MATERIALS & METHODS
3 MATERIALS & METHODS

3.1 BACTERIOLOGICAL ANALYSIS OF SAMPLES

3.1.1 Samples
Soil and liquid samples were collected from hot springs of Manikaran, India. The samples were suspended in water and analyzed for total plate count, thermophilic count and lipolytic thermophilic count.

3.1.1.1 Total Plate Count

3.1.1.1.1 Media Preparation

a) Nutrient broth (NB)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5 g</td>
</tr>
</tbody>
</table>

Distilled water to 1000 ml. pH 8.0

Media was sterilized by autoclaving at 121°C and 15 psi for 20 minutes.

b) Nutrient Agar

NB agar was made by adding 20 g agar per liter of NB. Then sterilized by autoclaving as above.

Appropriate dilutions of the samples were plated on nutrient broth agar according to standard techniques and the plates were incubated at 37 °C for 24 h.
3.1.2 Thermophilic Count

Suitable dilutions of the samples were plated on nutrient agar plates and the plates were incubated for 24 h at 60 – 65°C for thermophilic count.

3.1.3 Lipolytic Thermophilic Count

Tributyrin agar plates: One percent emulsion of tributyrin was mixed with NB agar to prepare tributyrin agar plates. Lipolytic thermophilic counts were obtained on tributyrin agar plates. The plates poured with appropriate dilutions of the samples were incubated at 60-65°C for 24 h and the colonies showing zone of clearance around them were counted.

3.1.2 Isolation Of Lipolytic Thermophilic Bacteria

Typical colonies showing comparatively bigger zone of clearance around them on tributyrin agar plates were selected and purified by repeatedly streaking on the same substrate plates. Isolated colonies, which appeared after overnight incubation at 60-65°C were transferred on to nutrient agar slants after ascertaining their purity. The selected isolates were maintained on nutrient agar slants and kept at 5°C in a refrigerator after growth at 60-65°C for 48 h. The cultures were transferred at monthly intervals.

3.1.3 Screening Of Thermophilic Isolates For Lipase Activity

The isolates were screened for the quantitative production of extracellular lipase in a medium containing 1.3% nutrient broth (pH 8.0), which supported both growth and enzyme production. One hundred milliliter aliquots of the medium in 500 ml, Erlenmeyer flasks were inoculated at 2% level from overnight broth culture and incubated on a rotary shaker for overnight at 60°C. The cells were harvested by centrifugation at 7,000 rpm for 30 min and the lipase activity in the supernatant was assayed.
Materials & Methods

3.1.3.1 Assay of Lipase Activity

3.1.3.1.1 Stock Solutions

a) Phosphate buffer [0.05 M] pH 8.0

0.05 M disodium hydrogen phosphate (Qualigens) was prepared and pH was adjusted to 8.0 with 0.05 M sodium dihydrogen phosphate (Qualigens).

b) Substrate solution [0.01 M pNP laurate].

3 mg p-nitrophenyl laurate (Sigma) was dissolved in one ml absolute alcohol (Merck). Dissolved substrate was stored at 4°C in dark.

c) Stop solution [0.1 M Na₂CO₃] (Qualigens).

1.06 g Na₂CO₃ was dissolved in 100 ml of distilled water.

Procedure

The lipase activity was determined according to the method of Sigurgisladottir et al (1993) with slight modification. To 800 µl of 0.05M phosphate buffer (pH 8.0), added 100 µl of culture supernatant or enzyme solution. The mixture was kept on water bath at 60°C for 10 min for pre incubation. Now added 100 µl of 0.01M pNP-laurate as substrate and after mixing, the reaction mixture was incubated at 60°C for 30 minutes. The reaction was terminated by adding 250 µl of 0.1 M Na₂CO₃. Then tubes were centrifuged at 10000 xg for 10 minutes and the absorbance was read at 420 nm in Beckman spectrophotometer. For blank, the enzyme was heat treated at 90°C for 60 min and then added to the reaction mixture as described above.
3.1.3.2 Unit of Activity

The lipase activity of the enzyme on p-nitrophenyl laurate was expressed in terms of units, where a unit is defined as the amount of enzyme, which liberates 1 μg p-nitrophenol from pNP-laurate as substrate in 30 minutes under standard assay conditions.

3.1.3.3 Standard Curve for p-nitrophenol

3.1.3.3.1 Stock Solutions

a) p-nitrophenol [10 μg ml⁻¹]

Ten milligram p-nitrophenol (Sigma) was dissolved in 10 ml of propane-2-ol. It was further diluted with propane-2-ol to prepare working range of 10 μg ml⁻¹.

b) Tris buffer [50 mM, pH 8.0]

50 mM Tris buffer (Qualigens) was prepared and pH was adjusted to 8.0 with 0.2 N HCl. (SD-fine)

A series of dilutions were then prepared from the above stock solution to have solutions with p-nitrophenol concentration ranging from 1-10 μg ml⁻¹. The absorbance was read at 420 nm.

The standard curve was drawn, by plotting the optical density values against the p-nitrophenol concentration (appendix 1).

3.1.4 Identification of Isolate

Lipolytic thermophilic culture showing comparatively high lipase activity were identified according to Bergey’s Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).
3.1.4.1 Morphological Characterization

Pure cultures of the selected isolate were studied for shape and size of the cells, motility, gram reaction and spore position by microscopic examination.

3.1.4.2 Scanning Electron Micrograph of J33-A

Isolate was grown on nutrient agar for 24 hrs. A piece of agar containing isolated small colonies was cut and placed in a clean petri plate. Then few drops of 1 % solution of OsO₄ were put around the agar piece. Petri plate was covered and sealed properly to prevent the loss of vapours of OsO₄ and kept at room temperature for 18-24 h. This step was done for vapour fixation of colonies. Petri plate was opened under exhaust hood and a single thin colony was taken out by cutting a portion of agar around it. The colony was mounted on the stub with the help of silver paint. The stubs were sputter coated with gold using Fine coat, ION SPUTTER JEC-1100. The gold-coated stubs were finally examined under scanning electron microscope JSM-6100 SM JEOL at 15KV.

3.1.4.3 Cultural and Biochemical Characterization

The following tests were conducted for cultural and biochemical characterization of the selected isolate.

