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Media Optimization for Lipase Production from *Pseudomonas otitidis* G5

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Abstract

Microbial lipases are enzymes capable of hydrolyzing the triglycerides to free fatty acids and glycerol. Microbial lipases are one of the most important groups of commercially and industrially produced enzymes. The lipase production was carried out by using shake flask fermentation by *Pseudomonas otitidis* G5 isolated from soil sample collected from a garage located near to Pondicherry University, Kalapet, India. Fermentation conditions (Incubation time, Temperature, pH, Carbon sources, Nitrogen sources and agitation) were optimized to increase lipase production by *Pseudomonas otitidis* G5. The results showed that lipase production by *Pseudomonas otitidis* G5 was increased under optimized conditions with a medium supplemented with glucose as carbon source, ammonium nitrate as a nitrogen source, pH 8 at 40ºC for 72 h of incubation of fermentation broth. These results in the optimization study on various parameters may be highly useful for further production of lipase in large scale by *Pseudomonas otitidis* G5.

Keywords: Lipase, Screening, Optimization, *Pseudomonas otitidis* G5

1. Introduction

Lipases (triacylglycerol hydrolases E.C.3.1.1) are important group of biotechnologically relevant enzymes produced by various microorganisms, plants and animal. Lipases of microbial origin, mainly fungal and bacterial play a crucial role in commercial sectors and have become key enzymes having enormous applications in food, dairy, textile, paper, leather, detergent, cosmetics and pharmaceutical industries (Hasan et al., 2006; Sharma et al., 2001;).

Microbial enzymes are often more useful than enzymes from plants and animals because they allow microbes to be easily cultivated and their lipases can catalyse a wide variety of catalytic activities , the high yields possible, ease of genetic manipulation, rapid growth, regular supply due to absence of seasonal fluctuations, more stable, convenient and safer. Lipase-producing microorganisms include bacteria, fungi and yeasts found in diverse various habitat such as industrial wastes, soil contaminated with oil, vegetable oil processing factories, dairies, compost heaps, decaying food, and hot springs (Joseph et al., 2008; Aly et al., 2012; Anbu et al., 2011).

A number of bacterial sources producing lipase are available, only a few are commercially exploited. Of these, the important are *Pseudomonas, Alcaligenes, Achromobacter, Bacillus, Arthrobacter, Burkholderia,* and *Chromobacterium* . Microbial lipases are mostly extracellular and excreted in the culture medium. Optimization of culture conditions for microbial lipases is of great important because the culture conditions influence the properties of the enzyme producer and the ratio between extracellular and intracellular lipases (Lin et al., 1996; Sarkar er al., 1998). The amount of lipase produced depends on several environmental factors such as temperature, incubation time, pH, nitrogen sources, carbon sources, lipid sources, inorganic salts and aeration and agitation (Shukla and Desai, 2016; Sirisha et al., 2010; Veerapagu et al., 2013)

In this study our aim to optimize the media condition for the maximum production of lipase from *Pseudomonas otitidis* G5 isolated from oil contaminated garage soil sample.

2. Materials and methods

2.1 Sample collection

The lipase producing bacteria were isolated from the oil contaminated garage soil. The garage is located near to the Pondicherry University, Kalapet, Pondicherry. The soil sample was immediately brought to the laboratory and kept in refrigerator.

2.2 Isolation of lipase producing bacteria

The serial dilution method followed by standard plate count assay was used for the isolation of bacteria from soil sample.

2.3 Screening of the best lipase producer

A total of 45 different single colonies were grown on nutrient agar media. These bacteria were assigned as (G1 to G45). Then, these isolated colonies were subjected for primary screening by Rhodamine olive oil agar plate assay (ROA) for the isolation of lipase producer. The olive oil is used as a substrate for the lipase and the presence of orange colour florescence zone indicates the production of lipase with the help of rhodamine dye on the agar plate (Verma and Prakash, 2015).

