"Isolation of Lipase Producing Bacteria from Oil Contaminated Soil for the Optimisation and Production of Lipase by Solid State Fermentation"

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ABSTRACT

The soil samples were collected around different petrol bunks and tributyrin agar was used as selective media for isolation of lipase producing bacteria. A comparative study on the production of extra cellular lipase by Solid State Fermentation (SSF) using Pseudomonas aeruginosa with various substrates has been made. The maximum extracellular lipase activity was obtained with niger seeds used as a substrate, at an incubation period of 72 h, moisture content of the ratio 1:1, incubation temperature of 45^{o}C, inoculum size 1%, maltose of 5% as a carbon source, ammonium sulphate of 0.5% as a nitrogen source, castor oil of 2% as a inducer.

Keywords: Lipase, *Pseudomonas aeruginosa*, Solid state fermentation

INTRODUCTION

Lipases are the enzymes capable of catalysing the hydrolysis and synthesis of esters formed from glycerol and long-chain fatty acids. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Only about 2% of world's microorganisms have been tested as enzyme sources[1]. Lipases are the most widely used enzymes in organic synthesis and more than 20% biotransformations are performed with lipases. In addition to their role in synthetic organic chemistry, these also find extensive applications in chemical, pharmaceutical, food and leather industries. Promising fields for the application of lipases also include the biodegradation of plastics and the resolution of racemic mixtures to produce optically active compounds. These functions of lipases owe to their broad specificity for a wide spectrum of substrates, stability in organic solvents and enantioselectivity[2]. Because of huge Variation in applications, the availability of lipases with specific characteristics is still a limiting factor. Thus to search for new lipases with different characteristics and improve lipase production continue to be important research topics.

A wide variety of Gram positive and negative bacterial species are reported to produce lipase but the most widely used enzymes originate from genus *Bacillus* and *Pseudomonas.*

Recently, several reports have been published indicating the application of SSF in upgrading the food and industrial wastes and in the production of fine chemicals and enzymes. The utilization of by-products and wastes from food and industrial sources has several advantages over submerged fermentation such as superior productivity, simple techniques, reduced energy requirements, low wastewater output, improved product recovery and the reduction in production costs. This paper reports the production of lipase by SSF using *Pseudomonas*

aeruginosa, isolated from oil contaminated soil and optimisation of various production parameters.

METHODOLOGY

Sample collection

Soil samples were collected at 4-5 cm depth with the help of sterile spatula in a sterile plastic bag around different petrol bunks in and around Unkal, Hubli, Karnataka.

Screening of microorganism

The collected soil samples were serially diluted and the diluents of 10^{-6} were used for plating to get only lipolytic isolates. Samples were spread on agar plates containing (g/l): yeast extract 5.0, peptone 10.0, sodium chloride 10.0, agar 20.0, and tributyrine l%. The tributyrine was added to media after autoclaving when it reaches to temperature of 55^{o}C. After incubation for 24 h a colony showing maximum tributyrine hydrolysis zone was picked and this selected isolate was maintained on agar slant.

Identification of isolate

The strain identification of isolate was done by 16S DNA sequencing. The sequence obtained was analyzed by NBLAST and was submitted to Genbank.

Screening of substrates

Different agro wastes and oil seeds used as substrates such as rice bran, pigeon pea waste, wood bark, paddy husk, cashew nut, safflower seed, groundnut seed, coconut, mustard, sesame seed, linseed, cotton seed, soybean, almond, sunflower seed, niger seed. Nutrient broth media was used as moisturising agent containing (g/l): peptone 10, sodium chloride 10 , yeast extract 5 . 5 g of substrates were suspended in 5 ml of nutrient broth media in 250 ml flasks. They were autoclaved at 15 lbs pressure, 120^{o}C for 20 min and cooled before using. The prepared mix was inoculated with 0.5 ml 24 h old culture. After thorough mixing, all the flasks were incubated for 24 h. After a stipulated period 10 ml of tris-HCl buffer (pH-8) was added to the fermented matter and squeezed through a wet muslin cloth. The

pooled enzyme extract was centrifuged at 10,000 rpm for 3 min and clear supernatant was used for the assay. The substrate in which lipase with maximum activity was produced, was used for optimisation study and large scale production.

