FERMENTATION AND PURIFICATION OF LIPASE ON A LABORATORY SCALE.

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1.0 INTRODUCTION

Lipases have been defined as enzymes which are capable of hydrolyzing triglycerides with long chain fatty acids (Brockerhoff and Jensen, 1974). Although lipases are widespread in nature, occurring in animals, plants, bacterial and fungi, microbial lipases (bacterial and fungal) are more important in commerce and research since they are usually available in large amounts in purified form. They are also generally more stable than lipases from other sources.

Microbial lipases are used for several purposes including cheese ripening (Otherholm *et al*, 1965); removal of excessive fat from fish (Burkholder *et al*, 1968); in cosmetics (Saphir, 1967) vegetable fermentation (Vorbeck, *et al*, 1963) Meat curing (Gilotti *et al*, 1971). Other applications of lipases include use as detergent additives (Kuehling *et al*, 1971), in pharmaceuticals (Berrebi *et al*, 1968), leather processing (Ivood et al and Law, 1912). and sewage treatment (Seitz, 1974). Also a variety of esters suitable for flavouring have been produced from ethanol and free fatty acids (FFA) using microbial lipases (Kanisawa, 1983) etc.

Culture conditions are important to microbial production of lipases. *Pseudomonas florescence* was found to produce lipases in broth culture between 4-30°C, but production was greatest at 8 °C, whereas optimum growth temperature was 20 °C (Anderson, 1980). Production of lipase by *Pseudomoncus aeruginosa* was found by Nadkarni (1971a) to be optimum at pH 7. However, Nashi and Nelson (1953b) found pH>7 to be best for lipase production by *Ps fragi*. Development of acidity in fermentation broth was accompanied by decreased lipase production. Like all other cultural

conditions, effect of aeration on lipase production depends on the specific microorganism. Nashi and Nelson (1953) found certain *Pseudomonas spp* to produce more lipase with modern aeration whereas high aeration favoured greater lipase production by *Ps mephitica va lipolytica* (Kosugi and Kanbayashi, 1971). Winkler and Stuckman (1979) found glycogen, gum arabic, hyalouronate, laminarin and pectin B to be more effective in stimulating lipase production out of 21 polysaccharide tested Chandlers et al (1979) found short chain fatty acids to stimute lipase production whereas long chains fatty acids inhibited it.

Production of lipases may be affected by the presence of other exo-cellular enzymes. Anderson (1980b) has observed the inactivation of *Pseudomonas* lipases by subtilisin at 20°C. This suggests that lipases may be destroyed by proteinases produced simultaneously in culture. Microbial lipases are purified by a combination of ion exchange and gel filtration as for the purification of other proteins. However, if microbial lipases are produced in a lipid abundant medium, the first step must be the extraction or precipitation with organic solvents such as ethanol or butanol (Sugiura *et al*, 1974). The molecular weight of purified lipases depends on the source. Inguina *et al* (1977) reported 32,000 for certain *Ps. florescence* lipases whereas Dring and Fox (1983) report 55,000 and 16,000 for *Ps. florescence* MC 50 and AFT respectively. *Pseudomonas* lipases are usually inactivated by heavy metal ions (Lu and Liska, 1969).

Measurement of lipases activities are often based on the rate of release of free fatty acids (FFA) from triglycerides. Both tributyrin and triolein are widely used as assay substrates. However, triolein better fulfils the definition of a substrate for lipase by virtue of its long chain fatty acids (Brockerhoff and Jensen, 1974).

The aim of the present work is to produce lipases from a suitable *Pseudomonas spp* in separate draught tube and stirred fermenters, and to determine the activation of the lipase so produced in crude, semi-purified, purified and immobilized forms.

2.0 MATERIALS AND METHODS

2.1 Selection of *Pseudomonas* Strain for Lipase Production

18 different *pseudomonas* strains were each plated on agar plates containing 4% agar, 0.5% peptone, 0.15% yeast extract, 0.01% calcium chloride and 10g of either tween 20, tween 40 or tween 80 as substrates. The plates were incubated at 30°C for 3d. Lipase production was indicated on the plates by the precipitation of calcium salts on the plates around the colonies.

2.2 Fermentation: Production of Extra Cellular Lipase using *Pseudomonas* ATCC No. 21808

i. Preparation of starter culture:

Two 1 litre flasks each containing 700ml of medium made up of 0.5% peptone, 0.5% yeast extract, 0.05% NaCl and 1% olive oil were each incubated with *Pseudomonas* ATCC 21808 which had proved positive for lipase production. The inoculated flask were incubated at 37°C for 24 h.

ii. Production of lipases in 100 litre stirred tank reactor and a 100 litre draft tube reactor

A 100 l stirrred tank fermenter containing 70 l of medium made up of 0.5% peptone, 0.5% yeast extract, 0.05% NaCl and 1% olive oil was inoculated with 0.7 l of the starter culture of *Pseudomonas* ATCC 21808. The parameters used to run the batch fermentation were - temperature: 20 °C, pH 7.0, initial pO_2 100% and stirrer speed: 250 rpm. pH was maintained at 7.0 by the addition of 2N NaOH, and antifoam was added when necessary. The stirrer speed was later increased to 500 rpm to increase oxygen uptake. A 100 litre Draft tube fermenter also containing 70 l of the same medium was inoculated with 0.7 l of the same culture and the fermentation run on batch basis using similar parameters.

The two fermenters were run for 3 d and samples taken at intervals to determine lipase activity, using the pH stat and the p-Nitrophenyl palmitate (PNPP) methods.

iii. **Determination of lipase activity by the P-Nitrophenyl palmitate method**

10 μl of the supernatant of the centrifuged sample was added to 1 ml of a solution containing p-nitophenylamine as a substrate. The splitting of the ester resulted in a colour change which was measured at 405 nm spectrophotometrically. This method measures esterase activities but is not specific for lipases.

2.3 PRODUCT RECOVERY

2.3.1 Centrifugation

The products from the two fermenters were combined and centrifuged to remove the cells from the medium.

2.3.2 Lipase Concentration

2.3.2.1 Ultra and Microfilteration

The product was filtered using ultra and microfilteration in order to recover the enzyme from the medium thereby reducing the volume of the product from 160 l to 5 l for easy handling and purification. Microfilteration was carried out using a filter of pore size 0.2 μm. Ultra filtration was carried our at 10,000 g cut off value.

