect the rate of the reaction and yield of the ester. The effect of octanoic acid concentration on lipase catalyzed synthesis of hexyl octanoate was first studied. The acid concentration was ranged from 0.1M to 1.5M with 1.0M n-hexanol in each case. The esterification activity of the enzyme was increased with increase in concentration of the acid upto 0.6M. However, a decrease in activity was observed when the acid concentration was increased beyond 0.6M (Figure 20). The increase in the reaction rate with increase in the initial concentration of the reactant is according to the Law of Mass Action[252]. However, the decrease in the esterification rate at higher concentrations of the acid is due to the interaction between the enzyme and its acidified hydration layer.

Similarly the effect of hexanol concentration on the esterification activity of the lipase was studied (Figure - 21). The concentration of hexanol

was varied between 0.1M to 1.5M whereas the concentration of octanoic acid in each case was 0.6M. It was found that esterification rate was increased with increase in the hexanol concentration. The highest esterification rate of 92 μmoles per minute per gram mycelium was achieved when the concentration of hexanol was 1.0 mole/dm^3. Further increase in the alcohol concentration was ineffective.

The equimolar concentrations of the octanoic acid and hexanol selected for the batch esterification reactions were 0.6M each. Using these concentrations of the two substrates in n-hexane as the solvent and 1.0% w/v mycelial lipase of *Rhizopus arrhizus* (300 lipase u/g), an esterification rate of 86 μmol/min./g mycelium was achieved.

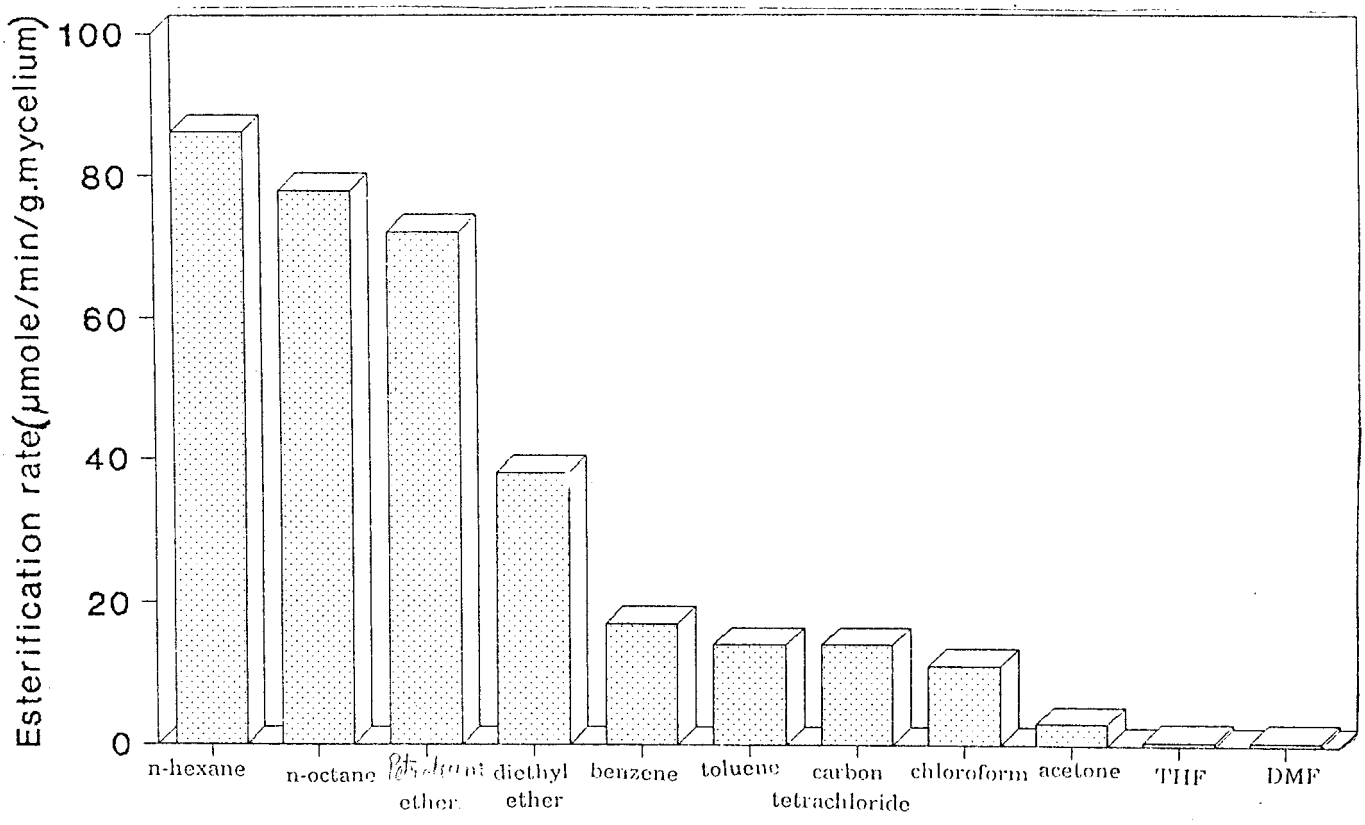


FIG. 19. EFFECT OF DIFFERENT SOLVENTS ON ESTERIFICATION ACTIVITY OF MYCELIAL LIPASE OF *RHIZOPUS ARRHIZUS*.

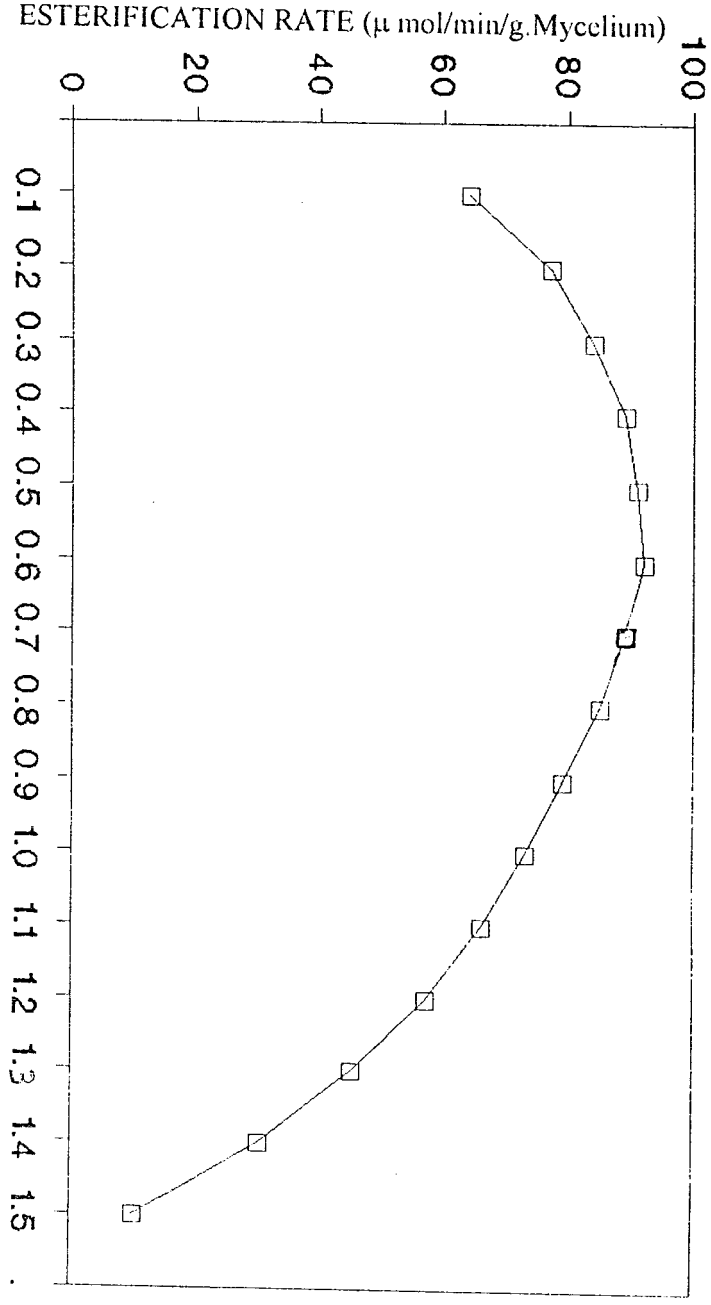


FIG. 20. EFFECT OF CONCENTRATION OF BUTANOIC ACID ON ESTERIFICATION RATE.