2.4 Production of crude lipase

The positive culture was inoculated in the production media (Mineral salt medium, supplemented with olive oil as a carbon source) and incubated for seven days at 37°C in a shaker at 150 rpm. Every 24 hrs of incubation (Till the 7^{th} day) the production medium was centrifuged at 6000 rpm for 30 minute at 4°C temperature. The cell free supernatant (crude enzyme) was collected and used for the lipase activity assay to check the maximum production of lipase.

2.5 Preparation of substrate emulsion for lipase assay

One ml of tributyrin oil was mixed with 10 ml of distilled water. Then 50mg of bile salt was added and stirrer it. After that 1 gm of gum arabic was added slowly into above solution and mixed properly. 10 ml of the above solution was taken and mixed with 2.5 ml of 0.05M phosphate buffer by using magnetic stirrer. The substrate emulsion was used for lipase activity assay.

2.6 Lipase assay

Lipase activity was determined titrimetrically using tributyrin oil as the substrate. 1000µl of substrate emulsion was mixed with enzyme (1000 µl) and kept in the shaker incubator at 37°C for 60 minute at 200 rpm. The blank also was prepared in which only 1000µl substrate emulsion was added and kept in same condition as test. After the completion of incubation, the reaction was terminated by using 1000µl acetone: ethanol (1:1) to both blank and test. Then same volume of enzyme was added into blank to make equal volume with test. After that 100µl phenolphthalein indicator (0.1%) was added and mixed properly. The blank and test was titrated with 0.05M NaOH. One unit of enzyme activity was defined as the amount of enzyme that released one µmole fatty acid per minute under standard assay condition (Jensen, 1983)

2.7 Protein estimation

The amount of protein in the sample was estimated by Bradford protein assay at 595 nm. Bovine serum albumin was used as a standard.

2.8 Identification of lipase producer

The best lipase producer (G5) was identified by physiological and biochemical characterization and 16S-rRNA sequencing. The DNA was extracted by phenol-chloroform extraction method followed by PCR amplification.

2.9 Media optimization for maximum lipase production

The media condition like incubation time, temperature, pH, carbon sources, nitrogen sources and agitation were used to optimize the media parameter for the maximum lipase production by the isolate (Anbu et al., 2011).

2.9.1Effect of incubation period on lipase production

The production media (MSM, pH 7) was inoculated with overnight grown culture (G5). The media was kept at 37ºC in a rotary shaker under submerge fermentation condition for 24h to 168h incubation (Day 1 to Day 7). Every 24h interval the cell free broth (crude enzyme) was obtained and the lipase activity was measured under the standard assay conditions.

2.9.2 Effect of Temperature

The production media was inoculated with overnight grown selected bacterial isolate. Then the media was incubated at various temperatures from 20, 30, 40, 50 and 60 °C for 72h in 150 rpm. After

the incubation time period, the cell free culture broth was used to check the lipase activity.

2.9.3 Effect of pH

The production media was inoculated with overnight grown selected bacterial culture. The media pH was adjusted to pH 4, 5, 6, 7, 8, and 9 by using 6N HCl and 10% Na_2CO_3. Then the broth was incubated at 40 °C for 72h in 150 rpm. After the incubation time period, the cell free culture broth was used to determine the lipase activity.

2.9.4 Effect of carbon sources

The production of lipase was optimized by substituting the production media with different carbon sources (1% v/v) like, glucose, coconut oil, castor oil, groundnut oil, sunflower oil, groundnut oil cake, sesame oil, and sesame oil cake. The lipase enzymatic assay was carried out by the standard assay for the lipase activity.

2.9.5 Effect of nitrogen sources

The production of lipase enzyme was optimized by substituting the production media with different nitrogen sources (0.1% w/v) like peptone, beef extract, yeast extract, ammonium nitrate, and ammonium phosphate. The media was incubated at 40 °C for 72 h in 150 rpm. Lipase activity was measured in cell free supernatant at the end of the incubation.