Enzyme activity of the centrifuged cells, filtrate and concentrated products were determined by pH stat method.

2.3.2.2 **Separation of lipase from the lipid substrate**

A few ml or grams of various chemical compounds were added to 2 ml of aliquots of the product to determine the most suitable reagent for liberating the lipase from the fat globules. The chemicals used were butanol, polyethyliglycol, (PEG), Ethanol, SDS, chloroform and certain detergents.

2.4 Purification of Lipase by Fast Protein Liquid Chromatography

The crude lipase was purified twice FPLC using Q-sepharose and Amino Hexyl Agarose gel columns.

2.4.1 Q-Sepharose Anion Exchange Chromatography

A 200 cm gel column of Q-sepharose was equilibrated with 3 bed volumes, 900 ml of Buffer A containing 20 ml of glycine adjusted to pH 9.0, to wash off any salts in the column. 500 ml of a crude lipase product was then run into the column to bind the proteins including the enzyme whilst contaminants remained unbound. The column was washed with 2 bed volumes, 600 ml of buffer A. The bound protein was eluted with 1,500 ml in a linear gradient of 0-100 % buffer B, which contained 20mM glycine, 1m Nacl, 0.05 %, Iuprol with pH adjusted to 9.0. The detergent, luprol was added to clear the solution. Elution was completed by running 400mls of buffer B at a concentration of 100 % through the column. The eluted product was collected in 95 20 ml aliquots, giving a total recovered volume of 1900 ml. The column was then washed with 900 ml of buffer A.

A qualitative lipase activity test was carried out on the different fractions collected to locate the fractions in which lipase was eluted. 10 μl of the fraction was reacted with 40 μl of P-nitrophenyl patmitate in microtubules. Substantial lipase activity was indicated by rapid development of yellow colour. The actual lipase activity of the fractions which contained the lipase was then determined by the PNPP method.

The range of fractions in which lipase was collected were combined and further purified by Hydrophobic Interaction Chromatography.

2.4.2 Hydrophobic Interaction Chromatography

This was carried out using a gel column of Amino Hexyl Agarose in the FPLC. The procedure followed was similar to the Q-sepharose Anion exchange chromatography using the following materials and volumes.

Buffer A:	20 mM glycine, 1m Nacl, pH adjusted to 9.0
Buffer B:	20 mM glycine, 1m Nacl, 0.05% improl, pH 9.
Bed volume:	103 ml of buffer A
Sample:	1000 ml
Wash:	300 ml buffer A.
Elution:	0-100 % buffer, 800 ml.
Hold:	200 ml of 100 % buffer B
Wash:	300 ml buffer A.

Qualitative and quantitative tests were carried out to identify the collected fractions which contained the lipase. The protein content of the product after anion exchange chromatography purification and after hydrophobic interaction chromatography were determined photometrically using bovine serum albumin standards to obtain a calibration curve. 50 µl of sample and 950 µl of sample were used and measurement made at 562 nm.

2.4.3 Polyacrylamide Gel Electrophoresis

Non-denaturation electrophoresis were carried out on the crude products and the final product to determine the success of purification and molecular weight of the unknown lipase. The gel

used was a 8-25 % gradient native polyacrylamide and standard protein solutions were run concurently. Thyroglobulin mol. wt. 669,000; ferritin mol. wt. 440,000 catalase mol. wt. 232,000, lactate dehydrogenase mol. wat, 140,000 and bovine serum albumin, mol. wt. 67,000. The gels were either silver stained or stained with fast red plus naphthylactate.

2.5 IMMOBILIZATION OF LIPASE AND LIPASE PRODUCING MICROORGANISMS

2.5.1 Immobilization of Lipase Enzyme

Lipase enzymes were immobilized on different carriers using different coupling compounds. The carriers with immobilized enzymes were tested for enzyme activity and immobilization yield.

Coupling Agent	Epoxy Group Carrier	
Phosphate	Polymentrager VA Epoxy	pH 7:
Glutaraldehyde	Silica D2	pH 7:
Glutaraldehyde	Silica D6	pH 7:
CMPT	Silica D6	PH 4:
EDC		
CMPT	STP	pH 4, pH 10
CMPT	EDC	pH 4:

2.5.2 Immobilication of Lipase Producing Whole Cells

Cells of *mucor michei* were immobilized in calcium alginate beads at cell loadings of 1,10,20 and 30 %. The beads were incubated in a nutrient medium at 45 °C for 3 d in a reciprocal shaker bath. After incubation the enzyme activity was determined in the culture filtrate.

2.6 REACTION OF LIPASE

2.6.1 Synthetic Reaction

2.6.1.1 Kinetic Resolution of Monoketal of Racemic 4-Acetoxy-2-Cyclopentene-1-one

The hydrolysis of racemic 4-Acetoxy-2-Cyclopentene-1-one into R and S alcohols enantiomorphs by two different lipases was studied. For each enzyme both the free and immobilized enzymes were studied. The products formed were converted into diastereomeric MTPA esters for detection by Nuclear Magnetic Resonance (NMR) in order to determine the concentrations of the R and S alcohols formed and the Enantic meric excess value (ee). The success of the initial hydrolysis was also studied by HPTLC.

2.6.1.2 Selective Esterification of Enantiomers by Lipase

5 lipases and Dowex 50 were investigated for their selectivity in using only one enantiomer in ester synthesis with 0.1m myristic acid and 0.1m myristic acid and 0.1m optically active R-S menthol in iso octane saturated with water. The products were analysed by HPTLC.

2.6.1.3 **Lipase Determination for Enantiomers of δ-0H Decanoic Acid and Conversion Rates**

The conversion rate and enzymatic discrimination of s-lipases for enantiomers of optically active δ-oH decanoic acid was studied in a reaction with butanol.

2.6.1.4 **Transesterification of Optically Active Fatty Acids**

The ability of 30 lipases to transesterify optically action δ-OH decanoic butanol and to secondary alcohols was studied in reactions with butanol and 2-pentanol.

