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Studies on nutritional requirements of *Pseudomonas aeruginosa* for lipase production

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ABSTRACT

A nutritional requirement for the lipase production by Pseudomonas aeruginosa strain was investigated. The bacterial strain was isolated from municipal sewage and it was culture with different nutritional composition. The maximum lipase activity was obtained with maltose as the sole carbon source 6.5 Uml^{-1}, followed by lactose 4.6Uml^{-1}. The various salts used the best results were observed with calcium chloride 4.6 Uml^{-1}as and sodium chloride an inorganic source. Among the various commercial grade oil highest activity was observed with coconut oil 8.1 Uml^{-1} and gingili oil 6.8 Uml^{-1}. Amino acids are also play a significant role for the lipase production. The maximum lipase activity was observed with glycine 5.1Uml^{-1} followed by tryptophan 4.6Uml^{-1} as a supplement. The organism grows optimally in the temperature of 40^{o}C and pH 8. The strain isolated from the municipal sewage is industrially important from in terms of ability. These findings will be helpful in prospect for increase the production of industrial enzyme.

Keywords: Lipase; *Pseudomonas aeruginosa*, optimization, lipase activity.

INTRODUCTION

Enzymes are biological catalysts that allow chemical reactions to occur in living organisms at ambient conditions. Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities such as the possibility of high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms with inexpensive media [Dharmsthiti S and Kuhasuntisuk,1998, Kojima, Y. *et al*.,1994]. Among microbial enzymes the lipase has been studied extensively [Karl-Erich J and Reetz, 1998].

Lipases (EC 3.1.1.3) comprise a group of enzymes which catalyze the hydrolysis of triglycerols. In the recent years, the interest on lipase has grown significantly. The development of technologies using lipase for the synthesis of novel compounds result in expanding into new areas and increase in number of industrial applications [Wiseman, A 1995]. Lipases are extremely versatile enzymes, showing many interesting properties in industrial applications. Microbial lipases have high demand due to their specificity of reaction, stereo specificity and less energy consumption than conventional methods. Microbial lipases have wide application in the processing of food, leather, domestic, industrial wastes, cosmetic, detergents and pharmaceutical industries [Houde *et al*., 2004, Wang *et al*., 1995] its mainly due to their ability to catalyze esterification, interesterificcation and transesterification reactions in aqueous media [Khyami-Horani H,1996]. Extracellular lipases have been produced from several microorganisms, such as fungi, yeast, and bacteria beside from plants and animals. Many commercial lipases produced from *Pseudomonas cepacia, Pseudomonas alcaligenes, Pseudomonas mendocina Burkholderia* studied by [Johnvesly and Naik 2001, Krieg and Holt, 1984].

The quality and quantity of the microbial enzymes mainly depend on the physical parameters and nutritional supplements. The present investigation is planned to focus on the nutritional requirement for the better production of lipase by *Pseudomonas aeruginosa*. The important growth parameters such as culture medium, carbon, amino acid, inorganic nitrogen source, certain lipid inducers and the physical parameters mainly the pH and temperature were optimized for the maximum production of lipase enzyme.

MATERIALS AND METHODS

Isolation and identification of bacterial strain

The bacterial strain used in this study was isolated from sewage sludge soil at Thiruninravur of Tiruvallur district of Tamilnadu, India. The soil was suspended in sterilized water and the suspension was spread directly on the plate. Grown colonies were collected for further studies. Thus isolated colonies were cultured in sterile Petri plates using Cetrimade agar medium and incubated at 37^{o}C. The developed colonies of *Pseudomonas aeruginosa* was isolated and screened for lipase production.

Species confirmation

The isolated strain was confirmed by criteria given in the Bergey's Manual of Systemic Bacteriology [Watanabe *et al*., 1977] and with the help of GC-MIS Gas Chromatograph Microbial Identification System Agilent Technologies (model- 6890 N).

