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Production and Immobilization of extracellular Lipase from *Pseudomonas aeruginosa*.

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Abstract: ***Lipases (Triacylglycerol acylhydrolases, EC 3.1.1.3) catalyzes the enzymatic hydrolysis of triacylglycerol into diacylglycerol & free fatty acids. Lipases act as a key enzyme that is used in various industries like Detergent, Food, Leather, Textiles, Pharmaceuticals, etc. The present study investigates the potentials of Pseudomonas spp. isolated from oil contaminated soil samples in the production and immobilization of Lipase enzyme. Submerged fermentation of SP45 (Pseudomonas aeruginosa) was performed for the production of Lipase. Extracellular SP45 Lipase was then partially purified by ammonium sulphate precipitation followed by dialysis. SP45 Lipase precipitated with 50% saturated solution of ammonium sulphate showed optimum Lipase activity of about 56.0433U/ml and it was increased upto 101.2400U/ml after dialysis. Purified SP45 Lipase was then immobilized using physical adsorption and microencapsulation methods. SP45 Lipase immobilized in sodium alginate beads showed greater enzymatic activity when compared with SP45 Lipase immobilized with ion exchange resins.***

Keywords: Lipase, *Pseudomonas aeruginosa*, Fermentation, Immobilization, Spectrophotometric assay

1. Introduction

Lipases (Triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze hydrolysis of Mono-, Di- & Tri-acylglycerides into glycerol & free fatty acids in presence of water-lipid interface. Lipase enzyme can be isolated and purified from a variety of sources like fungi, bacteria, yeast, animal and plant sources, out of which bacterial Lipases are considered as physiologically significant and commercially important [1], [2]. Extracellular Lipase enzyme has tremendous industrial applications in various industries like detergent, bakery, dairy, textiles, leather, cosmetic, paper / pulp, pharmaceutical, etc. [3]. In dairy industry, development of flavors in cheese, butter & milk was generally performed with the help of Lipase enzyme, while enzymatic prolongation of shelf life of bakery items and beverages was achieved in food industry [4], [5]. Lipase acts as a key enzyme in detergent industry for removal of oily stains from fabrics. In addition to this, Lipases are also used for the moisturizers / emulsifiers production in cosmetic industry [6]. Textile industry mainly uses Lipase enzyme for improving the absorbency of fabrics, while as far as leather industry is concerned, Lipase was routinely used for the enzymatic hydrolysis of leather products [3]. In addition to this, production of different products which are regularly used in pulp / paper industry & also in the pharmaceutical industry uses hydrolytic enzymes like Lipases [7].

Different bacteria and fungi like *Bacillus spp., Enterococcus spp., Streptococcus spp., Staphylococcus spp., Klebsiella spp., Proteus spp., Aspergillus spp., Candida spp.,* and *Rhizopus spp.* were routinely studied for industrial production of Lipases [1]. Among these microbes, the *Pseudomonas spp.* remains considerably less explored. The present study hence explores the potentials of *Pseudomonas* strains isolated from various oil contaminated soil samples in the production and purification of extracellular Lipase enzyme. The present study also aims at the immobilization of partially purified Lipase using different types of support materials.

2. Material and Methods

In our previous studies, around 50 lipolytic *Pseudomonas spp.* (SP01-SP50) were isolated from 29 oil contaminated soil samples collected from different regions of Mumbai, Solapur and Pune. It was also found that SP45 showed optimum Lipase activity (52.8867 U/ml) and thus SP45 was selected for media optimization studies. Media optimization studies reveals the fact that SP45 exhibits increased Lipase activity upto 66.2567 U/ml after optimization of various fermentation parameters like pH, temperature, carbon and nitrogen sources of fermentation medium [8].

The present study primarily aims at the production and immobilization of extracellular Lipase produced from SP45 (*Pseudomonas aeruginosa*) with the help of submerged fermentation for about 24 - 48 hours. Extracellular SP45 Lipase was produced with the help of ammonium sulphate precipitation followed by dialysis. The proposed study was carried out during the period commencing from September 2014 to January 2015.