2.9.6 Effect of agitation on lipase production

The production media was inoculated with overnight grown selected bacterial culture. Then the media was incubated at various rpm in shaker from 50, 150, 200, 250 and 300rpm for 72h at 40 °C. After the incubation time period, the cell free culture broth was used to check the lipase

3.0 Results and discussion

3.1 Isolation and screening of the protease bacteria

The lipase producing bacteria were isolated and showed clear orange fluorescence zone around colony on rhodamine olive oil agar plate as shown in Fig.1. Out of 45 bacterial isolate 13 isolates showed lipase production by plate assay. Based on the highest fluorescence zone diameter (29mm), the best isolate G5 was selected for lipase activity assay (secondary screening). It was observed that the bacterial strain G5 showed maximum lipase activity on third day of incubation (16.34 ± 0.11 U/ml) by tritremetric assay

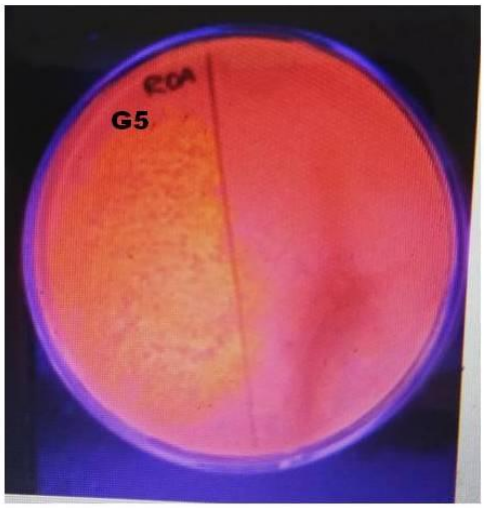

Fig.1: Fluorescence orange colour zone of bacterial isolate (G5) on rhodamine olive oil agar plate

3.2 Identification of lipase producing bacteria

The lipase producing bacteria was identified by the morphological, biochemical test and 16S rRNA sequencing. The morphological and biochemical result was listed in the Table 1. For the 16S r-RNA sequencing, the bacterial DNA was extracted by phenol-chloroform assay (Fig.2A) and it was amplified by PCR (Fig.2B). The amplified product was sent for sequencing in macrogen Korea. Based on the BLAST result it was matching with *Pseudomonas otitidis*. Therefore the isolated bacterium was *Pseudomonas otitidis* G5 which is represented by phylogenetic tree in Fig.3.

Table 1: Morphological and biochemical test of G5 isolate

Test/Character	Result
Gram staining	Gram negative rod
Motility	Motile
Catalase	Positive
Oxidase	Positive
Indole	Positive
Methyl Red	Positive
Voges Proskauer	Negative
Simmon Citrate	Positive