Estimation of growth and lipase production

The strain was inoculated in the enriched medium. The culture was incubated at 30^{o}C under static and shaking condition (200x g) for 48 hrs. The enriched medium used was nutrient broth. All stock cultures were stored at -10^{o}C. Growth of *Pseudomonas aeruginosa* was estimated through spectrophotometer at optical density of 540 nm at different time intervals. One growth unit is the amount of cells that cause a change in OD of 1 unit. The growth of *Pseudomonas aeruginosa* was investigated in basal medium with 0.2ml of the 6hrs old culture inoculums in nutrient broth inoculated for a period of 4 days at in both shaken and static conditions the pH of the medium was maintained at 7. The enzyme activity was assayed for every 4 hrs intervals in both shaken and static conditions.

Nutritional factors influencing the lipase production by *Pseudomonas aeruginosa*

The nutritional factors which are influencing the growth and lipase production of *Pseudomonas aeruginosa* were observed with two types of media which includes nutrient broth and minimal medium (MM). The minimal medium (g/l) contains peptone 2%, Starch 2%, KH_2PO_4 0.2%, (NH_4) NO3 0.5%, Na_2HPO $12H_2O$ 0.8%, $MgSO_4$ $7H_2O$ 0.001%, $CaCl_2$ $2H_2O$ 0.01%, 1 %(v/v) olive oil and the pH was adjusted to 7.5. The medium was inoculated with 2ml of overnight culture and incubated at 37^{o}C. After 24hrs of incubation the culture was harvested from the medium by centrifugation in a high-speed refrigerated centrifuge at 10,000x g for 10 minutes and supernatant was carefully removed and stored at 4^{o}C until used. The supernatant was then filtered with a Whatman No.1 filter paper. Bacterial growth was determined spectrophotometrically at absorbance 540nm. Each experiment was done in triplicates.

Lipase Assay

Titrimetric assay method

Lipolytic activity was measured according to [Jagtap *et al*., 2010]. The reaction mixture containing 5ml of olive oil emulsion composed of 25ml olive oil, 75ml 2% polyvinyl alcohol (PVA) solution, 4ml of 0.2M tris buffer, 1ml of 115mM $CaCl_2$ and 1ml of enzyme solution. The control containing boiled inactivated enzyme (at 100^{o}C for 5 minutes) was treated similarly. After the incubation the enzyme activity was blocked by 20ml of acetone and ethanol (1:1) mixture and liberated free fatty acid was titrated against 0.02 M NaOH using phenolphthalein as indicator. One unit of lipase was defined as the amount of enzyme, which liberates 1M of fatty acid/min (1ml of 0.02N NaOH is equivalent to 100 M fatty acid liberated per min).

Effect of culture variable on lipase production

The amount of lipase production was assessed with different carbon sources such as glucose, starch, fructose, lactose and maltose. These carbon sources were mixed with production medium at the level of 0.5 %. The lipase activity was estimated in the culture filtrate after the incubation period. Likewise different lipid sources at 1.0 % level were also added to the production medium which acts as inducers of lipase which includes Tween-80, tributyrin and some vegetable oils (Castor oil, groundnut oil, coconut oil, olive oil and gingili oil) then the strain *Pseudomonas aeruginosa* inoculated into the production medium and incubated as above.

Effect of inorganic nitrogen source on lipase production

The effect of inorganic nitrogen source was investigated without the presence of organic nitrogen source in the basal medium. The following inorganic nitrogen sources were substituted into the basal medium such as 0.1 % of ammonium nitrate, disodium hydrogen phosphate potassium, dihydrogen phosphate, sodium chloride, magnesium sulphate and calcium chloride. The lipase activity was measured from the each culture after the incubation period.

Effect of amino acids on lipase production

Alanine, glutamine acid, methionine, tryptophan, glycine and valine were added to the lipase production medium at 0.2 % level. Lipase activity was estimated from the each culture after the incubation period.

TABLE1. Taxonomic characteristics of lipolytic bacterial strain

Morphological characteristics	
Morphology	Gram negative rods, actively motile, non-sporing, non-capsulated and Facultative anaerobes.
Nutrient agar	*Pseudomonas aeruginosa* maintained at 4^{o}C in nutrient agar slants
Cetrimade agar	This is the selective medium that help in the isolation of *Pseudomonas aeruginosa.* The organism is grown in the medium and used for further Procedure. Green colour pigment are observed in cetrimade agar plate With earthy mawkish.
Temperature	37^{o}C
pH	7.2
Biochemical characteristics	
IMVIC	
Indole production	Negative
Methyl red test	Negative
Voges- Proskauer	Negative
Citrate utilization	Positive
Oxidase test	Positive
Triple sugar iron	Alkaline slant, Alkaline butt, No H_2S and No gas production
SUGARS	
Glucose	Positive
Fructose	Negative
Sucrose	Negative
Mannose	Negative
Lactose	Negative

Effect of temperature and pH

The lipase obtained from the culture was assayed for its activity at different temperatures (20-50^{o} C) and different pH (5-10).