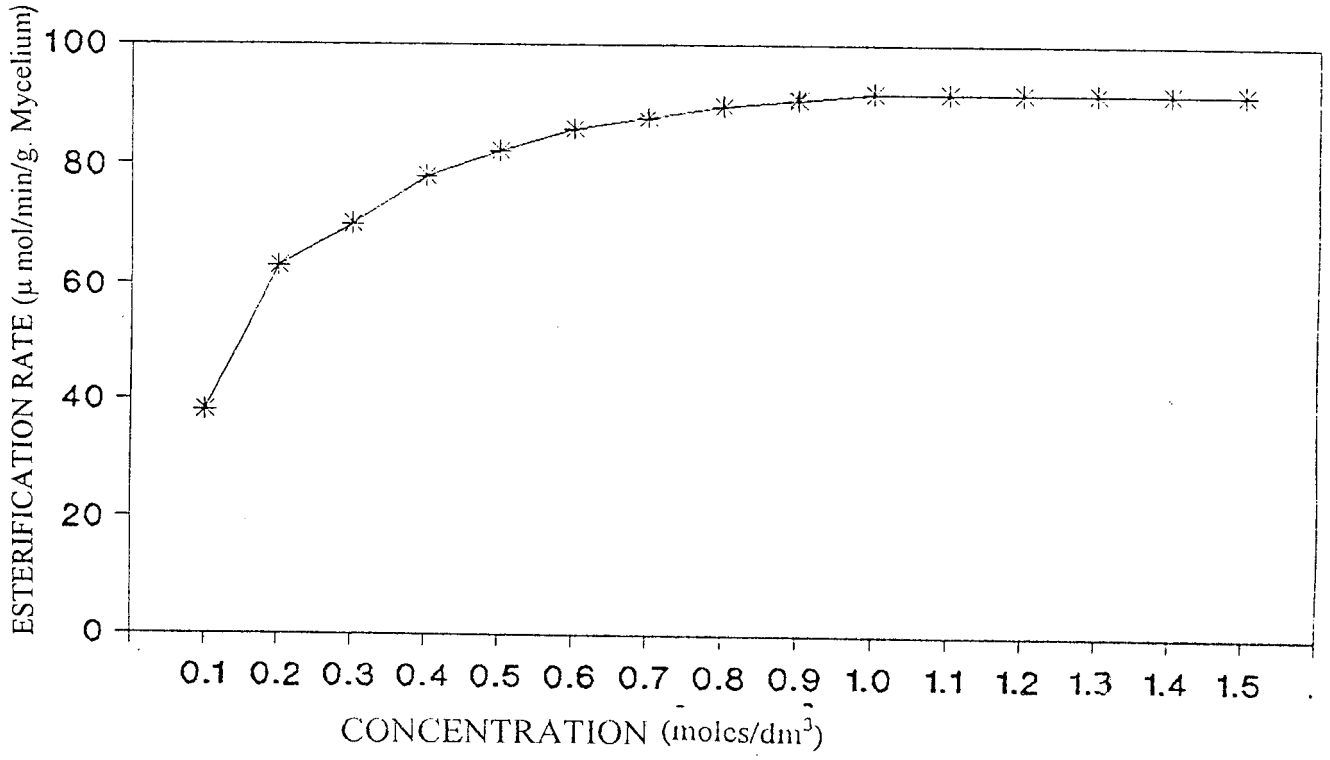


FIG. 21. EFFECT OF CONCENTRATION OF n-HEXANOL ON ESTERIFICATION RATE.

4.5.4 Effect of Acid Chain Length on Lipase Catalyzed Synthesis of Hexyl Octanoate

The chain length of a carboxylic acid was found to have great influence on the enzymic estrification. Fatty acids of different chain length (ranging from C_2 to C_{12}), were made to react with n-hexanol and initial rates of esterification of these acids were measured. In each case, the initial concentrations of n-hexanol and carboxylic acid were kept at 0.6M. Figure-22 shows that ethanoic acid was not esterified at all and beyond that the rate of esterification increased with increase in the chain length of the carboxylic acids. This may be due to the specificity of the enzyme for long chain fatty acids as pointed out by Miller *et al*[20] for the lipozyme. The low esterification rate with short chain fatty acids may also be due to higher acid strengths of short chain carboxylic acids. Owing to their strengths, these ionize in the presence of the moisture and acidify the environment thus rendering the enzyme less active or inactive. So, for the low esterifications activity of *Rhizopus arrhizus* lipase with short chain acids, pH specificity of the enzyme was also responsible, in addition to its substrate specificity for long chain fatty acids. To confirm this hypothesis, the mycelial powder (lipase activity. 300 u/g) was incubated with 0.6mol/dm^3 solution of butanoic acid or 0.6mol/dm^3 solution of octanoic acid in n-hexane at 30°C. The lipase bearing mycelia were separated from the reaction mixture after 24 hours, washed, dried and their hydrolytic activities were assayed. It was found that there was 13.3% loss in lipolytic activity of the mycelium, incubated with butanoic acid as compared with negligible loss in the lipolytic activity in case of incubation with octanoic acid(table 19). Thus the short chain fatty acids not only reversibly inhibited the

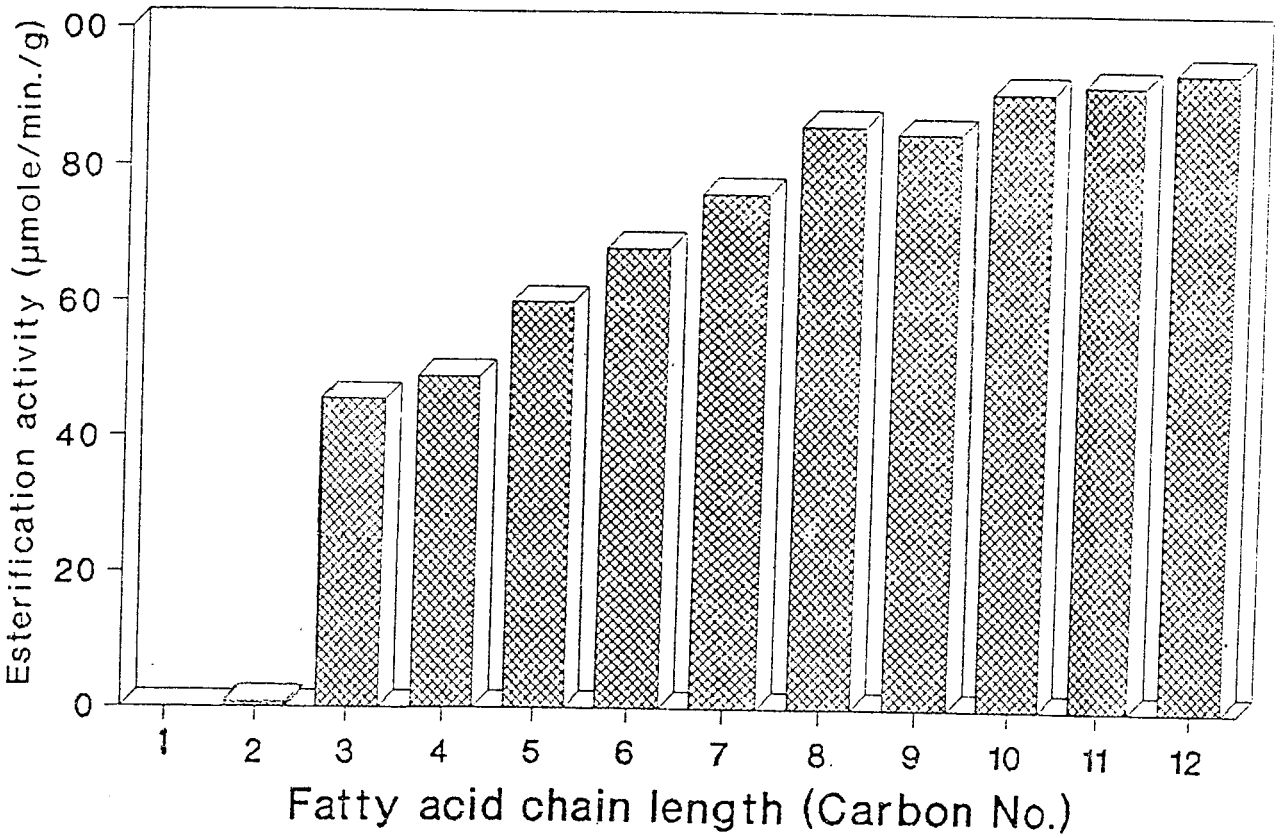


FIG. 22. EFFECT OF FATTY ACID CHAIN LENGTH ON THE ESTERIFICATION ACTIVITY OF THE MYCELIAL LIPASE OF *RHIZOPUS ARRHIZUS.*

TABLE 19

DENATURATION OF MYCELIAL LIPASE BY SHORT CHAIN FATTY ACIDS

Acid	Lipase activity before incubation (u/g)	Lipase activity after incubation (u/g)	Loss of activity (%)
n-Butanoic Acid	300	260	13.3
n- Octanoic acid	300	295	1.6

Note: The experiments were performed in triplicate and the data of the table represents means of the three experiments.

action of *Rhizopus arrhizus* lipase during the reaction but also cause some irreversible loss to the enzyme.

4.5.5 Effect of Chain Length and Nature of Alcohols on the Lipase Catalyzed Esterification

During an esterification reaction, an acyl radical from carboxylic acid is transferred to an alcohol. So the nature of the alcohol influences the esterification activity of the mycelial lipase. To study the effect of alcohol chain length, normal alohols of varying chain lengths were made to react with n-octanoic acid in the hexane. Initial concentrations of both reactants were kept 0.6 mol/dm^3. Figure-23 shows that the initial rate of esterification was increased with increase in the chain length of alcohols upto pentanol. The esterification rates remained more or less the same beyond pentanol. The low esterification rates with short chain alcohols may be explained on the bases of substrate specificity of the enzyme for long chain alcohols. This may also be related to the hydrophilic nature of the short chain alcohols due to which these penetrate the hydrosphere of the enzyme protein rendering it less active.

