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STUDIES ON THE LIPASE PRODUCTION BY *ASPERGILLUS NIGER* THROUGH SOLID STATE FERMENTATION

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Abstract

The current study includes isolation and screening of different fungal cultures from soil samples for lipase production by solid state fermentation. Seven different lipolytic fungal strains were isolated and screened for the purpose. These isolates were identified on the basis of morphological and microscopic studies. Among these fungal strains, *Aspergillus niger* gave best reproducible results and hence selected for further studies. Oil substrates were optimized and maximum lipase activity of 5.12±0.059 U/ml was observed using olive oil as substrate in a medium moistened with a diluent containing (g/ml): $MgSO_4$.7H2O, 0.05; KH_2PO_4, 0.1; KCl, 0.05; ; $FeSO_4$, 0.001; glucose, 0.8 and peptone, 2.0 (pH 6.5). Maximum lipase activity was observed for an incubation period of 72 hrs at 30ºC.

Keywords: *Aspergillus niger,* Lipase, Solid state fermentation, Optimization.

Introduction

Lipase (Triacylglycerol Lipase; EC 3.1.1.3), is a subclass of esterases and catalyzes the hydrolysis of triacylglyceroles and convert them to diacylglycerols, monoacyglycerols, fatty acids and glycerols (Thomoson *et al.,* 1999; Veeraragavan, 1990). During hydrolysis a lipase cleaves the acyl group from glycerides and transfers it to the OH. In non-aqueous conditions, lipases can transfer acyl group to nucleophiles other than water (Martinelle & Hult, 1995). There are region specific and non-specific lipases; region-specific as their name suggests acts on specific positions on lipid molecule while non-specific lipases can catalyze reaction at all positions (Sonnet & Gazzilo, 1991).

For last few decades, due to their vast usage as flavor enhancers in various foods, in preparation of medicinal digestive enzymes, diagnostic reagents and detergents, and in biosynthesis of biopolymers and biofuel, interest has been remarkably increased in lipases (Pandy, 1999). Region-specific lipases are found to be having vast industrial applications which includes flavor enhancement by lipid removal for fish, meat, dairy, bakery, alcoholic beverage products. Moreover, lipases also have applications in biosynthesis, lipases are involved in more than 20% biotransformations (Giltesen *et al*., 1997). Promising fields for the application of these enzymes include plastic biodegradation i.e. polyhydroxyalkanoates (PHA) (Gombert *et al*., 1999) and resolution of racemic mixtures for the production of optically active compounds (Muralidar *et al*., 2001).

Solid state fermentation has remarkable potential for lipase enzyme production and this method has been proven to be an effective enzyme producing method, especially by filamentous fungi because the organism is provided with an environment resembling their natural habitats (Durand, 2003; Pandey *et al*., 1999). SSF is preferred because it holds several advantages over submerged fermentation system, which includes productivity in high volume, less effluent generation, relatively high product concentration and simple fermentation requirements (Mitra *et al*., 1994). However SSF is a feasible substitute used for cost effective enzyme production at industrial level (Hoelker *et al*., 2004), due to availability of agricultural and industrial growth substrate residues. These substrates have been used for enzyme production and antibodies through fermentation (Sumitra *et al*., 2007). Hence, Solid state fermentation proved to be an economical for various enzyme production including lipases and esterases (Hoelker *et al*., 2004).

Different substrates including wheat bran, maize bran, gram bran, rice husk, rice straw, wheat straw, sugar cane bagasse, grapevine trimming dust, coconut coir pith, corncobs, tea waste, banana waste, aspen pulp, rapeseed cake, mustard oil cake, peanut meal, corn flour, wheat flour cassava flour, steamed willow, steamed rice and starch have been used for solid state fermentation (Selvakumar *et al*., 1998).

Fungi are the best producers of lipase enzyme among all microorganisms, especially for food industry. Lipase producing strains through solid state fermentation are *Penicillium camembertti* (Ortiz-vfazquez *et al*., 1993), *Penicillium restrictum* (Leal *et al*., 2000; Palma *et al*., 2000), *Penicillium simplicissimum* (Diluccino *et al*., 2004), *Rhizopus sp.* (Macedo *et al*., 2003), *Rhizomucor meihei, Rhizomucor pusillus* (Uvarani *et al*., 1998; Cordova *et al*., 1998), *Rhizopus rhizododiformis* (Cordova *et al*., 1998), *Mucor meihei* (Ortiz-vazquez *et al*., 1993), *Mucor racemosus* (Bogar *et al*., 2003) and *Yarrowia lipolytica* (Dominguez *et al*., 2003), *Aspergillus niger* (Mahadik *et al*., 2002; Olama *et al*., 1993; Kamini *et al*., 1998). *Aspergillus niger* is found to be among well-recognized producers of lipase enzyme and enzyme obtained from this fungi is preferred in many industrial processes (Macris *et al*., 1996; Mala *et al*., 2007).

