Research Journal of Pharmaceutical, Biological and Chemical Sciences

Isolation, optimization, production and molecular characterization of lipase from Bacillus and Pseudomonas species and protein profiling

Shivareddy DM[1], Raghavendra L Ksheerasagar[1], Abhilash M[2]*

[1]Department of Biotechnology, C.M.R.I.T, Bangalore, Karnataka, India
[2]Department of Biotechnology, The Oxford college of Engineering, Bangalore, Karnataka, India

ABSTRACT

Lipases are class of Enzymes which are having wide range of Application and is having lot of commercial applications. Bacillus and pseudomonas species were isolated and isolation of these were confirmed using confirmatory test including Tributent agar test, staining and various biochemical tests.Lipase production was estimated using Lowry's method and various tests were carried out for optimization of temperature, pH, Sources for Carbon, Nitrogen and metal salts. Optimal concentration of elution buffer for separation using Ion exchange chromatography was determined and insilico analysis of enzyme was carried out using Bioinformatics tools.

Keywords: Lipases, Bacillus & pseudomonas, Optimization of parameters,Insilco analysis.

*Corresponding author
(Cell) : +91 9036064200
Email: abhibiotek@gmail.com

INTRODUCTION

Enzymes are the proteins that catalyze biochemical reactions. Enzymes convert the substrate on which they act into different molecules, the products. Almost all the processes in cell need enzymes in order to function at significant rates. Enzymes are of various types like amylases, proteases, lipases etc. Today lipases stand amongst the most important biocatalysts carrying out novel reactions in both aqueous and non aqueous media. This is primarily due to their ability to utilize a wide spectrum of substrates, high stability towards extreme pH, temperature and organic solvents, chemeo-selectivity, regio-selectivity and enantio-selectivity [1]. More recently the determination of their three dimensional structure has thrown light into their unique structure- function relationship. Among lipases of plant, both animal and microbial origins, it is microbial lipases that find immense application. This is because microbes can be easily cultivated and their lipases can catalyze a wide variety of hydrolytic and synthetic reaction. Lipase is a water soluble enzyme that catalyzes the hydrolysis of ester bonds in water insoluble, lipid substrates that is necessary for absorption and digestion of nutrients in the intestine. Lipases are ubiquitous throughout living organism and genes encoding them are even present in certain viruses.

Most lipases act at specific position on the glycerol backbone of a lipid substrate (A1, A2, A3). Lipase acts to convert triglyceride substrates to monoglycerides and free fatty acids. Lipases of different origin show different affinities for tri, di, and monoglycerides, they may also hydrolyse esters of other aliphatic alcohols with different specificity for acyl chain length [2].

Some lipases work within the interior spaces of living cells to degrade lipids eg; lysosomal lipases. Other lipase enzymes work outside the cells and have roles in metabolism, absorption and transport of lipids throughout the body.Eg; pancreatic lipase. Lipases play an important role in cell biology. Lipases are also involved in diverse biological process ranging from routine metabolism of dietary triglycerides to cell signalling and inflammation. Lipids are insoluble in water and need to be broken down extracellularly into their more polar components to facilitate absorption if they are to function as nutrients to the cell. The majority of lipases are secreted extracellularly.

Types of lipases

Several different types of lipases are found in human body.

- Pancreatic lipase
- Hepatic lipase
- Lysosomal lipase
- Gastric lipase
- Endothelial lipase and
- Different phospholipases

Application of lipases

In present day industry, lipases have made their potential released owing to their environment in various industrial reactions either in aqueous or organic systems, depending on their specificity. Lipases are extensively used in the dairy industry for hydrolysis of milk fat. Current application includes flavour enhancement of cheese, acceleration of cheese ripening, manufacture of cheese like products and lipolysis of butter fat and cream [3]. Polyglycerol and carbohydrate fatty acid esters are widely used as industrial detergents and as emulsifiers in great variety of food formulations (low fat spreads, sauces, ice creams). Enzyme synthesis of functionally similar surfactants has been carried out at moderate temperature (60^{o}C-80^{o}C) with excellent regio-selectivity. Lipases are also used in the production of isopropyl myristate, isopropyl palmitate and 2 ethyl hexyl palmitate for used as emollient in personal care product like skin and sun- tan cream and bath oils. Lipases is very well recognized and used in the preparation of chiral synthesis. The resolution of 2 halopropionic acid, the starting methods for synthesis of phenoxypropionate herbicides, is a processes based on selection esterification of (S) isomer with butanol, which is catalysed by porcine pancreatic lipase in anhydrous hexane. Also used as industrial catalyst in the preparation of some prostaglandins,

