Optimization of Lipase Production by *Pseudomonas* spp. in submerged batch process in shake flask culture

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ABSTRACT

Extracellular lipase production by *Pseudomonas* spp a soil isolate was optimized for submerged batch process. The optimum time for lipase production was found to be 72hrs. Lipase production was enhanced when media was supplemented with mustard oil as carbon source and ammonium di-hydrogen phosphate as nitrogen source which was better supported lipase production than organic nitrogen sources tested. The lipase produced worked optimally at 50^0C, pH 8 and 15% Olive oil as substrate. In the assay instead of continuous pH readout mathematical calculations were used to calculate enzyme units.

Keywords: Lipase, *Pseudomonas*, mustard oil, olive oil, enzyme units.

INTRODUCTION

Lipases (E.C. 3.1.1.3) have emerged as key product of rapidly growing biotechnology industry. This enzyme has versatile applications by virtue of their unique properties. Lipases have been employed in a wide array of industrial applications, such as food technology, detergent, chemical industry and biomedical sciences (Gupta *et al,* 2004). Lipase catalyzes hydrolysis of lipids and liberates fatty acids and glycerol (Janeg Ilbertj *et al,* 1991)[1]. Additionally, they also catalyze the synthesis and transesterification of glyceride (Shuen-Fuh Lin *et al* 1996). Lipases are serine hydrolases which act at the lipid water interface. The catalytic triad is composed of Ser-Asp/Glu-His and usually also a consensus sequence (Glyx-Ser-x-Gly) is found around the active site serine. The three-dimensional structures of lipases reveal the characteristic α/β-hydrolase fold (Nardini *et al,* 1999).

Different genera of bacteria including *Streptomyces* spp. are known to produce lipase but *Achromobacter* spp, *Alcaligenes* spp, *Arthrobacter* spp, *Pseudomonas* spp and *Chromobacterium* spp have been well exploited for lipase production. But three important species of *Pseudomonas* i.e. *P. fragi*, *P. fluorescens* and *P. aeruginosa* have been extensively exploited (Ghosh *et al,* 1996). Microbial lipases have been exploited in the food and detergent industries (Pandey *et al,* 1999; Saxena *et al,* 1999), chemical and pharmaceutical industry owing to their activity in micro-aqueous media (Jaeger and Reetz, 1998).

The commercial exploitation of these enzymes requires their bulk production in a bioreactor. Lipase activity and production depend upon the composition of the fermentation medium (Kim *et al,* 1996). Major content of lipase production media is carbon source which also act as inducer for lipase production. Since microbial lipases are often inducible enzymes (Lotti *et al,* 1998). Whereas, nitrogen source and other media components regulate the growth of producer organism and thus the fermentation process (Rathi *et al,* 2002). There are many approaches for designing an ideal fermentation process. The traditional approach to the optimization problem is the one variable at a time method. In which all variables but one are held constant and the optimum level for this variable is determined. Using this optimum, the second variable's optimum is found, etc. (Henry *et al,* 1997). Despite certain disadvantages the simplicity of this method attracts workers. In the current communication we exploited same method for designing the simple media and process for lipase production by *Pseudomonas* spp.

Various methods of lipase assay have been classified as; Titrimetry, Interfacial tensiometry, Spectroscopy, Chromatography, Immunochemistry and Conductimetry (Frédéric *et al,* 2000; Kulkarni, 2002) of these methods titrimetry is the simplest method but needs continuous readout by pH meter; the so called *pH stat* method.

In this research paper we introduced mathematical calculation to determine micromolar concentration of product released; free fatty acids as a result of lipase action. As a result we could eliminate the need of pH meter for lipase assay. The modification is explained in material method section.

MATERIALS AND METHODS

Bacterial Culture: *Pseudomonas* spp. that produced greenish yellow fluorescent diffusible pigment (Fig 4) was used for lipase production. Its extracellular lipase production ability was checked by spot inoculating on tributyrin agar (Aaronson 1970) for 24 hrs at 30^0C.