RESULTS AND DISCUSSION

Table 1. **Production of Lipase on Different Fat Substrates by *Pseudomonas* spp.**

	Lipase production on		
	Tween 20 (c 2:0)	Tween 40 (c16:0)	Tween 80 (c18:1)
Pseudomonas acidocarius			
DSM 39	+	(+)	-
DSM 50057	+	+	-
Pseudomonas chlororamphis			
DSM 50135	++	+	+
DSM 50136	++	+	+
Pseudomonas flourenceus			
DSM 84	-	+	-
DSM 2005	+	++	-
DSM 50090	+	+	(+)
DSM 50106	-	-	-
DSM 50108	-	(+)	-
DSM 50117	+	++	+
DSM 50148	++	++	++
DSM 50415	-	+	-
Pseudomonas fragi			
ATCC 4973	(+)	(+)	-
DSM 38	+	+	(+)
Peudomonas testeroni			
DSM 1622	++	+	-
DSM 50241	+	+	+
DSM 50242	++	+	(+)
Pseudomonas spp.			
ATTC 2808	+++	+++	+++

3.1 **Production of Lipases by Different *Pseudomonas* Strains**

Table 1 shows the production of lipases by six different *Pseudomonas* strains screened for lipase production in media with 3 different fat substrates.

3.2 **Production of Lipase by fermentation Using *Pseudomonas* (ATCC No. 1208)**

The consumption of 0_2 as measured by decrease in the partial presure of 0_2 in the media in a batch stirred tank reactor and in a draft tuber reactor during fermentation are presented in tables 2 and 3.

Table 2.

Fermentation Control of a Batch Stirred Tank Reactor

Time(h)	pH	Temp °C	$p0_2$ %	Agitation (rpm)	Observation
0	7.0	20	100	250	heavy foam
3.5	7.0	20	95.2	230	development
8.3	7.1	19.5	93.9	260	antifoam
22.15	7.1	20.5	82.5	260	added
32.00	7.1	20.5	78.2	260	

Table 3

Fermentation Control of Draught Tube Reactor

Time(h)	pH	Temp °C	pO_2 %	Agitation (rpm)
0	7.0	20	100	250
3.5	7.5	20	98.5	230
8.3	7.1	19.5	93.9	260
22.15	7.1	20.5	82.2	260
32.0	7.1	20.5	78.2	260

The % PO_2 in the draft tube fermenter dropped to 0.6% in 22 h and was suspected to be due to poor oxygen supply into the medium rather than its utilization by the cells. An increase in the stirrer speed of this fermenter readily increased the PO_2 and it was 16.7% at the end of 32 h of fermentation. The final % PO_2 in the stirred tank reactor which showed a higher enzyme activity was 18.2 %.

The enzyme activity was found to be associated with the cells probably because the enzyme was adsorbed to the cell envelope. This behaviour can be observed by the development of lipase activity in the supernatant and cell suspension of media sample after centrifugation, as shown in figures 1 and 2.

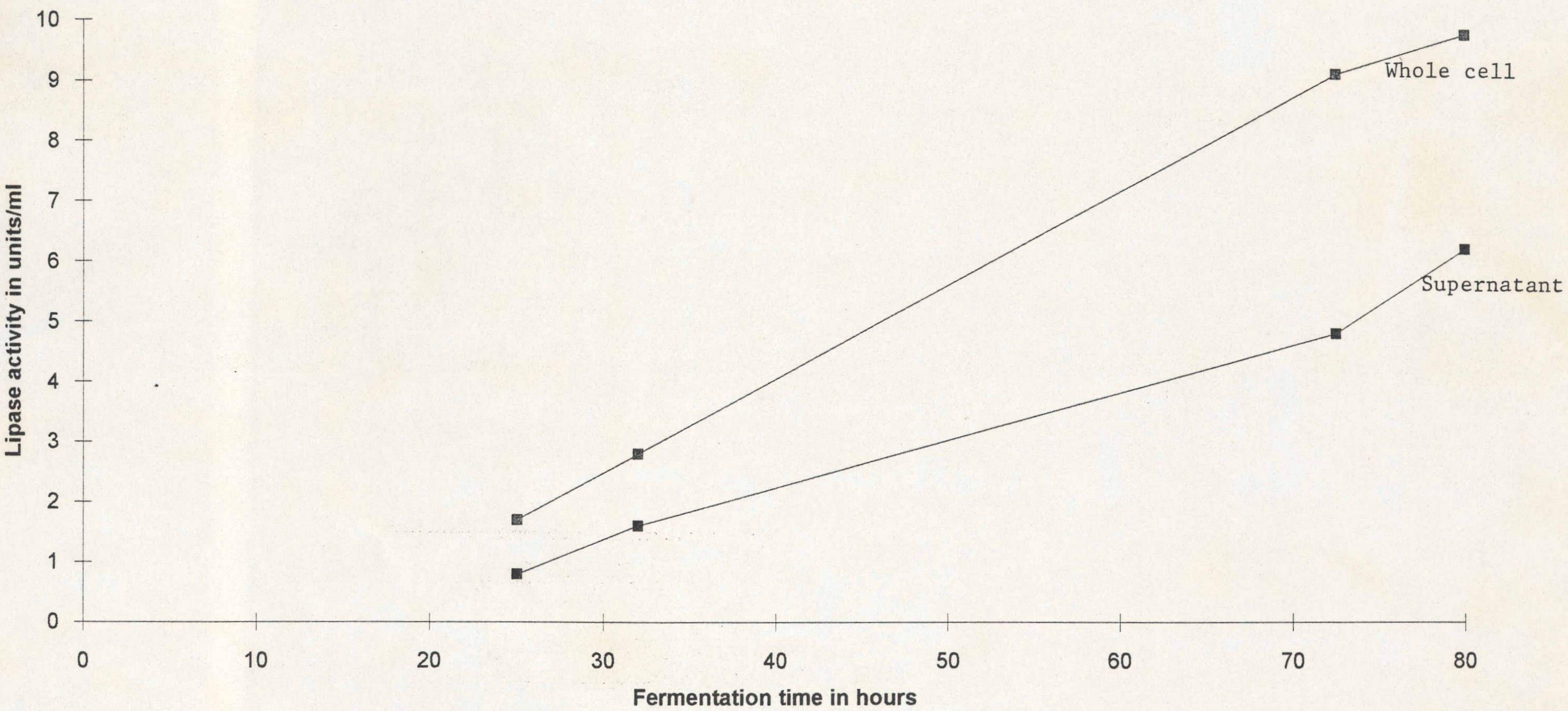


Fig 1. Lipase activity in Stirred Tank Reactor durnig fermentation
Lipase activity in units/ml
0
1
2
3
4
5
6
7
8
9
10
Fermentation time in hours
0
10
20
30
40
50
60
70
80
Whole cell
Supernatant

