Asian Journal of Pharmaceutical and Clinical Research

Vol 6, Suppl 3, 2013

ISSN - 0974-2441

Research Article

SCREENING SELECTION IDENTIFICATION PRODUCTION AND OPTIMIZATION OF BACTERIAL LIPASE FROM OIL SPILLED SOIL.

M.VEERAPAGU[1], DR .A. SANKARA NARAYANAN[1], K.PONMURUGAN[2], K.R.JEYA[3]

[1]PG Research Department of Microbiology, K.S.R.College of Arts & Science, Tiruchengode, Tamilnadu, India. [2]Department of Botany and Microbiology, College of Sciences, King Saud University, Riyadh, Kingdom of Saudi Arabia. [3]Department of Biotechnology, Bharathidasan University College, Kurumbalur, Perambalur, Tamilnadu, India. Email: mveerapagu@gmail.com

Received: 13 May 2013, Revised and Accepted: 29 may 2013

ABSTRACT

Lipases are glycerol ester hydrolases that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol. Bacterial Lipase producers were isolated from oil spilled soil from vegetable oil processing factories. One of the twenty isolated strain exhibited a greater zone of clearance than the others indicating higher lipase activity was selected and identified based on their morphological and physicochemical characteristics and 16s rRNA sequencing. The effect of incubation time, medium pH, temperature, agitation, inoculums concentration, carbon source and nitrogen source for the lipase production was studied. The lipase production was maximum at pH7, temperature 370C and incubation time 48 hours by the lipase producing bacteria BLP2 Pseudomonas gessardii. Increased enzymatic production was obtained when the organisms were cultured in medium supplemented with 1% protease peptone by Pseudomonas gessardii (168.7Uml-1). The results of the present study demonstrate that the Pseudomonas gessardii is ideal for extracellular lipase production at industrial level.

Keywords: lipase, Pseudomonas, screening, production, and oil spilled soil.

INTRODUCTION

Lipases are glycerol ester hydrolases that act on acylglycerols to liberate fatty acids and glycerol. Lipases can hydrolyze long chain water-insoluble triglycerides into diglycerides, monoglycerides, glycerol and fatty acids 1,2. Lipases are ubiquitous enzymes which are widely

Distributed in plants, animals and microbes3. The ability of lipases to perform very specific chemical transformation (biotransformation) has make them increasingly popular in the food, detergent, cosmetic, organic synthesis, and pharmaceutical industries4,5,6.

Lipases are produced by many microorganisms and higher eukaryotes. Most commercially useful lipases are of microbial origin. Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food, compost heaps, coal tips, and hot springs7,8.Lipase producing microorganisms include bacteria, fungi, yeasts, and actinomyces. Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food, compost heaps, coal tips, and hot springs8.

Microbial lipases have gained special industrial attention due to their stability, selectivity, and broad substrate specificity 9,10. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer 11 .

Many microorganisms are known as potential producers of extracellular lipases, including bacteria, yeast, and fungi12. A variety of extracellular lipases of bacterial origin with different properties and specificities have been described and characterized. Extracellular lipase was isolated from many different bacterial species, including Bacillus13 and Pseudomonas 14,15.

Particular attention is focused on specific classes of enzymes of species Pseudomonas, that are among the first studied and used in biotechnological production but also because of their involvement in bacterial pathogenesis. Lipases are found in a number of Pseudomonas 16,17,15. Enzymes of P. aeruginosa, P. cepacia and P. fluorescens obtained in industrial conditions and are used in organic synthesis, including catalysis of reactions in aqueous solutions18,19. The bacterial genus Pseudomonas secretes a number of extracellular enzymes, which include lipases, in response to fluctuating external nutrients. Interest in Pseudomonas lipases stems either from their potential usefulness in a variety of biotechnological applications or from their detrimental effect on stored food products such as refrigerated milk. The majority of the strains of Pseudomonas sp. are producers of lipase and phospholipase-C20. A simple and reliable method for detecting lipase activity in microorganisms has been described21. This method uses the surfactantTween 80 in a solid medium to identify a lipolytic activity. The formation of opaque zones around the colonies is an indication of lipase production by the organisms. Modifications of this assay use various surfactants in combination with Nile blue or neet's foot oil and Cu2 + salts.

Also, screening of lipase producers on agar plates is frequently done by using tributyrin as a substrate22 and clear zones around the colonies indicate production of lipase. Screening systems making use of chromogenic substrates have also been described23. Plates of a modified Rhodamine B agar was used to screen lipase activity in a large number of microorganisms8. Other versions of this method have been reported24.

Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition besides physicochemical factors such as temperature, pH, and dissolved oxygen. The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes. These enzymes are generally produced in the presence of a lipid such as oil or any other inducer, such as triacylglycerols, fatty acids,hydrolysable esters, Tweens, bile salts, and glycerol 25,26. However, nitrogen sources and essential micronutrients should also be carefully considered for growth and production optimization.

Generally, high productivity has been achieved by culture medium optimization. Optimization of the concentration of each compound that constitutes a cultivation medium is usually a time-consuming procedure 5. Microbial lipases are produced mostly by submerged culture but solid state fermentation methods can be used also. The use of by-products as substrates for lipase production, adds high value and low-cost substrates may reduce the final cost of the enzyme 27,28. Considering the importance of lipase enzyme, lipase producing Pseudomonas sp. have been characterized and optimized in the present study.

MATERIALS AND METHODS

Collection of soil sample

A soil auger was used in collecting soil sample for analysis. The auger was used to make a depth of 30 cm using a grid or Zig zag sampling system. The soil sample for physiological analysis was collected with unused plastic bag sealed with heavy-duty rubber bounds. All samples were labeled with a permanent waterproof maker, while the microbiological analyses were collected using 200 ml capacity sterile glass sampling container.

Isolation of lipase producers

The soil samples were collected from different oil mills located at Dharmapuri and Salem districts enriched by periodic subculturing of samples in Nutrient Broth (NB) media. They were aseptically subjected to serial dilutions and plated on Nutrient Agar (NA) and incubated at 37°C for 24, 48 and 72 h. Acceptable plate counts for bacteria were between 30 – 300 cfu/ml per plate. After incubation 200 predominant bacterial colonies were isolated and screened for lipase activity and then subjected to morphological, cultural and biochemical examinations.

Screening for lipase activity by Tributyrin Clearing Zone (TCZ)

The predominant bacteria in the nutrient agatr plate were isolated and screened for lipolytic activity. Lipolysis is observed directly by changes in the appearance of the substrate such as tributyrin and triolein, which are emulsified mechanically in various growth media and poured into a petri dish. The bacterial isolates were screened for lipolytic activity on agar plates containing tributyrin (1%, w/v), agar (2%, w/v) in Luria–Bertani medium 29. Lipase production is indicated by the formation of clear halos around the colonies grown on tributyrin- containing agar plates 30,31,13.

Characterization of Bacterial lipase producer

Halos around the colonies on tributyrin agar plates are considered as positive colonies for lipase enzyme production. Such colonies are isolated and identified by phenotypic characterization based on morphological, biochemical and physiological characters according to Bergeys Manual of Systematic Bacteriology32.Characterization and identification of the isolate with higher lipolytic activity was carried out both biochemically and by 16s r RNA sequencing.

Lipase Enzyme production

The composition of production medium used in this study was: (%w/v) peptone 0.2; $NH_4H_2PO_4$ 0.1; NaCl 0.25; $MgSO_4•7H_2O$ 0.04; $CaCl_2.2H_2O$ 0.04; olive oil 2.0 (v/v); pH 7.0; 1-2 drops Tween 80 as emulsifier. Overnight cultures were suspended in 5ml of sterile deionised water and used as the inoculum for pre culture to obtain an initial cell density to adjust the turbidity of 0.5 McFarland standard. Submerged microbial cultures were incubat-ed in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium on a rotary shaker (150 rpm) and incubated at 36°C. After 24 hours of incubation, the culture was centrifuged at 10,000 rpm for 20 min at 4ºC and the cell free culture supernatant fluid was used as the sources of extracellular enzyme. The lipase activity in the supernatant was determined by the colorimetric method. The bacterial isolate that produced maximum lipase was selected for further work.

Colorimetric Assay of Lipase Enzyme using Copper Soap method

Fatty acids liberated during hydrolysis of an olive oil substrate by lipase can be determined colorimetrically using a cupric acetate/pyridine reagent33. Fatty acids complex with copper to form cupric salts or soaps that absorb light in the visible range (λmax 715 nm), yielding a blue color. Quantification of fatty acid released by lipase is determined by reference to a standard curve prepared using oleic acid. Olive oil is used a substrate. The reaction mixture consists of 1ml of crude enzyme, 2.5ml of olive oil was incubated for 5 minutes. Then the reaction was stopped by adding 1.0ml of 6N HCLand 5ml Benzene. The upper layer 4ml was pipetted into a test tube and 1.0 ml of cupric acetate pyridine was added. The FFA dissolved in Benzene was determined by measuring the absorbency of Benzene solution at 715nm. Lipase activity was determined by measuring the amount of FFA from the standard curves of oleic acid. One unit of lipase activity is defined as the amount of enzyme that liberated 1μmol FFA in 1min at 37 °C.