Lipase Assay: Titrimetric method with modification was used. In usual assay method continuous pH readout is required instead of this we introduced mathematical calculations to calculate enzyme units and specific activity. The calculations are illustrated below. 10% Olive oil emulsion in 2% gum acacia was used as substrate. Reaction mixture composed of 0.5mL substrate emulsion, 0.4mL 0.1M Tris-HCl buffer (pH-7.2), 0.1mL lipase solution. In blank lipase solution was replaced with equal amount of distilled water. Reaction was carried out at 30^0C for 30min. Reaction was stopped by adding 2mL acetone. Liberated fatty acids were titrated with 0.05N NaOH using phenophthalene indicator. Amount of NaOH required to achieve end point (colorless to pink) was recorded. From this enzyme units and specific activity was calculated as follow,

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[NaOH required to titrate liberated fatty acids] = [NaOH required to titrate test sample] − [NaOH required to titrate blank]

The titration equation is (Bettelheim and Landesberg, 2000),
$N_1V_1 = N_2V_2$

Where, N_1 = Normality of liberated fatty acid solution (One normality unit = 10^{-3} µM), V_1 = Volume of reaction mixture, N_2 = Normality of NaOH solution (0.05N), V_2 = Volume of NaOH required to titrate (colorless to pink)
$N_1 = N_2V_2 / V_1$

One unit of lipase was determined as amount of lipase required to liberate one µM of fatty acids per minute under assay conditions. Specific activity was determined as enzyme unit per mg of total protein concentration. Protein concentration was determined by Biuret method (Harisha 2007).

Enzyme Unit (µM/min) = Fatty Acids Liberated (µM) / Time of Incubation (in min)

Specific Activity (µM.min^{-1}.mg^{-1}) = Enzyme Units / Protein Concentration

Optimization of fermentation conditions

Time course of lipase production: Mineral media ($NH_4H_2PO_4$ 0.1g, KCl 0.02g, $MgSO_4$ 0.02g, Yeast extract 0.3g, Olive oil 5ml, D/W 100mL, pH − 7.2) was used for time course optimization. 1mL of 24hr old *Pseudomonas* culture was inoculated in 100mL of mineral media and incubated on rotary shaker adjusted at 30^0C and 120rpm. After every 24hrs protein concentration and lipase activity was determined.

Effect of Carbon Source: Several carbon sources (Soybean oil, castor oil, vaanspati ghee, mustard oil, coconut oil, Safflower oil, sanflower oil, peanut oil, tributyrin, olive oil) were tested as substitute for olive oil in mineral media.

Effect of Nitrogen Source:
The media used for optimization of nitrogen source composed of mustard oil 5mL, Nitrogen source ($NH_4H_2PO_4$/ beef extract/ peptone/ yeast extract/ tryptone) 0.1g, KCl 0.02g, $MgSO_4$ 0.02g, D/W 100mL, pH − 7.2.

Characterization of lipase:
Optimum temperature, pH and substrate concentration for lipase action was determined. For temperature optimization the reaction mixture was exposed to various temperatures in the range 30, 40 to 80^0C. Effect of pH on lipase action was analyzed by substituting the buffer in reaction mixture with the different buffers for different pH [Acetate Buffer (0.1M) for pH − 3, 4, 5; Phosphate Buffer (0.1M) for pH − 6, 7, 8; Ammonia Buffer (0.1M) for pH − 9, 10]. In substrate optimization experiment olive oil (Substrate) emulsion of various percentages (3, 6, 9, 12, 15%) was used.

