

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

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### 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, inoculum concentration, carbon source and nitrogen source for the lipase production was studied. The lipase production was maximum at pH7, temperature 37°C 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* (168.7U/ml-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 microbes<sup>3</sup>. The ability of lipases to perform very specific chemical transformation (biotransformation) has made them increasingly popular in the food, detergent, cosmetic, organic synthesis, and pharmaceutical industries<sup>4,5,6</sup>.

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 springs<sup>7,8</sup>. Lipase producing microorganisms include bacteria, fungi, yeasts, and actinomycetes. 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 springs<sup>8</sup>.

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

Many microorganisms are known as potential producers of extracellular lipases, including bacteria, yeast, and fungi<sup>12</sup>. 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 *Bacillus*<sup>13</sup> and *Pseudomonas*<sup>14,15</sup>.

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*<sup>16,17,15</sup>. 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 solutions<sup>18,19</sup>. 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 described<sup>21</sup>. This method uses the surfactant Tween 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 Cu<sup>2+</sup> + salts.

Also, screening of lipase producers on agar plates is frequently done by using tributyrin as a substrate<sup>22</sup> and clear zones around the colonies indicate production of lipase. Screening systems making use of chromogenic substrates have also been described<sup>23</sup>. Plates of a modified Rhodamine B agar was used to screen lipase activity in a large number of microorganisms<sup>8</sup>. Other versions of this method have been reported<sup>24</sup>.

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<sup>25,26</sup>. 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<sup>5</sup>. 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 agar 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 Bacteriology<sup>32</sup>. 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<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> 0.1; NaCl 0.25; MgSO<sub>4</sub>•7H<sub>2</sub>O 0.04; CaCl<sub>2</sub>•2H<sub>2</sub>O 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 incubated 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 4°C 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 reagent<sup>33</sup>. Fatty acids complex with copper to form cupric salts or soaps that absorb light in the visible range ( $\lambda_{max}$  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 HCL and 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 1 $\mu$ mol 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 inoculum 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 amino acids alanine, arginine, aspartic acid, Glutamine, Histidine, leucine, lysine, valine, threonine and tryptophan, organic nitrogen sources 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 etc<sup>34</sup>. 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 springs<sup>35</sup>.

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<sup>-1</sup> was selected for further research while others showed less than 5U ml<sup>-1</sup>. Identification

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 *Pseudomonas 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

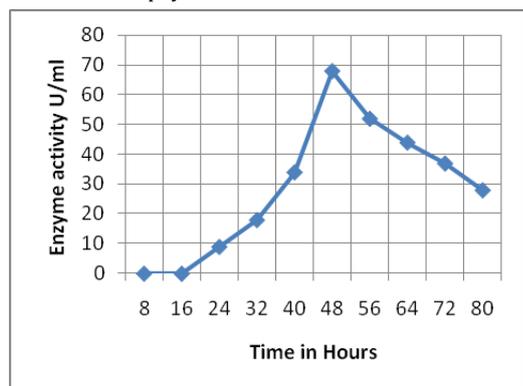


Fig 1: Optimization of incubation time for lipase production

medium was optima for 67 hours for *Pseudomonas*<sup>36</sup>. Maximum lipase production was at 72 hours for *Pseudomonas spp*<sup>37</sup> and *Bacillus coagulans*<sup>38</sup> and 48 hours for *staphylococcus*<sup>39</sup> and *Trichoderma viride*<sup>40</sup> 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<sup>-1</sup> to 114Uml<sup>-1</sup>. The bacteria *Pseudomonas gessardii* has optimum lipase production at pH 7.0 (114.0U ml<sup>-1</sup>). 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 pH 7.0 and temperature 37°C by *staphylococcus*<sup>39</sup>.

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.