Optimization of fermentation conditions

Time course of lipase production

The time course of lipase production was studied in the enzyme production medium in shake flasks incubated for 60 h. A 5% inoculum was added to 50 ml of medium, in 500-ml Erlenmeyer flasks and incubated at 150 rpm on a rotary shaker, at 36°C, for 80 hrs. Samples were removed periodically at 8 hr interval and bacterial growth as well as lipase activity in the culture supernatant were determined.

Effect of the medium pH and incubation temperature

The effect of pH and temperature of the fermentation medium for lipase production was performed by varying pH of the medium from 4 to 10 whereas the other parameters were unaltered. For selection of optimum temperature for the production of lipases, the temperatures varying from 20 °C to 50°C were selected by keeping the remaining parameters same.

Effect of Agitation

Effect of agitation on lipase production was performed by incubating the enzyme production medium with inoculated culture in an orbital shaking incubator at 36°C at varying agitation speed from 110rpm to 200 rpm for 24 hrs. The enzyme was assayed by colorimetric method after incubation.

Effect of Inoculum concentration

Optimum inoculum concentration for lipase enzyme production was studied by preparing the inoculum as described and varied inoculums concentration (1% to 10 %) were added to the enzyme production medium in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium on a rotary shaker (150 rpm) and incubated at 36°C for 24 hrs and the enzyme was assayed.

Effect of Carbon source

Effect of carbon source on the lipase production was analysed by replacing the olive oil with different carbon sources maltose,Glucose, sucrose, starch, mannitol, Lactose , Fructose, Mannose, arabinose, galactose at a concentration of (1% w/v) were added into the production medium in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium on a rotary shaker (150 rpm) and incubated at 36°C for 24 hrs and the enzyme was assayed.

Effect of nitrogen sources

Effect of nitrogen sources on the lipase production was studied by replacing the nitrogen source with aminoacids alanine, arginine, asparatic acid, Glutamine, Histidine, leucine, lysine, valine, threonine and tryptophan, organic nitrogen soures Beef extract, casein, peptone, proteose peptone, yeast extract, tryptone , meat extract , malt extract soyapeptone, soyabean meal and inorganic nitrogen sources ammonium nitrate, ammonium sulphate ,ammonium dihydrogen phosphate, ammonium chloride, ammonium acetate , ammonium oxalate, calcium nitrate, potassium nitrate, sodium nitrate and urea at a final concentration of 1 % (w/v) were added to the medium and incubated at 36°C for 24 hrs in a rotary shaker (150 rpm).

RESULTS AND DISCUSSION

Isolation and Screening

Lipases are currently used in different industrial products and processes and new areas of applications are constantly being added, which include the production of single cell protein, cosmetics, pulping, lubricants etc34.Lipase producing microbes have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food , compost heaps, coal tips, and hot springs35.

During the period of research oil spilled soil samples collected from different oil mills of Dharmapuri and Salem districts were processed and serially diluted and plated on Nutrient agar medium. After incubation predominant bacterial colonies were observed. A total of 200 bacterial colonies were selected and isolated. They were screened for lipase activity by cultivating them on tributyrin agar medium and observed for the presence of clear halos (zone) around the colonies. Among the 200 isolates 20 shows clear zone in the tributyrin medium. The bacterial isolate which showed lipolytic activity was screened for lipase production in the screening medium. In the screening medium the isolate Pseudomonas gessardii shows maximum lipase production which produced 12U ml-1 was selected for further research while others showed less than 5U ml-1. Identificiation

The bacterial isolate which showed maximum lipase production was further characterized and identified by morphological, biochemical characteristics and by 16srRNA sequencing as Pseudomonas gessardii.

Optimization of fermentation

Time course of lipase production

The incubation time for enzyme production is governed by the characteristics of the culture and is based on growth rate. In the present study the production of lipase starts only after 24 hours of incubation .Lipase was not produced by the Pseduomonas gessardii at 8 hr and 16 hr of incubation time. The lipase production decreases after 48 hours. It was reported that maximum lipase activity was obtained when the physical environment of the fermentation

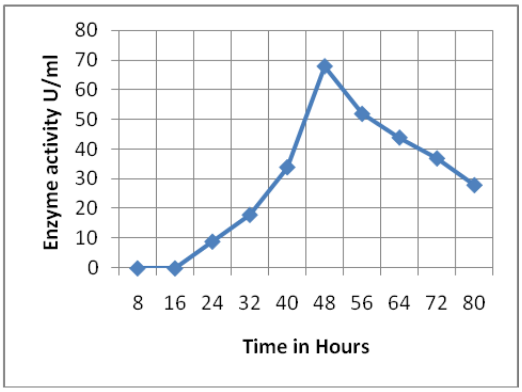

Fig 1: Optimization of incubation time for lipase production

medium was optima for 67 hours for Pseudomonas[36]. Maximum lipase production was at 72 hours for Pseudomonas spp[37] and *Bacillus coagulans*[38] and 48 hours for staphylococcus[39]and *Trichoderma viride* [40] respectively.