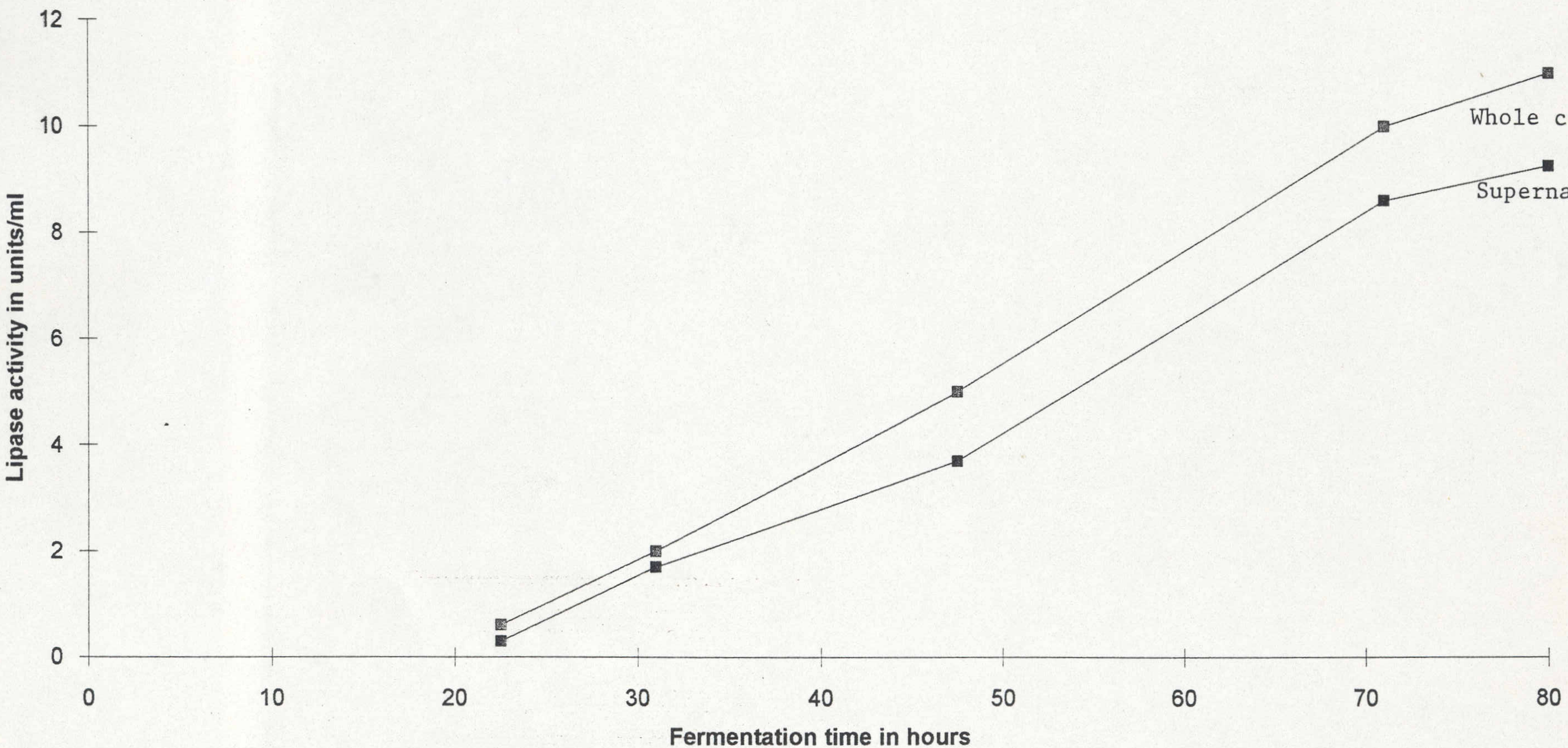


Fig 2. Lipase activity in Draft Tube Reactor during fermentation
Lipase activity in units/ml
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70
80
Fermentation time in hours
Whole cell
Supernatant

The final enzyme activities and the total enzyme activity in the supernatants of the two fermentations and combined products are shown in table 4.

Table 4.

Final lipase activities in the stirred and draft-tube fermenters

Fermentation	Final enzyme assay (units/ml)	Total enzyme activity (units)
Draft tube fermenter	6.4	384,000
Stirred tank fermenter	9.25	555,000
Combined product (supernatant)		1,050,000

3.3 PRODUCT RECOVERY

Fermentation broth from reactors I and II were pooled and the resulting 120 l broth was centrifuged and activity determined in the supernatant. Total activity in the supernatant was 10,800,000 units.

After centrifugation, the liquid was passed through an ultre filtration unit (cut-off value 10,000 δ) to achieve separation of enzyme from fat emulsion. However, no separation was achieved as the membrane pores were blocked due to fat aggregates. The suspension was then filtered through a microfiltration unit of pore size 0.2 μm. The first run gave a turbid filtrate, however, a film of fat developed on the surface of the membrance improving its filtration capacity and the filtrate was clear but with little enzyme activity. The membrance was washed intermittently

with 20 mM glycine buffer (pH 9). The filtrate with 5.31 enzyme activity was discarded and the retentate having the major part o the enzyme activity (ie. 11,25,000 units) was stored at 5 °C. The enzyme form aggregates with oil drops and these agreegates were larger than 0.2 μm, hence most of the activity was retained in the filteration unit.