Several factors such as substrate, moisture and particle size play a major role in enzyme production. More surface area is provided by smaller sized particles for microbial attack (Pandey & Ashok, 1992). Several environmental factors such as pH, temperature, lipid source, agitation, nitrogen and dissolved oxygen concentration are also important in lipase production (Nahas & Gen, 1988). Availability of lipids and triglycerides enhanced lipase production (Suzuki & Takahiro, 1988). Presence of free fatty acids, bile salts, hydrolysable esters and glycerols also stimulate the lipase production (Lawrence *et al*., 1967).

Materials and Methods

Isolation of lipolytic fungi: Several soil samples taken from different sources were processed for the isolation of lipolyitc fungi using a selective medium containing 10% soil extract, 1.5% agar and 0.02% urea. To prepare soil extract sieved soil sample (100g) mixed with 0.2g $CaCO_3$ was taken and then water added with continuous stirring and final volume raised to 1000ml. Muslin cloth was used for mixture filtration and clarified by centrifugation at 6000 rpm for 30 min. In 10 ml of soil extract 0.02g urea and 5g agar was added and final volume raised to 100 ml and mixed by shaking. Then After preparing the medium flasks were plugged and sterilized. After sterilization medium was allowed to cool upto 40°C and then poured into petri plates along with a few drops of chloramphenicol and allowed to solidify.

Soil suspension (0.5ml) was poured (10^{-4} to 10^{-6}) on media containing petri plates and incubated at 30°C for 3-4 days. Fungal colonies obtained after incubation were transferred onto PDA slants and incubated for 3-4 days at 30°C. After incubation period slants were stored at 4°C and transferred the culture to new PDA slants, for maintenance of cell viability, every two weeks.

Solid state fermentation (SSF): In 250ml flask 10g of substrate (wheat bran) was taken and 10ml of diluents and 2ml of Tween 80: olive oil (1:1) emulsion was used for moistening. To homogenize the mixture flasks were agitated and autoclaved for sterilization. After sterilization flasks were allowed to cool down and then inoculated with 1.0ml of spore inoculum from 5-7 days old slant. Then flasks were incubated with shaking twice a day for mixing for 72h at 30 ± 1˚C. All experiments were performed in triplicates.

After 72 hrs incubation, phosphate buffer (40 ml) was added and flasks were kept in shaker for 30 min. After incubation contents of the flask were filtered using muslin cloth or filter paper followed by centrifugation at 10,000 rpm for 15 min. Supernatant was taken to calculate the lipase activity as lipase was present in supernatant.

Lipase assay: Lipase activity was calculated titrimetrically on the basis of oil hydrolysis. Olive oil emulsion method was employed for the estimation of lipase activity in fermentation broth. In a conical flask oil emulsion (1.0ml), 0.5ml phosphate buffer (0.5ml) having pH 7.0 and enzyme (0.5ml) mixed and incubated for 15 min at 37°C. Two ml of ethanol: acetone (1:1) was used for reaction terminattion. NaOH (0.01N) was used to calculate the amount of lipase released, phenolphthalein was used as indicator. End point was light pink color.

One unit (U) of lipase activity was defined as the amount of lipase which yields 1.0 µ mole fatty acids per min per ml under assay conditions (Arima *et al*., 1972).

$$\text{Lipase activity} = \frac{\Delta V \times N \times 1000}{V\ (\text{sample}) \times 60}$$

where $\Delta V = V2 - V1$
V1 = volume of NaOH used against control flask
V2 = volume of NaOH used against experimental flask
N = NaOH normality
V (sample) = Sample volume

Diluents: Different diluents were used for moistening the substrate for lipase production by *Aspergillus niger*. These include:

D1 (g/l): NH_2PO_4, 6.0; $(NH_4)_2SO_4$, 5.0; $MgSO_4.7H_20$, 3.0; KH_2PO_4, 2.0; $CaCl_2$, 3.0
D2 (%w/v): KH_2PO_4, 0.05; $CaCO_3$, 0.5; $MgSO_4.7H_2O$, 0.025; yeast extract, 0.1; soluble starch, 2.0;
D3 (g/l): Na_2HPO_4, 12.0; $MgSO_4.7H_2O$, 3.0; KH_2PO_4, 2.0; CaCl2, 0.25
D4. (g/l): KCl; 0.5; KH_2PO_4, 1.0; $FeSO_4$, 0.01; peptone, 20 (pH6.5); glucose, 0.8
D5 (%w/v): $(NH_4)_2SO_4$, 0.9; $MgSO_4$, 0.2; KH_2PO_4, 0.5
D6 : Water