steroids, and carbocyclic nucleoside analogue. Regio-selective modification of polyfungal organic compounds is yet another rapidly expending area of lipase application particularly in the field of AIDS treatment [4].

The stereo-selectivity of lipase is useful for the synthesis of optically active polymer. These polymers are symmetric reagents and are used absorbents.

Lipases have been successfully employed in the food industry as well as in high- tech production of five chemicals and pharmaceuticals. Lipases have also been successfully used in paper manufacturing for the treatment of pulp with lipase leads to a higher quality product and reduced cleaning requirement [5].

Enzyme has also been used in association with a microbial cocktail for the treatment of fat-rich effluents from an ice cream plant which could also be utilized in waste processing of many food industries [6]. In general, lipase supplements are thought to help the body absorb food more easily, keeping nutrients at appropriate, healthy levels throughout the body [7].

A relatively small number of bacterial lipases have been well studied compared to plant and fungal lipases. Bacterial lipases are glycoprotein, but some extracellular lipases are lipoprotein. Most of the bacterial lipases are constitutive and are non specific in their substrate specificity and a few bacterial lipases are thermostable [8]. Examples for bacterial lipases are *Pseudomonas, Staphylococcus, Alcaligens, and Bacillus* etc.

MATERIALS AND METHODS

Method employed for isolation of bacteria

Soil sample was collected from nearby place for Bacillus isolation and stale raw meat it was taken for Pseudomonas isolation and the method of serial dilution was followed. To make serial dilution, we start with organism in liquid medium. Adding 1 ml of this medium to 9ml of sterile water makes a 1:10 dilution; adding 1ml of the 1:10 dilution to 9 ml of sterile water a 1:100 dilution; and so on. The number of bacteria per millilitre of fluid is reduced by 9/10 in each dilution. Subsequent dilutions are made in ratios of 1:1000, 1:10,000, 1:1,00,000, 1:1,000,000, or even 1:10,000,000if the original culture contained extremely large number of organisms.

From each dilution, usually with the 1:100, 1 ml of the culture is transferred to an agar plate. (One milliliter of the 1:10 dilution typically contains too many organisms to yield countable colonies when transferred to a Petri plate.) The transfer can be done by spread plate method. In this method diluted sample is first placed on the center of a solid, cooled agar medium. The sample is then spread evenly over the medium's surface with a sterile, bent glass rod. After incubation, colonies develop on the agar surface.

Spread plate method

For this technique, the nutrient agar and pseudomonas isolation media was prepared and was autoclaved. Then the media was taken and was poured into 5 petriplates (previously autoclaved) inside the laminar air- flow. After the media had been solidified, 100µl soil sample from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4},10^{-5} solutions were taken and were spreaded onto each Petri plate respectively with the help of a spreader. Then the petriplates were incubated for 24 hours at 37°c.

Identification and Screening

The Gram Stain, a differential stain method developed by Dr. Hans Christian was used for identification of micro-organisms. In this process, the fixed bacterial smear is subjected to four different regents in the order listed:

1. Crystal violet (Primary stain)
2. Iodine solution (mordant),
3. Alcohol (decolorizing agent) 95% ethyl alcohol.
4. Safranin (counter stain)

The bacteria which retain the primary stain (appear dark blue or violet) (i.e. not decolorized when stained with grams' method) are called gram-positive, whereas those that lose the crystal violet and counter stained by safranin (appear red) are referred to as gram-negative.