2.1 Production of SP45 Lipase by submerged fermentation:

2.1.1 Inoculation and incubation:

The basal fermentation medium containing tributyrin as a Lipase inducer was inoculated with 1% (v/v) of freshly prepared SP45 inoculum (cells density equivalent to 0.5 McFarland's standard). Then the inoculated medium was kept in an incubator shaker at agitation speed of 110 rpm at 37^0C for 24 - 48 hours of fermentation.

2.1.2 Recovery of extracellular SP45 Lipase:

After 24 hours & 48 hours of fermentation, 10ml medium was taken out for centrifugation at 4000rpm for 15 minutes to get the culture supernatant which can be used in the

spectrophotometric Lipase enzyme assay. The culture supernatant was treated as the source of crude Lipase enzyme for spectrophotometric assay.

2.2 Spectrophotometric Lipase assay

Polyoxyethylene sorbitan ester (Tween 80) was used as substrates for determining the activity of extracellular Lipase enzyme using the method described by Tirunarayanan and Lundbeck with slight modifications. Briefly, the reaction mixture contains 0.1ml of 1M $CaCl_2$ in Tris buffer, 0.1ml of 10% Tween 80 in 50mM Tris hydrochloride buffer (pH 7.6), 0.5ml of concentrated culture supernatant as a source of enzyme, and 2.3ml of Tris buffer (pH 7.6). Reaction mixture containing 0.5ml of deionized water instead of supernatant was treated as a blank. Enzyme assay for each isolate was performed in triplicates. Then the reaction mixtures were incubated in an incubator for 2 hours at 37°C. In this assay, Tween was cleaved and a fatty acid and an alcohol were produced. The presence of calcium in the reaction mixture results in the formation of an insoluble fatty acid salt, giving a precipitate which can be measured spectrophotometrically at 400nm. One unit of Lipase activity was defined as that amount of enzyme resulted in an increase of optical density at 400nm (OD400) of 0.01 after 2 hours under the assay conditions [9].

2.3 Purification of extracellular SP45 Lipase:

2.3.1 Ammonium sulphate precipitation:

Extracellular Lipase enzyme produced from fermentation of SP45 (*Pseudomonas aeruginosa*) was purified by ammonium sulphate precipitation using the methods described by C.K. Sharma et al. and M. Chouhan et al. with slight modifications. For ammonium sulphate precipitation, culture supernatant was fractioned into 10ml parts in centrifugation tubes for making 10% to 90% saturation of ammonium sulphate solution. After preparing 10% to 90% saturated solution of ammonium sulphate, the centrifuge tubes were kept in a refrigerator at 4^0C for 20 minutes of incubation which was required for protein precipitation. Then these precipitated proteins were separated by centrifugation at 5000rpm for 15 minutes. Then the precipitated proteins were dissolved in 50mM Tris Buffer (pH7.6) and stored at 4^0C for further use [10], [11]. These precipitated fractions were used for spectrophotometric Lipase assay. Fraction showing optimum Lipase activity was then selected for further Lipase production studies.

2.3.2 Purification of crude SP45 Lipase by dialysis:

The precipitate (Crude Lipase enzyme) produced after ammonium sulphate precipitation method was then further purified with the help of dialysis treatment against Tris HCl buffer (pH 7.6) by the method described by V. Ramdas and R. Lavanya Ramadoss with slight modifications. The crude Lipase enzyme (precipitated protein) re-suspended in 50mM Tris buffer (pH 7.6) was dialyzed in the pretreated dialysis membrane (Hi-Media) against 50mM Tris HCl buffer (pH 7.6) for about 24 hours at 4^0C with 3 changes of buffer. The dialysis buffer was regularly changed after a time intervals of 06, 12 and 24 hours. After 24 hours of stirring incubation, the dialysis assembly was taken out. All the contents (Dialyzed Enzyme) present in the dialysis bag was taken in a sterile 5ml storage vial [12], [13]. This dialyzed Lipase enzyme was then stored at 4^0C for further immobilization studies. The dialyzate was also assayed for Lipase activity by spectrophotometric Lipase assay.

