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ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF LIPASE ENZYME FROM PSEUDOMONAS AERUGINOSA FROM SOIL AND SEWAGE WASTE

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ABSTRACT A lipase producing Pseudomonas sp. is isolated from soil and sewage waste water in Hyderabad. The growth curve of Pseudomonas aeruginosa on growth medium is plotted. It is capable of producing the enzyme lipase where the specific enzyme activity is maximum. The optimum hydrolysis conditions for lipase are pH 7.0 to 9.0 and temperature at 450 C. These results show that Pseudomonas aeruginosa may have potential for industrial sewage waste water treatment and recycling of sewage wastes from the environment.

KEYWORDS : Lipase, pH, Sewage, Temperature And Waste Water.

INTRODUCTION

Fossil fuels are consumed so fast and the planet resources will be going to be exhausted soon. There is a need to develop inexpensive and alternative new technologies to produce sustainable green fuels. Besides hydrolytic reactions lipases undergo esterification reactions or transesterification reactions thereby useful for the production of biodiesel, an alternative energy. The selection of the lipases producing organism that perform transesterification reactions is not easy. There is few biodiesel producing lipases from micro organisms are currently available for industrial purpose (Escobar-Niño et al.,2014).

The application of enzymes in the transesterification reaction or process of triglycerides making integration of glycerol to form monoglycerides. There is an increasing yield in the process and also the environmental sustainability of the conventional biodiesel production. This is called as Ecodiesel (Luna, Cet al.,2017). Lipases occur widely in nature but microbial lipases are commercially significant. Microbial lipases have wide applications in industries because of their shorter generation time, ease of production (that further enhanced with advancement in fermentation technologies) and ease of manipulation (either genetically or environmentally). Microorganisms that produce lipase are found in diverse habitats such as dairies, vegetable oil processing factories, industrial wastes and soil contaminated with oil. Many microorganisms such as bacteria, yeast and fungi are known to secret lipases but bacterial lipases are more economical and stable. Several lipases from *Bacillus thermoleoverans, B.stearothermophilus, B.acidocaldarius, thermoacidophilic bacteria* and *Bacillus* sp. strain A 30-1 (alkaliphilic bacteria) have been isolated and characterized. Bacterial lipases are used extensively in food and dairy industry for the hydrolysis of milk, fat, cheese ripening, flavor enhancement and lipolysis of butter fat (Arpigny JL and Jaeger KE, 1999).

Bacterial lipases are extracellular, they are greatly influenced by physico-chemical and nutritional factors, such as temperature, pH, nitrogen & carbon sources, inorganic salts, agitation and dissolved oxygen concentration. The carbon sources accounted for 50% of the production cost in the lipase production. In order to reduce the cost of production, usage of cheaper substrates like oil seed cakes, peanut oil cake, groundnut oil cake, palm oil effluent etc., is advisable. In further to these substrates, oil mill effluent can also be used as substrate for lipase production since it is rich in oil, suspended solids, organic and inorganic nutrients, carbohydrates etc.

Microbial lipases gained prominence in industry mainly because of its hydrolytic and synthetic activities, high yield, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and easy cultivation of microbes on inexpensive media (K.E. Jaeger et al., 1994, F. Hasan et al., 2006). Lipases of fungal and bacterial origin are widely used in industrial applications. Some important lipase producing bacterial genera include *Bacillus*, *Pseudomonas* and *Burkholderia*. Lipase/esterase-producing bacteria have been found in diverse habitats such as soil contaminated with oil, dairy waste, industrial wastes, oil seeds and decaying food, compost heaps, coal tips and hot springs (J. Vakhlu et al., 2006, Y. Wang et al., 1995). The demand for the biocatalysts with novel and specific properties such as specificity, stability, pH, and temperature is increasing (K.E. Jaeger et al., 1998, Y. Wang et al., 1995).

The industrial demand for highly active preparations of lipolytic enzymes continues to stimulate the search for new enzyme sources. Topal et al., 2000 in Turkey first isolated lipase from *Trichoderma harzianum* IDM14D but the enzyme was not characterised. It produces some hydrolytic enzymes such as chitinase, β-glucanase, amylase and cellulase. It is also used as a biocontrol agent.

In the present study bacterial species from wastewater was isolated and screened for the best lipolytic strain (for lipase production, oil mill effluent was used as substrate) by optimizing its environmental factors.

MATERIALS AND METHODS

Screening Of Lipolytic Bacteria

Lipase producing microbial cultures was isolated from different soil and sewage industrial sites in Hyderabad. They are enriched by periodic subculturing of samples in Nutrient Broth (NB) media containing 20% (v/v) and 40% (v/v) wastewater in successive samples. The composition of NB medium is (per liter) 5g peptone and 3g yeast extract. The pH of the medium was adjusted to 7 with 0.1M NaOH.