3.1.4.3.1 Media Preparation

a) Luria Broth Medium (per liter)

Tryptone 10 g
Yeast Extract 5 g
Sodium Chloride 10 g
adjusted pH to 8.0
Materials & Methods

b) Nitrate Broth
Peptone 5.0 g
Potassium nitrate 0.2 g
Distilled Water to 1000 ml
pH 8.0

c) Gelatin Agar
Peptone 5.0 g
Beef Extract 3.0 g
Gelatin 3.0 g
Agar 15.0 g
Distilled Water to 1000 ml
pH 8.0

d) Acid Mercuric Chloride
Mercuric Chloride 12.0 g
Distilled water to 80 ml
Conc HCl 16.0 ml

e) Basal Medium for Sugar Fermentation
Peptone 10.0 g
Sodium chloride 5.0 g
Bromothymol blue 12.0 g
(0.2% aqueous solution)
Distilled Water to 1000 ml
pH 7.4

The percent solutions of different sugars were separately sterilized by millipore filter and added to the basal medium at 1% level before use.
Materials & Methods

f) Starch Agar
Peptone 10.0 g
Beef Extract 10.0 g
Sodium chloride 5.0 g
Agar 16.0 g
Soluble starch 2.0 g
Distilled Water to 1000 ml
pH 8.0

g) Lugol's Iodine
Iodine 5.0 g
Potassium iodide 10.0 g
Distilled Water to 1000 ml
The solution was diluted 1/5 with distilled water before use.

h) VP Broth
Proteose Peptone 7.0 g
Glucose 5.0 g
Sodium chloride 5.0 g
Distilled Water to 1000 ml
All media unless otherwise stated were sterilized by autoclaving at 121°C and 15 psi for 20 minutes.

3.1.4.3.2 Catalase Test

Twelve-hour slant cultures of the isolate incubated at 60°C was taken and 2-3 drops of 3% hydrogen peroxide solution were poured over the growth. Liberation of gas bubbles due to decomposition of \( \text{H}_2\text{O}_2 \) was taken as the positive catalase reaction.

3.1.4.3.3 Voges-Poskauer Test

The organism was grown in 5ml aliquots of VP broth. Added 3ml of 40% (w/v) sodium chloride and 0.5 - 1.0 mg of creatine to the
culture broth kept at room temperature for 30-60 minutes and observed for the appearance of red color.

3.1.4.3.4 Nitrate Reduction Test

The isolate was inoculated into nitrate broth and incubated at 60°C for 12 h, subsequently; 0.1 ml of nitrate test reagent (equal volume of 0.8 % sulphanilic acid in 5 N-acetic acid and 0.5 % naphthylamine in 5 N-acetic acid) was added to the culture. Reduction of nitrate was indicated by the development of red color within a few minutes.

3.1.4.3.5 Gelatin Hydrolysis

Hydrolysis of gelatin was examined on gelatin agar. The culture was streaked on the gelatin agar and incubated at 60°C for 12 h. Observations were made for the clearing around the line of growth when acidified mercuric chloride solution was poured over the plates.

3.1.4.3.6 Tolerance to Sodium chloride

The isolate was inoculated in nutrient broth containing 5% sodium chloride. The tubes were incubated at 60°C for 12 h and observed for the growth.

3.1.4.3.7 Fermentation of Sugars

The culture was inoculated into different tubes of the basal medium containing 1% of one of the following sugars, glucose, xylose, arabinose or mannitol. After incubation at 60°C for 12 h, the tubes were examined for acid and gas production as indicated by the change in colour of the indicator from blue to yellow, and accumulation of gas in Durham tube.
3.1.4.3.8 Oxidase Test
The organism was grown on nutrient agar. Cells were removed with a toothpick and applied on a filter paper. 2-3 drops of 1% aqueous solution of tetramethyl-p-phenylene diamine dihydrochloride were put on the cells. A positive reaction was shown by development of a dark purple colour within 10 seconds.

3.1.4.3.9 Hydrolysis of Starch
The isolate was streaked on the starch agar plate. After incubation at 60°C for 12 h, the plates were flooded with iodine solution and the appearance of clear areas around the colonies against a blue-black background was noted.

3.1.4.3.10 Hydrolysis of Casein
The isolate was streaked on the skim-milk agar plates. After incubation at 60°C for 12 h, the plates were observed for the appearance of clear areas around the colonies.

3.2 Growth and Lipase Production of Bacillus J 33-A

3.2.1 Growth Curve of Bacillus J 33-A
One hundred milliliter aliquots of the sterile nutrient broth medium (pH 8.0) dispensed into 500 ml Erlenmeyer flasks were inoculated with 2% inoculum and incubated at 60°C in a rotary shaker. Samples were drawn aseptically at regular intervals and analyzed for lipase activity and growth.

3.2.2 Heat Resistance of Bacillus J33-A lipase
The culture supernatant from Bacillus J 33-A was treated at 60, 65 & 70°C temperatures for different time points respectively. The residual lipase activity was determined by pNP-laurate assay over a time period of 7 days (168 h).
3.2.3 Factors Influencing Lipase Production

The effect of various nutrients and environment factors were studied on the production of extracellular lipase by the Bacillus J33-A. Basal medium consisting of 1.3% (w/v) nutrient broth (pH 8.0) was inoculated with a loopful of the slant culture and incubated at 60°C on a rotary shaker for 14-16 h. The lipase activity was determined in culture supernatant. Two percent inoculum was used throughout the studies. The purity of the inoculum was always ascertained microscopically.

3.2.3.1 Effect of Nutrients

20 ml aliquots of the different media in 100 ml Erlenmeyer flasks were seeded with 2% inoculum and incubated at 60°C on a rotary shaker. The lipase activity was assayed after 14 – 16 h incubation.

3.2.3.1.1 Nutrient Broth Concentration

The organism was grown from 0.325 to 2% (w/v) nutrient broth at pH 8.0, temperature 60°C for overnight. Growth and lipase production was then checked.

3.2.3.1.2 Nitrogenous Nutrients

Various nitrogenous ingredients incorporated individually in the nutrient broth medium as a substitute for studying their effect on the enzyme production were – sodium nitrate, yeast extract, ammonium sulphate, tryptone, ammonium chloride, potassium nitrate, peptone, beef extract, urea, malt extract at 1% level.
Materials & Methods

3.2.3.1.3 Sugars

Various mono and disaccharide sugars like glucose, fructose, maltose, rhamnose, sucrose, galactose, dulcitol, xylose, arabinose, sorbitol at 1% level were added to nutrient broth medium to find their modifying effect on enzyme production. The sugar solutions were sterilized by autoclaving at 10 psi for 30 min.