Lipase Assay

Lipase activity was assayed as described in literature[5]. The reaction mixture consisted of 0.5 ml of 10% tween in 50 mM tris HCl buffer (pH 8), 0.25 ml of $CaCl_2$ solution of 1 M, 0.05 ml of enzyme and 50 mM tris HCl buffer. The crude sample obtained was used for the assay. Reagent blank was prepared with buffer instead of $CaCl_2$ and substrate. This as enzyme incubated at 40^oC for 1 h. As a result of tween hydrolysis the fatty acids released during the reaction forms an insoluble fatty acid salt giving a precipitate and that can be measured spectrophotometrically at 400 nm. 1 unit of lipase activity was defined as that of the amount of enzyme which after 1 hour under the condition of the assay resulted in an increase at 400 nm of 0.01.

Estimation of protein

The protein estimation for all the enzyme extracts was carried out according to Bradford method using BSA as standard.

Optimization of medium parameters

Different parameters such as substrate selection, pH of the medium, inoculums size, incubation temperature, incubation time, effect of moisture content of substrate, effect of carbon and nitrogen source, effect of inducer were optimized.

Incubation period: Fermentation media was prepared in four flasks as described above and incubated for period of 24, 48, 72 and 96 h. After every period of incubation the contents of the flasks were harvested and assayed.

Moisturising agent: In nutrient broth which was used as moisturising agent, instead of three ingradients, combination of only two different ingradients was taken and fermentation was done and contents of flasks were harvested and assayed.

Mineral salt solution: In place of nutrient broth media as moisturising agent used

above, the five different mineral salt solutions, with detailed compositions (g/l) given below were used and fermentation was done and the contents of flasks were harvested and assayed.

Mineral salt solution 1- K_2HPO_4, 6.0; NaCl, 6.0; $MgSO_4.7H_2O$, 0.4; $CaCl_2$, 0.2.

Mineral salt solution 2- K_2HPO_4, 4.0; NaCl, 4.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1.

Mineral salt solution 3- K_2HPO_4, 2.0; NaCl, 1.0; $MgSO_4.7H_2O$, 0.1; $CaCl_2$, 0.05.

Mineral salt solution 4- K_2HPO_4, 2.0; NaCl, 1.0; $MgSO_4.7H_2O$, 0.05; $CaCl_2$, 0.03.

Mineral salt solution 5- K_2HPO_4, 1.0; NaCl, 0.5; $MgSO_4.7H_2O$, 0.05; $CaCl_2$, 0.02.

Moisture Content: The effect of moisture content on production of lipase was studied by varying the ratio of substrate to moisturising agent (1:0.5, 1:1, 1:2 and 1:5).

Incubation temperature: The influence of temperature on the production of lipase was studied by incubating the fermentation flasks at different temperatures (room temperature, 45^{o}C and 55^{o}C).

Inoculum size:

Flasks containing 5 g of substrate moistened with 5 ml of nutrient broth were autoclaved and inoculated with 1, 5, 10, 25 and 50% of 24 h old inocula. The inoculated flasks were incubated and then enzyme was extracted and assayed.

Effect of pH: At different pH of moisturising agent such as pH 5, 6, 7, 8 and 9, the fermentation and assay was performed in order to evaluate the effect of pH on the lipase production.

Effect of Additives

Carbon source: The effect of carbon source on the enzyme production was studied by adding different carbon sources of 1% (starch, maltose, dextrose, lactose, sucrose, fructose, xylose) to the fermentation media. The enzyme was extracted and assayed.

Concentration of maltose: The influence of concentration of maltose on the lipase production was studied by adding different concentration of maltose such as 0.5%,

1%, 2%, 2.5% and 5% to the fermentation media.

Nitrogen source: The role of different nitrogen source on lipase production was evaluated by adding different nitrogen source of 1% such as ammonium sulphate, ammonium nitrate, peptone, beef extract, potassium nitrate to the fermentation media.

Concentration of ammonium sulphate: The effect of concentration of ammonium sulphate was studied by adding different concentration of ammonium sulphate such as 0.5%, 1%, 2%, 2.5% and 5% to the fermentation media.

Effect of Inducer: The influence of inducer on lipase production was studied by adding different inducers at 2% (sunflower oil, groundnut oil, gingelly oil, castor oil, soybean oil, olive oil, coconut oil and mustard oil) to the fermentation media.

Concentration of inducer: By adding different concentration of optimised inducer such as 0.5%, 1%, 2%, 2.5% and 5% to the fermentation media, the optimum concentration of optimised inducer was found at optimum conditions of above parameters.