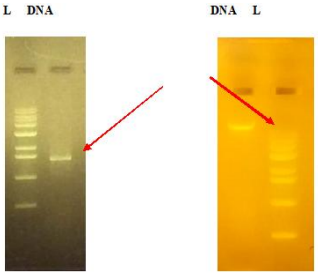

Fig.2A: Extracted DNA from the isolate G5 Fig.2B: PCR amplified DNA product

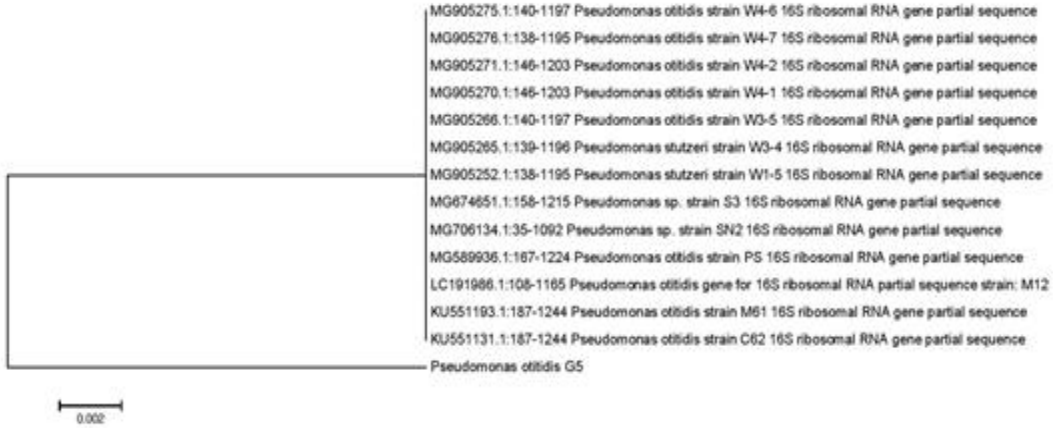

Fig.3: Phylogenetic tree of the isolate G5

3.3 Optimization of culture conditions for maximum lipase production

3.3.1 Effect of incubation time on lipase production

The lipase production medium was inoculated with overnight grown bacterial culture (G5). The production broth was kept in shaker over period of 24-168 hours (Day 1to Day7) at 37°C. At the end of incubation period the cell free culture broth (crude enzyme) was used for lipase activity. The results are presented in Fig.4.

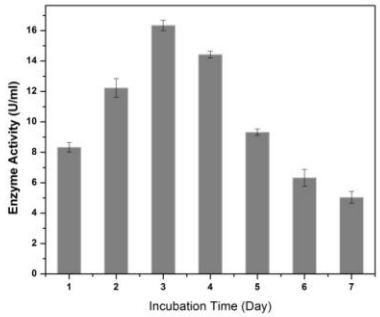

Fig.4: The effect of incubation time on lipase production

The outcomes represented in Fig.4 showed that *Pseudomonas otitidis* G5 produced lipase enzyme in a different level with the different time period of time. At the beginning of incubation time (Till 48h) the lipase production was not much significant. But after 72 hrs of incubation the lipase activity was remarkable (16.34 ± 0.34 U/ml). Thereafter the enzyme activity was gradually decreased. The highest lipase activity by *Pseudomonas otitidis* G5 has been observed on the third day of incubation (16.34 ± 0.34 U/ml).

3.3.2 Effect of temperature on lipase production

Temperature has a significant role in microbial growth. Every enzyme producing bacterium has its own optimum temperature for their growth and maximum enzyme production. In our study the enzyme activity was recorded while the bacteria were growing at different temperatures (20-60 °C) (Fig.5). The highest enzyme activity was observed at 40 °C (18.23±0.97 U/mL).

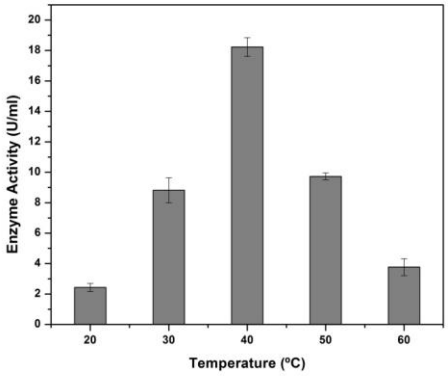

Fig. 5: Effect of temperature on lipase production

3.3.3 Effect of pH on lipase production

The result of the pH optimization study was depicted in the Fig. 6. Based on the result it was observed that the Bacteria *Pseudomonas otitidis* G5 showed maximum lipase activity at pH 8 (21.74±0.57 U/ml). It was noticed that there was a continuous increase in lipase activity from pH 4.0-8.0.Therafter, the lipase activity was started decreasing. A decline in the enzyme activity was observed at both lower and higher pH.

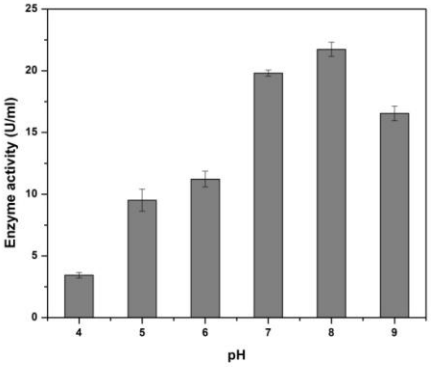

Fig. 6: Effect of pH on lipase production

3.3.4 Effect of carbon sources on lipase production

Results showed in Fig.7 clearly indicated that glucose is the best carbon source when compared to other carbon sources for the production of lipase. This may be due to the reason of water soluble nature of glucose. The highest lipase activity was 25.49±0.75 U/ml after 72 h of incubation at pH 8. The oils are insoluble in nature as a result the bacteria take up the oils very slowly as a carbon source for the production of lipase.