Clarification of Emulsion

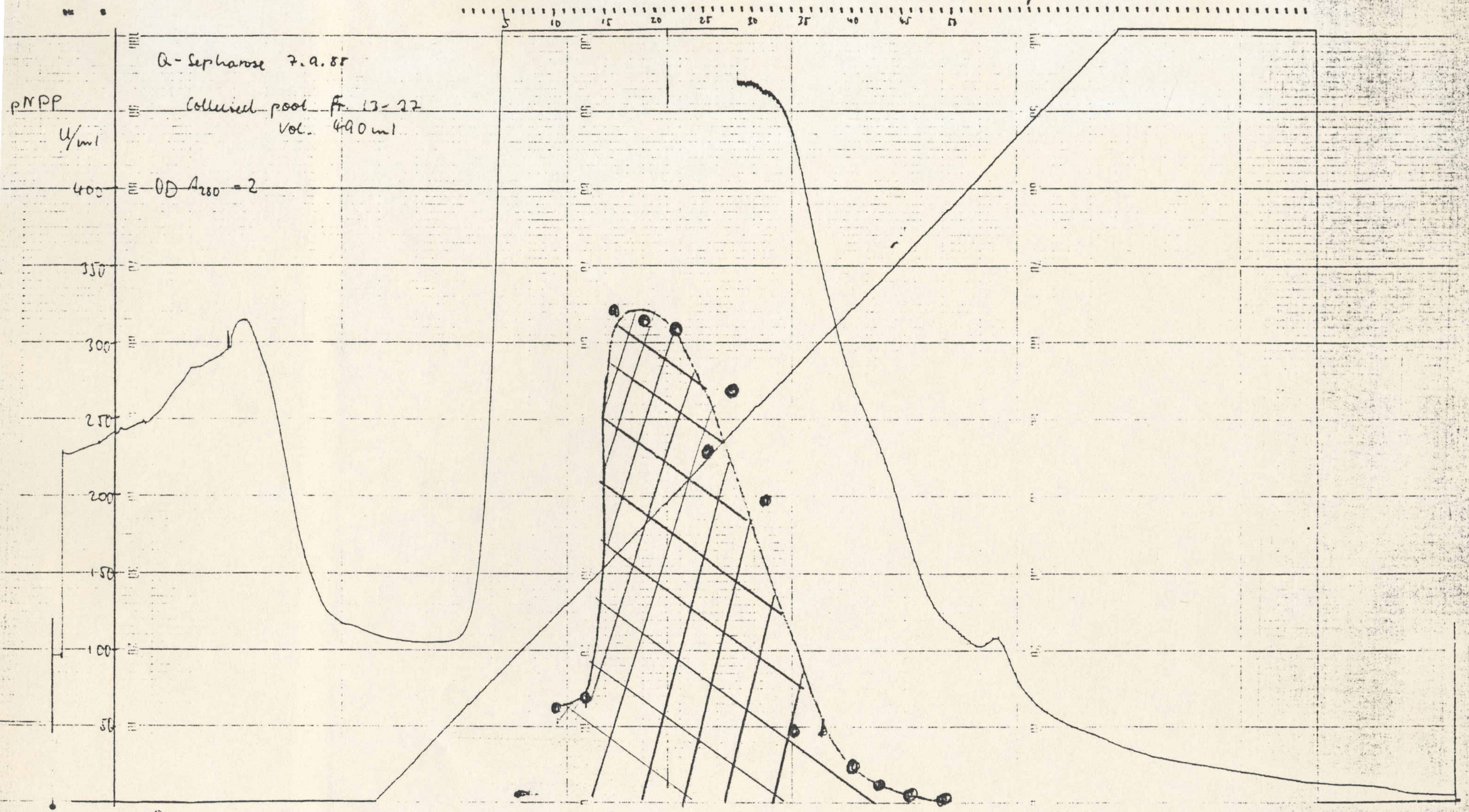
The fat emulsion was very stable and no clarification could be achieved with detergents and solvents like chloroform, hexane, butane, and polyethylene glycol, SDS etc. The enzyme could be recovered by precipitation with ethane (first with 20% and then increased up to 80 % in three steps. Temp of ethane was - 28 °C), the steps were carried out at O°C. The enzyme yield was 56 % with 36 % of the enzyme being denatured and 8 % activity in the supernatant.

3.3.1 Enzyme Purification

The crude enzyme sample was clarified by adding a detergent Luprol PX 100 (sigma) to remove greasy material adjusted to pH 9.0 and run over the Q-sepharose column. The column was first equilibrated with 3-bed volumes (ca 900 ml) buffer A, sample was run over the column and a linear gradient from 0.100 % buffer B with salt was applied for elution. A total of 95 fractions (20 ml each) were collected. Fractions Nos. 13-37 which were in the region of maximum activity (figure 3) were pooled together and the total activity determined.

The enzyme was further purified on amino hexyl agarose column as follows: The column was equilibrated with 3 bed volumes of buffer A. The sample was run over the column and a linear

FIGURE 3 : Q - SEPHAROSE CHROMATOGRAPHY



gradient from 0-100% of buffer B was applied for elution. A total of 90 fractions were collected and fractions 20-31 which were in the region of maximum activity (as shown in figure 4) were pooled together and the total activity determined.

During the hydrphobic interaction chromatography, enzyme activity was detected in the run-off and wash off solution of the column. This was probably due to the excess luprol added to the sample for clarification. To get rid of the luprol, the enzyme sample was passed through ultra filteration unit and applied to the HIC column. A similar result was obtained. This was probably due to the fact that the reach the limit of the binding capacity of the column had been reached. The results of the protein determination of all samples are shown in figure 5.

3.3.2 **Gel Electrophoresis**

The enzyme samples were subjected to native polyacrylamide Gel electrophoresis using an 8-25 % gradient gel (PhastGel, pharmacea). A standard protein solution containing thyroglobulin, ferrtin, catalase, lactate dehydrogenase and albumin was run along with the crude enzyme, enzyme from q-sepharose and from Hydrophobic Interaction chromatography (undiluted and diluted 1:10 and 1:100 respectively). Two gels were run in this fashion, one was silver stained and the other was stained to detect enzyme activity using naphthylacetate and fast red.

By comparison, the molecular weight of the lipase was determined as shown in figure 6.

