Studies on lipase productivity of *Pseudomonas aeroginosa* and *Staphylococcus aereus* using cheap substrates

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SUMMARY

In the present study the lipase-producing organisms were isolated from the soil sample using olive oil containing medium. The isolates were identified based on the morphological and biochemical characteristics. The isolated *Pseudomonas aeroginosa* and *Staphylococcus aereus* were inoculated into inoculum medium and incubated at 37°C for 24hours used as the inoculum. The inoculums mixed with fermentative substrate like molasses and soybean, after incubation, the lipase was estimated. High lipase activity was observed in *Pseudomonas aeroginosa* inoculated medium. Maximum productivity was noted in pH 7 and temperature 35-40°C. Lipase productivity was maximum in the immobilized cell (0.49± 0.11µ/ml/min).

Key words : *Pseudomonas aeroginosa, Staphylococcus aereus,* Lipase

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Lipase has become one of the prominent industrial enzymes for its specificity in hydrolysis and interesterification. It catalyzes both the hydrolysis of triglycerides and the synthesis of esters from glycerol and long chain fatty acids (Jager and Reetz, 1998). In addition, it also serve as biocatalyst for alcoholysis, acidolysis, esterification and aminoacids (Pandey *et al*., 1999). Lipase is produced by various microbes, such as bacteria, fungi, yeast, and also in the pancreas of mammals, like pigs and humans. It has also been reported in higher plants, such as castor bean **(***Ricinus communis*) and rapeseed (*Brassica napus*) (Hellyer *et al*., 1999).

Numerous lipases have been characterized and efforts have been made to improve their stability in organic solvents for varied applications (Hung *et al*., 2003; Soumanou, and Bornscheuer, 2003). The most important commercial use of lipases are added to 13 billion tons of detergents produced every year (Jager and Reetz, 1998). Lipases are also emerging as important enzymes in the field of biopolymers. They are used in the synthesis of polymers (Gross *et al*., 2001). Immobilized *Pseudomonas fluorescence* lipase has been used for the production of bio diesel fuel from triglycerides and alcohols (Iso *et al*., 2001).

Transesterification of oils catalyzed by lipase have fuel (Fukuda *et al*., 2001). Use of organic solvents in transesterification reactions by lipase in producing methyl esters from sunflower oil showed improved conversion (Soumanou and Bornscheuer, 2003). Another major industrial application of lipases is in resolving racemixtures (Kamal *et al*., 2002; Paloma *et al*., 2003; Shibatani *et al*., 2000).

Optimization of the enantioselective resolution reactions in various bioreactors, like biphasic enzyme membrane reactors (Sakakai *et al*., 2001) and packed bed reactors (Sanchez *et al*., 2000) which favours large-scale production. Applications of lipases also extend to the field of waste management and improving tanning technique (Benjamin and Pandey, 1997) and in separation, which are difficult-to-separate mixtures of organic acids (Dai, 2000).

MATERIALS AND METHODS

Enrichment of lipase producing microorganism:

One gram of soil sample was suspended in 10ml sterile water. After shaking, 5ml suspension was added in 250ml Erlenmeyer flask containing 25ml of Enrichment medium. The medium was incubated at 30°C on a rotator shaker at 200 rev. /min for 3-5days, then aliquot was transferred to fresh medium and cultured again under the same condition. The above incubation and transfer operation were repeated for 5 to 6 times until microbial cells in the culture became nearly uniform (same were periodically observed under microbes). The enrichment cultivation on olive oil was carrier out on the assumption that microorganisms capable of growth on olive oil are capable of

producing lipases; and high alkaline pH was employed on the assumption that only bacteria could grow under this condition.

Isolation and screening of lipase producing microorganism:

The microorganism in the enrichment culture was isolated on nutrient agar plates containing olive oil (2.5%) and Victoria Blue B (4mg/100ml) with an initial pH of 9.5-10.0. Growing colonies with blue colour zone were isolated and subsequently screened by the double layer tributtyrinr agar method of colony with large clear zone were collected. Subsequent screening tests were then made recording lipase fermentation activity. Morphological and biochemical characteristics of bacteria were done by Gram staining, Motility, shape, indole test, methyl red test, voges proskaeur test, citrate utilization test, oxidase test, catalase test, starch hydrolysis test, gelatin test, sucrose, dextrose, xylose, galactose, maltose and mannitol test.

Substrate used for lipase productivity:

In this study molasses and soybean extract was used as a medium for fermentation the best solid substrate achieved by this step was fixed in subsequent experiment.