3.2.3.1.4 Oils

Various oils like cod liver, cotton seed, sunflower, soyabean, mustard, castor oil, groundnut, coconut, olive oil, awla oil, desi ghee, butter and dalda at 1% level were added to nutrient broth medium for studying their effect on enzyme production. Emulsions of different oils were prepared and then sterilized by autoclaving.

3.2.3.1.5 Additives

Various additives were added to nutrient broth medium for studying their effect on the enzyme production were – Tween-20, Tween-60, Tween-80, glycerol, starch, and skim milk at 1% concentration.

3.2.3.1.6 Starch Concentration

Starch was added 0.5% to 5% in the nutrient broth medium (pH 8.0).

3.2.3.2 Effect of Environmental Factors on Lipase Production

3.2.3.2.1 Effect of pH

The initial pH of the broth medium was adjusted to different values ranging from 6.0 to 11.0 and after inoculation; the flasks were incubated on a rotary shaker at 150 rpm and 60°C. The growth and lipase activity was determined after 14 – 16 h of incubation.
3.2.3.2.2 Temperature

The culture flasks of nutrient broth medium were incubated on a rotary shaker at 30, 40, 50, 60, 70 and 80°C and the lipase activity was determined after 14 – 16 h.

3.2.3.2.3 Size of Inoculum

The nutrient broth medium was inoculated with different sizes of inoculum (1% to 10%) and incubated on a rotary shaker at 150 rpm and 60°C. The enzyme activity was determined after 14 – 16 h of incubation.

3.2.3.2.4 Aeration

The nutrient broth medium was inoculated (2% v/v) and incubated on a rotary shaker at different rpm (50, 100, 150, 200 rpm) overnight (14 – 16 h) at 60°C.

3.2.3.3 Lipase Production in Different Media

Various media like nutrient broth, Luria broth, brain heart infusion broth (BHI), wheat bran, rice bran, soybean casein digest were tried at 1% concentration for lipase production at 60°C on a rotary shaker. The lipase activity was determined at different time points.

3.2.3.4 Lipase Production in Wheat Bran with Different Variables

3.2.3.4.1 Effect of Different Inoculum Percentage on Lipase Production in Wheat Bran

The wheat bran medium (1%) alone was inoculated with different sizes of inoculum 1%, 2%, 5% and 10% and incubated at 60°C. The enzyme activity was determined after 24 h, 48 h, 72 h and 96 h.
3.2.3.4.2 Lipase Production in Wheat Bran in Combination with Different Media

Wheat bran medium (1%) was supplemented with 1% various media like BHI, NB and LB. It was inoculated with 2% of over night culture followed by growth at 60°C under stationary conditions. The lipase activity was determined after 24 h and 48 h.

3.2.3.4.3 Effect of Incubation Time on Lipase Production in Wheat Bran / Nutrient Broth media

Wheat bran (1%) / nutrient broth (1%) was inoculated (2% inoculum) and incubated at 60°C under stationary conditions. Samples were drawn aseptically at regular intervals and analyzed for lipase activity.

3.2.3.4.4 Effect of Different % of Wheat Bran on Lipase Production

The organism was grown in 1%, 2%, 5% and 10% wheat bran (w/v) and nutrient broth (w/v) for different time points under stationary conditions at 60°C. Samples were checked for lipase activity by p-nitrophenyl laurate assay.

3.2.3.4.5 Effect of Incubation time on the production of crude lipases of **Bacillus J33** and **Bacillus J33-A**.

The lipase production by both the above strains was compared by growing the cultures in wheat bran (1%) and nutrient broth (1%) w/v, pH 8.0 under stationary conditions at 60°C. The samples were withdrawn after 12 h interval and checked for lipase production by pNP-laurate assay.

3.2.3.4.6 Solid Substrate Cultivation of Lipase of **Bacillus J 33-A**

5 gm of wheat bran in distilled water (5 ml) was taken in 125 ml flask and the contents were mixed uniformly and pH was adjusted to 8.0. Media was sterilized. It was 2% inoculated and then incubated at 60°C for four days. After 24 h interval for 4 days, the lipase activity was checked by taking out 1 gm of solid substrate from the flask.
3.2.3.4.7 Immobilization of *Bacillus* J 33-A on polyurethane foam.

Polyurethane foam (PUF) along with wheat bran was used as the inert support material for immobilization of *Bacillus* J33-A for lipase production. Wheat bran (2%) and PUF in three different pieces i.e. 2,5,10 PUF cubes were used and they were added to 20 ml of distilled water (pH 8.0). After sterilization the media was 2% inoculated. Lipase activity was checked after 24 h and 48 h.

3.3 PURIFICATION OF EXTRACELLULAR LIPASE FROM *BACILLUS* J 33 – A

3.3.1 Preparation of Supernatant

The culture (*Bacillus J33-A*) was grown under optimal conditions for lipase production. A series of 500 ml, Erlenmeyer flasks containing 100 ml wheat bran and nutrient broth medium, (1% w/v each) pH 8.0 were seeded with 2% inoculum and incubated at 60°C for 48 h. The culture was then centrifuged at 10,000 rpm to remove the cells. The clear supernatant containing the extracellular lipase was used for further studies.

3.3.2 Ammonium Sulphate Precipitation

To 900 ml of the cold culture supernatant, ammonium sulphate was added with constant stirring to achieve 70% saturation and kept on the magnetic stirrer at 4°C overnight to facilitate complete precipitation. The pH was adjusted to 8.0. It was subsequently centrifuged at 12,000 ×g in a refrigerated centrifuge at 4°C for 20 min and the precipitate were dissolved in 0.05 M phosphate buffer (pH 8.0). The lipase activity and the protein concentration were determined.

3.3.3 Hydrophobic Interaction Chromatography

Phenyl Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) liquid gel was dispensed in 0.05 M phosphate buffer (pH 8.0) and the
fines were decanted. The final gel slurry was then packed in 4.0 x 4.5 cm glass column. The column was flushed with 10 volumes of the 0.05 M phosphate buffer (pH 8.0). The (NH₄)₂SO₄ precipitated enzyme (276 ml) preparation was then carefully layered over the surface of the gel with the pipette. The column was first washed with the two volumes of 0.05 M phosphate buffer (pH 8.0) followed by two volumes of 0.001 M phosphate buffer (pH 8.0). The elution of the enzyme protein was done first by 40% ethylene glycol (80 ml) in 1 mM phosphate buffer (pH 8.0) and then by of 80% ethylene glycol (80 ml) in 1 mM phosphate buffer (pH 8.0). The enzyme protein was eluted from the column at a flow rate of 45 ml h⁻¹. Eight-milliliter fractions were collected and analyzed for lipase activity. Fractions with high lipase activity were pooled for further purification.