FIGURE 4: HYDROPHOBIC INTERACTION CHROMATOGRAPHY

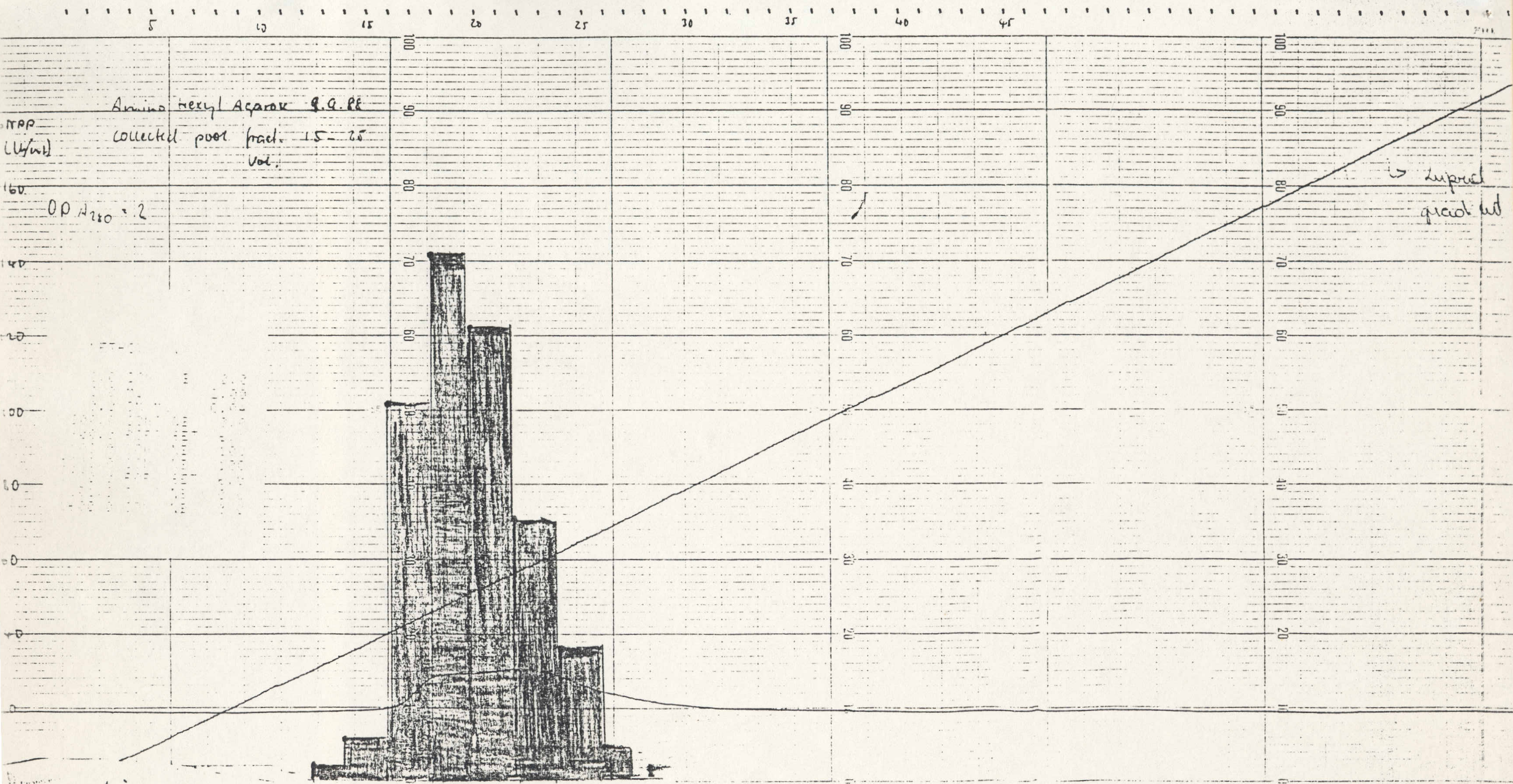
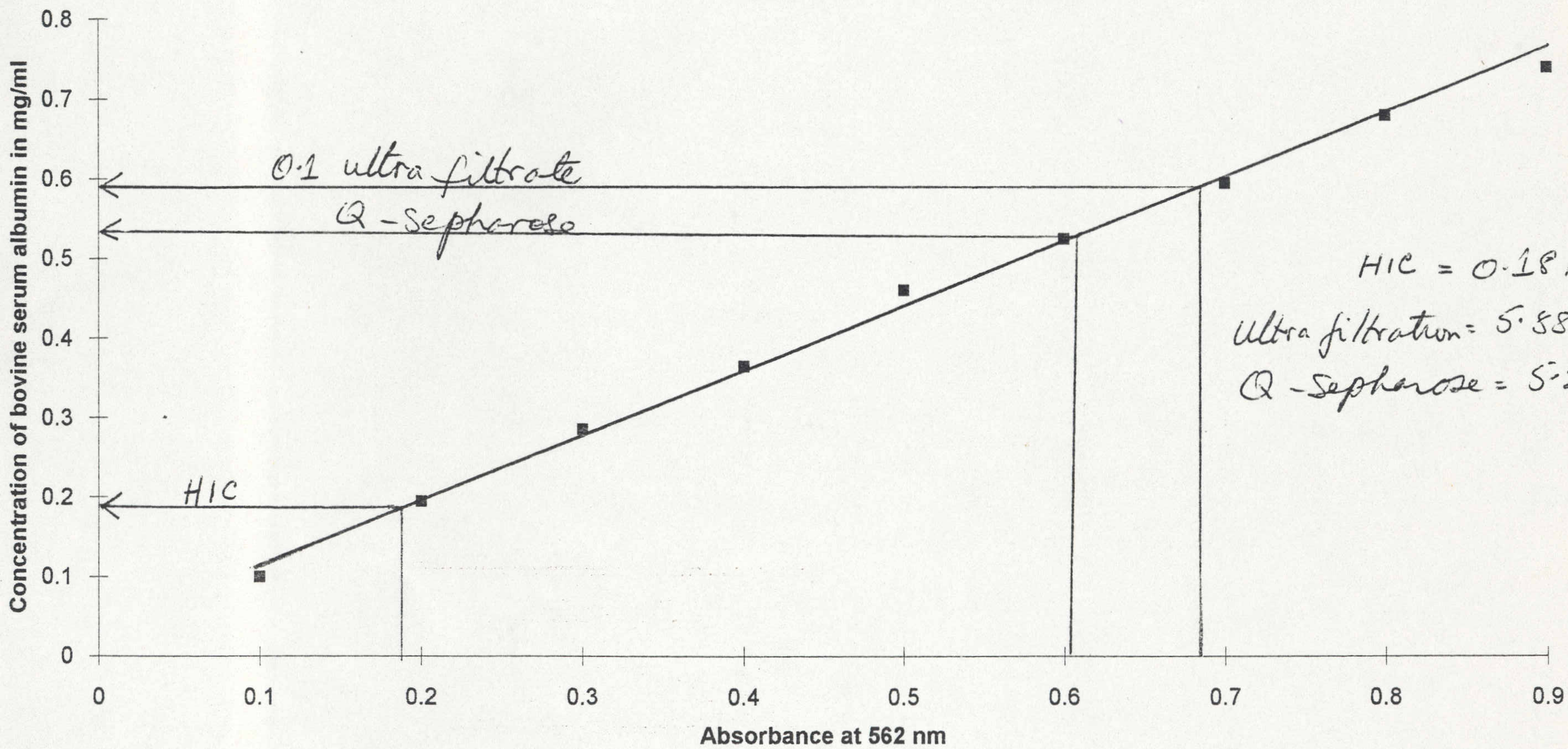