The esterification rates were more affected by the position of the hydroxyl group in alcohols than their chain length. Only primary alcohols were esterified at reasonable rates. The rates of esterification of secondary alcohols were very low, whereas tertiary alcohols were not esterified at all. The data of the table-20 shows that the tertiary pentanol (2-methyl butan-2-ol) was not esterified and the rate of esterification of the secondary pentanol (pentan-2-ol) was 19 μmol/min./g mycelium. Among primary pentanols n-

amyl alcohol (pentan-1-ol) was esterified at the rate of 86 μmol/min./g mycelium, whereas the rate of esterification of isoamyl alcohol (3-methyl butan-1-ol) was 59 μmol/min./g mycelium. This showed that the lipase of the present strain of *Rhizopus arrhizus* is specific for hydroxyl groups at primary positions and it preferably esterified unbranched alcohols as compared to branched chain alcohols. The lipase of *Rhizopus arrhizus* has already been reported to attack at 1,3- positions of triacylglycerols during its hydrolytic action[153,196]. The resistance of tertiary nucleophiles (alcohols and amines) in accepting acyl radical have also been observed in case of lipase mediated acyl transfer reactions[18].

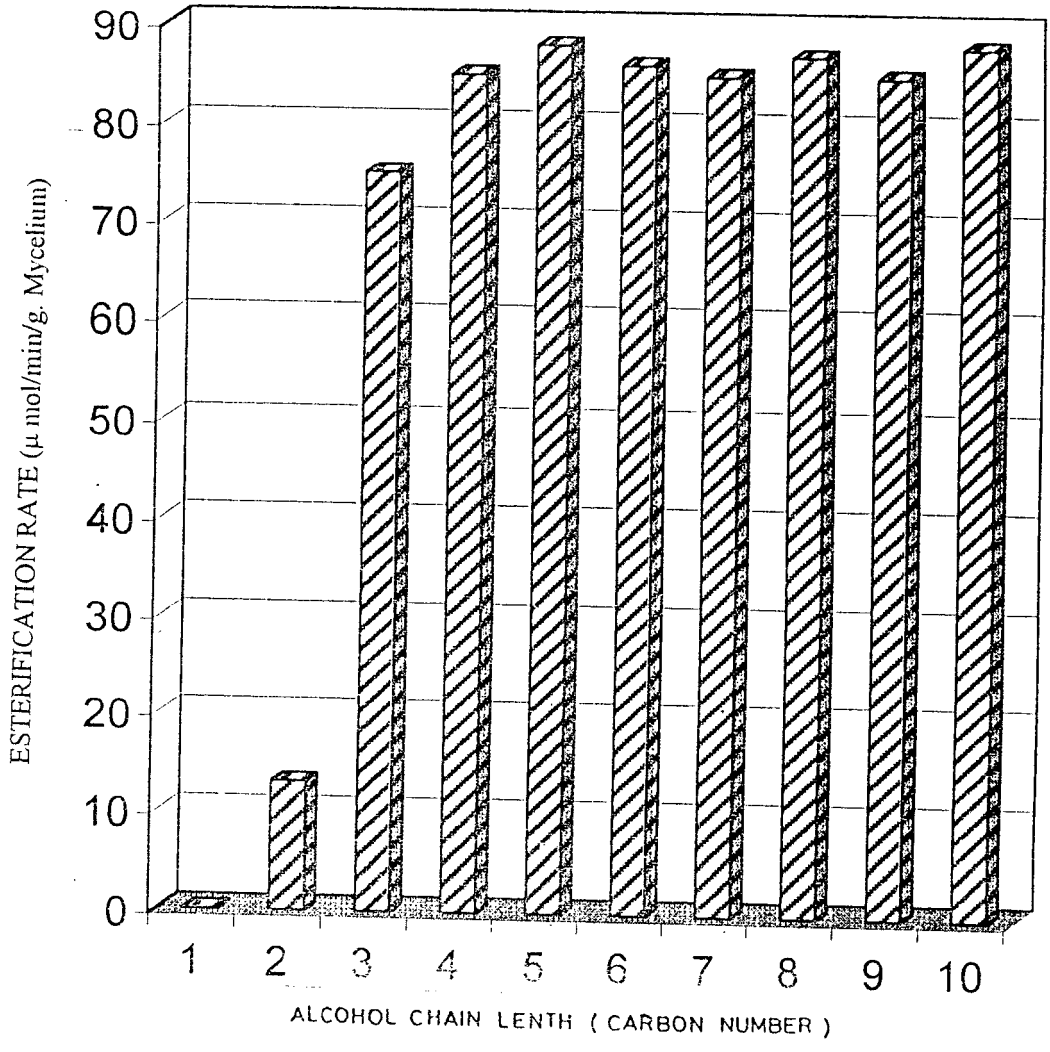


FIG. 23. EFFECT OF ALCOHOL CHAIN LENTH ON ESTERIFICATION ACTIVITY OF MYCELIAL LIPASE.

TABLE 20

EFFECT OF THE NATURE OF ALCOHOLS ON ESTERIFICATION ACTIVITY OF MYCELIAL LIPASE OF *RHIZOPUS ARRHIZUS*

No. of carbon atoms in the alcohol	Esterification rate (μmol/min./g mycelium)			
	Primary Alcohols		Secondary Alcohols	Tertiary Alcohols
	Unbranched	Branched		
3	75	--	10	--
4	85	44	14	0.0
5	88	59	19	0.0

Note: The experiments were performed in triplicate and the data of the table represents means of the three experiments.

4.5.6 Effect of Moisture Level on Lipase Mediated Esterification

The esterification is a reversible reaction and the position of the equilibrium depends on water activity. To avoid hydrolytic reaction and pull the equilibrium of the reaction in forward direction, removal of water from the reaction mixture is necessary. The presence of some moisture, however, is vital for all enzymic activities. Water is involved in the structural integrity of the enzyme proteins. Forces such as salt bridges and hydrophobic interactions, which are responsible for active conformation of the proteins are very much related to water. If water is completely removed, the enzymes may lose their catalytic activity. So level of water in the reaction mixture is very critical to achieve a reasonable esterification rate and the ester yield.

Acetone dried mycelium used for the biocatalysis was found to retain 4.0% (w/w) moisture, on the average. So when 1.0 g of this mycelium was added to 100cm^3 anhydrous reaction mixture, 0.04% moisture level was achieved. Different amounts of water were added to the reaction mixture containing 1.0% mycelium to give rise to the moisture levels ranging from 0.04% to 1.0%. Figure 24 shows that maximum yield of the ester was observed at a moisture level of 0.04%) i.e. without further addition of water. Increase in moisture level, beyond 0.04%, lowered the acid consumption by shifting the equilibrium in reverse direction (hydrolysis side).

$$C_6H_{13}OH + C_7H_{15}COOH \underset{\text{Water}}{\overset{\text{n-Hexane}}{\rightleftharpoons}} C_7H_{15}COOC_6H_{13} + H_2O$$