Effect of the medium pH and incubation temperature

The initial pH of the growth medium influences the rate of lipase production. It was inferred from the results that the bacteria is capable of producing lipase from the initial pH of medium from pH 4.0 to pH 10.0. The enzyme production varied considerably from 12.0Uml^{-1}to114Uml^{-1}. The bacteria Pseudomonas gessardii has optimum lipase production at pH 7.0(114.0U ml^{-1}). However it was noted that the lipase production was declined with increase in pH from pH 7.0 to pH 10.0. Maximum lipase activity was observed at pH7.0 and temperature 37°C by staphylococcus [39].

Temperature is a critical parameter that has to be controlled and it varies from organism to organism. Temperature influences secretion of extra cellular enzymes by changing the physical properties of the cell membrane.

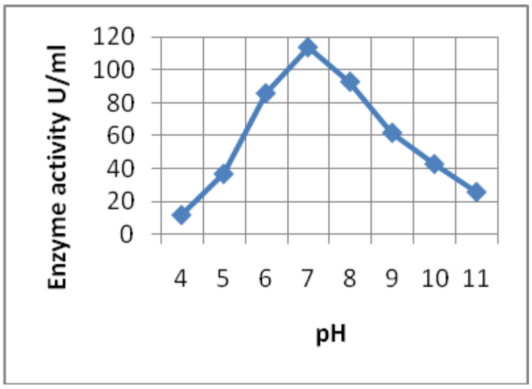

Fig 2: Optimization of medium pH for lipase production.

Studies conducted for the optimization of temperature shows that the bacteria produces lipase in wide range of temperature from 20 °C to 50°C.The lipase enzyme produced at different range of temperature was from 34.2 U ml^{-1} to 108.0U ml^{-1}. The optimum temperature for lipase enzyme production was at 37°C (108.0 U ml^{-1}) and the enzyme production was affected and decreased after increase of temperature above 37°C to 50°C. It was also noted that the lipase enzyme production was ceased at temperature 50°C.Similar result was reported that the maximum lipase production was at 37°C by Pseudomonas *xinjiangensis* [41]. It was also reported that the growth and lipase enzyme production of Pseudomonas fluorescens was maximum at the temperature 36^{0}C [42]. Kulkarni and Gadre[43] reported that maximum lipase production was at 25^{0}C for Pseudomonas sp.4.

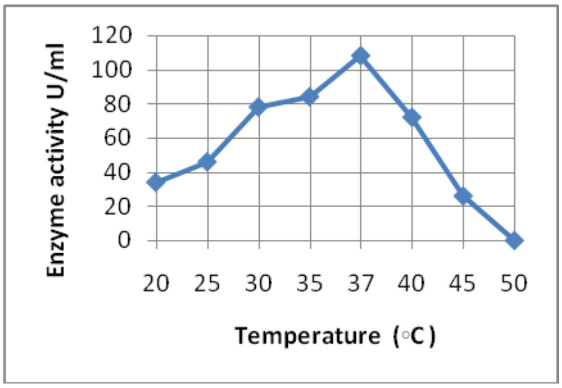

Fig 3: Optimization of Incubation temperature for lipase production

Effect of Agitation and Inoculum concentration

From the results it was clearly evident that agitation is required for the bacteria to produce lipase since there was no lipase production at stationary condition. Agitation at 110 rpm to 160rpm enahanced the lipase enzyme production .The optimum agitation speed for the production of lipase by the bacteria was 160rpm (121.6 U ml^{-1}). The rate of agitation speed above 160rpm led to decrease in the enzyme production. The increase in lipase production could be attributed by increased oxygen transfer rate, increased surface area of contact with the media components and better dispersability of the oil substrate during fermentation under agitated condition. However, at higher agitation rates, there was a reduction in growth as well as lipase production [44, 45]. Studies conducted for the optimization of inoculums concentration indicated that the variation in the level of concentration of inoculum from 1 % to 10 % influence the lipase enzyme production. The enzyme activity varied from 94.3U ml^{-1} to 108.0Uml^{-1}. The optimum inoculums concentration for lipase production was 6%(108.0Uml^{-1}).YU Hong-wei et al[36]. reported that highest lipase was produced when the inoculums concentration was 6% for Pseudomonas Lip35.