3.3.4 **Sepharose-6B Column Chromatography**

Preswollen Sepharose-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) was dispensed in 0.05 M phosphate buffer (pH 8.0) and the fines were decanted. This gel slurry was then packed in (30 x 2.0 cm) glass column (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was flushed with 2-3 volumes of the eluting buffer. The enzyme sample containing ethylene glycol recovered from the Phenyl Sepharose column was chromatographed on Sepharose-6B column. The enzyme was eluted from the column at a flow rate of 1 ml min⁻¹. The fractions of 7 ml were collected. The enzyme activity of the fractions was determined. The fractions containing highest enzyme activity were pooled and its enzyme activity and protein concentrations were determined.

3.3.5 **Q-Sepharose Ion-Exchange Chromatography**

Q-Sepharose was equilibrated with 0.05 M phosphate buffer (pH 8.0) and was packed in a 7 x 4.5 cm glass column. The enzyme sample recovered from the gel filtration step was loaded onto the column and
then column was washed with ten column volumes of 0.05 M phosphate buffer (pH 8.0). The enzyme was eluted first with 25 ml of 0.05 M phosphate buffer (pH 8.0) containing 0.05 M NaCl and subsequently a linear gradient of 0.05 – 0.5 M NaCl was applied. Fractions were collected with a flow rate of 36 ml h⁻¹. The fractions were analyzed for lipase activity and those exhibiting higher lipase activity were pooled and used for the characterization studies.

3.3.6 Dialysis

Purified enzyme was dialyzed overnight against 0.05 M phosphate buffer (pH 8.0) to remove sodium chloride.

3.3.7 Concentration of the Enzyme

The enzyme was concentrated by filtration through 10 kDa cut off membrane filters.

3.3.8 Determination of Protein

Reagents

a) Solution A

Prepared 20 g l⁻¹ sodium carbonate in 0.1 mole l⁻¹ NaOH.

b) Solution B

5 g l⁻¹ CuSO₄ 5 H₂O was dissolved in 10 g l⁻¹ sodium – potassium tartarate. (Prepare fresh).

c) Solution C

Solution A and B were mixed in the ratio 50: 1. It should be prepared fresh.

d) Folin – Ciocalteau reagent

Commercially available reagent is diluted with distilled water in 1:1 ratio.
e) Standard Protein solution

Bovine serum albumin 1 mg ml⁻¹ was prepared as standard protein.

Procedure

Protein was determined according to the method of Lowry et al. (1951). To 1.0 ml of sample added 5.0 ml of solution C, and mixed well. This mixture was allowed to stand for at least 10 min at room temperature. Add 0.5 ml of solution D, and mixed immediately. After 30 min at room temperature, the absorbance was measured at 660 nm. A standard curve was prepared by using bovine serum albumin 0.05 – 0.5 mg ml⁻¹ as standard protein (appendix II).

3.3.9 Determination of Specific Activity

Specific activity of enzyme is defined as units of lipase activity per mg of protein.

3.3.10 Determination of Homogeneity of the Purified Lipase

3.3.10.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Stock Solutions

a) 30 % Acrylamide Stock

30 g acrylamide (Sigma) and 0.8 g bisacrylamide (Sigma) was dissolved in distilled water and volume was made to 100 ml with distilled water.

b) Lower 1.5 M Tris buffer (pH 8.8)

18.17 g Tris (Qualigens) was dissolved in distilled water and its pH was adjusted to 8.8 with 6 N HCl. Final volume was made to 100 ml.

c) Upper 0.5 M Tris buffer (pH 6.8)

6.06 g Tris (Qualigens ) was dissolved in distilled water and its pH was adjusted to 6.8 with 6 N HCl. Final volume was made to 100 ml.
d) Tris glycine reservoir buffer

3 g Tris base (Qualigens) and 14.4 g glycine (Qualigens) was dissolved in distilled water. Final volume was made to 1000 ml.

e) Sample buffer (2 x)

The denaturing sample buffer was prepared by dissolving 200 mg bromophenol dye (Sigma) in 25.0 ml of upper tris buffer. Then added 20 ml glycerol, (Sigma), 10 ml β mercaptoethanol (Sigma) and 30 ml 20% SDS (Sigma). Finally volume was made to 100 ml with distilled water. For native sample buffer, in place of β-mercaptoethanol and SDS, same volume of water was added.

f) Ammonium persulphate

100 mg ammonium persulphate (Sigma) was dissolved in one ml distilled water. It was prepared fresh.

g) TEMED

It was used as supplied by Sigma company.

e) Staining solution

It was prepared by dissolving 1 g coomassie brilliant blue R-250 (Sigma) in 400 ml methanol (SD fine), 100 ml glacial acetic acid (SD-fine) and 500 ml distilled water.

f) Destaining solution

It was prepared by mixing 100 ml glacial acetic acid, 400 ml methanol and 500 ml distilled water.

g) Standard marker proteins

i) SDS MW electrophoresis marker protein (Sigma)
Materials & Methods

ii) SDS MW electrophoresis marker protein (Promega)

SDS marker proteins mixture was used as supplied.

Procedure

Gel was prepared according to the method of Laemmli (1970). 10% separating gel (30 ml) consisted of 10 ml acrylamide stock solution, 7.5 ml lower Tris buffer pH 8.8, 0.3 ml SDS (10%), 12.1 ml distilled water, 150 µl ammonium persulphate, 20 µl TEMED. Stacking gel (4%) consisted of 0.67 ml acrylamide stock solution, 1.25 ml Tris-HCl (0.5 M) pH 6.8, 0.05 ml SDS (10%), 3.0 ml distilled water, 25 µl ammonium per sulphate and 2.5 µl TEMED. Lipase sample were loaded along with molecular weight marker proteins. Electrophoresis was carried out in SDS-Tris glycine buffer for 20 minutes at 5 mA constant current and then for 1 h at 20 mA constant current. When tracking dye reached about 1 cm above the gel end, the electrophoresis was stopped. The gel was then removed. The gel was stained in the staining solution for overnight. Excess dye was then removed by washing and incubation with destaining solution. The gel was subsequently examined for separation of protein bands and photographed.