RESULTS AND DISCUSSION

Optimization of fermentation conditions:

The strain of Pseudomonas used showed maximum lipase production after 72 hours of incubation. The specific activity of lipase at this point of time was found to be 0.052 $\mu M.min^{-1}.mg^{-1}$ of protein. Further incubation was found to be negatively affecting the yield (Table 1). Similar results were obtained by Gerritse *et al*. in their studies on *Pseudomonas alcaligenes* but its lipase activity was even at 96hrs of incubation (Gijs Gerritse *et al*., 1998). The mustard oil was found to be far better stimulant for lipase production than other carbon sources tested. The lipase yield in media supplemented with mustard oil was 0.276 $\mu M.min^{-1}.mg^{-1}$ of protein. Whereas, soybean oil completely inhibited liapse production (Table 2). Sirisa *et al*. also found mustard oil better stimulant than sunflower oil but poorer than peanut oil, olive oil and coconut oil (Sirisha *et al.*, 2010). The hydrogenated form of vegetable oil (vanaspati ghee) very poorly stimulated lipase production. In the tested nitrogen sources the inorganic nitrogen source Ammonium di-hydrogen phosphate was well suited than organic nitrogen source (Table 3). And peptone was found to be best of the tested organic nitrogen sources. This result is in agreement with the finding of Sirisa et al, according to their studies lipase production by Staphylococcus was better when peptone was used in place of yeast extract and tryptone as nitrogen source (Sirisha *et al*, 2010).

Table 1: Time course of lipase production	
Incubation Time (In Hours)	Lipase activity ($\mu M . min^{-1}. mg^{-1}$)
24	0.014
48	0.020
72	0.052
96	0.020

Table 2: Effect of Carbon Source on Lipase Production	
Carbon Source	Lipase activity ($\mu M . min^{-1}. mg^{-1}$)
Mustard Oil	0.276
Peanut Oil	0.123
Safflower Oil	0.114
Olive Oil	0.085
Tributyrin	0.085
Coconut Oil	0.085
Sunflower Oil	0.057
Castor Oil	0.053
Vanaspati Ghee	0.010
Soybean Oil	0

Table 3: Effect of Nitrogen source on Lipase Production	
Nitrogen Source	Lipase activity ($\mu M . min^{-1}. mg^{-1}$)
$NH_4H_2PO_4$	0.223
Peptone	0.123
Beef Extract	0.114
Yeast Extract	0.085
Tryptone	0.053

Characterization of lipase

The enzyme worked optimally at slightly higher temperature; 50^0C, at which lipase activity was 0.357 μM.min^{-1}.mg^{-1}. At still higher temperatures the specific activity was reduced drastically may be due to denaturation of enzyme molecules (Fig 1). Similarly pH also affected lipase action. Lipase worked better in slightly alkaline pH (8). The specific activity at this pH was 0.276 μM.min^{-1}.mg^{-1} near about equal activity was obtained at pH 7. Acidic and extreme alkaline pH negatively affected lipase action (Fig 2). The lipase of used Pseudomonas strain was found to working at 15% substrate concentration, till this point the specific activity was steadily rising (Fig 3). At 15 % concentration the specific activity remain unchanged from 0.223μM.min^{-1}.mg^{-1}. Gilbert et al reported in their studies that lipase of *Pseudomonas aeruginosa* EF2 worked optimally at 50^0C and pH 8.5 (Janeg Ilberta *et al* 1991).[2]
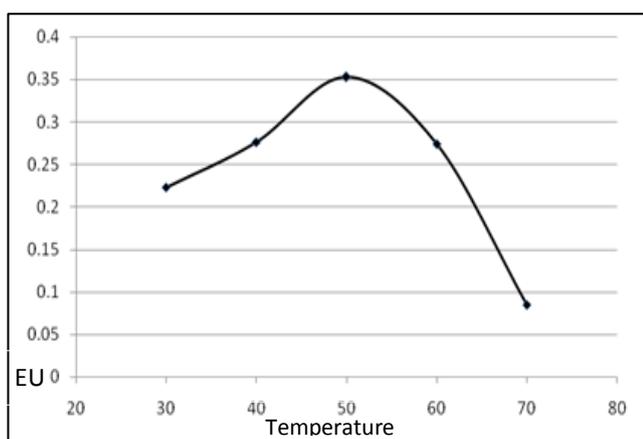