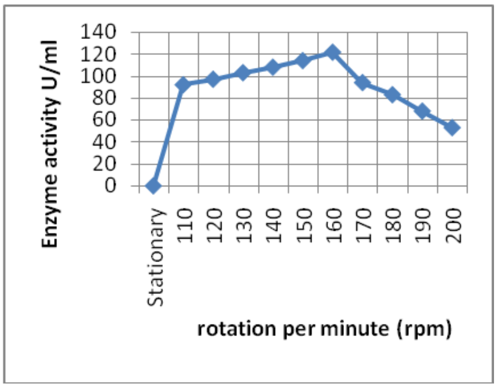

Fig.4. Effect of agitation on lipase production by P. gessardii.

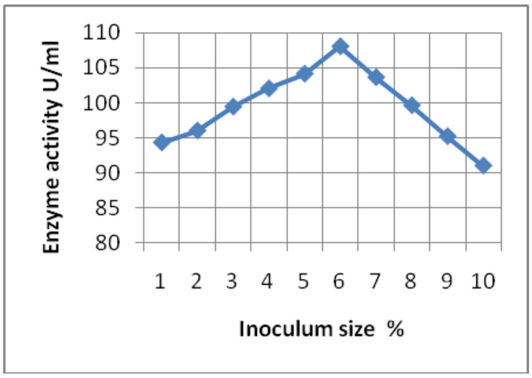

Fig. 5 .Effect of Inoculum size on lipase production by P.gessardii.

It is desirable to produce maximum enzyme activity with lower concentration of inoculums for industrial application.

Effect of carbon source

Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth, temperature, and the dissolved oxygen concentration [45].Studies conducted on the effect of sugars supplied as additional carbon source have not enchaned the lipase production when compared to control medium. Galactose, maltose and sucrose inhibited the lipase production. while other sugars tested decreased the level of lipase enzyme production compared to control medium. It was reported that starch was the best carbon source for lipase production by P. fluorescens[46]. Reduction in the lipase production in the prescence of sugars as carbon sources could be due to catabolite repression by readily available carbon sources in the medium[14, 36].

Effect of Nitrogen Source

Aminoacid as additional nitrogen source has influenced the lipase production. Among the 10 aminoacid Histidine (135.7 U ml^{-1}) and lysine (126.8 U ml^{-1}) has influenced lipase production. On the other hand asparatic acid, tryptophan, valine, glutamine and alanine caused a considerable reduction in enzyme production.

On the other hand asparatic acid, tryptophan, valine, glutamine and alanine caused a considerable reduction in enzyme production. The effect of additional organic and inorganic nitrogen source 1 % (w/v) on the production of lipase was studied. Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used, such as peptone and yeast extract, which have been used for lipase production by various thermophilic *Bacillus sp.* and various Pseudomonas [47,48]. Among the ten different organic nitrogen sources proteose peptone (168.7 U ml^{-1})and peptone (132.5 U ml^{-1}) enchanced lipase production whereas lipase production was very low with casein, soyabean meal and soy peptone. Similar results was reported for organic nitrogen source by Pseudomonas fluorescens NS2W[41]. Among the different nitrogen sources used peptone (2 g/l) was found to be the most suitable source for maximum lipase activity[49]. Similarly inorganic nitrogen sources ammonium sulphate 129.4Uml^{-1} and ammonium chloride 121.6Uml^{-1}enchanced lipase production.

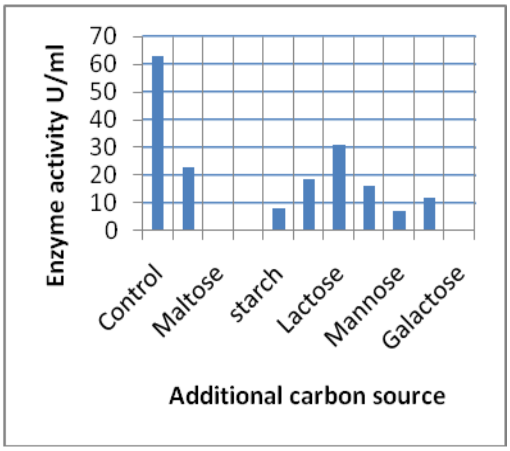

Fig. 6.Effect of sugars as additional carbon source on lipase production by P.gessardii.

Lipase production was very low with urea.

The results of the present study provides useful information for the optimization of culture conditions such as pH ,temperature, fermentation time, carbon sources and nitrogen sources to provide the best lipase production by Pseudomonas species.