3.3.10.2 Native Polyacrylamide Gel Electrophoresis

Native gel (10%) was prepared according to the above method of SDS-polyacrylamide gel except that instead of SDS, distilled water was used in all preparations. In the sample buffer also, β-mercaptoethanol and SDS were replaced by distilled water.

3.3.10.3 Zymography

The lipase was run on native PAGE (10%, without SDS) for zymography. After electrophoresis was over, gel was transferred on a 2% agar plate containing 1% tributyrin emulsion in 10 mM Tris-HCl, pH
8.0 for lipase activity detection. After incubation for 3 h at 60°C lipase activity was visualized as a band of clearance on the tributyrin plate.

3.3.10.4 Detection of Esterase Activity

Stock solutions

a) Staining solution

0.02 g of α-naphthylacetate was dissolved in 1 ml acetone and then mixed with 1 ml distilled water. To this added 50 mg fast blue BB and 50 ml 0.05M Tris buffer (pH 8.0).

b) Destaining solution

It was prepared by mixing 100 ml ethanol (Merck), 40 ml glacial acetic acid, 20 ml glycerol and 80 ml distilled water.

Esterase staining was done by the method of Bornscheuer et al, 1994 with slight modifications. The gel was left in staining solution at room temperature in the dark for up to 90 minutes. Stained gel was then washed in water and then destained with destaining solution. Destained gel was then stored in 20% glycerol.

3.3.11 Effect of incubation time on the production of peak I and peak II lipase

The Bacillus J 33 A was grown for different time periods (24 h, 48 h, 72 h and 96 h) at 60°C in WB (1% w/v) and NB (1%w/v) pH 8.0. After each particular time period, the culture was centrifuged and processed for purification (only by ammonium sulphate followed by Phenyl Sepharose column chromatography). The total activity of peak I and peak II lipase was assayed by pNP laurate and by tributyrin hydrolysis separately for each culture. The production of peak I and peak II at different time points was compared.
3.4 CHARACTERIZATION OF THE PURIFIED LIPASE

3.4.1 Determination of Molecular Weight of Native Enzyme

<table>
<thead>
<tr>
<th>Molecular Weight Markers (Sigma)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C (M W 12,400 Da)</td>
<td>5 mg ml⁻¹</td>
</tr>
<tr>
<td>Carbonic anhydrase (M W 29,000 Da)</td>
<td>5 mg ml⁻¹</td>
</tr>
<tr>
<td>Ovalbumin (M W 45,000 Da)</td>
<td>5 mg ml⁻¹</td>
</tr>
<tr>
<td>Serum albumin (M W 67,000 Da)</td>
<td>5 mg ml⁻¹</td>
</tr>
<tr>
<td>Blue Dextran (M W 2 x 10⁶ Da)</td>
<td>5 mg ml⁻¹</td>
</tr>
</tbody>
</table>

The molecular weight of the purified enzyme preparations was determined by the gel filtration method of Andrews (1964) using Sephadex G-200 (52 x 1.6 cm) column equilibrated with 0.05 M phosphate buffer (pH 8.0). The blue dextran (mol. wt. 2 x 10⁶) used for determining the void volume ($V_0$) of the column and the standard proteins were obtained from Sigma. Sephadex G-200 column was calibrated with standard marker proteins by elution with 0.05 M phosphate buffer pH 8.0 at a flow rate of 30 ml h⁻¹. Lipase samples were applied to Sephadex G-200 column and then eluted with the same 0.05 M phosphate buffer (pH 8.0). Three ml fractions were collected and the absorbance at 260 nm (Beckman Spectrophotometer) was taken as a measure of their protein content. The elution volume ($V_e$) of the standard protein and the lipase preparations were measured at the protein peak. The molecular weights of the purified enzymes were extrapolated from the graph plotted with $V_e/V_0$ against log molecular weight values of standard proteins.
3.4.2 Optimum pH

Purified enzyme was assayed in different pH buffers (0.05 M) ranging from 6.0 – 11.0 pH using phosphate buffer (6.0-7.0), Tris-HCl (8.0-9.0) and carbonate-bicarbonate buffer (10.0-11.0) at 60°C.

3.4.2.1 Stock Solutions

a) Citric acid / Sodium citrate buffer (0.05 M, pH 3.0)

91.0 ml of 0.05 M citric acid was taken in a volumetric flask and made up to the 100 ml mark with 0.05 M trisodium citrate.

b) Acetate buffer (0.05 M, pH 4.0, 5.0)

For the preparation of pH 4.0, 83.0 ml of 0.05 M acetic acid was taken in a 100 ml volumetric flask and made up the volume with 0.05 M sodium acetate.

For pH 5.0, 32.0 ml of 0.05 M acetic acid was taken in a 100 ml volumetric flask and made up to the mark with 0.05 M sodium acetate.

c) Phosphate buffer (0.05 M, pH 6.0, 7.0)

For pH 6.0, added 5.8 ml of 0.1 M sodium hydroxide to 50 ml of 0.01 M sodium dihydrogen phosphate and diluted to 100 ml.

For pH 7.0, added 30 ml of 0.1 M sodium hydroxide to 50 ml of 0.01 M sodium dihydrogen phosphate and diluted to 100 ml.

d) Tris buffer (0.05 M, pH 8.0, 9.0)

For pH 8.0, added 29.0 ml of 0.1 M hydrochloric acid to 50 ml of 0.1 M Tris and made to 100 ml.

For pH 9.0, added 6.0 ml of 0.1 M hydrochloric acid to 50 ml of 0.1 M Tris and made to 100 ml.
Materials & Methods

e) Carbonate / bicarbonate buffers (0.05 M, pH 10.0, 11.0)

For preparation of pH 10.0, 49.0 ml of 0.05 M sodium bicarbonate was taken in a 100 ml volumetric flask and made up to the mark with 0.05 M sodium acetate.

For pH 11.0, 5.5 ml of 0.05 M sodium bicarbonate was taken into a 100 ml volumetric flask and made up to the mark with 0.05 M sodium acetate.