Procedure for gram stain

Make thin smears of samples on separate glass slides. Let the smears air dry. Heat fixes the smears. Hold the smears using slide rack or clothes pin. Cover each smear with crystal violet for 30 seconds. Wash each slide with distilled water for a few seconds, using wash bottle. Cover each smear with grams iodine solution for 60 seconds. Wash off the iodine solution with 95 per cent ethyl alcohol. Add ethyl alcohol drop by drop, until no more colour flows from the smear. (The gram-positive bacteria are not affected while all gram-negative bacteria are completely decolorized).Wash the slides with distilled water and drain. Apply safranine to smears for 30 seconds (counter-staining).Wash with distilled water and blot dry with absorbent paper .Let the stained slides air dry.

The IMViC tests consisting of four different tests were carried out for Bacterial Identification i) Indole production ii) Methyl-red iii) Voges-Proskauer iv)Citrate utilization

Confirmatory Test

Tributyrin agar test

Prepared 20ml of tributent agar medium containing 0.06g of tributyrin agar ,0.32g of agar ,0.2ml of tributyrin oil and 90.8ml of distilled water and autoclaved. After autoclaving it pour in to two petriplate each with 10ml of the medium. Then allow for solidification. After solidification inoculate one plate with Bacillus and the second plate with Pseudomonas. Then incubate inside the bacterial incubator for 24 hours.

Production of Lipase

Preparation of production media

Initially 50ml of production media containing 0.25g of NaCl,0.25g of $CaCl_2$, 0.25g of yeast extract and 0.25ml of tween80 , pH 8 is transferred in to two conical flasks and autoclaved.

Preparation of inoculum

Prepare 20ml of nutrient broth containing 0.1g of peptone, 0.1g of NaCl and 0.06g of beef extract and pour in to two test tubes and autoclave it. After autoclaving one tube inoculate with Bacillus and second tube inoculate with Pseudomonas and incubate inside the shaker incubator for 6 hours.

Lipase assay

Procedure

90ml of phosphate buffer of pH-8 was prepared containing 0.207 g of sodium deoxycholate and 0.1 g of gum acacia .10 ml of isopropanol containing 0.03 g of p. nitrophenylpalmitate was added to the above mixture. The mixture was warmed at 37°C in water bath for 15 minutes. From this 2.4 ml of freshly prepared substitute was added with 0.1 ml of cell free supernatant fluid in vials. The vials were incubated at 37°C in water bath for 45 minutes. The absorbances of the incubated samples were taken at 410nm in UV-VIS Spectrophotometer [9].

Protein estimation by Lowry's Method

The Lowry reaction for protein estimation is an extension of the biuret method. The method developed by Lowry is about 10 times more sensitive than biuret method. Hence it is largely followed to determine the protein content of enzyme extract.

Optimization of production media

- *Optimization of temperature* – Both the flask inoculated with Bacillus and Pseudomonas is incubated in both room temperature (28°C) and 37°C. Then the enzyme activity is checked by the previous method [10].
- *Optimization of pH* – For both the organism enzyme activity is checked by using different pH of the media. i.e. pH 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5
- *Optimization of Carbon sources* - For both the organism enzyme activity is checked by using different Carbon sources of the media. i.e. Olive oil, Mustered oil, Sunflower oil, Coconut oil, Castor oil,Tween 80 [10].
- *Optimization of Nitrogen sources*- For both the organism enzyme activity is checked by using different Nitrogen sources of the media. I.e. Yeast extract, Peptone, Soyabean Tryptone, Gelatin,Urea [11].
- *Optimization of Metal Salt sources*- For both the organism enzyme activity is checked by using different Metal salt sources of the media. i.e. $MgCl_2$, CH_3COONH_4, $ZnCl_2$, $MgSO_4$, $CaCl_2$, NaCl.

Purification of lipase

The supernatant obtained containing the enzyme lipase was subjected for purification. The main steps of purification were: Salting out, Dialysis, Ion-exchange chromatography, SDS PAGE. After SDS PAGE the gels were separated.

RESULTS

Results of gram staining

1) A purple coloured rods – Gram positive Bacillus.
2) A pinkish coloured rods – Gram negative Pseudomonas.

Result of tributyrin agar test

A clear zone appeared around the inoculated area of Bacillus and Pseudomonas culture. It was cleared that lipase produced from Bacillus and Pseudomonas degrade the oil.