RESULTS AND DSCUSSION

In this study carried out lipase production [Fig 1-7] and *Pseudomonas aeruginosa* identified as [Table-1].

Fig-1 Growth of incubation *Pseudomonas aeruginosa* static and shaking

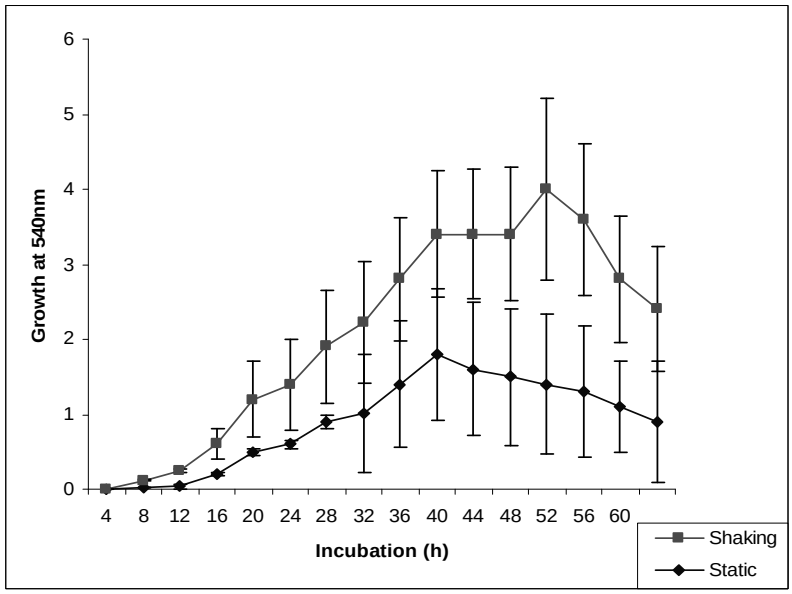


Fig-2 Effect of different carbon source on extracellular lipase production by *Pseudomonas aeruginosa*

Fig-3 Effect of inducers on extracellular lipase production by *Pseudomonas aeruginosa*

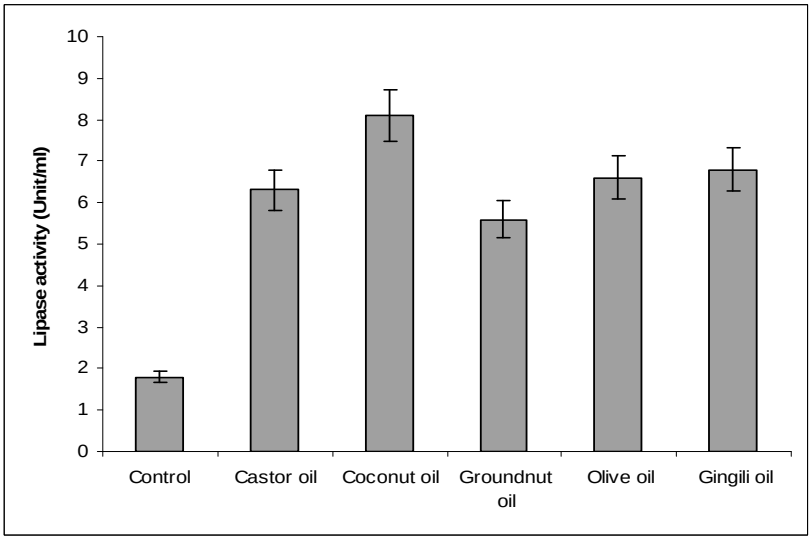


Fig-4 Effect of different inorganic salts on extracellular lipase production by *Pseudomonas aeruginosa*