3.4.3 pH Stability

The purified enzyme was incubated in different pH values ranging from 3.0 – 11.0 pH using 0.05 M citrate, acetate, phosphate, Tris-HCl, carbonate-bicarbonate buffers at 37°C for 90 min and the lipase activity was subsequently determined at pH 8.0 and temperature 60°C.

3.4.4 Optimum Temperature

The lipase activity of the purified enzyme was assayed at different temperatures ranging from 50°C to 90°C. Before addition of the enzyme, the substrate and buffer was pre incubated at the respective temperatures for 10 min.

3.4.5 Temperature Stability

Aliquots of the enzyme solution in 0.05 M phosphate buffer (pH 8.0) were exposed for 1 h at different temperature ranging from 50°C to 90°C then they were immediately transferred to chilled water bath and the residual lipase activity was determined at 60°C. A control sample, kept at 0°C, was assayed under similar conditions.

3.4.6 Half Life of Lipases

The enzymes were incubated at 60, 70 and 80°C for 120 min at pH 8.0. Samples were withdrawn after regular intervals and immediately cooled in ice bath. The residual lipase activity was then determined by pNP-laurate assay to determine half-life.
3.4.7 Shelf Life of Lipases
The purified enzymes were incubated at 4, 25, 37 and 60°C. The residual lipase activity was then determined up to 120 days at regular intervals.

3.4.8 Effect of Substrate Concentration
Varying substrate concentrations from 0.1 – 5 mM were used for assay of the lipase activity keeping the enzyme concentration constant in the reaction mixture.

3.4.9 Effect of Metal Ions
The influence of different metal ions CaCl₂, BaCl₂, CoCl₂, CuSO₄, KCl, FeCl₃, MgCl₂, HgCl₂, MgSO₄, NaCl, CdCl₂ and LiCl on enzyme stability was determined at a concentration of 10 mM. The purified enzyme in 0.05 M phosphate buffer (pH 8.0), was incubated with the selected metal salts at 37°C for 30 min. Subsequently, it was diluted and assayed for the residual lipase activity.

3.4.10 Effect of Organic Solvents
The effect of different organic solvents ethanol, methanol, acetone, DMSO, dioxane, benzene, hexane, propanol, pyridine, acetonitrile, glycerol, ethylene glycol on enzyme stability was determined at 30% (v/v) concentrates. The purified enzyme in 0.05 M phosphate buffer (pH 8.0), was incubated with the organic solvents at 37°C for 21 h. Subsequently, it was diluted so that the solvent was only up to 1% and then assayed for residual activity.

3.4.11 Effect of Various Chemical Reagents
The enzyme solution was preincubated at 37°C for 21 h at pH 8.0 with various chemical reagents MgCl₂, PMSF, EDTA, DTT, SDS and (EDTA+MgCl₂) (100 mM), eserine (10 mM), βME (1 M), DEPC (100
mM) and Urea (1 M). The residual lipase activity was subsequently assayed.

### 3.4.12 Effect of proteolytic enzymes on lipase

The enzyme solution was incubated with trypsin, chymotrypsin and thermolysin (10 mg ml⁻¹ each) at 37°C and the enzyme activity was checked by p-nitrophenyl laurate assay.

### 3.4.13 Effect of NaCl

Varying concentration of NaCl (50 mM to 200 mM) was tried to check its effect on lipases. The enzyme activity was checked by pNP-laurate.

### 3.4.14 Substrate Specificity

Substrate specificity was determined by using tributyrin (C₄), tricaprin (C₆), tricaprylin (C₈), tricaprin (C₁₀), trilaurin (C₁₂), tripalmitin (C₁₆), tristearin (C₁₈) and triolein (C₁₈ : 1) as substrates. Their emulsions were prepared by using different solvents and then plates were prepared. The rate of hydrolysis of different substrates was analyzed (Sommer et al., 1997) by measuring the diameter of the zone of hydrolysis.

### 3.4.15 Positional Specificity

Positional specificity was determined by thin layer chromatography of the enzymatic reaction products obtained with pure triolein as the substrate. The reaction mixture containing 5 mg triolein, 1.5 ml Tris (0.1 M) (pH 8.0) and 100 µl of peak I and peak II enzyme solution was incubated for 10 min at 60°C with constant stirring. In another set, 50 µl of both enzymes were taken for reaction. After incubation, reaction was stopped by adding chloroform, and then reaction products extracted with chloroform at least 3 times. Products were completely dried. Aliquots of the extracts were applied to a silica-gel-60 TLC plate (Merck) and developed with a solvent mixture of chloroform : acetone : acetic acid (96 : 4 : 1). Pure 1(3)-monolein, 1,3/1,2-diolein and triolein
were used as reference glycerides. Spots were visualized by I₂ vapors (Sugihara et al., 1991).

3.4.16 Amino Acid Analysis

The amino acid analysis was carried out using an Applied Biosystems 420/H PTC amino acid Analyzer/Hydrolyzer. The sample was hydrolyzed in a vapour-phase automated hydrolyser. The resulting amino acids were derivatized with PITC to form PTC amino acids that are extracted and transferred to an online 130/A HPLC for analysis. Generally 0.1 to 0.2% of the sample analyzed resulted in about 100-300 p mole, which falls within the range of the instrument accuracy. Standard peptide was run and treated exactly same manner before applied for amino-acid analysis to calibrate instrument accuracy.

3.5 IMMUNOLOGICAL STUDIES

3.5.1 Production of polyclonal antibody

Stock Solution

Phosphate buffer saline (PBS)

Sodium chloride 8.0 g
Potassium chloride 0.2 g
Na₂HPO₄ 1.44 g
KH₂PO₄ 0.24 g

In 800 ml double distilled water. Its pH was adjusted to 7.2 and volume was made to 1 litre. Then the solution was autoclaved.

For primary immunization, rabbits were injected at multiple subcutaneous sites with a total of 150 μg lipase emulsified in Freund's complete adjuvant. This was followed by the three series of...
subcutaneous booster injection of lipase (~60 μg) in freund’s incomplete adjuvant at an interval of 3 weeks. Antilipase serum fraction was collected at 1 week after the last booster. Presence of antibodies was checked by ouchterlony double immnodiffusion-assay and interfacial ring test. Glass plates coated with 1.5 mm layer of 1% melted agar in PBS are punched out. In the control well (1), BSA (0.1%) was used in place of antigen. The outer wells (2), (3) and (4) were filled up to the brim with supernatant, ammonium sulfate and peak I fraction of lipase respectively. The central well with antibody solution. The plates were incubated in humid boxes and development of precipitin pattern was checked.