Results of biochemical tests

Staining characters

They were observed as gram positive rods arranged in chain forms. The bio-chemical characters of *Bacillus* are given in the table.

Results of enzyme assay

The absorbance of lipase enzyme produced by free Bacillus cells at pH-8 was 1.443
The absorbance of lipase enzyme produced by free Pseudomonas cells at pH-8 was 3.00
The absorbance of lipase enzyme produced by Bacillus cells at different pH and temperature were as follows:

Table 1. Bio-chemical characters of the isolatedmicro-organism

S.No	Differential reactions of the isolated micro organism sp.	Results
1	Anaerobic growth	Negative
2	Cirtate utilization	Positive / Negative
3	Gas (Gibson & Abd-el- Malek Test)	Negative
4	Catalase	Positive
5	Glucose	Positive
6	Xylose	Positive
7	Strach	Positive
8	Casein	Positive
9	Gelatin	Positive
10	Nitrate reduction	Positive
11	VP	Positive
12	Urease production	Negative

Table 2 : OD readings at different pH
Optimization of pH

Organism	pH	6	6.5	7	7.5	8	8.5	9
Bacillus	Optical density(O.D.) at 410 nm	1.61	1.68	1.63	1.62	1.73	1.3	1.67
Pseudomonas		1.6	1.73	1.75	1.71	1.65	1.62	1.58

Figure 1: Showing optimal pH for Bacillus as 8 and for pseudomonas 1.75

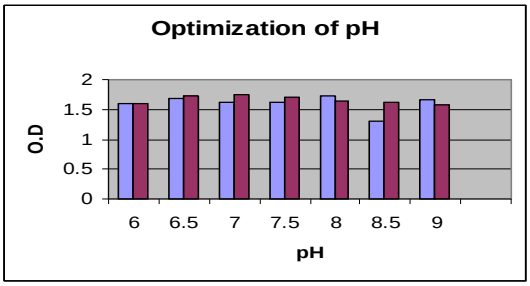


Table 3: OD readings at different temperature
Optimization of temperature

Temperature	Organism	28°C	30°C	32°C	34°C	36°C	37°C
Optical density(O.D.) at 410 nm	*Bacillus*	1.5	1.54	1.52	1.43	2.18	2.197
	Pseudomonas	2.2	2.18	1.97	1.9	1.87	1.8

Figure 2: Showing optimal temperature for growth of *Bacillus* as 37°C and for pseudomonas 2.2

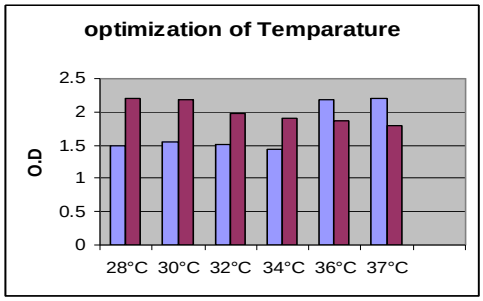


Table 4: OD readings using different Carbon Sources
Optimization of Carbon Sources

Carbon sources	Organism	Olive oil	Mustered oil	Sunflower oil	Coconut oil	Castor oil	Tween 80
Optical density(O.D.) at 410 nm	*Bacillus*	2.61	2.51	2.58	2.4	2.6	2.69
	Pseudomonas	2.5	2.46	2.52	2.54	2.48	2.6

Figure 3: Showing optimal C-Sources for both *Bacillus* and *Pseudomonas* as Tween 80

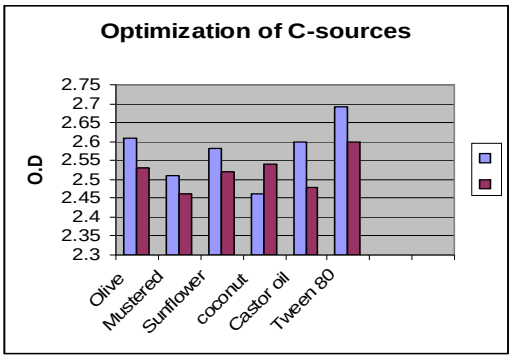


Table 5: OD readings using different Nitrogen sources
Optimization of Nitrogen Sources

Nitrogen sources	Organism	Yeast extract	Peptone	Soyabean	Tryptone	Gelatin	Urea
Optical density(O.D.) at 410 nm	*Bacillus*	2.15	2.2	2.1	2.12	2.17	2.15
	Pseudomonas	2.09	2.13	2.1	2.4	2.1	2.2

Figure 4: Showing optimal Nitrogen Source for Bacillus as peptone and for pseudomonas was Tryptone.