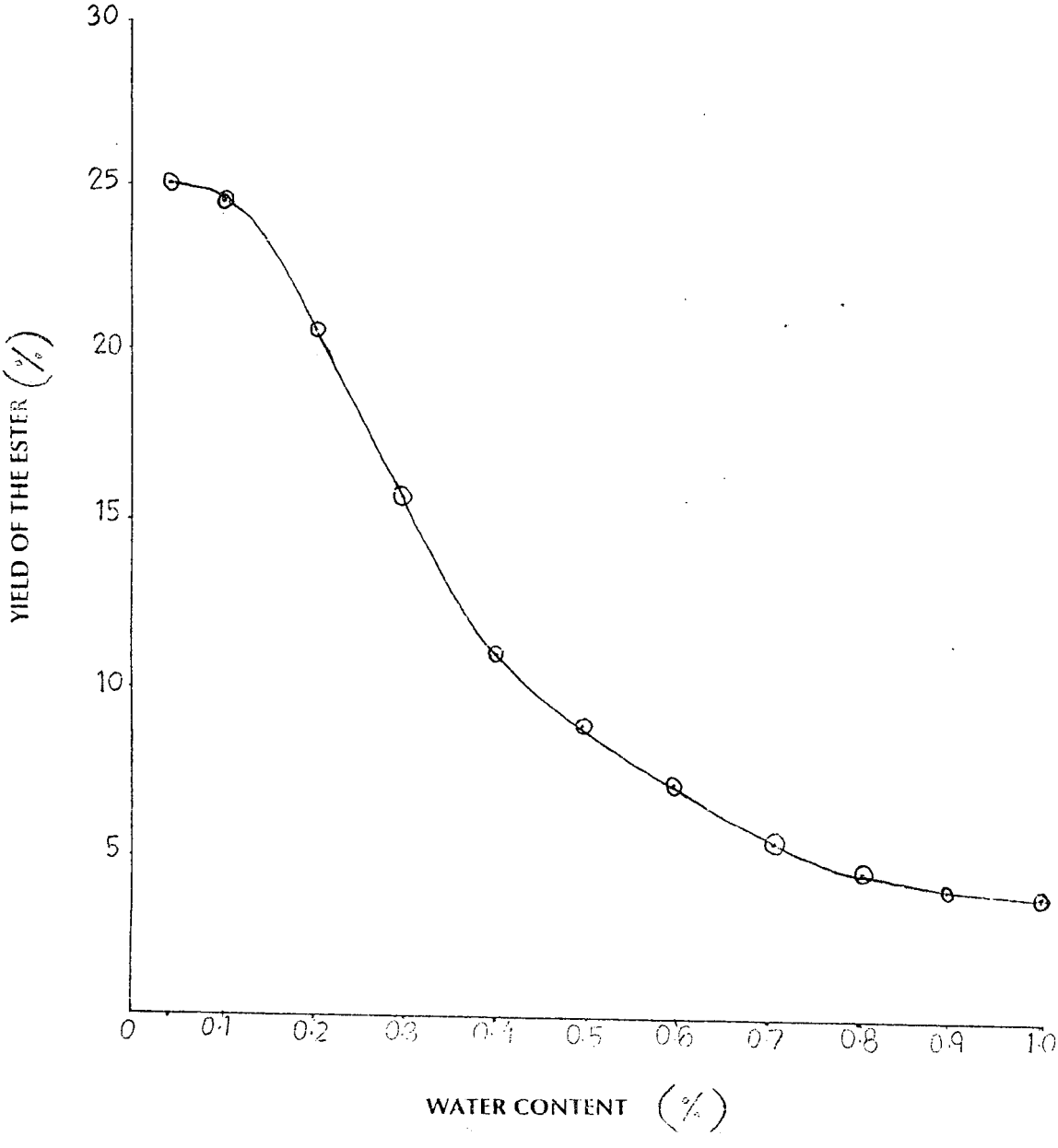


FIG. 24. EFFECT OF WATER CONTENT ON ESTERIFICATION ACTIVITY.

4.5.7 Effect of Temperature on Lipase Mediated Esterification

The effect of temperature on both the synthetic activity and the stability of the fungal lipase was determined. The influence of temperature on the enzyme activity was studied over a range of 20 to 60°C by measuring the initial rates of synthesis of hexyl octanoate in n-hexane. Maximum initial rates were obtained at 45°C (Figure-25). At temperatures higher than 45°C, the reaction rates decreased rapidly and at temperatures below 45°C reaction rates decreased very slowly so that there was only a slight difference in the initial rates of esterification at 45°C and 30°C. This shows that the optimum temperature for the esterification in n-hexane is different from the optimum temperature for the hydrolysis of fats in the aqueous medium. A similar difference between optimum temperatures for esterification and hydrolysis have not already been reported by any other worker. The experiment was repeated many times and the optimum temperature for esterification ranged from 43°C to 46°C. The reported optimum temperature of 45°C is the mean of six independent experiments.

To study the enzyme denaturation during the catalysis of esterification at different temperatures, one gram mycelium was incubated for 24 hours, with 100cm^3 n-hexane containing 0.6 mol/dm^3 n-hexanol and 0.6 mol/dm^3 octanoic acid. After 24 hours, the enzyme was recovered, washed and analyzed for its hydrolytic activity. It was found that during 24 hours incubation at 45°C, the mycelial enzyme lost about 55% activity as compared to 1.6% loss during 24 hours incubation at 30°C (Table - 21).

A value of 30°C was used in all other experiments to avoid excessive evaporation of the organic solvent and to minimize enzyme denaturation.

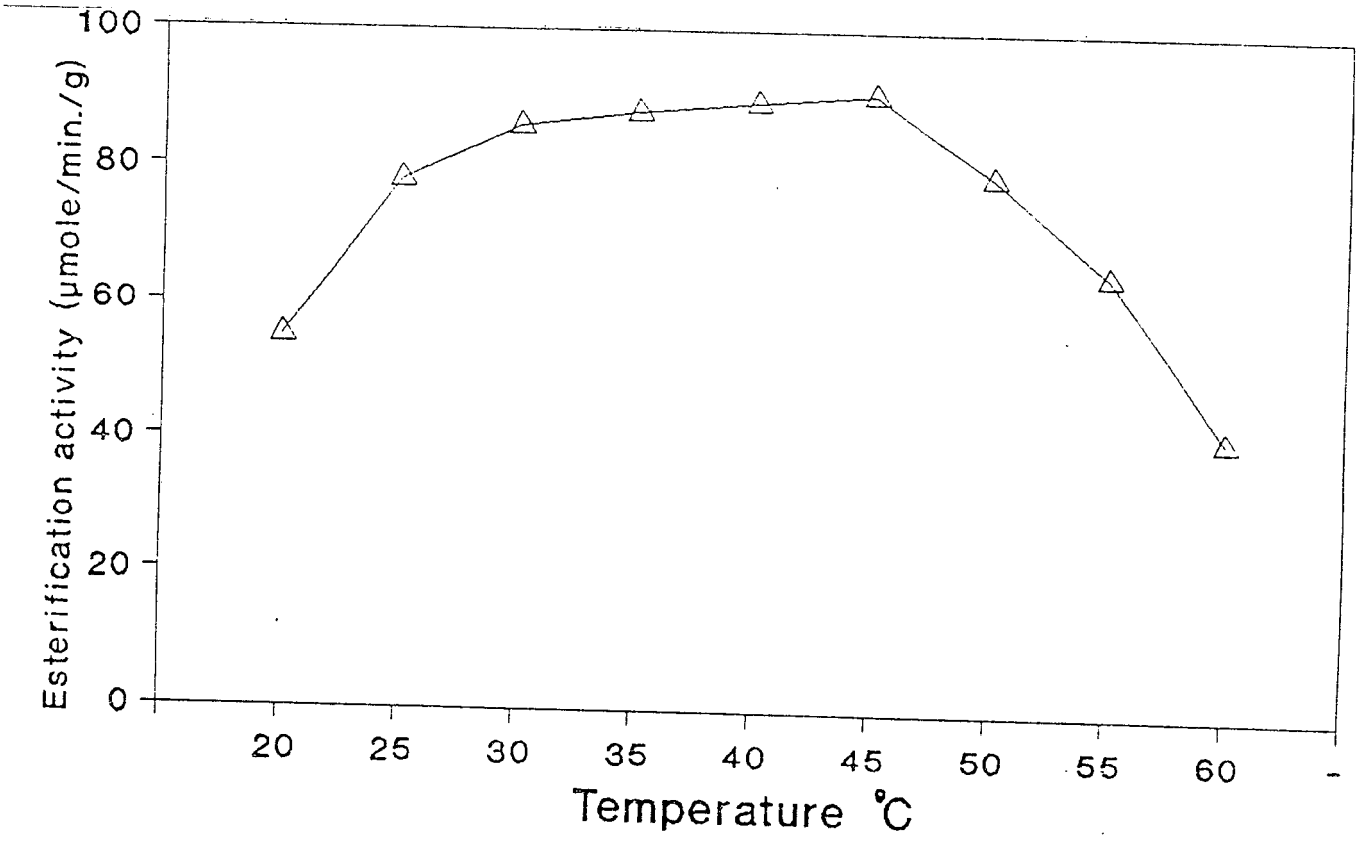


FIG. 25. EFFECT OF TEMPERATURE ON ESTERIFICATION ACTIVITY OF MYCELIAL LIPASE OF *RHIZOPUS ARRHIZUS*.

TABLE 21

DENATURATION OF THE MYCELIAL LIPASE OF *RHIZOPUS ARRHIZUS* DURING INCUBATION IN NON-AQUEOUS ENVIRONMENT

Incubation Temperature (°C)	Loss of Lipase activity during incubation (%)
20	1.0
25	1.0
30	1.6
35	11.5
40	27.0
45	55.0
50	89.0
55	100
60	100

4.6. PRODUCTION OF FLAVOURING ESTERS FROM FUSEL OIL

Fusel oil is the by-product of alcohol manufacturing industry. It has little use and is easily available at thrown away price. It is composed of a large number of alcohols mainly having three to five carbon atoms. These alcohols can be esterified with short chain fatty acids through enzymic or chemical route to produce fragrant esters. The enzymic esterificaiton has certain advantages over the chemical method, being less energy consuming and a cleaner route. It is carried out at ambient conditions of temperature and pressure. The specificity of the enzyme, together with the mild reaction conditions, prevents side reactions resulting in purer products. Ravi Rayon (Pvt.) Ltd. kindly supplied the fusel oil, used in the present research, and it was mainly consisting of propanols, butanols and pentanols. The mole fractions of these alcohols in the fusel oil were 0.032, 0.103 and 0.863, respectively.

Esters of the fusel oil formed with short chain fatty acids such as ethanoic acid, propanoic acid or butanoic acid find application in food indusry as flavour and aroma constituents. Out of the three above mentioned short chain fatty acids only butanoic acid could be esterified at a reasonable rate, using mycelial lipase of *Rhizopus arrhizus* (figure-26). So conditions for the synthesis of butanoate esters of the fusel oil were optimized.