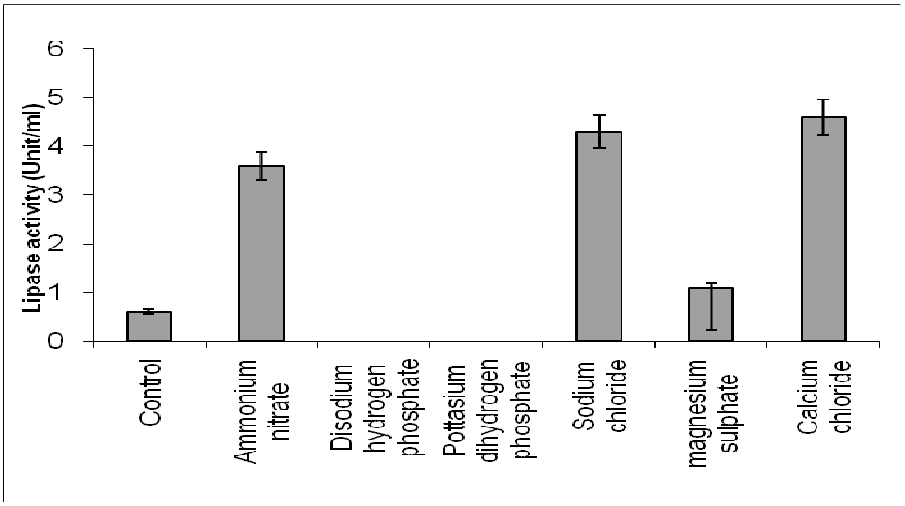


Fig-5 Effect of different amino acids on extracellular lipase production by *Pseudomonas aeruginosa*

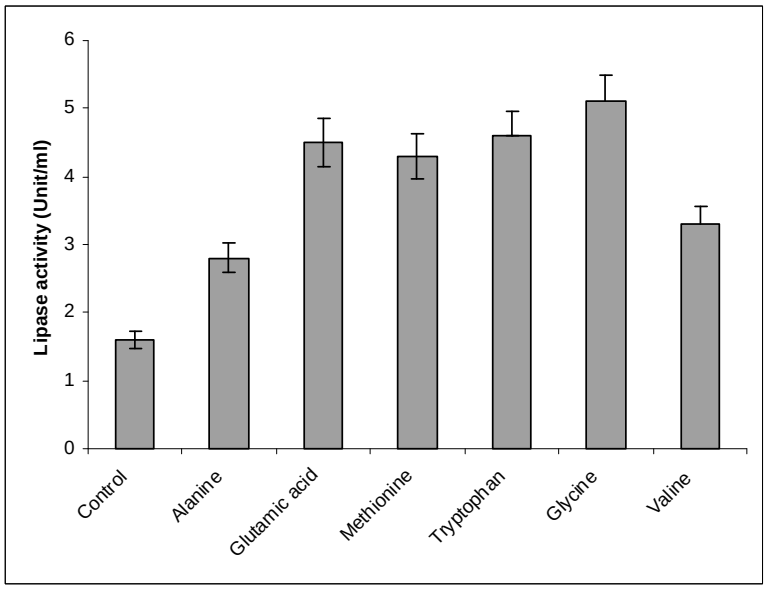


Fig-6 Effect of different temperatures on extracellular lipase production by *Pseudomonas aeruginosa*