Results and Discussion

Screening of microorganisms: About seven 'fungal cultures including three stock cultures (*Aspergillus niger, Rhizopus oryzae and Mucor meihei*) were screened for the lipase production. *Aspergillus niger* was found to be the best producer with maximum lipase production of 5.12± 0.059 U/ml in camparison to other microbes which gave comparatively less enzyme production. Minimum lipase activity (0.89± 0.096 U/ml) was calculated in *Rhizopus oryzae Aspergillus niger* is reported by Olama *et al*. (1993) and Mala *et al*. (2007) as well-recognised producer of lipase through solid state fermentation. Therefore, *Aspergillus niger* was selected for lipase production using solid state fermentation.

Table 1. Screening of microorganisms for the lipase production by solid state fermentation.

Sr. No.	Microorganisms	Lipase activity (U/ml/min)
1	Isolate I	1.13± 0.081
2	Isolate II	1.56± 0.071
3	Isolate III	2.44± 0.092
4	Isolate IV	2.98± 0.056
5	*Aspergillus niger*	5.12± 0.059
6	*Rhizopus oryzae*	0.89± 0.096
7	*Mucor meihei*	3.44± 0.064

The values are mean of three replicates and differ significantly ($p \leq 0.5$). ± indicates the standard deviation from the mean.

Diluent Selection: To moisten the fermentation substrate used for the production of lipase using *Aspergillus niger*, diluents ranging from D1 to D6 were used. D4 containing (g/l) magnesium sulfate ($MgSO_4$), 0.5; dihydrogen potassium phosphate (KH_2PO_4) 1.0; ferrous sulfate ($FeSO_4$), 0.01; potassium chloride (KCl).0.05; glucose, 0.8 and peptone, 20 (pH6.5) gave the maximum production while all the other diluents did not support good enzyme production.

Maximum production of lipase by *Aspergillus niger* was obtained when D4 was used as moistening agent might be due to the accessibility of growth favoring nutrients. Ingredients of D4 include glucose, peptone, and ferric sulfate, a most important growth ingredient and potassium chloride that acts as a fungal growth enhancer. These ingredients were not present in any other diluents.

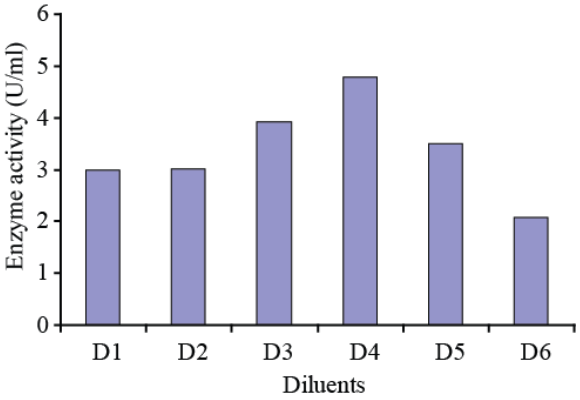

Fig. 1. Diluent selection for the lipase lipase by *Aspergillus niger* through solid state fermentation. Error bars are too small to visible.

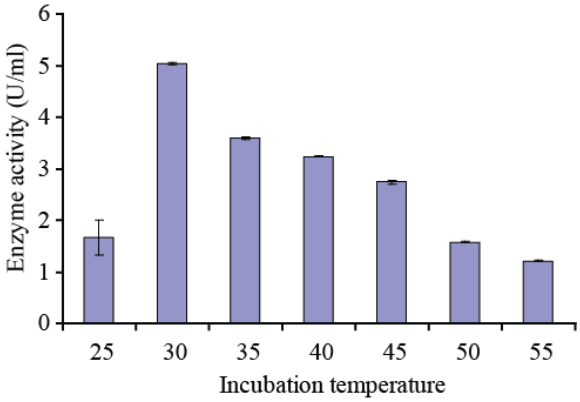

Fig. 2. Effect of temperature on production of lipase by *Aspergillus niger* through solid state fermentation.The values are mean of three replicates and differ significantly ($p \leq 0.5$).