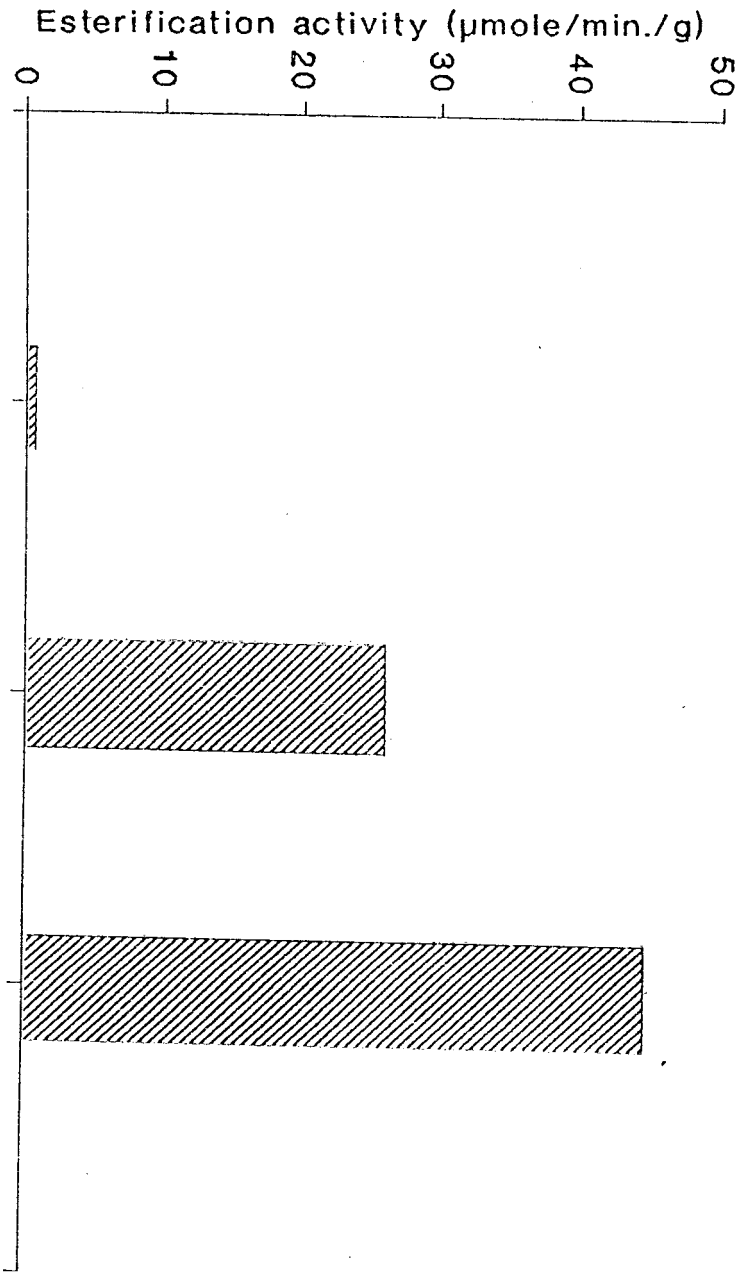


FIG.26. EFFECT OF ACID CHAIN LENGTH ON ESTERIFICATION OF FUSEL OIL.

4.6.1 Effect of Fusel Oil Concentation on Esters Synthesis

To study the effect of fusel oil concentration on the rate of esterification, different concentrations of the fusel oil (0.1 mol/dm^3 to 1.0mole/dm^3) were made to react with butanoic acid in n-hexane as a non acqueous medium. The concentration of butanoic acid, in each case was 0.4 mol/dm^3 and 1.0% mycelial powder of *Rhizopus arrhizus* was used as the catalyst.

The data of the fig-27 shows that the rate of esterification was increased with increase in the cocentration of the fusel oil. Maximum rate of esterification was observed when the concentration of fusel oil was 0.5 mol/dm^3. Further increase in the concentration of fusel oil was found to lower the rate of esterification. This may be due to the enzyme inactivation as discussed in article 4.5.3.

4.6.2. Effect of Butanoic Acid Concentration on the Esterfication of the Fusel oil.

The effect of changes in butanoic acid concentration on esterification was studied by changing the initial concentration of butanoic acid from 0.1mol/dm^3 to 1.0 mol/dm^3. The initial concentration of the fusel oil in each case was kept 0.5 mol/dm^3. Fig-28 shows that the maximum initial rate of ester synthesis was achieved when the concentration of butanoic acid was 0.4 mol/dm^3. Beyond 0.4 mol/dm^3 there was a decrease in the acid consumption due to inactivation of the enzyme as discussed in article. 4.5.3.

So initial concentraitons of 0.4 mol/dm^3 for each of the fusel oil and the butanoic acid were used for further studies.

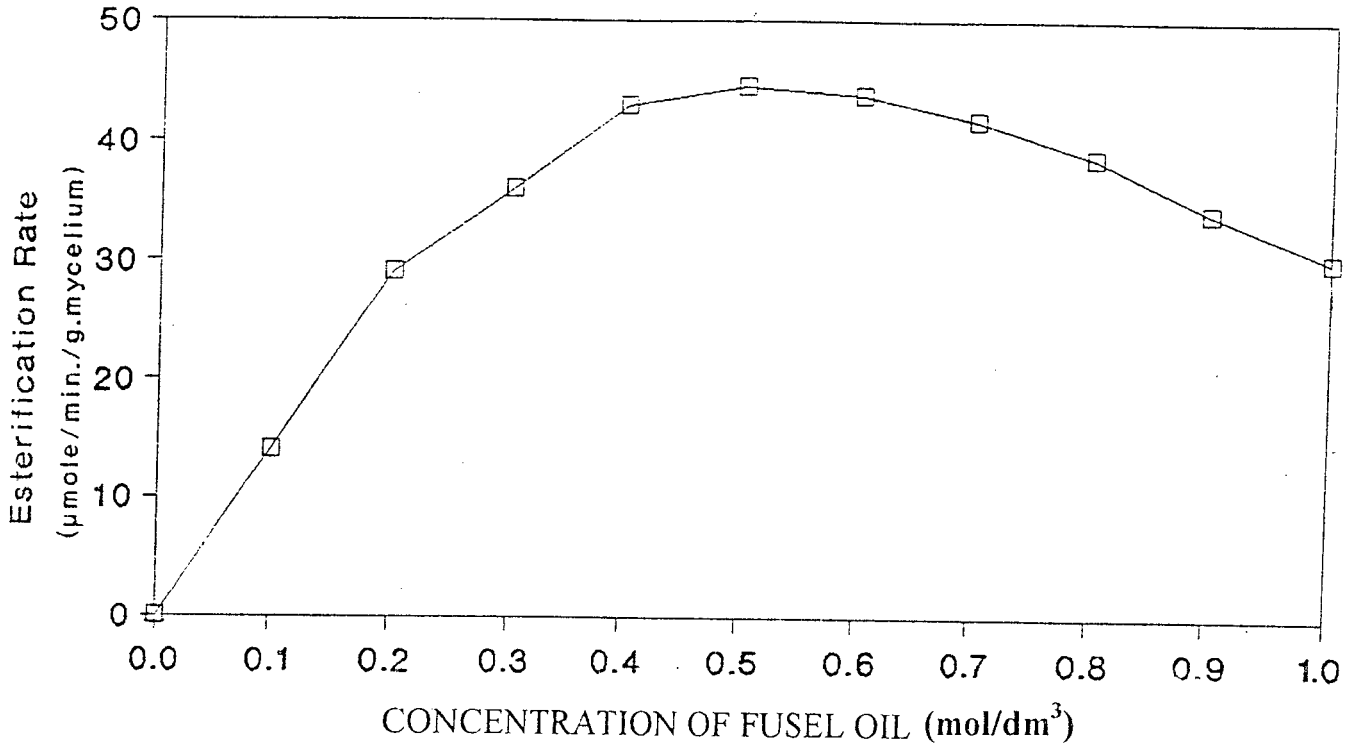


FIG.27. EFFECT OF CONCENTRATION OF FUSEL OIL ON ESTERIFICAITON RATE.