3.5.2 Purification of Antibody

Stock solutions
a) Phosphate Buffer (1 M, pH 8.0)
b) Glycine-HCl buffer (0.1 M, pH 3.0)
c) Serum
d) Tris buffer (1 M, pH 7.0)

Procedure

Protein A covalently coupled to the CNBr activated Sepharose 4 B (Sigma) has been used for antibody purification. It was equilibrated in 0.1 M phosphate buffer (pH 7.0). Diluted serum (in three volumes of phosphate buffer) was applied to the column. After thorough washing with the same buffer, bound antibodies were eluted with 0.1 M glycine – HCl buffer (pH 3.0). The pH of the eluted sample was immediately adjusted to 7.0 with 1 M Tris buffer. The fractions having high protein content were pooled and analyzed by SDS-PAGE.
Materials & Methods

3.5.3 Enzyme Linked Immunosorbent assay

Stock Solutions

a) Coating buffer [0.1 M carbonate-bicarbonate buffer pH 9.6]
0.1 M sodium carbonate was prepared (Qualigens), pH was adjusted to 9.6 with 0.1 M sodium bicarbonate (Qualigens) and stored at 4 °C.

b) Blocking Solutions
5 gm of casein was dissolved in 100 ml of PBS and stored at -20 °C in small aliquots.

c) Wash buffer [0.05M PBS, pH 7.6, 0.05% Tween-20]
0.05 M PBS, (pH-7.6) was prepared and then 0.05% Tween-20 (Hi-media) was added.

d) Substrate buffer [0.1M citrate phosphate buffer pH 5.0]
0.1 M citric acid (Himedia) was prepared and pH was adjusted to 5.0 with 0.1 M disodium hydrogen orthophosphate (Merck).

e) Substrate Solution
The solution was prepared by dissolving 4 mg of orthophenylene diamine dihydrochloride OPD (SIGMA) in 5.5 ml of substrate buffer and then added 5.5 μl of 30% hydrogen peroxide (Qualigens). Prepared immediately before use and protected from light.

f) Stop Solution
2 N sulphuric acid.

Procedure
ELISA was performed according the slightly modified method of Voller et al (1976). 0.25 micro gram equivalent protein of purified lipases i.e. peak I & peak II, in 50 μl of 0.1 M carbonate-bicarbonate buffer (pH 9.6), was coated on each well of microtitre ELISA plate (Tarson, India). First two column of the ELISA plate wells were used as blank and negative controls for peak I & II. Rest of the wells in remaining columns
were used for test samples (table 3.5.3.1A). After overnight incubation of the plate at 4 °C, it was given four washes with 0.05 M Tween-20 (PBST). Then unoccupied sites of each well on the plate were blocked for about 2 hours at 37 °C, with 200 µl of 5% casein in PBS. This was followed by three PBST washes of 5 min each. Then 50 µl of (1:25) diluted primary antibody was added in the wells (table 3.5.3.1A) including control and excluding blank sets. After incubating above, the unbound primary antibody was washed with PBST and plate was incubated further with the addition of 50 µl of 1:1000 diluted antirabbit immunoglobulin conjugated to horse raddish peroxidase (Bangalore genei India), in each well (including blank and control) for about 1 hour at 37 °C. After giving an extensive wash with PBST, the colour reaction was developed by adding 50 µl of orthophenyl diamine (OPD) in 0.1 M citrate phosphate buffer (pH 5.5 containing 0.01% H₂O₂). Finally by adding 50 µl of 2 N sulphuric acid in each well, the reaction was terminated and the optical density was measured at 450 nm in an ELISA reader (BIORAD).

Antigenic determinants sites in native and heat denatured peak I and II lipase were determined by using 20 µg ml⁻¹ of lipase in each well (table 3.5.3.1B) and then ELISA was performed as in the above method.

### Table 3.5.3.1A: Determination of antigenic determinants in peak I and II lipase

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Sample</th>
<th>Blank</th>
<th>Control</th>
<th>Antibody Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/4</td>
</tr>
<tr>
<td>Peak I</td>
<td>1</td>
<td>do</td>
<td>do</td>
<td>do</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>do</td>
<td>do</td>
<td>do</td>
</tr>
<tr>
<td>Peak II</td>
<td>1</td>
<td>do</td>
<td>do</td>
<td>do</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>do</td>
<td>do</td>
<td>do</td>
</tr>
</tbody>
</table>
Table 3.5.3.1B: Determination of antigenic determinants sites in native and denatured peak I and II lipase

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Sample</th>
<th>Blank</th>
<th>Control</th>
<th>Native</th>
<th>Denatured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak I</td>
<td>1</td>
<td></td>
<td></td>
<td>50μl</td>
<td>50μl</td>
</tr>
<tr>
<td>(20μg/ml⁻¹)</td>
<td>2</td>
<td></td>
<td></td>
<td>50μl</td>
<td>50μl</td>
</tr>
<tr>
<td>Peak II</td>
<td>1</td>
<td></td>
<td></td>
<td>50μl</td>
<td>50μl</td>
</tr>
<tr>
<td>(20μg/ml⁻¹)</td>
<td>2</td>
<td></td>
<td></td>
<td>50μl</td>
<td>50μl</td>
</tr>
</tbody>
</table>

The level of antigens in supernatants of J 33 and J 33-A was estimated by taking 1X and 10X dilutions (table 3.5.3.1C). ELISA was performed as in above method.