Preparation of inoculam:

Agar slant of pure culture was used as source of preparing inoculum. Erlenmeyer flask (250) containing 50ml of medium incubated at 35°C in an incubator shaker at 250 rpm for 16hrs.

Lipase fermentation:

One loop from the nutrient agar slant was inoculated in 30ml nutrient broth medium and incubated at 30^0C on the rotatory shaker at 150 rev/min for 24h. 1ml of this pre-culture fluid was inoculated in 250 ml Erlenmeyer flask containing 100ml of each substrate medium with initial pH 7. The mixture was incubated in rotary shaking for 72 hrs, the lipase activity of the culture broth supernatant (centrifuged at 3000 rev/min for 20min) was measured.

Assay of lipase:

Modified protocol of Elad *et al*. (1982) was followed for the estimation of lipase secreted by the antagonists in culture. Crude enzyme (2ml) was diluted with 8ml of distilled water and mixed well with 500ìl of vegetable oil. This reaction mixture was incubated at 37°C for 2h in a rotary shaker (200rpm). Ethanol was added to it to get a final concentration of 30%. Free fatty acids were extracted with 25ml of pure petroleum ether and the extract was evaporated in a rotary evaporator. The free fatty acids were dissolved in 15ml of neutralized ethanol containing phenolphthalein at 60°C. Each sample was titrated with ethyl alcohol containing 0.5 N NaoH. Free fatty acid was neutralized and one lipolytic unit (LU) was defined as micromoles of NaOH /mg portion/h.

Optimization of enzyme productivity:

pH vs productivity:

Optimized initial pH of the basal medium the pH aqueous solution varied from 6.0 to 11.0 within HCl or IN NaOH. The fermentation was carried out at 37°C to study the effete on enzyme production.

Temperature vs productivity:

The fermentation was carried out at various temperatures such as 25°C, 30°C, 37°C and 45°C to study their effect on enzyme production keeping all other conditions at the optimum level.

Immobilization of Pseudomonas aeroginosa and Staphylococcus aerues strain in sodium alginate:

Entrapment of cells in non-toxic alginate is one of the simplest, cheapest and most frequently used of immobilized (Kierstan and Bucke, 1977). Sodium alginate and calcium chloride were used to prepare the alginate beads containing the whole cells. Sodium alginate solution (3%wt/vol) was prepared by dissolving sodium alginate in 100 ml hot water. The contents were stirred vigorously for 10 minute to obtain thick uniform slurry without any undissolved lumps and then sterilized by autoclaving. Both alginate slurry cell suspensions (equivalalent to 0.03 gm dry cell weight) were mixed and stirred for 10 minute to obtain a uniform mixture. The slurry was taken into a sterile stringe, added drop-wise into 0.2m $CaCl_2$ solutions from 5cm height and kept for curing at 4°C for 1 hour. The cured beads were washed with sterile water 3 to 4 times. When the beads are not used, these are preserved in normal saline solution in a refrigerator (Farid *et al*., 1994).

RESULTS AND DISCUSSION

Isolation and screening of lipase producing microorganisms:

After 24h incubation, plates were observed for lipase producing colonies based on the clear zone formed around the growth. Growing colonies with blue colour zones were isolated and subsequently screened by double layer tributrin agar method.

Morphological and biochemical characteristics of bacterial isolate:

The screened two colonies were identified by morphological and biochemical characteristic and the results (Table 1) were compared with Bergey's bacteriological classification and the S_1, S_2 were named as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively.

Table 1 : Morphological and biochemical characteristics of bacteria

Sr. No.	Morphological and biochemical characters	*Pseudomonas aerogenosa* (S_1)	*Staphylococcus aureus* (S_2)
1.	Gram staining	'-'Ve	'+'Ve
2.	Motility	'+'Ve	'-'Ve
3.	Shape	Rod	Coccius
4.	Indole test	'-'Ve	'-'Ve
5.	Methyl red test	'-'Ve	'+'Ve
6.	Voges proskaeur test	'-'Ve	'-'Ve
7.	Citrate utilization test	'+'Ve	'-'Ve
8.	Oxidase test	'+'Ve	'-'Ve
9.	Catalase	'-'Ve	'+'Ve
10.	Starch hydrolysis	'-'ve	'-'Ve
12.	Gelatin test	'+'Ve	'+'Ve
Carbohydrate fermentation			
13.	Sucrose	'-'Ve	'-'Ve
14.	Dextrose	'-'Ve	'-'Ve
15.	Xylose	'-'Ve	'-'Ve
16.	Galactose	'+'Ve	'-'Ve
17.	Maltose	'V'	'-'Ve
18.	Mannitol	'-'Ve	'+'Ve

Lipase productivity in various substrates:

Lipase productivity of *Pseudomonas aeroginosa* and *Staphylococcus aureus* was analyzed in molasses and soybean substrate following (Table 2) Elad *et al.* (1982).