These results shows clearly that lipase producing bacteria are widespread in oil contaminated soil. The optimized growth conditions developed in this study can be used for a large scale in industrial purposes.

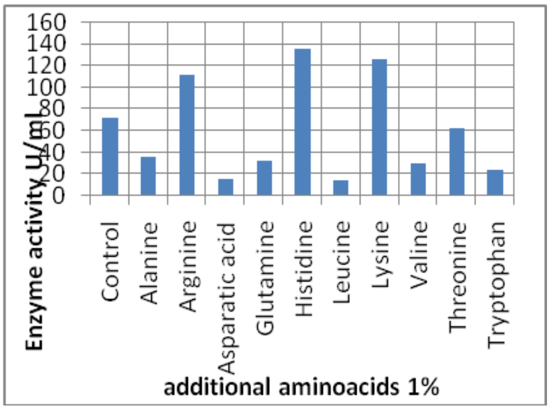

Fig.7.Effect of additional aminoacid as nitrogen source on lipase production by P.gessardii.

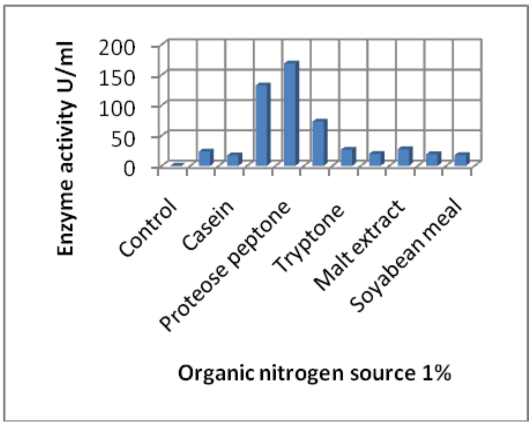

Fig.8.Effect of organic nitrogen source on lipase production by P.gessardii.

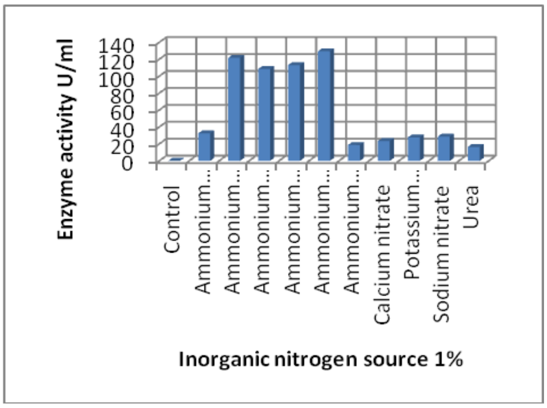

Fig.9.Effect of inorganic nitrogen source on lipase production by P.gessardii.