Fig 5. Calibration curve for protein determination



3.4 IMMOBILICATION OF LIPASE

Table 5.

Activity of Lipase after immobilization on different carriers

Carrier	Yield	Activity in units/g		
		Enzyme used	Wet beads	Dry beads
Carrier with epoxy groups				
Polymetruger VA epoxy washed with				
(i) 150 ml buffer	19.4	2059.8	112.8	400.0
(ii) 300 ml buffer	18.9	2059.8	9.0	389.0
Silica carrier M1	12.7	2059.8	113.4	261.0
Silica carrier M4	5.5	2059.8	55.9	114.3
Carrier groups amino groups				
D2 silica with glutaraldehyde, pH 7	7.12	1152.0	37,5	83.5
D6 silica with glutaraldehyde, pH 4	5.7	1152.0	53.72	66.6
D6 silica with EDC*	5.0	1152.0	25.0	58.18
Carriers with carboxl groups				
STP#, pH 4	2.5	1152.0	20.0	29.85
STP, pH 10	3.4	1152.0	17.85	40.0
STP pH 4 with EDC	3.1	1152.0	17.0	35.9

* 1-ethyl-3-dimethyl aminopropyl carboamine

activity of free enzyme - 11.52 Units/mg enzyme powder in 0.5 M phosphate buffer.

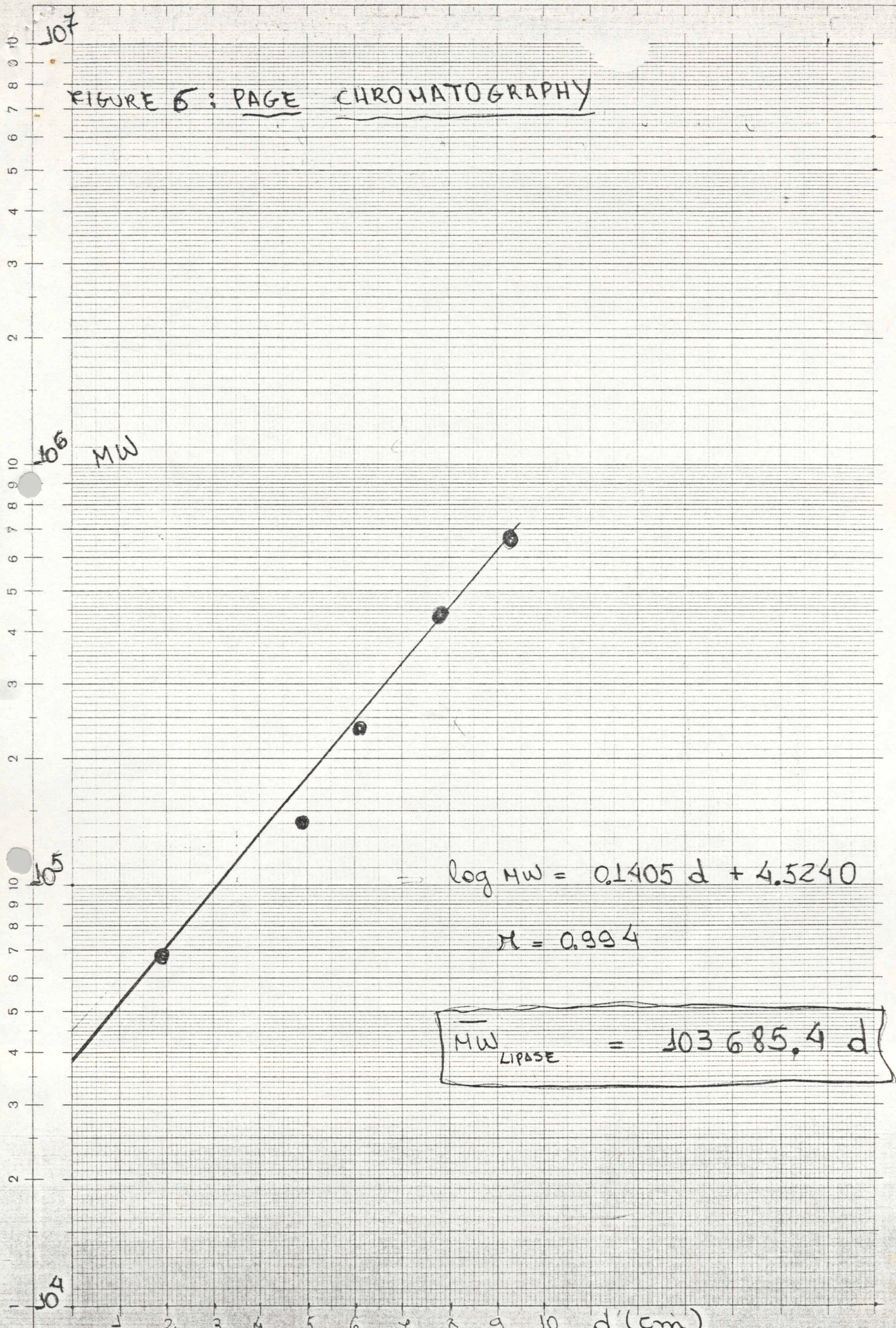


FIGURE 5 : PAGE CHROMATOGRAPHY
10^7
10^6
MW
10^5
10^4
log MW = 0.1405 d + 4.5240
r = 0.994
MW LIPASE = 103 685.4 d
1 2 3 4 5 6 7 8 9 10 d (cm)