RESULTS AND DISCUSSION

Isolation and identification of lipase producing organism

On the basis of larger clear zone formationon on Tributyrin agar, the lipase producing organism was isolated (Figure 1). By 16S rDNA sequencing and NBLAST analysis, the isolated strain was found to *Pseudomonas aeruginosa* and the 16S rDNA of isolate was submitted to Genbank [Accession No-HQ658761]. This isolate was further subjected to SSF studies.

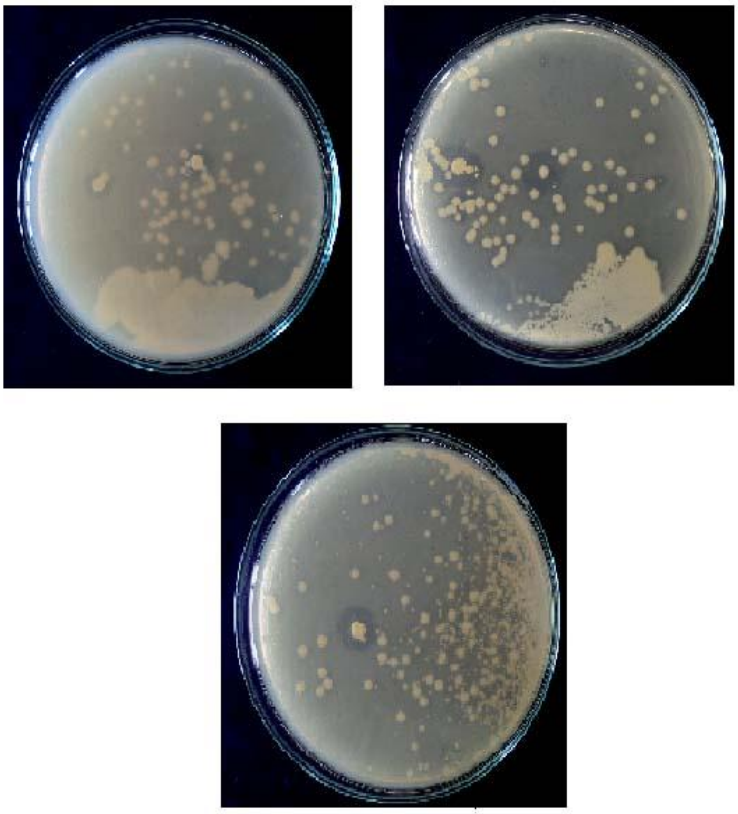

Figure 1: Tributyrin hydrolysis zone obtained by inoculating soil sample on Tributyrin agar media

Screening of substrate

The enzyme activity of *Pseudomonas aeruginosa* on different substrates is shown in figure 2. The different agrowastes used as substrates such as wood bark, pigeon pea waste, paddy husk, rice bran had given the negative results for lipase production perhaps due to insufficient oil content in the above agrowastes for the production of lipase. Therefore different oil seeds such as sunflower seeds, groundnut seeds, niger seeds, sesame seeds, safflower seeds etc were screened for lipase production. Among these oil seeds tested the niger seed produced better lipase.

Optimization of SSF Parameters

A low level of lipase activity detected in the earlier periods of incubation and enzyme activity reached a maximum level by 72 h. At longer incubation periods, the lipase activity decreased which might be due to the depletion of nutrients,

accumulation of toxic end products or loss of moisture (Figure 3).

The maximum production of enzyme was found when the substrate moistened with the moisturising agent containing only NaCl and yeast extract (1010 U/ml) when compared to the combination of peptone and yeast extract (398 U/ml) and peptone and NaCl (932 U/ml) in a 1:1 substrate-to-moisture ratio (Figure 4). Higher moisture would lead to decrease porosity, promotes development of stickiness and increases the chances of contamination.

The mineral salt solutions were used of composition mentioned earlier, have not given significant for lipase production using *Pseudomonas aeruginosa.* But mineral salt solutions found to be good moisturising agent for the production of xylanase from *Baccilus* species and α-amylase production by Bacillus licheniformis M-27.

Pseudomonas aeruginosa produced maximal level of lipase at 45°C (1020 U/ml) in an incubator when compared to room temperature (380 U/ml) and 55°C (180 U/ml) (Figure 5).