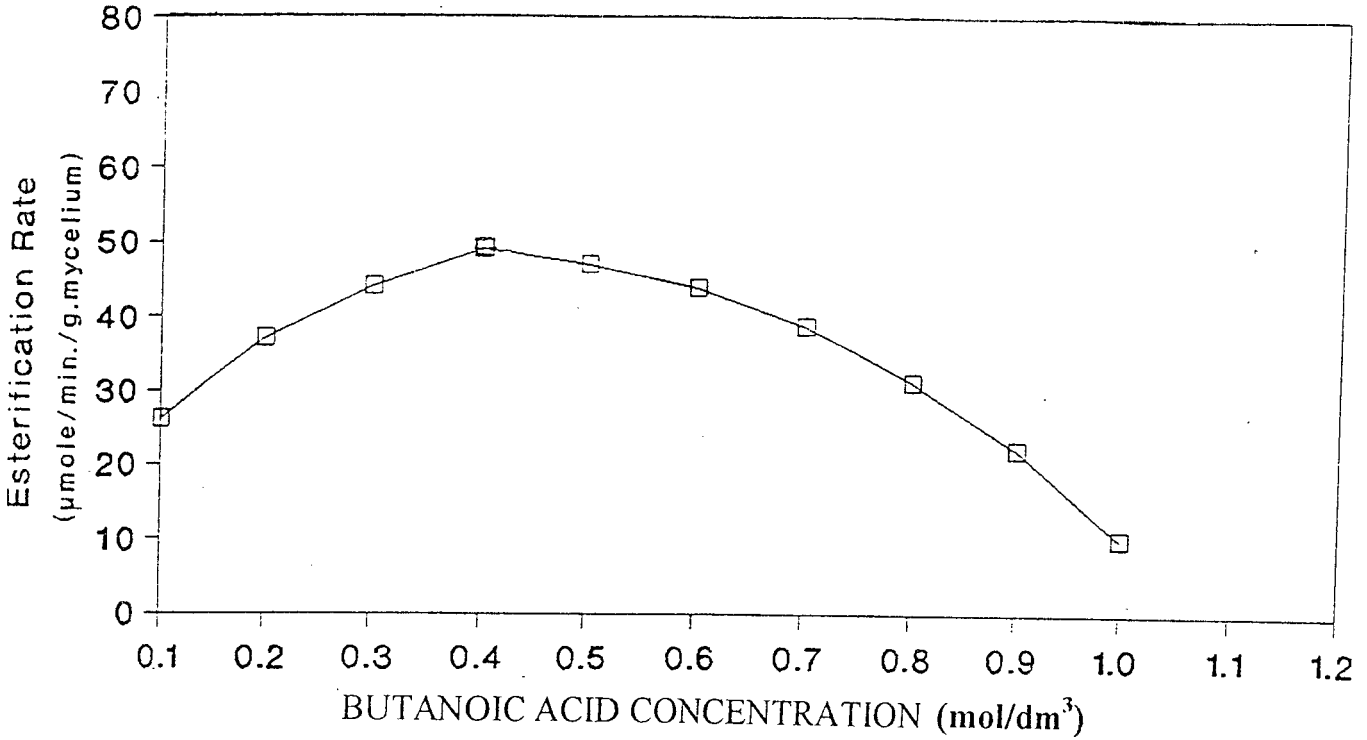


FIG.28. EFFECT OF BUTANOIC ACID CONCENTRATION ON ESTERIFICATION RATE.

4.6.3. Repeated use of Mycelium

The potential for reusing the mycelium was considered because the economics of the process would be enhanced if the enzyme could be reused. The mycelium lost 50% of its syhnthetic activity after seven cycles. However, when concentrations of the substrates were lowered from 0.4M to 0.1M each, the mycelium retained more than 95% activity upto 10 cycles.

4.6.4. Optimization of Enzyme - Filler Ratio in Packed Column for the Production of Butanoate Esters of Fusel Oil.

The esterification of the fusel oil was also carried out in the packed column reactor as shown on page 53. The reaction mixture containing 0.1 mol/dm^3 each of the butanoic acid and fusel oil, in n-hexane, was passed through the column (as described in the Methodology) at a rate of 0.5cm^3/min. The column was maintained at a temperature of 30$\pm$1^{o}C and it contained a mixture of mycelial powder (300 lipase unit/g) and neutralized sand (mesh size 60-80) as filler. Effect of different ratios of the enzyme and the sand such as 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9 on the ester yield was studied. Figure 29 shows that maximum of 56% acid was esterified while passing through the column containing 100% mycelial powder (10:1). However, a decrease in ester yield was not observed until the proportion of mycelium was below 60% (6:4). Below 60%, the enzyme became the rate limiting factor.

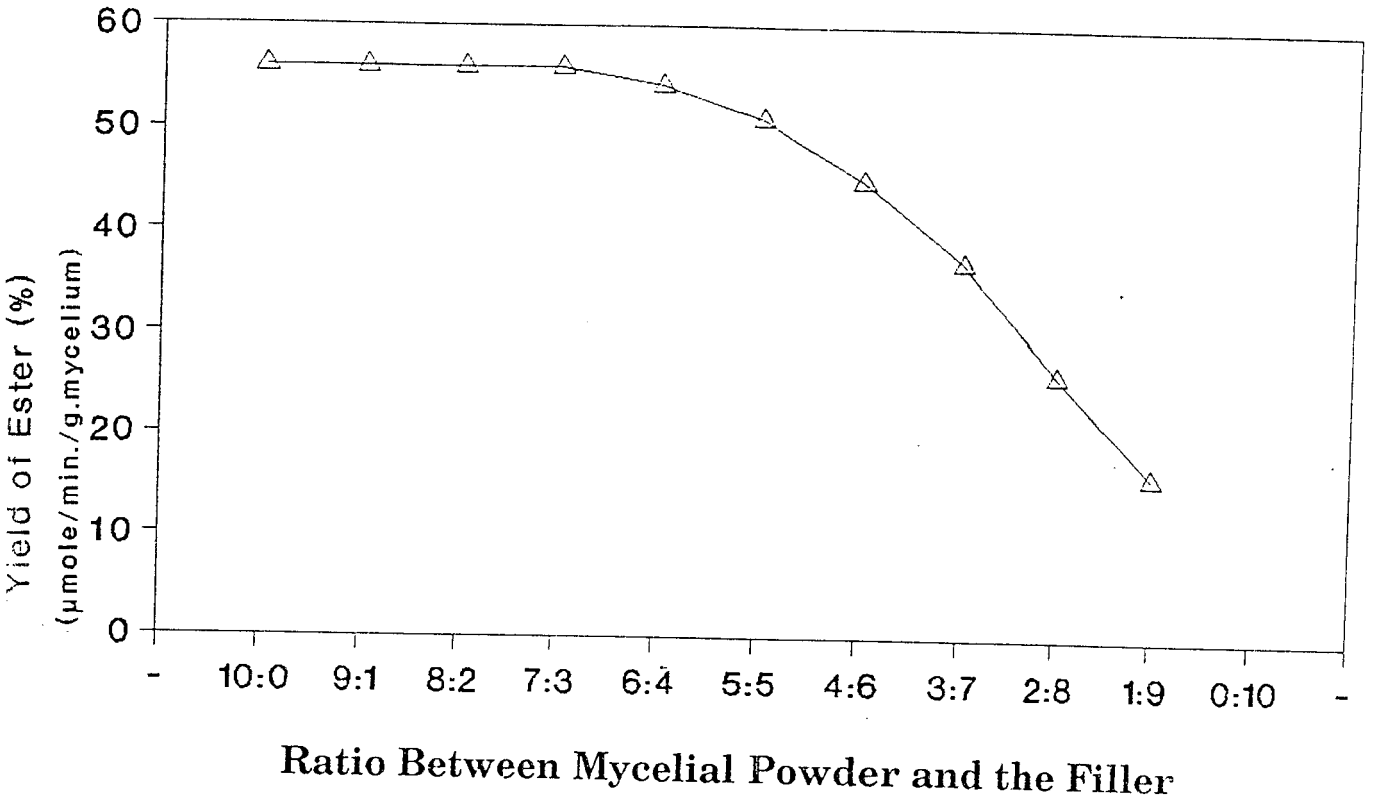


FIG.29. Optimization of the Enzyme - Filler Ratio for the Production of Fusel Oil Butyrates

4.6.5. Effect of the Flow Rate and Recycling of the Reaction Mixture.

The contact time between the enzyme and the reactants was changed by altering the flow rate of the reaction mixture through the column containing the mycelium and the sand (6:4 w/w). The reaction muxture containing 0.1mol/dm^3 butanoic acid and 0.1mol/dm^3 fusel oil was passed through the column at different rates ranging from 0.5cm^3/minute to 4.0cm^3/minute. Maximum conversion (56%) was observed at the lowest flow rate i.e. 0.5cm^3 per minute. The ester yield began to decrease when the flow rate was increased. At a flow rate of 4.0 cm^3 / minute a conversion rate of 6.0% was observed (Fig- 30)

To achieve the maximum possible yield of the ester the flow rate was kept low (0.5 cm^3/min.) and the reaction mixture was recycled through the column. After three cycles, the reaction mixture was analyzed by titrating residual butanoic acid. The analysis showed the presence of 13% residual acid, indicating 87% acid consumption.

Gas cromatography (fig-31) of the reaction mixture was done after removing the biocatalyst and the solvent. The peaks were identified using external standards. It was found that the reaction mixture contained 85% esters including 81.5% isoamyl butanoate (peak no.9). Peaks 12, 13, and 14 correspond to isomers of butanoic acid (8.2%). The reaction mixture also contained 6.7% alcohols including 4.86% isoamyl alcohol (peak no.3).