2.4 Immobilization of dialyzed Lipase enzyme:

2.4.1 Immobilization of Lipase by entrapment method:

The purified Lipase enzyme produced from SP45 (*Pseudomonas aeruginosa*) was immobilized by gel entrapment method with the help of formation of sodium alginate beads in calcium chloride solution. The dialyzed Lipase was mixed with 2% (w/v) sodium alginate solution previously heated to 80^0C & cooled to 40^0C. Then this solution of sodium alginate containing Lipase was added drop-wise in 2% (w/v) solution of chilled calcium chloride with the help of sterile glass pipette. The beads thus formed were then washed with Tris Buffer (pH 7.6). The activity of immobilized enzyme was calculated by performing spectrophotometric Lipase assay [14].

2.4.2 Immobilization of Lipase by adsorption method:

The purified Lipase enzyme produced from SP45 (*Pseudomonas aeruginosa*) was also immobilized by physical adsorption method. The ion exchange resins were used for the immobilization of Lipase. About 1gram of resins were soaked in 10ml Tris Buffer (pH7.6) containing 1ml dialyzed Lipase enzyme in sterile conical flask. Then this flask was kept in an incubator shaker at 110rpm for 24 - 48 hours at 37^0C. After 48 hours of incubation, resins were washed twice with distilled water. The activity of immobilized enzyme was calculated by performing the spectrophotometric Lipase assay. Remaining ion exchange resins were then air dried for about 36 hours for further use [15]. Lipase assay of crude enzyme along with dialyzed enzyme was also performed for effective comparison between the two.

3. Results and Discussion

3.1 Production of SP45 Lipase by Submerged Fermentation:

It was observed that SP45 exhibits increased Lipase activity after optimization of different fermentation parameters as shown in Figure 1. Thus submerged fermentation of SP45 was carried out in optimized conditions for enhanced production of Lipase.

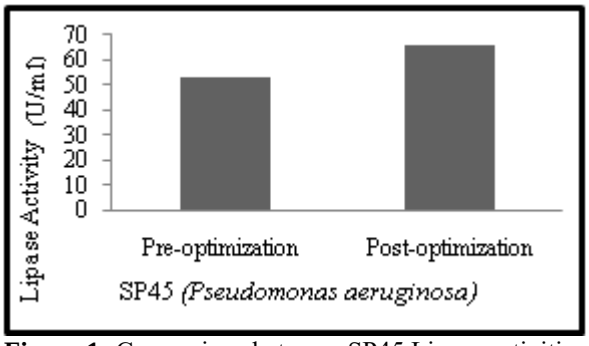

Figure 1: Comparison between SP45 Lipase activities

3.2 Purification of extracellular SP45 Lipase by ammonium sulphate precipitation:

Fractions of saturated solutions of ammonium sulphate starting from 10% to 90% were prepared for precipitation of Lipase in the fermentation medium. Spectrophotometric Lipase assay of these ammonium sulphate precipitate fractions indicates that 50% saturated solution of ammonium sulphate showed optimum Lipase activity of 56.0433 U/ml as indicated in Figure 2. These precipitated proteins were then dissolved in 3ml Tris Buffer (pH 7.6) for further use.