Effect of temperature: Effect of temperature was investigated for the estimation of lipase production by the selected strain of *Aspergillus niger* and a temperature range of 25-55˚C was selected for experimentation. Flasks having olive oil and wheat bran along with D4 diluent were incubated at different temperatures after inoculating with *Aspergillus niger* spores. At 30^{o}C maximum production was obtained while at other temperatures a comparatively less production of lipase was obtained.

Enzyme activity was low at 25˚C but it steadily increased and maximum activity was calculated at 30˚C (4.95 ± 0.02 U/ml) and then decreased above 30˚C and reached minimum at 55˚C (1.80 ± 0.03 U/ml). Enzyme production increases with increase in temperature but at very high temperature, enzyme activity decreases due to denaturation of enzyme. Lipase producing strains of *Aspergillus niger* which have shown their activity between a range of 40-55˚C have been reported (Kamini *et al*., 1998; Namboodiri *et al*., 2002).

Effect of incubation period: Production of lipase was calculated at different incubation periods ranging from 24-120 hr. Fermentation flasks were incubated at 30oC for different time intervals by inoculating with *Aspergillus niger* spores. As depicted in the fig. 3.0 in the start of the process the graph move steadily as the enzyme activity was low which then increased gradually and reached at maximum at 72 hrs and then started decreasing after 96 hr. The activity of lipase enzyme produced by *A. niger* after 72 hr was 4.98 ±0.0543 U/ml. Thus, according to the results shown in graph optimum time for maximum production of lipase in *Aspergillus niger* was 72 hr.

As the incubation period increased biosynthesis of lipase was also increased and reached at maximum at 72 hr and when the incubation period further increased production started decreasing gradually after 96 hr. This might be due to the continuous decrease in availability of nutrients to the microorganism and accumulation of by products and enzyme proteolysis. Best yield (13U/ml) of lipase from *Penicillium aurantiogriseum* after 72 hr was reported by Lima *et al.* (2006).

Substrate selection: For the lipase production various oil substrates such as mustard oil, olive oil, coconut oil, castor oil, sunflower oil and almond oil were tested. Flasks containing wheat bran and some other substrates along with D4 were incubated at 30^{o}C by inoculating with *Aspergillus niger* spores. The result showed that maximum lipase production (5.75 ± 0.031 U/ml) was observed when olive oil was used while minimum lipase activity was obtained (0.98±0.01 U/ml) when coconut oil used. Cordova *et al.* (1998) also reported the lipase production during solid state fermentation using olive oil as lipid' substrate by *Rhizomucor pusillus.*

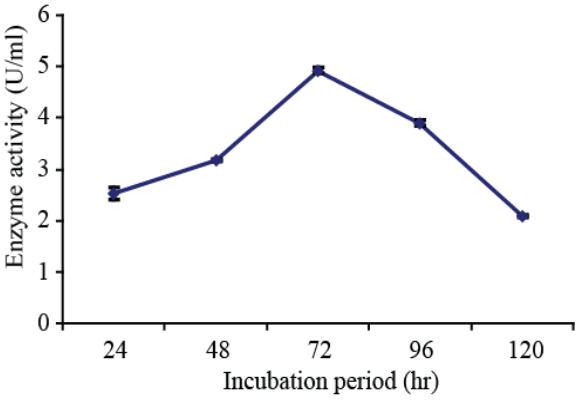

Fig. 3.Effect of incubation period on lipase production by *Aspergillus niger* in solid state fermentation. The values are mean of three replicates and differ significantly ($p \leq 0.5$).

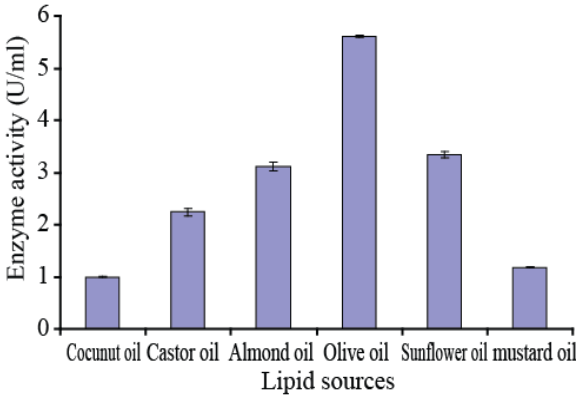

Fig. 4. Effect of lipid substrate for lipase production by *Aspergillus niger* in solid state fermentation. The values are mean of three replicates and differ significantly ($p \leq 0.5$).

Conclusion

Present study concludes that *Aspergillus niger* was a good producer of lipase during solid state fermentation. The production of enzyme was enhanced by optimizing some fermentation parameters. The productivity can be further improved by optimization of some other parameters.

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