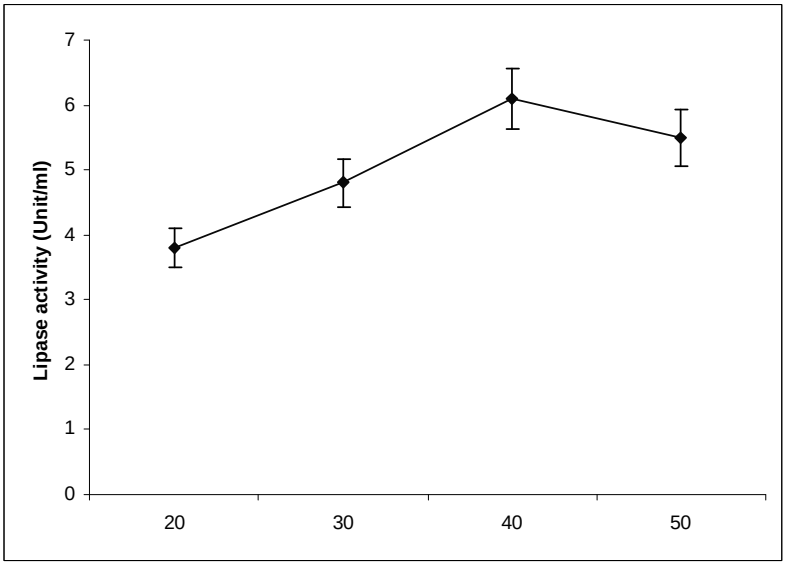
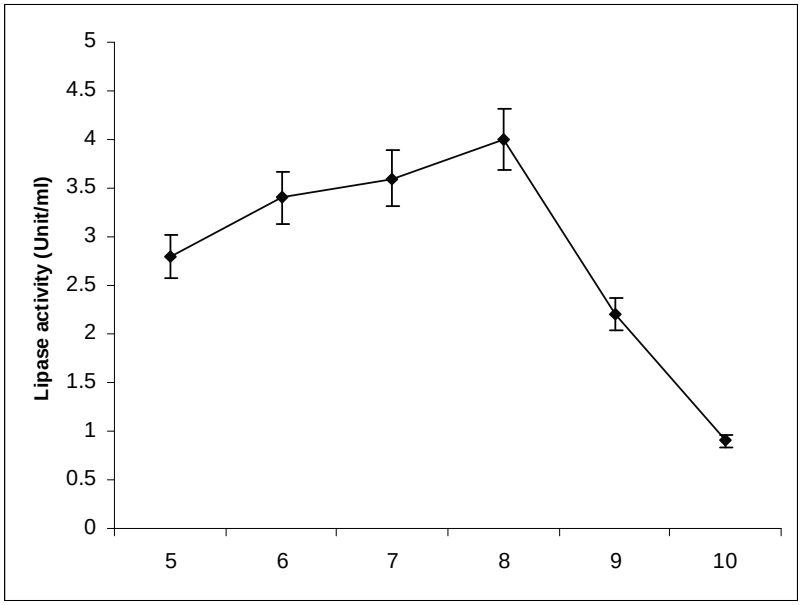


Fig-7 Effect of different pH on extracellular lipase production by *Pseudomonas aeruginosa*



Isolation and identification of the bacterial strain

In the present investigation, the lipolytic bacterial strain was isolated from sewage sludge sample and it is identified as *Pseudomonas aeruginosa* based on the morphological and biochemical characteristics of this organism as listed in Table-1.

Bacterial growth and lipase production

The present study shows that the shaken culture yields maximum lipase production when compared to static culture. This study also emphasizes that the incubation period is crucial for enzyme elaboration. A four day incubation offered good yield of the enzyme in the test organism. The different environmental conditions were used to determine the growth in every four hrs intervals. Maximum growth measured as optical density of this bacterium was achieved after 36hrs in static condition whereas in shaking condition it was achieved after 52 hrs incubation. In basal medium the logarithmic phase commenced at 8hrs and continued for another 12hrs. Lipase production was detected in the late logarithmic phase after (16hrs) and it increased until its optimum production after 48-hrs incubation. The fall of activity might be due to the absorption of the enzyme by the substrate. Similar studies have been carried out with *Pseudomonas aeruginosa EF2* [Chigusa *et al*., 1996]. The enzyme activity was found declined in subsequent days (Fig-1). The fall of enzyme activity might be due to the absorption of the enzyme by the substrate or by the proteolytic activity. Similar studies have been carried out with thermophilic *Bacillus spp*. [Litthauer *et al*., 2002], *Pseudomonas putida* [Pabai *et al*., 1995] and *Aspergillus niger* [Lesuisse *et al*., 1993], where maximum lipase activity was also produced during the late logarithmic phase and then decreased hastily once growth had ceased.