A lower inoculum may give insufficient biomass causing reduced product formation, whereas a higher inoculum may produce too much biomass leading to the poor product formation[10]. In our study, the maximum lipase activity (724 U/ml) was obtained with 1% inoculum size. Some researchers have used different levels of inoculum for lipase production employing different microorganisms. Maximum lipase production by *Yarrowia lipolytica* NCIM 3589, was achieved by 2.5% of inoculum[4]. An inoculum concentration of 1.07×10^8 spores/ 10 g of substrate was found to be optimal for lipase production by *Aspergillus niger*.

As pH is the important parameter required for the growth of bacterial culture in respective media so lipase activity got affected with basic pH, this indicates that suitable pH is responsible for bacterial growth in the media. The data obtained clearly indicates that there is a strong influence of pH on lipase enzyme

production. Thus the maximum activity was reported at pH 8. Similar result was found for the lipase production by *Bacillus subtilis*.

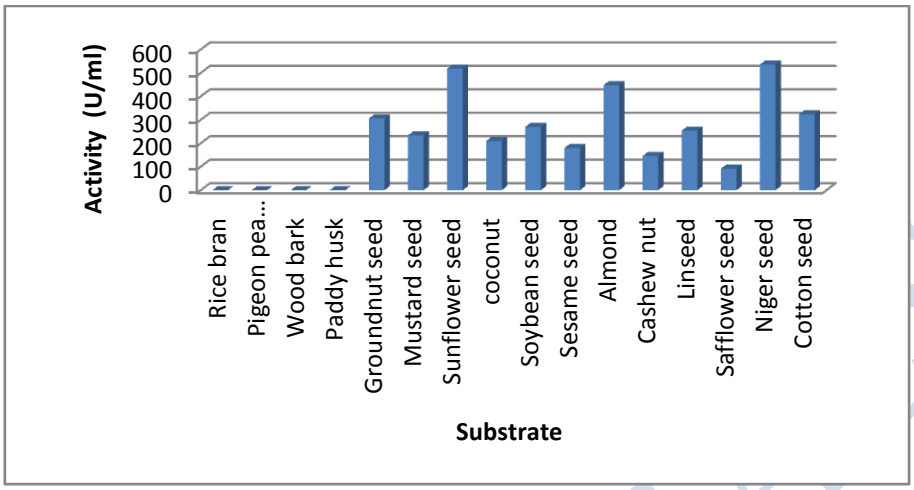

Figure 2: Effect of different substrates on lipase production

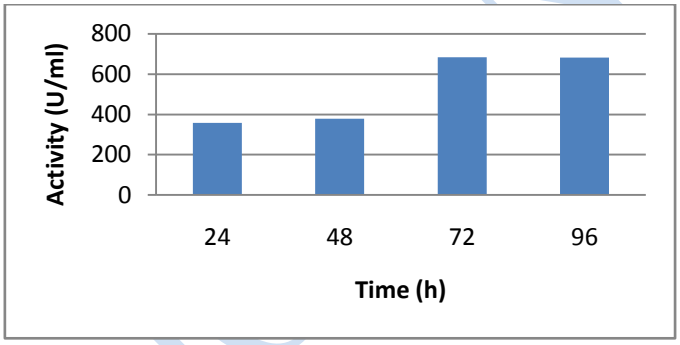

Figure3:Effect of incubation time on lipase production

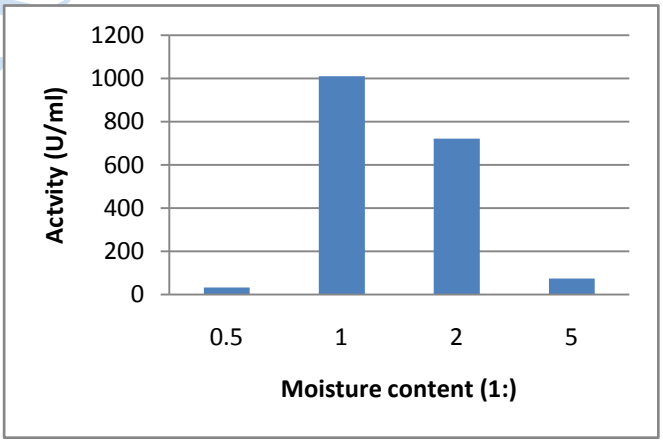

Figure 4: Effect of Moisture content on Lipase production

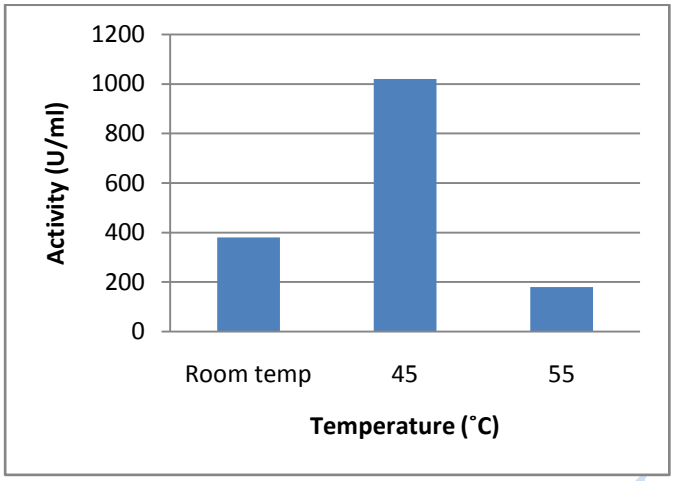

Figure 5: Effect of incubation temperature on lipase production

The imperative role of different carbon sources on lipase production by the *Pseudomonas aeruginosa* was elucidated by incorporating the selected carbon source (1%) to the substrate. Table 1 and 2 presents the results of different carbon sources and their concentration on lipase activity. Among all the carbon sources, maltose (5%) had better impact on productivity and among all the nitrogen sources, ammonium sulphate (0.5 %) yielded maximum lipase activity (Table 1 and 3). Similar result was found for lipase production by *Penicillium aurantiogriseum* using ammonium sulphate as nitrogen source[12].

Table 1: Effect of various additives on lipase production

Effect of carbon source		
Sl. No	Carbon sources	Activity [U/ml]
1	Starch	714
2	**Maltose**	**1128**
3	Dextrose	826
4	Lactose	702
5	Sucrose	778
6	Fructose	744

7	Xylose	722
Effect of nitrogen source		
8	**Ammonium sulphate**	**982**
9	Ammonium nitrate	704