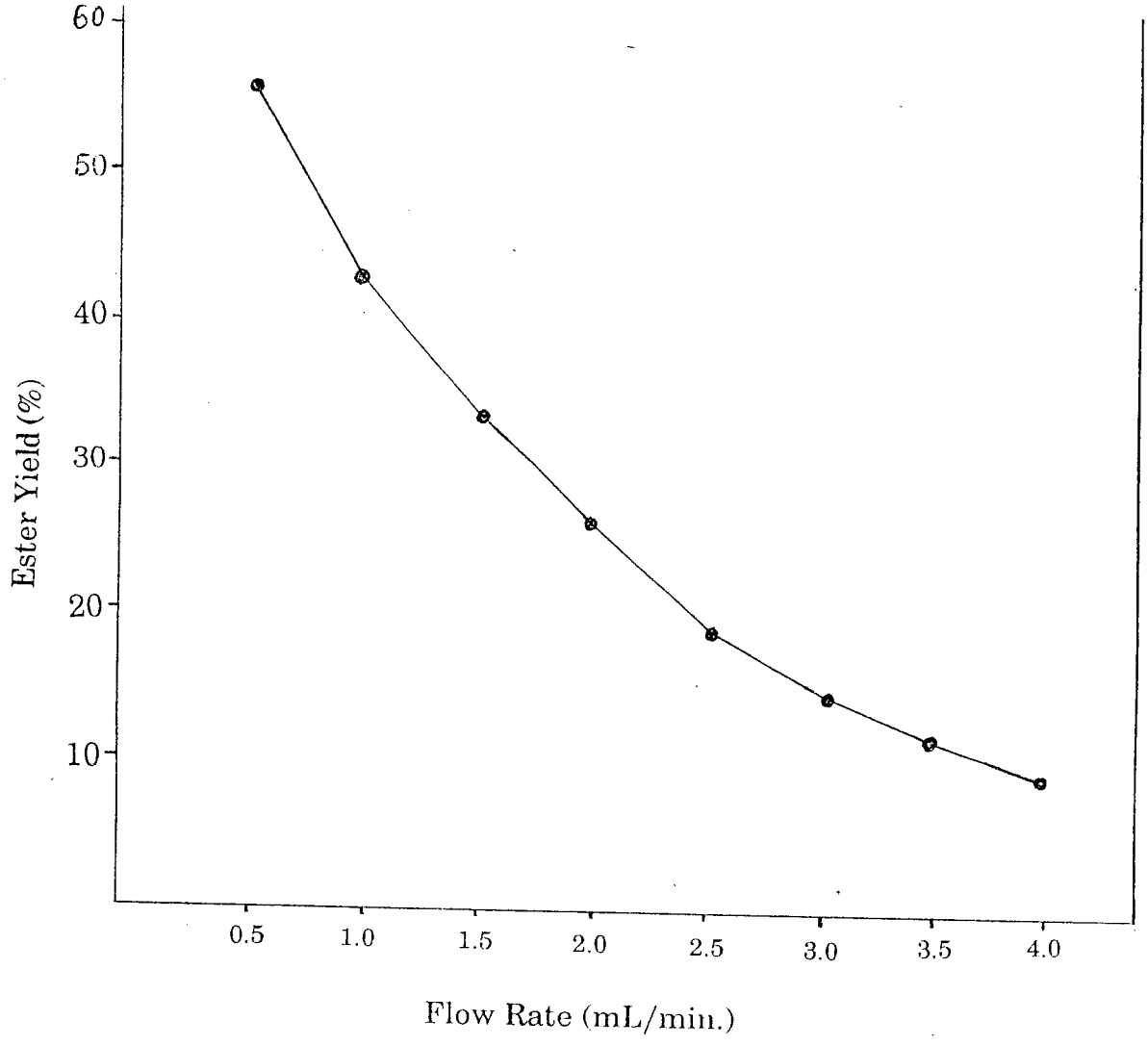


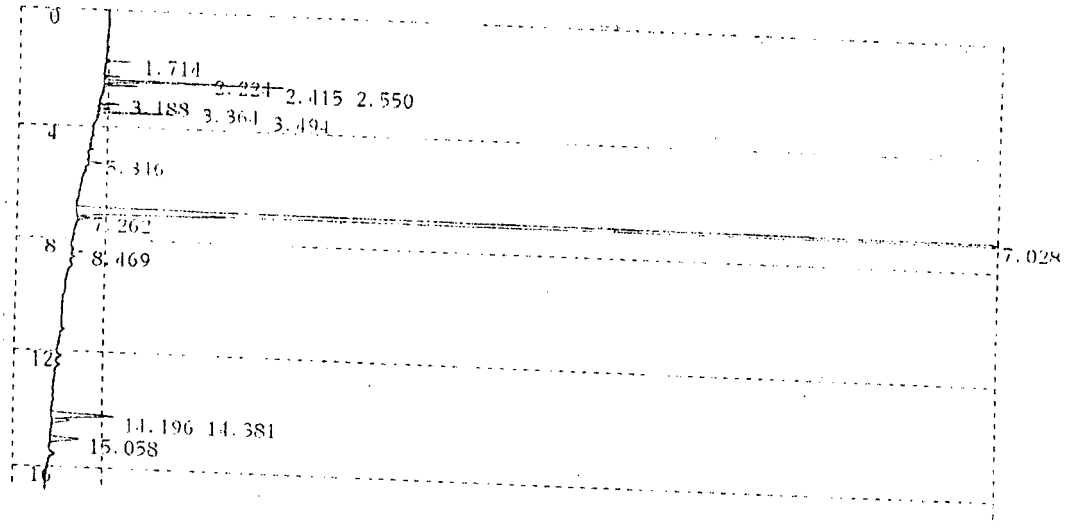
FIGURE - 30

EFFECT OF FLOW RATE ON ESTERIFICATION YIELD

C-R4A CHROMATOPAC CH=1 REPORT No.=11 CHROMATOGRAM=1:¥CHRM1.C04 91 08 10 12:31:5

Analysis File : 1:¥FILE1

FUSEL OIL ESTER
BP1.C.T.100



** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC
1	1	1.714	81	56			0.3531
	2	2.224	64	36			0.2788
	3	2.415	1112	396			4.8605
	4	2.55	150	76	V		0.6561
	5	3.188	107	47			0.4663
	6	3.364	59	28			0.2596
	7	3.191	396	170			1.7297
	8	5.346	126	32			0.5489
	9	7.028	18655	3739			81.5265
	10	7.262	131	80	V		0.5839
	11	8.469	105	24			0.1606
	12	14.196	1123	142			4.9059
	13	14.381	283	41	V		1.2346
	14	15.058	489	64			2.1353
		TOTAL	22883	4879			100

FIG.31. **GAS CHROMATOGRAM OF THE REACTION MIXTURE**

4.6.6. Purification of the Fusel Oil Esters

The free acid, from the mixture, was removed by $NaHCO_3$ treatment. The unreacted alcohols and other impurities were removed by column chromatography. TLC of the fractions, eluted with a mixture of n-hexane-ether (95:5), showed the presence of esters only. There was no spot corresponding to R_f values of acids or alcohols. Infra-red spectrum of this fraction (Figure-32) shows strong peaks at 2970 cm^{-1}, 2948 cm^{-1} and 2860 cm^{-1} (C-H-bond stretching), 1740 cm^{-1} (Carbonyl group), a medium peak at 1470 cm^{-1} (C-H-bending) and 1180 cm^{-1} (Ester group). There are no peaks at 3600 cm^{-1} and 3200 cm^{-1} indicating that eluted fraction did not contain acid or alcohol.

4.6.7. Enzyme Reactivation

During the process, the packed mycelial lipase absorbed water and efficiency of the column was dropped gradually. It was observed that after continuous use for six days the esterification activity of the system was reduced to 50% of the original. About 94% of the original activity was regained by dehydrating the column by passing 100cm^3 chilled acetone, 100cm^3 chilled acetone-diethyl ether mixture and 100cm^3 dry n-hexane. A similar technique for the regeneration of lipozyme have been used by Negishi and Minoshina[161]. They claimed 70% regeneration of the original synthetic activity of the enzyme. So the naturally immobilized cell bound lipase of *Rhizopus arrhizus* is more resistant than lipozyme-the immobilized lipase of *Mucor miehei*.