REFERENCES

1. Gilham.D. andR.Lehner. Techniques to measure lipase and esterase activity *in vitro*. Methods. 2005;36: 139-147.
2. Angkawidjaja, C. and S. Kanaya. Family I.3 lipase: Bacterial lipases secreted by the type I secretion system. Cell. Mol. Life Sci. 2006; 63: 2804-2817.
3. Dutta, S. and L. Ray. Production and characterization of an alkaline thermostable crude lipase from an isolated strain of *Bacillus cereus* C7. Appl. Biochem. Biotechnol. 2009; 159: 142-154.
4. Park, H., Lee, K., Chi, Y., & Jeong. S.Effects of methanol on the catalytic properties of porcine pancreatic lipase. Journal of Microbiology and Biotechnology. 2005; 15(2): 296–301.
5. Gupta, N., Shai, V., & Gupta, R. Alkaline lipase from a novel strain Burkholderia multivorans: Statistical medium optimization and production in a bioreactor. Process Biochemistry. 2007;42(2): 518– 526.
6. Grbavcic, S. Z., Dimitrijevic-Brankovic, S. I., Bezbradica, D. I., Siler- Marinkovic, S. S., & Knezevic, Z. D. Effect of fermentation conditions on lipase production by Candida utilis. Journal of the Serbian Chemical Society. 2007; 72(8–9): 757–65.
7. Sztajer H, Maliszewska I, Wieczorek J. Production of exogenous lipase by bacteria, fungi and actinomycetes. Enzyme Microb Technol. 1988; 10:492 –7.
8. Wang Y, Srivastava KC, Shen GJ, Wang HY. Thermostable alkaline lipase from a newly isolated thermophilic Bacillus strain, A30-1 (ATCC 53841). J Ferment Bioeng.1995;79:433 –8.
9. Dutra, J. C. V., Terzi, S. C., Bevilaqua, J. V., Damaso, M. C. T., Couri,S., Langone, M. A. P. Lipase production in solid state fermentation monitoring biomass growth of Aspergillus niger using digital image processing. Applied Biochemistry and Biotechnology. 2008; 147:63–75.
10. Griebeler, N., Polloni, A.E., Remonatto, D., Arbter, F., Vardanega, R., Cechet, J.L. et al. Isolation and screening of lipase producing fungi with hydrolytic activity. Food and Bioprocess Technology.2011; 4:578-586.
11. Hasan, F., A.A. Shah and A. Hameed. Industrial applications of microbial lipases. Enzyme Microb. Technol.2006; 39: 235-251.
12. Abada, E. A. E.Production and characterization of a mesophilic lipase isolated from Bacillus stearothermophilus AB-1. Pakistan Journal of Biological Sciences. 2008; 11: 1100–1106.
13. Ertuğrul S, Dönmez G, Takaç S. Isolation of lipase producing Bacillus sp. from olive mill wastewater and improving its enzyme activity. J Hazard Mater 2007;149(3):720–4.
14. Kiran GS, Shanmughapriya S, Jayalakshmi J, Selvin J, Gandhimathi R,Sivaramakrishnan S, Arunkumar M, Thangavelu T,Natarajaseenivasan K. Optimization of extracellular psychrophilic alkaline lipase produced by marine *Pseudomonas* sp. (MSI057). Bioprocess Biosyst. Eng. 2008; 31: 483-492.
15. Wang S-L, Lin Y-T, Liang T-W, Chio S-H, Ming L-J, Wu P-C. Purification and characterization of extracellular lipases from *Pseudomonas monteilii TKU009* by the use of soybeans as the substrate. J. Ind. Microbiol. Biotechnol. 2009; 36: 65-73.
16. Kim K, Kwon D, Yoon S, Kim W, Kim K. Purification, refolding, and characterization of recombinant *Pseudomonas fluorescens* lipase. Protein Expr. Purif.2005;39:124-129.
17. Singh S, Banerjee U. Purification and characterization of *trans*-3-(4-ethoxyphenyl) glycidic acid methyl ester hydrolyzing lipase from *Pseudomonas aeruginosa.* Process. Biochem. 2007; 42: 1063-1068.
18. Karadzic I, Masui A, Zivkovic L, Fujiwara N. Purification and characterization of an alkaline lipase from *Pseudomonas aeruginosa* isolated from putrid mineral cutting oil as component of metalworking xuid.J.Biosci.Bioeng.2006;102:82-89.
19. Reetz M, Jaeger K. Overexpression, immobilization and biotechnological application of *Pseudomonas* lipases. Chem. Phys. Lipids. 1998; 93: 3-14.
20. Stoyanova Vasilena, Stanka Georgieva ,Ivan Iliev ,Mariana Marhova ,Sonya Trifonova. Lipolytic activity of genus *Pseudomonas. J. BioSci. Biotech.* 2012; 163-168.
21. Sierra G. A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. Antonie van Leeuwenhoek 1957;23:15–22.
22. Cardenas J, Alvarez E, de Castro-Alvarez M-S, Sanchez-Montero J-M, Valmaseda M, Elson SW, Sinisterra J-V. Screening and catalytic activity in organic synthesis of novel fungal and yeast lipases. J Mol Catal B: Enzym. 2001;14:111–23
23. Yeoh HH, Wong FM, Lin G. Screening for fungal lipases using chromogenic lipid substrates. Mycologia. 1986;78: 298–300.
24. Kouker G, Jaeger KE. Specific and sensitive plate assay for bacterial lipases. Appl Environ Microbiol. 1987; 53:211–3.
25. Gupta, R., Gupta, N., & Rathi, P. Bacterial lipases: An overview of production, purification and biochemical properties. Applied Microbiology and Biotechnology. 2004;64:763–781.
26. Sharma, R., Chisti, Y.& Banerjee, U. C.Production, purification, characterization and applications of lipases. Biotechnology Advances. 2001;19:627–662.
27. Menoncin.S, N.M. Domingues, D.M.G. Freire, G. Toniazzo, R.L. Cansian and J.V. Oliveira et al., Study of the extraction, concentration and partial characterization of lipases obtained from Penicillium verrucosum using solid-state fermentation of soybean bran. Food Bioprocess Technol. 2010;3:537- 544.
28. Rodriguez, J.A., T. Bhagnagar, S. Roussos, J. Cordova and J. Baratti *et al*., Improving lipase production by nutrient source modification using *Rhizopus homthallicus* cultured in solid state fermentation. Process Biochem. 2006; 41: 2264-2269.
29. Fakhreddine L, Kademi A, Ait-Abdelkader N, Baratti JC. Microbial growth and lipolytic activities of moderate thermophilic bacterial strains. Biotechnol Lett 1998;20: 879–83.
30. Jaeger KE, Ransac S, Dijkstra BW, Colson C, Vanheuvel M, Misset O. Bacterial lipase.FEMS Microbiol Rev 1994;15:29–63.
31. Kim EK, Jang WH, Ko JH, Kang JS, Noh MJ, Yoo OJ. Lipase and its modulator from Pseudomonas sp. strain KFCC 10818: proline-to-glutamine substitution at position 112 induces formation of enzymatically active lipase in the absence of the modulator. J Bacteriol 2001 ; 183(20):5937–5941.
32. Sneath , P.H.A , Bergeys Manual of Determinative Bacteriology.2nd ed. Baltimore: Wiliams and Wilkins. (s) 1986: 1105-1138