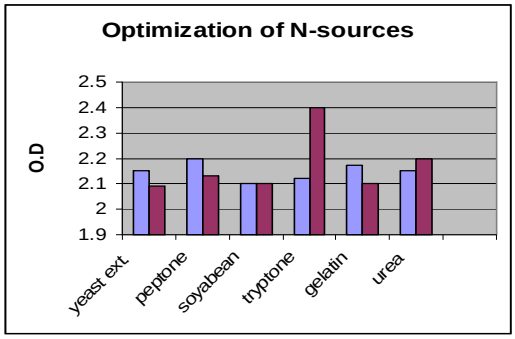
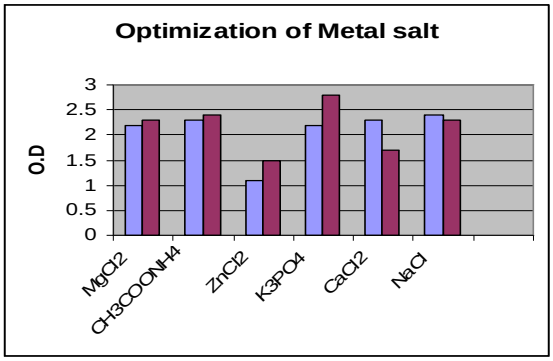


Table 6 : OD readings using different metal sources

Optimization of Metal salt Sources

Metal salts	Organism	MgCl2	CH3COONH4	ZnCl2	K3PO4	CaCl2	NaCl
Optical density(O.D.) at 410 nm	*Bacillus*	2.2	2.3	1.1	2.2	2.3	2.4
	Pseudomonas	2.3	2.4	1.5	2.8	1.7	2.3

Figure 5: Showing optimal metal salt for Bacillus as Na Cl and for pseudomonas as K_3PO_4



Results of purification

Table 7: Results of Ion-Exchange Chromatography (Bacillus Species)

Concentration of elution buffer (mM)	Optical density(O.D.) at 410nm (Enzyme assay)	Optical density(O.D.) at 280 nm(protein estimation)
50	1.68	1.853
100	1.47	0.86
150	1.54	0.811
200	1.59	0.695
250	1.49	0.534
300	1.45	0.397
400	1.44	0.327
500	1.43	0.396

Figure 6: Results of Ion-Exchange Chromatography (*Bacillus* Species)

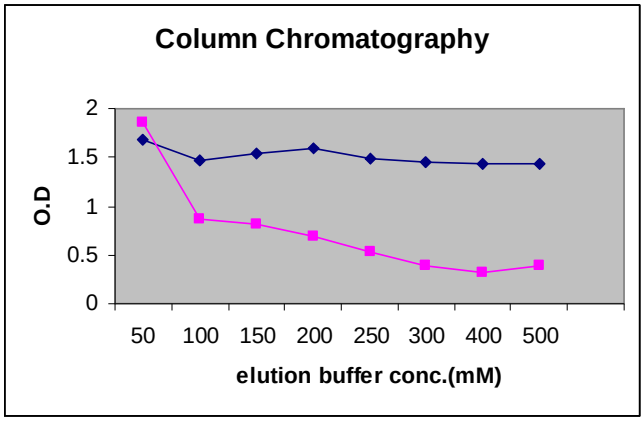


Table 8: Results of Ion-Exchange Chromatography (*Pseudomonas* Species)