Table 3.5.3.1C: Estimation of the level of Antigens in supernatants of Bacillus J 33 and J 33-A

<table>
<thead>
<tr>
<th>S.No.</th>
<th>1</th>
<th>2</th>
<th>J33</th>
<th>J33-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank</td>
<td>-ve Control</td>
<td>1X</td>
<td>1X</td>
</tr>
<tr>
<td>2.</td>
<td>-do-</td>
<td>-do-</td>
<td>1X</td>
<td>1X</td>
</tr>
<tr>
<td>3.</td>
<td>-do-</td>
<td>-do</td>
<td>10X</td>
<td>10X</td>
</tr>
<tr>
<td>4.</td>
<td>-do-</td>
<td>-do</td>
<td>10X</td>
<td>10X</td>
</tr>
</tbody>
</table>

3.5.4 Dot Blot

Stock solutions

a) Preparation of 1st Ab (1:100 dilution)

40 μl antibody in 20 ml PBS / milk.

b) Preparation of 2nd antibody – HRP conjugate

c) DAB (3, 3’ - Diaminobenzidine) substrate

10 mg of DAB in 0.2 ml of distilled water.
Materials & Methods

Procedure

The enzyme was blotted on the nitrocellulose paper. After blotting, it was placed in tray containing skim milk (1%) in PBS (pH 7.4) at room temperature for 3-4 h. The milk solution was poured off then added 1st antibody in PBS/milk (1:100 dilution) and incubated at room temperature for 3-4 h. Then discarded 1st Ab solution and nitrocellulose was washed four times for 15 min with 0.1 % Tween 20-PBS. The nitrocellulose was incubated in 1% PBS/milk for 15 min. Second antirabbit IgG-HRP conjugate (1:5000) in PBS/milk was added and incubated for 2 h at room temperature. The nitrocellulose was then washed three times for 15 minutes with 0.1 % tween-20 in PBS. Nitrocellulose was further quickly washed with PBS. Nitrocellulose was developed by soaking it in DAB substrate till fine coloured bands developed. The developing solution was poured off and nitrocellulose paper was washed twice with distilled water.

3.5.5 Immuno- Interference

Antibodies purified by Protein A - Sepharose was taken in three different dilutions (1 x, 10 x, 100 x) and incubated with lipase at 37 °C for 1 h. Then corresponding amount of phosphate buffer and substrate was added. The whole mixture was again incubated at 40°C (and not at 60 °C) to avoid the effect of temperature on Ag – Ab interactions. The optical density was read at 420 nm. Reaction mixture having only enzyme and no antibody served as 100 % activity. The experiment was carried out in triplicates and mean value had been reported.

3.5.6 Western Blotting

Stock Solutions

Transfer Buffer

25 mM Tris HCl
192 mM glycine containing 20 % (w/v) methanol (pH 8.3)

**Procedure**

Equal amount of protein concentration (2-3 μg) of peak I and peak II lipases were loaded in different wells of SDS-PAGE. After removing gel from electrophoresis apparatus, the lanes were cut for protein staining. The other half of gel having peak I and peak II lipase were blotted on the nitrocellulose paper at 220 mA current for 6 h at 4°C in transfer buffer. The membrane was shifted to a tray containing skim milk (1%) in PBS (pH 7.4) at room temperature for 3-4 h. The milk solution was poured off then added 1st antibody in PBS/milk (1:100 dilution) and incubated at room temperature for 3-4 h. Then discarded 1st Ab solution and nitrocellulose was washed four times for 15 min with 0.1% Tween –20 in PBS. The nitrocellulose was incubated in 1% PBS/milk for 15 min. Second antirabbit IgG - HRP conjugate (1:10000) in PBS/milk was added and incubated for 2 h at room temperature. The nitrocellulose was then washed three times for 15 minutes with 0.1% tween-20 in PBS. Nitrocellulose was further quickly washed with PBS. Nitrocellulose was developed by soaking it in DAB substrate till fine coloured bands developed. The developing solution was poured off and nitrocellulose paper was washed twice with distilled water.

3.6 IMMobilization of Lipase

3.6.1 Immobilization of Lipase on different matrices

The peak I lipase (in 100 mM phosphate buffer pH 8.0) was immobilized on different matrices. These matrices were first treated with following ways for immobilization.
Materials & Methods

**Sepa beads 825:**
They were first swelled in water overnight then in 25% glutaraldehyde in borate buffer, pH 8.0 (0.1 M borax and boric acid in NaOH) 2 h to 4 h. For immobilization, 1.5 gm of swelled Sepa beads was mixed with 4.5 ml of enzyme. Then the lipase activity was checked after 24 h, 48 h and 60 h by p-nitrophenyl laurate method.

**CNBr activated Sepharose 4 B:**
It was used as supplied. 0.31 g dry powder of CNBr activated Sepharose 4 B was swelled in phosphate buffer (0.05 M, pH 8.0) and mixed with 1.5 ml of lipase and incubated overnight at 20 –24 °C. The lipase activity was checked after 24 h, 48 h and 60 h.

**HP 20:**
Resin HP 20 was first washed with water then three times with isopropyl alcohol for three times. Again it was washed three times with water. Now it was incubated with 2 to 3.5% glutaraldehyde solution for 30 minutes. Again it was washed with water. After such treatment 0.89 g net weight of HP20 was taken and 1.5 ml of lipase solution was added. This mixture was incubated overnight at room temperature (25 °C). Then the lipase activity was checked after 24, 48 and 60 h.

**Silica:**
1 g of silica was taken and 1.5 ml of lipase solution was added directly. This mixture was incubated overnight at room temperature (25 °C). Then the lipase activity was checked after 24, 48 and 60 h.

**Phenyl Sepharose:**
The resin was washed with 50mM phosphate buffer(pH 8.0) and incubated with ammonium sulfate precipitated enzyme dissolved in phosphate buffer.
3.6.2 Retention of Activity by Immobilized lipase

Enzyme immobilized Sepa, HP-20, Silica, CNBr-activated Sepharose, Phenyl-Sepharose matrices, each 100 mg were assayed for 50 days after 2 days interval. The enzyme was stored at room temperature. For each cycle of assay, 1.0 ml of reaction mixture containing substrate (pNPL) was added to the immobilized support and incubated for 30 min with continuous shaking at 60 °C. After centrifugation of reaction mixture, the supernatant was used for measuring adsorption at 420 nm. Enzyme immobilized pellet after each cycle was washed 2 times with 0.05 M phosphate buffer (pH 8.0). Washed pellet was then used for the next assay by same procedure.

3.7 ESTERIFICATION STUDY

3.7.1 Esterification by peak I and peak II lipase

The esterification reaction was carried out by taking 0.4 M methanol along with 0.25 M different fatty acids (butyric acid, caprylic acid, stearic acid and oleic acid) in the presence of 4 ml hexane. Peak I and peak II lipases (40 U ml⁻¹) were used to carry out the reaction at 60°C for 14 h under stirring conditions. The total amount of ester formed was calculated by using alkalimetric method of titrating unreacted acid with 0.1N NaOH using phenolphthalein as an indicator. The percentage of conversion in ester was based on acid consumed.