Improvement of the Enzymatic Performance of lipase from *Pseudomonas sp.* ADT3 via entrapment in alginate hydrogel beads

Arpita Dey[*], Tushar Kanti Maiti[**] and Pranab Roy[***]

[*]Department of Biotechnology, The University of Burdwan, India
[**]Department of Botany, The University of Burdwan, India
[***]Department of Biotechnology, Haldia Institute of Technology, India

Abstract- Extracellular lipase producing psychrotrophic *Pseudomonas* ADT3 (NCBI GenBank Acc.no.JX914667) isolated from soil sample of Ny- Alesund, Svalbard, Arctic region produced maximal lipase activity of 527.8U/mg after 48 hours at pH 8.5 and temperature 22°C in presence of 1.2mM lead as cofactor. It was partially purified 2.9 folds by ammonium sulphate precipitation (80%). Enzymatic performance was improved by immobilization of enzyme on various carriers viz. Alginate and polyacrylamide gel. The immobilization yield of enzyme immobilized in polyacrylamide gel was low (40.0%) in comparison to that immobilized with alginate (70.0%). Different concentrations of alginate and calcium chloride were studied to acquire stable beads. Optimum concentration of alginate and calcium chloride was 2% and 0.12M respectively. The immobilized enzyme was found to be stable in alkaline pH. The maximal activity for immobilized enzyme was found at pH 8.5. Broader pH tolerance could be achieved by immobilization. Temperature optima of the enzyme showed no changes before and after immobilization i.e. 22°C. But the thermal stability was enhanced after immobilization. Immobilized enzyme remained active up to 50 °C while the activity of the free enzyme started to decrease from 40 °C. Even at 70°C immobilized enzyme retained 25% of residual activity but free enzyme totally loses enzyme activity. The storage stability of entrapped lipase up to 50% was found after 5 days at 4°C, while at 30°C the enzyme lost 60% of its activity after 2 days. The enzyme can be reused up to 5 cycles which is a promising technique for large-scale preparation of immobilized lipase for industrial applications.

Index Terms- Extracellular, psychrotrophic, arctic, immobilization

I. INTRODUCTION

In the natural environment, lipases (EC 3.1.1.3) catalyze the hydrolysis of esters formed from glycerol and long chain fatty acids [1, 2]. However, under appropriate experimental conditions, these enzymes are also very active biocatalysts for the esterification of fatty acids and transesterification reactions [3, 4]. Many lipases from microbial sources have been investigated and found to be promising catalysts for the hydrolysis and synthesis of fats and oils. Versatility of lipase leads to multiple industrial applications in food and flavour making, pharmaceuticals, synthesis of carbohydrate esters, amines and amides, biodetergents, cosmetics and perfumery. The use of free lipases is limited by their considerably unstable nature and the resulting requirement of stringent conditions, such as particular pH and temperature [5, 6, and 7]. In order to use them more economically and efficiently in aqueous as well as in non-aqueous solvents, their activity, selectivity and operational stability can be modified by immobilization [8]. Immobilization of enzymes is the key to expand the applications of these natural catalysts by enabling easy separation and purification of products from reaction mixtures and efficient recovery of enzyme proteins. The enzyme immobilization is to entrap the enzymes in a semi-permeable supports, which prevents the enzyme from leaving while allowing substrates and products to pass through [9].With immobilized enzymes, improved stability, reuse, continuous-operation, possibility of better control of reaction, product yields and hence more enzymatic activity over a broad range of pH and temperature can be expected[10].

Entrapment of enzyme in calcium alginate is one of the important methods of immobilization. Alginates are commercially available as water-soluble sodium alginates and they have been used for more than 65 years in the food and pharmaceutical industries as thickening, emulsifying and film forming agent. Entrapment within insoluble calcium alginate gel is recognized as a rapid, nontoxic, inexpensive and versatile method for immobilization of enzymes as well as cells [11].

The present research was based on the entrapment of extracellular cold active lipase in calcium alginate beads obtained from a newly isolated strain of psychrotropic *Pseudomonas sp ADT3* from soil samples collected from Ny- Alesund (78°55'N, 11°54'E) Svalbard, Arctic region during the Indian Arctic Expedition 2009, organized by National Centre for Antarctic and Ocean Research, Goa. and also the characteristic of immobilized enzyme. The effect of independent variables such as alginate concentration, $CaCl_2$ concentration, and effect of pH and temperature on immobilization yield and activity of immobilized enzyme were investigated. Repeated use of the immobilized lipases was also studied with storage stability. The present communication would enhance the enzyme stability via immobilization and hence leads to multiple industrial applications in food and flavour making, pharmaceuticals, biodetergents, cosmetics and perfumery.

II. Materials and Methods

2.1 Chemicals

All chemicals used were of analytical grade, obtained from Himedia (India) and Sigma-Aldrich Company.