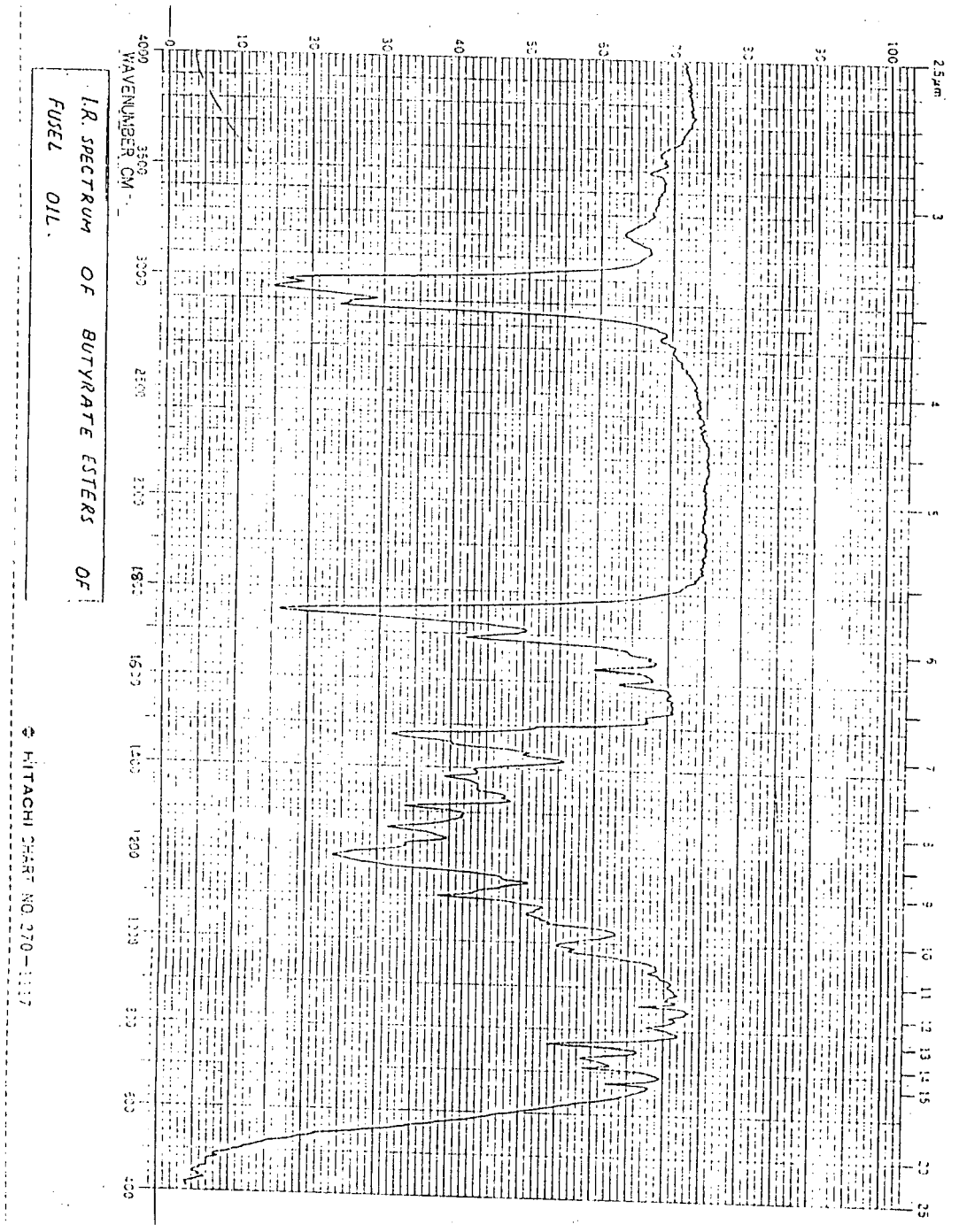


FIGURE - 32 INFRARED SPECTRUM OF BUTYRATE ESTERS OF FUSEL OIL

4.7. TERPENE ESTERS SYNTHESIS

Esters of terpene alcohols with short chain fatty acids are valuable flavour and fragrance compounds. These are major constituents of numerous essential oils such as rose oil, lavender oil and lime oil etc. Terpene esters are obtained either from their natural sources or are synthesized by esterification of terpene alcohols of plant origin. Fragrance of these compounds is very much dependent upon their stereospecific nature.

As enzymes are stereospecific in their action, enzeymic esterification of terpene alcohols was preferred for the production of stereospecific terpene esters. Different terpenols such as geraniol, nerol, citronellol, menthol, borneol, linolool and α-terpineol were esterified with butyric acid. Data of the Table-26 shows that terpene alcohols having hydroxyl group at primary position, such as geraniol, nerol and citronellol were esterified at reasonable rates. But secondary and tertiary terpenols such as menthol, borneol, linalool and α-terpineol were not esterified. There is notable difference in the behaviour of *Rhizopus arrhizus* lipase towards secondary terpene alcohols and saturated secondary alcohols. The saturated secondary alcohols were esterified but seconday terpene alcohols could not be esterified.

In contrast to the reports by other workers[253], the lipase of the present strain of *Rhizopus arrhizus* esterified both nerol and geraniol at comparable rates.

Table-22

ESTERIFICATION OF TERPENE ALCOHOLS WITH BUTYRIC ACID USING MYCELIAL LIPASE OF *RHIZOPUS ARRHIZUS*

Terpene alcohols	Esterification Rate (μmol/min./g mycelium)
Geraniol	34
Nerol	37
Citronellol	25
Menthol	0
Borneol	0
Linolool	0
α-Terpineol	0

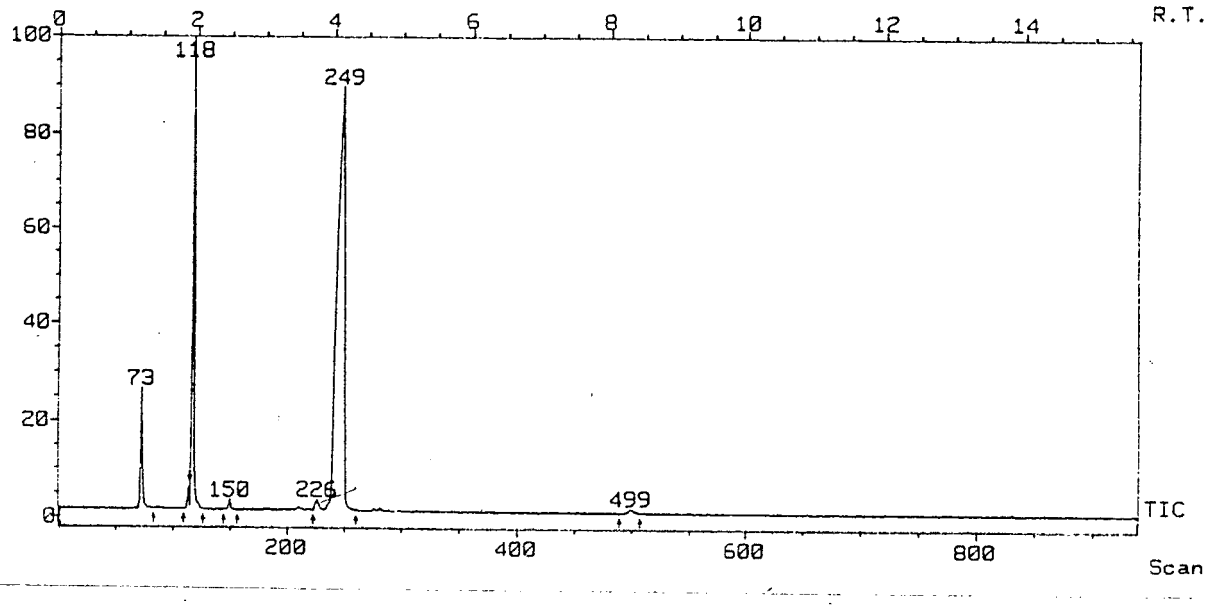
The reactions were carried out at 30 °C in n-hexane as the reaction medium. The concentrations of alcohols and acid were 0.6 mol/dm^3 each. The enzyme dose was 1.0% w/v.

4.7.1. Production of Geranyl Butanoate Using Packed Column of Mycelial Lipase of *Rhizopus arrhizus.*

A mxiture of 0.1 mole/dm^{3}geraniol and 0.1 mole/dm^3 butyric acid in n-hexane was circulated through the packed column at the rate of 0.5cm^3/min.. The ratio of the mycelium and the neutralized sand in the column was 1:1. The free fatty acid titration of the reaction mixture showed that in the first cycle 38% acid was consumed, in the second cycle 23% more acid was consumed (residual free fatty acids 39%). At the end of the third cycle percentage of residual fatty acid was 30% which was reduced to 23% after 4th cycle. Further recycling of the mixture, even after reactivation of the column, did not enhance percent yield of the ester. The experiment was repeated many times. The results varied over a range of about 5% at the maximum, and the values given above are the means of four experiments.

G.C.M.S. of the mixture (Figure-33) after removal of the hexane, showed the presence of 75.7% geranyl butanoate and 22.5% unreacted geraniol.

TIC Data File: GERAN 12-OCT-94 15:41
Sample: GERANYL BUTYRATE, BP5, CT170
Scan# 1 to 938 (938) RT 0'00" to 15'36" (15'36") EI (Pos.) Lv 0.00
Operator: Analytical



No.	R.Time	Top	Start	End	Type	Area	Rel	%	Height	Rel	%
1	1'12"	73	69	78	IN	1393.1240	7.50	5.4	712.0703	25.48	11.6
2	1'53"	114	111	115	PL	247.6243	1.33	1.0	126.5358	4.53	2.1
3	1'57"	118	115	126	PR	5502.4720	29.61	21.4	2794.6270	100.00	45.5
4	4' 8"	249	234	255	IN	18584.8600	100.00	72.2	2504.1780	89.61	40.8

FIG.33. GCMS

Note : The chromatogram shows the presence of about 5.0% solvent (n-hexane) in the sample due to its incomplete evaporation. So in the text percentages of esters and unreacted alcohols have been reported after correction.

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