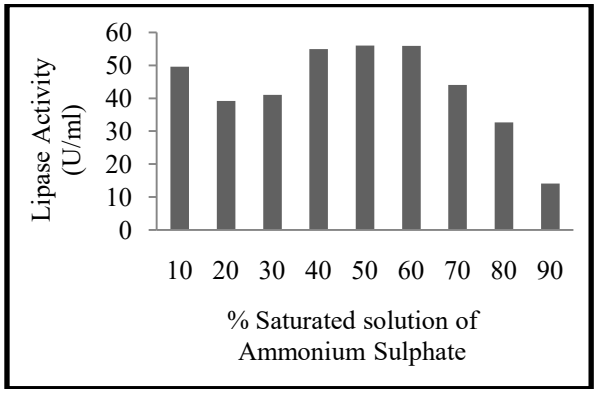

Figure 2: Effect of saturated solutions of ammonium sulphate on SP45 Lipase activity

3.3 Partial purification of crude Lipase by dialysis:

Crude Lipase enzyme precipitated by using 50% saturated solution of ammonium sulphate was then dialyzed against Tris Buffer (pH 7.6). On comparing the Lipase activity of dialyzed SP45 Lipase with that of the crude SP45 Lipase, it was found that the dialyzed SP45 Lipase showed the increased activity than that of the crude SP45 Lipase. Dialyzed SP45 Lipase exhibits the enzymatic activity of 101.2400 U/ml as indicated in Figure 3.

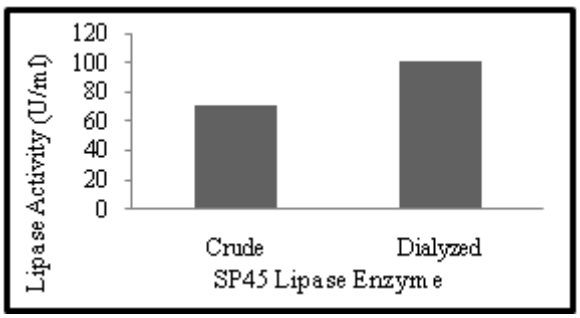

Figure 3: Comparison between Crude and Dialyzed SP45 Lipase activity

3.4 Immobilization of dialyzed SP45 Lipase enzyme

Immobilization of dialyzed SP45 Lipase was performed by the method of entrapment in sodium alginate beads (Encapsulation) and also by using ion exchange resins (Adsorption). It was observed that SP45 Lipase immobilized in sodium alginate beads showed higher Lipase activity than that of ion exchange resins as represented in Figure 4. SP45 Lipase immobilized in sodium alginate beads showed Lipase activity of about 43.1733 U/ml.

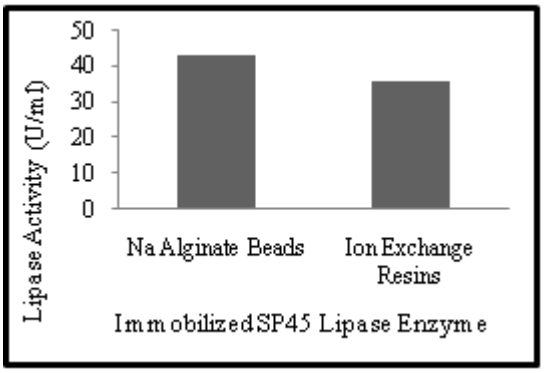

Figure 4: Effect of Immobilization of Dialyzed SP45 Lipase on Lipase activity

4. Conclusion

Lipase enzyme produced from microbial origin has tremendous applications in multiple industries [1]. The present study helps us to understand that microbial extracellular Lipase enzyme can be produced in significant quantities cost-effectively. In the present study, extracellular SP45 Lipase was produced with the help of submerged fermentation. Ammonium sulphate precipitation showed that 50% saturated solution exhibits optimum Lipase activity of 56.0433 U/ml after 48 hours of fermentation. The precipitated crude Lipase enzyme was then partially purified by using dialysis method. The dialyzed SP45 Lipase showed increased Lipase activity of about 101.2400 U/ml. The present study also revealed the fact that immobilization of partially purified SP45 Lipase gave us a comparatively stable enzyme system. However there is an ample scope for further research on efficient use of immobilized SP45 Lipase in the production of Biodiesel which is the most essential demand of human mankind.

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