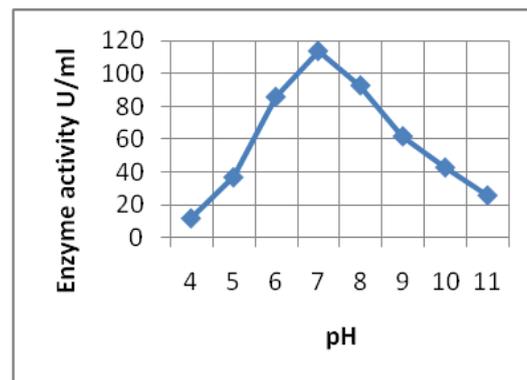


Fig 2: Optimization of medium pH for lipase production.

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<sup>-1</sup> to 108.0U ml<sup>-1</sup>. The optimum temperature for lipase enzyme production was at 37°C (108.0 U ml<sup>-1</sup>) 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*<sup>41</sup>. It was also reported that the growth and lipase enzyme production of *Pseudomonas fluorescens* was maximum at the temperature 36°C<sup>42</sup>. Kulkarni and Gadre<sup>43</sup> reported that maximum lipase production was at 25°C for *Pseudomonas sp.4*.

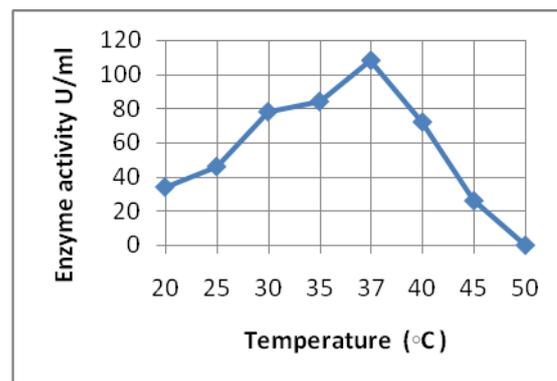


Fig 3: Optimization of Incubation temperature for lipase production

#### 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 enhanced the lipase enzyme production. The optimum agitation speed for the production of lipase by the bacteria was 160rpm (121.6 U ml<sup>-1</sup>). 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<sup>44, 45</sup>. Studies conducted for the optimization of inoculum 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<sup>-1</sup> to 108.0Uml<sup>-1</sup>. The optimum inoculum concentration for lipase production was 6% (108.0Uml<sup>-1</sup>). YU Hong-wei et al<sup>36</sup>. reported that highest lipase was produced when the inoculum concentration was 6% for *Pseudomonas Lip35*.

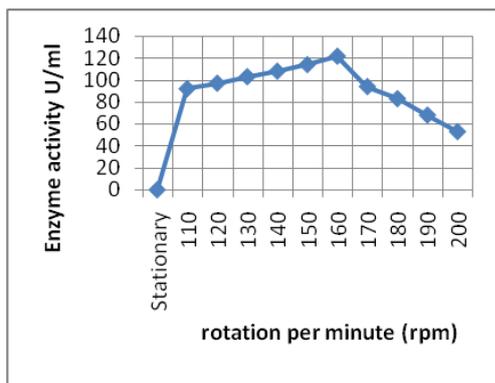


Fig.4. Effect of agitation on lipase production by *P. gessardii*.

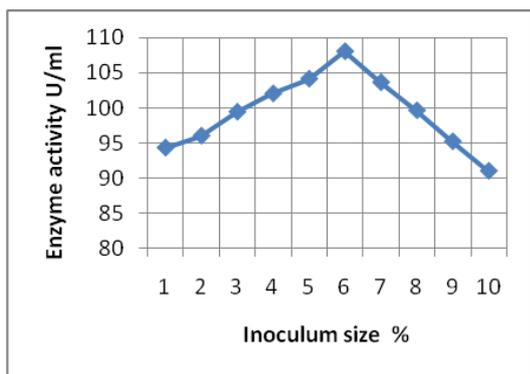


Fig. 5. Effect of Inoculum size on lipase production by *P.gessardii*.

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<sup>45</sup>. Studies conducted on the effect of sugars supplied as additional carbon source have not enhanced 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*<sup>46</sup>. Reduction in the lipase production in the presence of sugars as carbon sources could be due to catabolite repression by readily available carbon sources in the medium<sup>14, 36</sup>.