10	Peptone	630
11	Beef extract	836
12	Potassium nitrate	518
Effect of inducer		
1	**Castor oil**	**1326**
2	Olive oil	846
3	Coconut oil	1082
4	Groundnut oil	426
5	Gingelly oil	838
6	Sunflower oil	236
7	Soybean oil	364
8	Mustard oil	688

Table 2: Effect of concentration of maltose on lipase production

Sl. No.	Concentration of maltose	Activity [U/ml]
1	0.1%	588
2	0.5%	638
3	**1%**	**924**
4	2%	760
5	5%	419

Table 3: Effect of concentration of ammonium sulphate on lipase production

Sl. No.	Concentration of ammonium sulphate	Activity [U/ml]
1	0.1%	284
2	**0.5%**	**1305**
3	1%	982
4	2%	564
5	5%	258

Table 4: Effect of concentration of castor oil on lipase production

Sl. No.	Concentration of castor oil	Activity [U/ml]
1	0.5%	426
2	1%	782
3	**2%**	**1341**
4	2.5%	1009
5	5%	836

Various oils have beneficial effects on lipase production and they act as an inducer for lipase production[13]. In the present study castor oil (2%) (Table 1 and 4) showed maximum enzyme production. *Aspergellus niger* produced maximum lipase in presence of 2% of olive oil as inducer in SSF medium containing wheat rawa as a substrate. An increase in oil amount did not correspond to an increase in lipase activity, but on the contrary, lipase production was inhibited. This inhibition was also observed during the production of an alkaline lipase by

Acinetobacter radioresistens in medium containing only olive oil or the mixture of olive oil and *n*-hexadecane.

With all the above optimised factors, the productivity of lipase was 1324 U/ml. The optimum factors in the SSF medium include niger seed with maltose (1%), ammonium sulphate (0.5%), castor oil (2%), nutrient broth as moisturising agent of pH 8 containing NaCl and yeast extract, incubation time of 72 h, incubation temperature of 45^{o}C, substrate-to-moisturising agent of ratio 1:1 and 1% inoculum.

CONCLUSION

The best conditions for lipase production by *P. Aeruginosa* were determined. The optimization of concentration of media components was done in order to increase more lipase production. Thus this study has proved that the optimization of growth parameters in a suitable solid-state medium has significant effect on improved production. Further research will focus on purification of lipase, biochemical characterisation and molecular study of lipase gene of *P. Aeruginosa.*

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