Table 5 shows the activities of free lipase and lipase immobilized on different carriers. The lipase enzyme used in the immobilization studies was a crude commercial enzyme available from 'Amano' Japan. From the above table, the highest immobilization yield was obtained with polymetragerun, probably due to the mild condition of immobilization and small diameter (50-200 μm) of particle. The reaction of epoxy group and the yield was lower due to their larger diameter (0.3 - 1.4 mm) and the resulting lower surface area to volume ratio. Carriers D2 (diameter 0.3 -0.4 mm) and D6 (diameter 0.05 - 0.3mm) which are silica carriers with amino groups showed lower immobilization yield as compared to carriers with epoxy groups. This could be related to the drastic conditions of treatment of carriers with cross linking agents and possibly some of these were left behind and affected the enzyme activity. The different yield with D2 and D6 also could be related to the different methods used to produce these carriers.

The carriers with carboxy group showed the lowest immobilization yield and were not found suitable for use. It is therefore clear from the above that carriers with epoxy groups are most suitable for immobilization of lipase. This process has already been optimized at the Gesellschaft Biotechnologische Forschung. There appeared to be no direct relationship between enzyme activity and the percentage of cell immobilized on calcium alginates beads (table 6).as well as other carriers.

3.5 Fat Hydrolysis reactions

The lipase from *Mucor miehei* showed higher activity on short chain and medium chain fatty acids which commonly occur in milk (table 7) and hence it was decided to use this enzyme for further experiments on fat hydrolysis. The lipase activity on different substrates was determined using the pH stat a very sensitive method for working with natural substrates. The activity of lipase in a natural system depends on the interfacial area (between a fat phase and ageous phase) available for activity and does not necessarily follow Michaeli-Menten kinetics.

Table 6. **Lipase Activity of free cells and immobilized cells on different carriers**

Cell Loading (% Wet weight basis)	Activity (Units/ml Enzyme)
1	0.293
10	0.24
20	0.24
30	17.04
Free Culture	1.36

The Lipase from *Mucor miehei* showed different behaviour with different substrates as evident from the titration curves obtained by the pH stat method. In the reaction with butter fat A (table 7), the curve obtained was very noisy due to the fact that butter fat is a non-hetergenous processed substrate where there exits different interphases between fat and water molecules and also has different chain lengths of fats. When more enzyme was added to the system, the curve was straight due to increase interphacial area.

In reaction with butter fat containing water, the curve was less noisy due to improvement in interface between fat and water. In reaction with cream powder containing 40 % fat, a straight line was obtained because of a homogenous fat-water interface. Similar results (straight line) were obtained with milk powder (roller dried - D and spray dried) because of homogeneity of interphase. It must be noted that milk is a natural system containing sugar, protein, fat, salt etc. in a natural emulsion and these constituents become concentrated in the milk powder. Hence, the enzyme activity in such natural systems depends on interaction of these components especially the interactions of proteins and lipid.

Table 7.

Avtivity of Lipase From *Mucor meihei* on Different Fat Substrates

Substrate	Activity of enzyme in units/ml
Buffer fat, water free containing 99% fat	282.3
Butter fat containing 76.1% fat and water	349.8
Cream powder containing 40.1% fat	402.0
Milk powder, roller dried containing 25% fat	444.4
Milk powder, spray dried containing 25% fat	394.4
Triolein	109.6

The milk protein is bipolar (with both hydrophilic and hydropholic interaction) thus providing a better interface for lipase action. As a result a better enzyme activity was observed in milk powder as compared to pure fat. In case of triolein which is an artificial substrate, noisy curve was obtained.

3.6 Synthesis reactions with lipases

The immobilized enzyme types A and B showed good hydrolysis of the racemic acetate to a mixture of R and S alcoholcs (table 8). The NMR of the MTPA - esters of the alcohols indicated an enantiomer which is useful in the synthesis of prostaglandin. In case of free enzyme 'Amano' type AY the racemic actate was almost completely hydrolysed (as indicated by a faint spot on HPLTC plates) to yield a racemic mixture of R & S alcohols.

The 30 different lipase enzymes which were screened for their ability to transesterify the fatty acid (δ-OH secanoic acid to a secondary alcohol (2 Butanol, 2 pentanol) yielded an ester in most cases. The esters were detected by using HPLTC plates.

Table 8.

Kinetic Resolution of Monoketal of Racemic 4-Acetoxy-2-cyclopentene-1-one.

Enzyme Used for Hydrolysis	Enantiomeric excess of S-alcohol %
Immobilized enzyme type A	90
Immobilized enzyme type B	90
Free enzyme, Amano type AY	Racemic mixture of alcohols was obtained

4.0 **CONCLUSION**

- In both fermenters, a high yield of lipase activity of about 1,100.000 units was obtained. However, a lot of enzyme activity was found to be associated with the cells, probably attached to the cell envelope.

- The enzyme yield was very high but the enzyme formed an emulsion with oil droplets. Hence most of this activity was retained in the microfilteration step.

- The emulsion could be broken by precipitation with ethanol and 96 % of the enzyme activity was recovered and around 36 % of the enzyme denatured.

- A purification factor of seven fold was achieved after two steps purification with ion exchange chromatography and hydrophobic interaction chromatography.

- The partially purified enzyme had a specific activity of 362.8 units/mg and a molecular weight of 103,685 δ.

- The highest immbilized yield of about 20 % was obtained on the carriers with epoxy groups which had small diameters (50-200 μm) and mild conditions of immobilization.

- The *Mucor miehei* lipase showed highest activity when it was assayed with milk substrates. This kind of lipase had more affinity towards short chain and middle chain fatty acids available in milk.

- The immobilized enzymes types A and B showed good hydrolyses of the racemic acetate to a mixture of R and S alcohols. The NMR of the MTPA esters of the alcohols indicated an enantiomeric excess of 90 % for the S-alcohol.

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