**Effect of Nitrogen Source**

Amino acid as additional nitrogen source has influenced the lipase production. Among the 10 amino acid Histidine (135.7 U ml<sup>-1</sup>) and lysine (126.8 U ml<sup>-1</sup>) 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*<sup>47,48</sup>. Among the ten different organic nitrogen sources proteose peptone (168.7 U ml<sup>-1</sup>) and peptone (132.5 U ml<sup>-1</sup>) enhanced 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<sup>41</sup>. Among the different nitrogen sources used peptone (2 g/l) was found to be the most suitable source for maximum lipase

activity<sup>49</sup>. Similarly inorganic nitrogen sources ammonium sulphate 129.4Uml<sup>-1</sup> and ammonium chloride 121.6Uml<sup>-1</sup> enhanced lipase production.

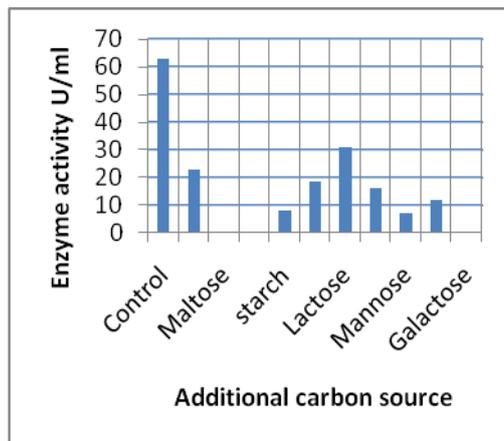


Fig. 6. Effect of sugars as additional carbon source on lipase production by *P.gessardii*.

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.

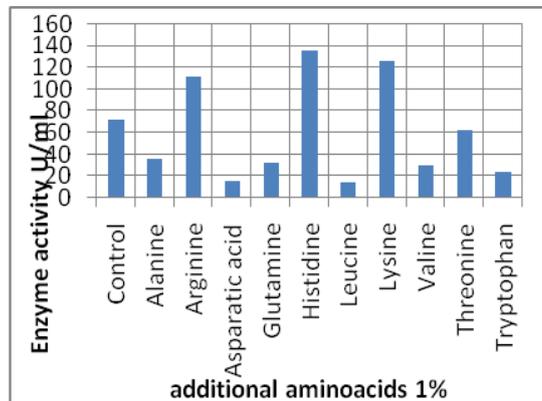


Fig.7. Effect of additional amino acid as nitrogen source on lipase production by *P.gessardii*.

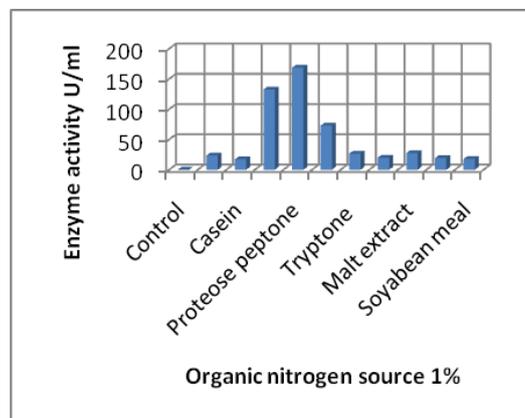


Fig.8. Effect of organic nitrogen source on lipase production by *P.gessardii*.

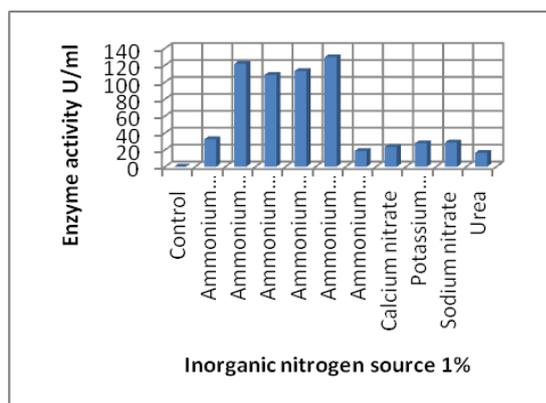


Fig.9.Effect of inorganic nitrogen source on lipase production by *P.gessardii*.

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