33. Lowry, R.R. and Tinsley, I.J. Rapid colorimetric determination of free fatty acids. *J. Am. Oil Chem. Soc.* 1976; 53:470-472.
34. vijay Gunasekaran and Debabrata Das, Lipase fermentation : progress and prospects. Indian Journal of Biotechnology.2005;4: 437-445.
35. Rohit S, Yusuf C, Ullamchand B. Production,purification, characterization and application of lipases. Biotechnol.Adv.2001;19: 627- 662.
36. YU Hong-wei, HAN Jun, LI Ning, QIE Xiao-sha and JIA Ying-min. Fermentation Performance and Characterization of Cold-Adapted Lipase Produced with *Pseudomonas* Lip35. Agricultural Sciences in China. 2009; 8(8): 956-962.
37. V R Tembhurkar, M B Kulkarni and S A Peshwe. Optimization of Lipase Production by *Pseudomonas* spp. in submerged batch process in shake flask culture . Science Research Reporter.2012; 2(1):46-50.
38. M.P.PrasanthKumar, A.K.Valsa. Optimization of culture media and cultural conditions for the production of extracellular lipase by *Bacillus coagulans.* Indian J. Biotechnol. 2007; **6:**114-117 .
39. E. Sirisha ,M. Lakshmi Narasu , and N. Rajasekar. Isolation and Optimization of Lipase Producing Bacteria from Oil Contaminated Soils. Advances in Biological Research. 2010; 4 (5): 249-252.
40. M. A.Kashmiri, Ahmad Adnan and Beenish Waseem Butt. Production, purification and partial characterization of lipase from *Trichoderma Viride.* Afr. J. Biotechnol.Vol.2006;5(10): 878-882.
41. KhemikaLomthaisong, Angkhameen Buranarom and Hataichanoke Niamsup. Investigation of Isolated Lipase Producing Bacteria from Oil-contaminated Soil with Proteomic Analysis of its Proteins Responsive to Lipase Inducer. *Journal of BiologicalSciences*2012;12:161-167.
42. N.Kulkarni and RV.Gadre. Production and properties of an alkaline ,thermophilic lipase from Pseudomonas fluorescens NS2W. Journal of Industrial Microbiology & Biotechnology.2002; 28: 344 – 348.
43. M. Kavitha and C. Shanthi. Isolation and Characterization of Cold active lipase producing *Pseudomonas* sp. 4 from Marine samples of Tamilnadu Coast. *Research Journal of Biotechnology.* 2013; 8 (4) : 57 -62.
44. Gulati. R, Saxena. R K. and Gupta. R. Fermentation and downstream processing of lipase from *Aspergillus terreus. Process Biochemistry.* 2000; *36:*149-155.
45. E1ibol, M., and Ozer, D. Influence of oxygen transfer on lipase production by *Rhizopus arrhizus. Process Biochemistry .2001;*36: 325-329.
46. Sztajer H and I Maliszewska. The effect of culture conditions on lipolytic productivity of microorganisms. Biotechnol Lett. 1988;10(3): 199– 204.
47. Sharma R, Soni SK, Vohra RM, Jolly RS, Gupta LK,Gupta JK. Production of extracellular alkaline lipase from a *Bacillus sp.* RSJ1 and its application in ester hydrolysis. *Ind J Microbiol* 2002; 42: 49-54.
48. Sugihara A, Tani T, Tominaga Y. Purification and . characterization of a novel thermostable lipase from *Bacillus sp. J. Biochem* 1991;109: 211-215.
49. Kasra-Kermanshahi.R, Mobarak-Qamsari.E,Moosavi-nejad.Z. Isolation and Identification of a novel, lipase producing bacterium, Pseudomonas aeruginosa KM110 Iran.J.M.icrobiol. 2011; 3(2) : 92-98.