Concentration of elution buffer (mM)	Optical density(O.D.) at 410nm (Enzyme assay)	Optical density(O.D.) at 280 nm(protein estimation)
50	1.54	2.12
100	1.51	0.981
150	1.46	0.844
200	1.4	0.804
250	1.56	0.458
300	1.5	0.418
400	1.49	0.333
500	1.5	0.235

Figure 7: SDS-PAGE Analysis of Purified Protein Fraction of lipase

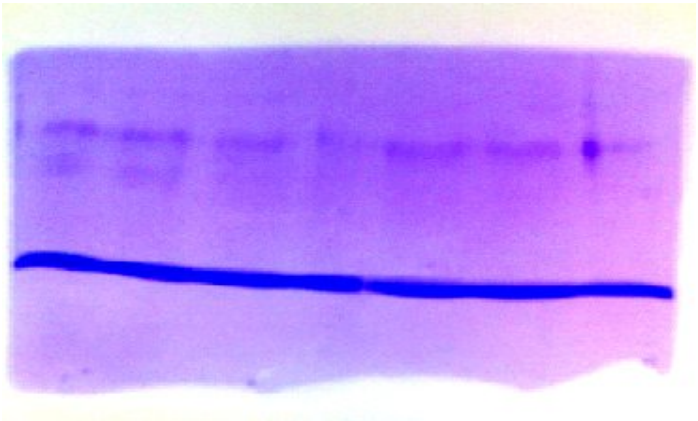
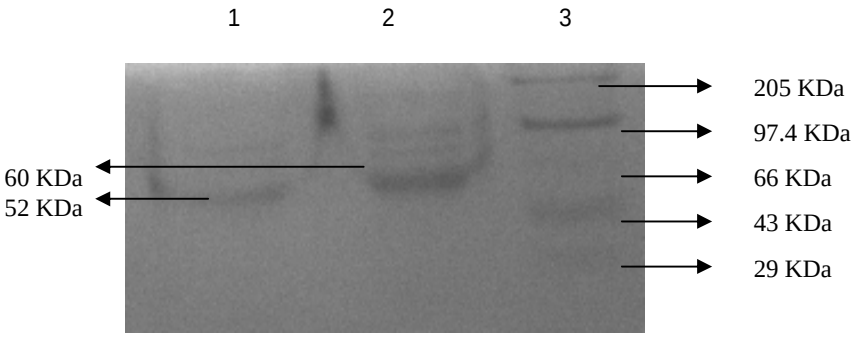


Figure 8: SDS PAGE for both Bacillus & Pseudomonas with reference marker.



Lane 1 *Bacillus*, Lane 2 *Pseudomonas* and Lane 3 *Protein Marker*

Molecular Characterization by using Bioinformatics Tools

Lipase in *Bacillus*

Genomic sequence for lipase (Lip A) in Bacillus

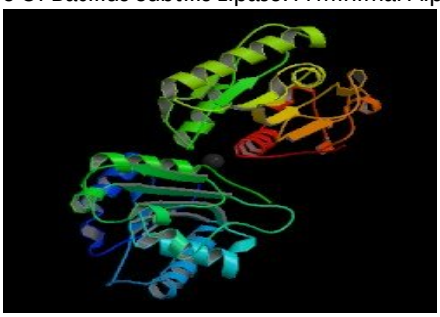
Entry name	LIP_BACSU
Primary accession number	P37957
Protein name	Lipase [Precursor]
Synonyms	EC 3.1.1.3 Triacylglycerol lipase

- FUNCTION: Active toward p-nitrophenyl esters and triacylglycerides with a marked preference for esters with C8 acyl groups.
- CATALYTIC ACTIVITY: Triacylglycerol + H_2O = diacylglycerol + a carboxylate.
- ENZYME REGULATION: Strongly inhibited when incubated with the serine reagent phenylmethylsulfonyl fluoride. Activated by the addition of calcium to the reaction mixture. When calcium was incubated with the lipase but not added to the reaction mixture, its effect is lower but still observable. Magnesium, manganese and strontium are not able to replace calcium with full retention of activity.
- BIOPHYSICOCHEMICAL PROPERTIES:

pH dependence:	Optimum pH is8 The activity decreases strongly above pH 10.5 or below pH 6.5. The enzyme is remarkably stable at alkaline pH, showing maximum stability at pH 12 and retaining more than 65% of its activity after 24 hours at pH 13;
Temperature dependence	Optimum temperature is 37degrees Celsius. Stable for at least 30 minutes at 40 degrees Celsius. Virtually no activity remains after 30 minutes at 55 degrees Celsius;

- SUBCELLULAR LOCATION: Secreted protein.