Table 2 : Assay of enzyme activity *Pseudomonas aerogenosa*

Sr. No.	Organisms	Substrate	Enzyme activity unit /ml/min
1.	*Pseudomonas*	Molasses	0.30±0.03
	aerogenosa	Soybean	0.27±0.07
2.	*Staphylococcus*	Molasses	0.20±0.03
	aureus	Soybean	0.19±0.04

Optimization of lipases productivity:

The effete of pH and temperature on lipase production during growth were studied using mineral medium.

Lipase productivity in various pH range:

The lipase productivity was optimized by changing initial pH of the medium, adjusting from 5 to 10, after fermentation the lipase activities were estimated and the results are presented in Table 3.

Table 3 : Lipase productivity of *Pseudomonas aerogenosa* in various pH ranges

Sr. No.	Substrate	pH range	Enzyme activity μ/ml/min
1.	Molasses	5	0.19±0.05
2.		6	0.34±0.41
3.		7	0.43±0.52
4.		8	0.39±0.10
5.		9	0.22±0.07
6.		10	0.13±0.04

Lipase productivity at various temperatures:

The culture inoculated medium was incubated at different range of temperatures from 20-45°C and the results are presented in Table 4.

Table 4 : Lipase productivity of *Pseudomonas aeroginosa at*n various temperatures

Sr. No.	Substrate	Temperature (^{0}C)	Enzyme activity μ /ml/min
1.	Molasses	20	0.13±0.09
2.		25	0.15±0.02
3.		30	0.20±0.07
4.		35	0.39±0.15
5.		40	0.31±0.13
6.		45	0.23±0.05

Lipase productivity in immobilized cell:

The lipase enzyme productivity was estimated in the immobilized *Pseudomonas aeroginosa* and *Staphylococcus aureus*. The immobilized organism was inoculated in the fermented medium. After fermentation, the lipases productivity was studied and the results are presented in Table 5.

Many researchers have found that among lipase of various organisms (animal, plant, microorganism) those from bacteria, especially *Pseudomonas aerogenosa* exhibit the highest versatility, reactivity and stability in catalyzing reaction in organic phase. In the present study lipase production of *Pseudomonas* sp. and *Staphylococcus aureus* were estimated in various substrates such as molasses and soybean extract. High lipase productivity was observed in molasses using *Pseudomonas aerogenosa.*

Table 5 : Lipase enzyme productivity in immobilized cell

Sr. No.	Test immuno organism cells	Enzyme activity free	Immobilized
1.	*Pseudomonas aeroginosa*	0.35±0.01	0.49± 0.11
2.	*Staphylococcus aureus*	0.27± 0.22	0.25±0.1

Physiochemical conditions were analyzed for the productivity of lipase enzyme in the molasses using *Pseudomonas aerogenosa*. The growth and lipase production were found to be optimal between pH ranges of 7-8 and at temperature 35ºC ± 1. The optimum pH and temperature determined in this study are in agreement with the findings of lipase by different microbial strain (Saxena *et al*., 1999). The optimal production of lipase by *Bacillus* was also reported at pH 8.0 (Nawani *et al*., 1998).

The enzyme productivity was analyzed in the free and immobilized *Pseudomonas aerogenosa* and *Staphylococcus aureus* in this study. The maximum lipase productivity was observed in immobilized *Pseudomonas aerogenosa, Staphylococcus aureus* (0.51±0.28u/ml/min). Batch and repeated batch (fed batch type) experiments have been carried out for lipase production using immobilized *C. rugosa* cell in expensed bed reactors, having enriched medium (Benjamin and Pandey, 1997). *Rhizopus. arrhizus* cells have been immobilized on polyurethane foams and effect of parameters like glucose concentration, pH, inoculums size and agitator speed have been studies on lipase production. Positive effect of corn oil as induced on the process and the storage stability has been investigated (Elibol and Ozer, 2000). Finally to conclude, the isolated *Pseudomonas aerogenosa* can be used for commercial production of lipase in molasses substrate. This lipase provides great opportunity in biotechnology of which has important industrial significance.

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