Growth conditions of Pseudomonas aeruginosa

The isolation was done by serial dilution of samples on tributyrin agar plates. The composition of tributyrin agar medium per liter is 5 g peptone, 3 g yeast extract, 10 ml tributyrin and 15 g agar. Culture plates were incubated at 30ºC. Colonies showing clear zones around them were picked out and purified on tributyrin agar plates, later it has been transferred to agar slants. The organism identified was *Pseudomonas aeruginosa.* Isolates having clearing zone were grown in the liquid culture and the level of lipase production was determined from the cell free culture supernatant fluid. Characterization and identification of the isolate with higher lipolytic activity was carried out biochemically. The identification of the microorganism *P. aeruginosa* was performed using positive oxidase test, the absence of indole production, a positive urease test, the oxidative metabolism of glucose and complete nitrate reduction to nitrogen (Szita and Biró 1990; Szita et al. 1995) have revealed that the organism is *Pseudomonas aeruginosa.*

Enzyme Production

The composition of production medium used in this study was: (% w/v) peptone - ±0.2; NH_4 H_2PO_4 - 0.1; NaCl - 0.25; $MgSO_4 \cdot 7H_2O$ - 0.04; $CaCl_2.2H_2O$ - ± 0.04 ; olive oil - 2.0 (v/v); pH - ±7.0; 1-2 drops Tween 80 as emulsifier. In 5ml of sterile deionised water, overnight cultures were suspended and used as the inoculum for pre-culture so that an initial cell density to adjust the turbidity of 0.5 McFarland standard is obtained. Submerged microbial cultures were incubated in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium on a rotary shaker (150 rpm) and incubated at 30°C. The culture is incubated for 24 hours and was centrifuged at 10,000 rpm for 20 min at 4ºC, the cell free culture supernatant fluid was collected and used as the sources of extracellular enzyme.

Assay Of Lipase Activity

Lipase activity was determined spectrophotometrically at 30°C using

p-nitrophenol palmitate (pNPP) as substrate. The reaction mixture was composed of 700 µl *p*NPP solution and 300 µl of lipase solution. The *p*NPP solution was prepared by adding the solution A (0.001 g *p*NPP in 1ml isopropanal) to solution B (0.02 g Sodium deoxycholate, 0.01 g gum arabic, 9 ml of 50 mM Tris-HCl buffer, 50 µl Triton X-100, pH 8). The absorbance was measured at 410 nm for the first 2 min of reaction. One unit (1U) was defined as that amount of enzyme that liberated 1µmol of *p*NPP per minute under the test conditions. Assay of Lipase was done with p-nitrophenly fatty acyl esters at various chain lenghts. Absorbance reading at 400nm was taken with the certain time interval. The curve which has a raising behavior was drawn according to absorbance versus minutes data.The highest enzyme activity data assumed 100% and relative enzyme activity estimated for each data. To calculate specific enzyme activity (units/mg protein), Bradford assay was performed. Specific enzyme activity was obtained by dividing enzyme activity value to protein content in mililiter. At optimum pH and temperature one unit releases one nanomole (10^{-9} mole) of p-nitrophenol per using p-nitrophenyl fatty acyl esters at various chain lenghts.

Effect Of pH On Lipase Activity

Optimal pH was determined at 30°C in buffer solutions of pH values ranging from 5 to 11 (0.05 M citrate-phosphate, pH 5-7; 0.05 M Tris–HCl, pH 8-9; 0; 0.05 M Glysin – NaOH, pH 11). By the spectrophotometric assay the effect of pH on enzyme stability was analyzed after pre-incubation of 300 µl of enzyme solution for 1 h at 30°C in 700 µl of the above mentioned buffer solutions (pH 5–11). Enzymatic activity was measured according to a standard protocol with *p*NPP as the substrate.

Effect Of Temperature On Lipase Activity

The crude enzyme used for assay was the culture broth after separation of cells and particles. The enzyme was stored at 4°C until used**.** The optimal temperature for the activity of enzyme was determined by performing assay at different temperatures (30–70°C), at pH 8.0 for 10 min. For determination of temperature stability, the reaction mixtures containing the enzyme in 50mM Tris–HCl buffer (pH 8.0) was incubated at different temperatures (37, 45, 50, 55, 65 and 70°C) for 3 h and immediately cooled. Residual enzyme activity was measured under standard enzyme test conditions.