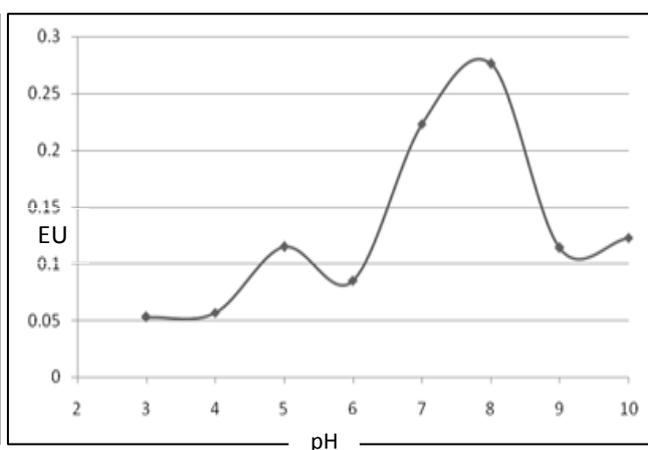**Fig 1: Effect of temperature on lipase activity.**

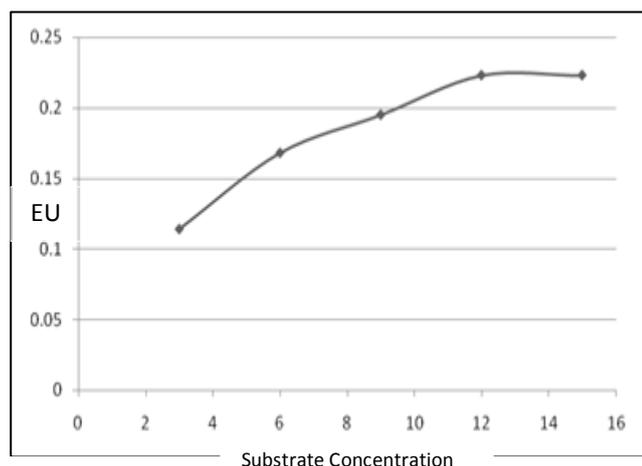**Fig 2: Effect of pH on lipase activity.**

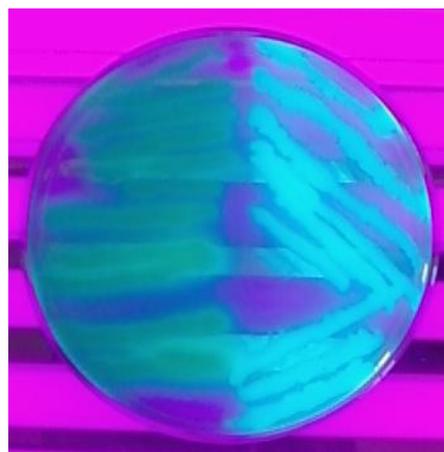**Fig 3: Effect of substrate concentration on lipase activity.**

Fig 4: Greenish yellow fluorescent pigment production (right) by Pseudomonas spp

CONCLUSION

Laboratory scale optimization of some of fermentation conditions for lipase production by Pseudomonas spp in submerged batch process was carried out. The optimized fermentation media composed of mustard oil 5%, $NH_4H_2PO_4$ 0.1%, KCl 0.02%, $MgSO_4$ 0.02%, pH 7.2. In this media the time of single batch was 72hrs. The inorganic nitrogen source is better stimulant for lipase production rather than organic nitrogen sources. The used strain of Pseudomonas used wide range of carbon sources but the best one as our finding is mustard oil. The lipase produced is moderately alkalotolerant and also could work in elevated temperature. The modification of titrimetric lipase assay method is good option for pH stat method and also circumvents the need continuous pH readout by pH meter but works with similar efficiency.

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