Effect of different carbon sources on lipase production

The study of lipase production with various carbon sources such as glucose, starch, lactose, fructose and maltose was observed with the medium. Among different carbon sources, the maximum enzyme production was observed with maltose (6.5 Uml^{-1}) while the low level of lipase production was observed with lactose (4.6 Uml^{-1}) (Fig-2). A study made by [Oh *et al*., 1999], that the carbon source was the best for lipase production by [Lee *et al*., 1999], *Pseudomonas fluorescens.* Similar to the present study was carried out by in *Psychrotrophic bacteria* as carbon was found to be best source for lipase production.

Lipolysis of commercial grade oils

The lipolytic activity of lipase enzyme produced by *Pseudomonas aeruginosa* on various commercial food grade fats and oils was determined at 37^{o}C (optimal temperature for activity) for 30min by titrimetric method. The enzyme was active against all kinds of lipids tested (Fig-3). Among the various source of inducers used in this study the highest activity (8.1 Uml^{-1}) was observed in the coconut oil followed by gingili oil (6.8 Uml^{-1}). The activities towards castor oil, olive oil, and groundnut oil were 6.3, 6.6 and 5.6 Uml^{-1} respectively. Similar trend as in the case of the present study was observed by [Bjorkling *et al*., 1991].

Effect of different inorganic salts on lipase production

The different inorganic salts were tested for the assessment of lipase productivity by *Pseudomonas aeruginosa*. The calcium chloride and sodium chloride got highest lipase yield as 4.6 and 4.3 Uml^{-1} respectively (Fig-4). Co-factors are generally not required for lipase activity, but divalent cations such as calcium often stimulate enzyme activity they are acting as co-factors for catalytic activity. The inorganic nitrogen compounds 0.5% resulted in good growth with the exception of ammonium acetate, potassium nitrate and sodium nitrate. Interestingly, ammonium citrate gave good growth but no lipase activity, while the oxalate gave good growth and poor lipase activity. Ammonium acetate on the other hand, suppressed both growth and lipase production. The effect of citrate could be due to catabolite repression, although this is unlikely to explain the effect of oxalate. However both anions could affect calcium availability by precipitation or chelation and therefore reduce lipase production. Both citrate and acetate were found to be inhibitory for lipase production when present as the sole of carbon [Makhzoum *et al*., 1995].

Effect of different amino acids on lipase production

Incorporation of amino acids in the basal medium has been found to enhance lipase production was observed by [Louwriver A, 1998]. The organism resulting in good growth with arginine, threonine and lysine, whereas, asparagines aspartic acid and serine gave the lowest lipase production [Alford and Pierce, 1963]. Likewise, in present observation all amino acids such as alanine, glutamic acid, methionine, tryptophan, glycine and valine appeared to stimulate lipase production (Fig-5). The maximum activity was noted in glycine and tryptophan as found maximum lipolytic activity as 5.1-4.6 Uml^{-1} respectively.

Effect of different pH and temperature for lipase production

The enzyme activities were observed at 20^{o}C, 30^{o}C, 40^{o}C and 50^{o}C. The optimum temperature was observed at 40^{o}C for maximum production (Fig-6). In the same manner the thermal stability of lipase ranging from 30-60^{o}C. Stability of organic solvents is desirable in synthesis reaction most of bacterial lipase are stable in organic solvents [Stead D, 1987 and Dong *et al*., 1999]. However, reports exist on bacterial lipases with optima in both lower and higher ranges [Sztajer *et al*., 1991, Nadakarani SR, 1971 and Sunna *et al*., 2002]. The effect of pH on lipase production was studied by using phosphate buffer with various pH values ranged from pH 5-10 (Fig-7). In the present observation, the enzyme functioned optimally at pH level of 8. In general, bacterial lipases possess stability over a wide range of pH (4-11) but it was most stable at the pH of 7-8 [Ghosh *et al*., 1996, Gilbert *et al*., 1991 and Sidhu *et al*., 1998b].

CONCLUSION

In this study the *Pseudomonas aeruginosa* isolated from sewage sludge identified as a one of the suitable species for the production of lipase enzyme. The nutritional factors of the production medium were optimized with olive oil as the substrate, and then the crude enzyme was very stable in various inducers. The screening of microorganism for the production of lipolytic enzyme will open a new and simple route for synthetic processes and consequently the present work will be a very good baseline for the large scale production.

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