RESULTS AND DISCUSSIONS

Isolation Of Pure Cultures Of Pseudomonas Aeruginosa

The isolation was done by serial dilution of samples on tributyrin agar plates. Culture plates were incubated at 30ºC. Colonies with clear zones around them were picked out and purified on tributyrin agar plates, later transferred to agar slants. The organism identified was *Pseudomonas aeruginosa. Pseudomonas aeruginosa* is a gram-negative rod shaped bacteria measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. maximum strains are motile by means of a single polar flagellum. The smooth and mucoid colonies are presumed to play a role in colonization and virulence. Natural isolates from soil or water typically produce a small, rough colony. In general clinical samples yield one or another of two smooth colony types (Fig. 1).

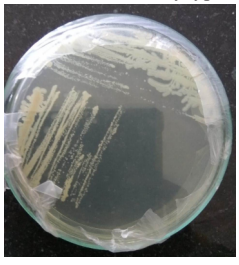

Fig 1 : Culture Of Pseudomonas Aeruginosa

Enzyme Assay Of Lipase

One unit (1U) was defined as that amount of enzyme that liberated 1µmol of *p*NPP per minute under the test conditions. Assay of Lipase was done with p-nitrophenly fatty acyl esters at various chain lenghts. Absorbance reading at 400nm was taken with the certain time interval. The maximum enzyme activity is obtained.

Effect Of pH On Lipase Activity

The effect of pH on the activity of lipase enzyme was determined using four different buffers with pH 3.0 to 12.0. The enzyme was most active at pH 6.0 & 9.0 increasing activity from pH 7-9 can be characterized as an alkalophilic enzyme but high lipase activity at pH 6 shows its activity in acidic conditions. Bacterial lipases are stable between pH 4-11. Lipase obtained in our study was stable at basic conditions from pH 7.0 to 10.0 but its stability was low at acidic pH. The remarkable stability of *Psudomonas aeruginosa* lipase in this range has proved it to be a potential alkaline lipase (Fig. 2). The optimal activity of lipases extracted from *Bacillus thermoleovorans* ID1 was at pH 9 [Lee DW et al., 2001]. Thermophilic bacteria like *Bacillus* that produce lipases showed optimal activity between pH 7.2–8.5 (Lee DW et al., 2001; Schmidt DC et al., 1994; Kim HK et al., 1994; Nawani et al., 1998; Lee DW et al., 1999; Dharmsthiti et al., 1999; Imamura S et al., 2000; Handelsman T et al., 1994)

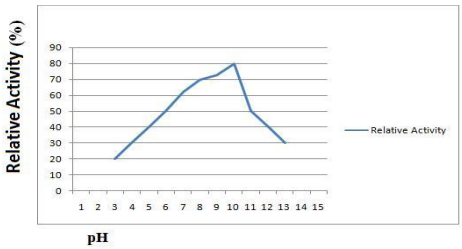

Fig2: Effect Of pH On Lipase Enzyme Activity

Effect Of Temperature On Lipase Activity

The temperature preference of this enzyme reveals higher activity values at temperature from 35 to 45°C. The lower activity at 35°C compared to 45°C, probably is due to the kinetics of enzymatic reaction as the enzyme is more conductive at 45°C. Assessment of the thermostability of lipase was performed by measuring the residual activity at various times, following incubation at different temperatures. *P. aeruginosa* lipases seem to be more thermostable than others from this genus (Fig. 3). *B. thermoleovorans* ID-1 showed optimal activity between 60–65°C (Lee DW et al., 2001). *Bacillus sp.* J33 showed optimal lipase activity at 60°C (Nawani et al., 1998), *Bacillus sp.* A30-1 also showed maximum activity at 60 ºC (Handelsman T et al., 1994).

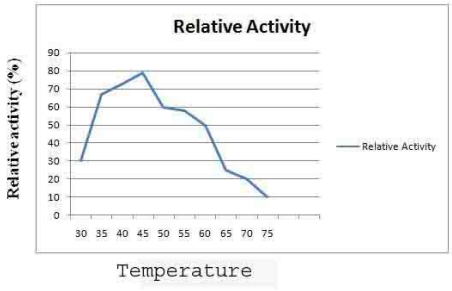

Fig 3: Effect Of Temperature On Lipase Enzyme Activity

CONCLUSION

The results obtained in this study show that the isolate *P. aeruginos* has produced a maxium concentration of lipase enzyme. Further studies are needed to enhance lipase production in this strain. Also, *Pseudomonas aeruginosa* lipase is a potential alkaline lipase. The enzyme exhibited maximum activity and stability between pH 7.0 and 10.0 and temperature of 45°C.The maxium enzyme activity is found as 50 units. The remarkable stability of *P. aeruginosa* lipase in this range has proved it to be a potential alkaline lipase similar to other and a candidate for industrial applications such as detergent, leather and fine chemical industries.

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