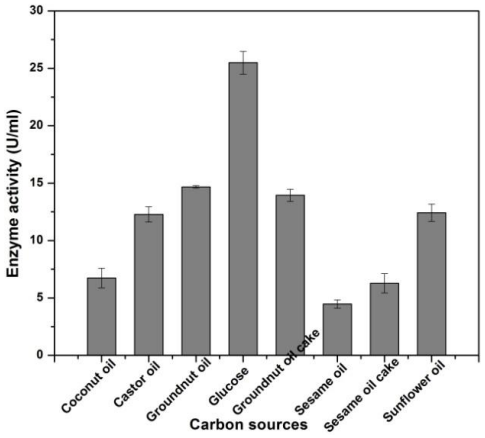

Fig. 7: Effect of carbon sources on lipase production

3.3.5 Effect of nitrogen sources on lipase production

Results presented in Fig.8 showed that among the various nitrogen sources tested, ammonium nitrate was found to be the excellent nitrogen source for the highest lipase production by *Pseudomonas otitidis G5* under previously optimized conditions. The highest activity was 29.86±0.45 U/ml by using ammonium nitrate as a nitrogen source. Basically ammonium nitrate acts as a very good inducer for lipase production.

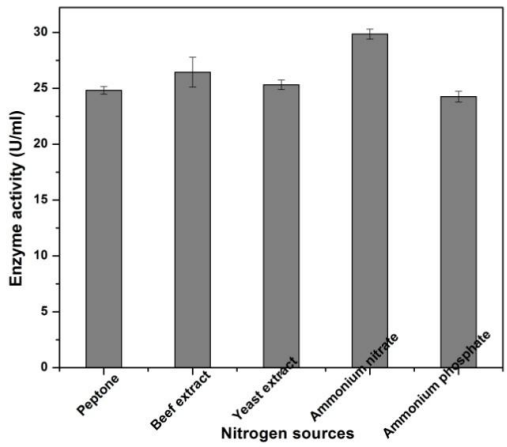

Fig. 8: The effect of nitrogen sources on lipase production

3.3.6 Effect of agitation on lipase production

The result of the agitation optimization study was depicted in the Fig. 9. Based on the result it was clearly observed that the *Pseudomonas otitidis* G5 showed maximum lipase activity at 200 rpm (34.78 ±0.55 U/ml). It was noticed that there was a continuous increase in lipase activity from 50-200 rpm.Thereafter the lipase activity was started decreasing. A decline in the enzyme activity was observed at both lower and higher rpm.

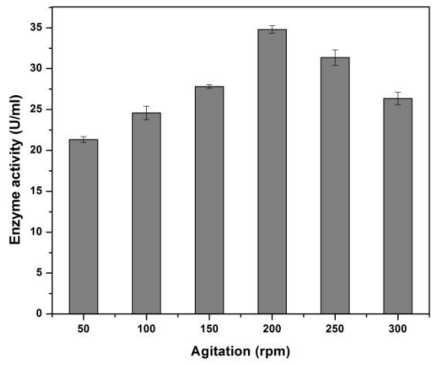

Fig. 9: The effect of agitation on lipase production

4. Conclusion

In this study the various fermentation conditions (Incubation time, Temperature, pH, Carbon sources, Nitrogen sources and agitation) were optimized in order to increase lipase production by *Pseudomonas otitidis* G5. The results showed that lipase production by *Pseudomonas otitidis* G5 was increased under optimized conditions with a mineral salt medium (MSM) containing glucose as carbon source, ammonium nitarte as nitrogen source, pH 8, at 40 ºC in 200 rpm for 72 h of incubation of culture broth. The various optimized parameters may be highly useful in future in the production of lipase in a large scale by *Pseudomonas otitidis* G5.

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