Figure 9: The Crystal Structure Of Bacillus Subtilis Lipase: A Minimal Alpha/Beta Hydrolase Enzyme.



Lipase of Pseudomonas
Length: 311 AA
Molecular weight: 60KDa

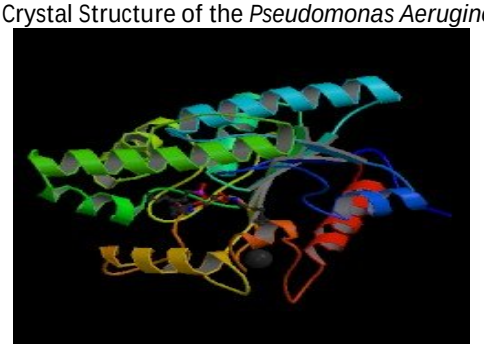
Entry name	LIP_PSEAE
Primary accession number	P26876

- FUNCTION: Catalyzes the hydrolysis of triglycerides. Catalyzes the synthesis of macrocyclic lactones in anhydrous organic solvents.
- CATALYTIC ACTIVITY: Triacylglycerol + H_2O = diacylglycerol + a carboxylate.
- COFACTOR: Binds 1 calcium ion per subunit.
- SUBCELLULAR LOCATION: Cell surface. Secreted protein; extracellular space. Note=During early stationary growth phase about 10% of the enzyme molecules remain cell-bound while about 90% are released into the growth medium.

CONCLUSION

Bacillus and *pseudomonas* species were isolated and isolation of these was confirmed using confirmatory test including Tributyrin agar test, staining and various biochemical tests. Lipase production was estimated using Lowry's method and various tests were carried out for optimization of temperature, pH, Sources for Carbon, Nitrogen and metal salts. Optimal temperature for growth of Bacillus as 37°C and for pseudomonas was observed to be 2.2.Optimal pH for growth of Bacillus was 8 and for *pseudomonas* 1.75.Best C-Sources for both *Bacillus* and *pseudomonas* was Tween. Best Nitrogen Source for growth of Bacillus was peptone and for pseudomonas was Tryptone. Na Cl was observed to be the best metal salt for Bacillus and $K_3PO_4$for pseudomonas. Insilco analysis for determination of various properties of isolated lipases was carried out using Bioinformatics tools.

Figure 10: Crystal Structure of the *Pseudomonas Aeruginosa* Lipase



REFERENCES

[1] Liebeton K, Zacharias A, Jaeger KE. J Bacteriol 2001; 183:597-603.

[2] Fultan JE, Noble NL, Bradley SS, Awad WM. Biochemistry 1974; 13 2320-2326.

[3] Adams DM, JD Barach, ML Speck. Dairy Science 1975; 58,828-834.

[4] Vadehra DB and Hanmon LG. J Bacteriol 1989; 32, 147-152.

[5] Linefield WM, Banauscas RA, Sievieri L Serotas and Stevenson SRW. JAOLS 1984;61:191-195.

[6] Garcia ML, B Sanz, P Garcia-collia and JA Ordonez. Milchwissenschaft 1984;,44:547-550.

[7] Gilbert EJ, Orozd JW and Jones CW. J Gen Microbiol 1991;137:2215-2221.

[8] Garcia ML, B Sanz, P Garcia-collia and JA Ordonez. J Microbiology 1984; 54:123-129.

[9] Rohit Sharma, SK Soni, RM Vohra, RS.Jolly, LK Gupta and JK Gupta. J Microbiol 2002; 42:49-54.

[10] Patrovic SE, Skrinjar M and Bekarevic. Biotechnol Lett 1990; 12:299-307.

[11] CordenonsA, Gonzales R, Kok R. Biotechnol 1996; 18:633-638