2.2 Bacterial isolate

Pseudomonas ADT3 was isolated in our laboratory from soil samples collected from Ny- Alesund (78°55'N, 11°54'E) Svalbard, Arctic region during the Indian Arctic Expedition 2009, organized by National Centre for Antarctic and Ocean Research, Goa under the Ministry of Earth Science, Govt of India. The molecular identification and lipase activity studies of this isolate were done by 16S rDNA sequencing and titration assay. The ADT3 isolate could grow on olive oil as the sole carbon source [12].

2.3 Growth conditions

The ADT3 isolate could grow on minimal medium containing: 0.3%(w/v) KH_2PO_4, 0.6%(w/v) Na_2HPO_4, 0.05% (w/v) NaCl, 0.1% (w/v) NH_4Cl, 2 mM $MgSO_4$, 0.1mM $CaCl_2$ with 1.0% olive oil as the sole carbon source and 2% (w/v) agar [13] at pH 8.0 by spread plate method and the culture was incubated with shaking at 22°C for 48 hrs. Pure cultures of the isolate were maintained on M9 minimal medium agar slants containing olive oil and were routinely maintained by sub culturing.

2.4 Production of lipase

ADT3 cells were grown in Erlenmeyer flasks (250 mL) with 50 mL of M9 minimal medium containing 1% olive oil as substrate and flasks were incubated in shaker incubator with 120 rpm agitation at 22°C for 48 h. Cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant obtained was filtered using 0.45 µm cellulose acetate filter membrane (Sigma-Aldrich). The cell free filtrate was used as the extracellular crude enzyme which was stored at −20°C for further studies.

2.5 Partial purification of extracellular lipase by Ammonium Sulphate Fractionation

The online program ammonium sulphate calculator from EnCor Biotechnology, Inc, Gainesville, Florida [14] was used for calculating the amount of solid ammonium sulphate to be added for getting desired fractions. Precipitation was done at 0-10%, 10-20% and 20-30% saturation for 2-3 hours and at 40-50%, 50-60%, 60-80%, and 80-90% saturation of ammonium sulphate for overnight incubation, with magnetic stirrer at 4◦C. After each precipitation step, the fraction was centrifuged at 12,000 rpm for 15 minutes at 4◦C. All the precipitates obtained were resuspended in a minimal amount of buffer (10 mM Tris-HCl, pH 8.0) and dialyzed against large volume of the same buffer by successive change in buffer after 2 hours at 4◦C. The process was continued till the last trace of ammonium sulphate was removed. The obtained precipitates (partial purified enzyme) were dissolved in Tris-HCl buffer (50 mM, pH 7.5) and used for entrapment.

2.6 Lipase assay

Extracellular lipase activity in bacterial culture supernatants after centrifugation (12,000 g for 20 min) was determined by titrimetric assay using olive oil as substrate. Lipase activity was examined by titrating free fatty acids liberated from triglycerides with alkali [15, 16]. The reaction mixture contained 1ml of 0.1 M Tris- HCl buffer pH 8.0, 50mM KCL, 200µl Tween 20, 1 ml olive oil, 1ml of culture supernatant at 22°C for 3 hrs. The reaction mixture was mixed well on a reciprocal shaker at 60 rpm. After incubation, the mixture was shaken vigorously with 3 mL of ethanol to stop the enzyme reaction and break down the emulsion. The amount of fatty acids liberated during the reaction was estimated by titrating with 10 mM NaOH using phenophathalein indicator. Blank contained the same components except enzyme solution.

One unit of lipase activity was defined as the amount of enzyme required to liberate 1µMole equivalent of free fatty acid per minute. Specific activity was defined as units of lipase activity per milligram protein. The results obtained were means of triplicates for each experiment.

$$\text{Specific activity} = \frac{\text{ml titrant x molarity of titrant x 1000}}{\text{Amount of protein (mg)}}$$

2.7 Immobilization of partially purified lipase

2.7.1 Calcium alginate beads

The partially purified enzyme solution was mixed with sodium alginate solution (2%) in 1:1 ratio. The lipase-alginate mixture was added dropwise into calcium chloride (0.12 M) solution with continuous stirring with magnetic stirrer at 4°C. As soon as the drop of lipase-alginate solution mixed with $CaCl_2$ solution, Na ions of Na-alginate were replaced by the Ca^{+2} ions of $CaCl_2$ solution, which finally formed Ca-alginate beads. The lipase immobilized in beads of calcium alginate was stirred in the calcium chloride solution for another hour at 20°C [17]. The beads thus formed were washed 3-4 times with deionized water to remove any traces of chloride and finally suspended in 50 ml solution of 0.05 mM Tris of pH 7.5. These beads were dried and weighed for further studies.

2.7.2 Polyacrylamide gel entrapment method

Potassium phosphate buffer (pH 7.0, 0.2 M) was used to dissolve acrylamide 2.85 g, bisacrylamide 0.15 g, ammonium persulphate 10 mg and 0.1 mL TEMED. Equal ratio of chilled partially purified lipase solution and chilled potassium phosphate buffer were mixed well and poured into sterile flat bottom 4 inch diameter Petri plates. After polymerization the acrylamide gel was cut into equal size cubes. The acrylamide cubes were placed in sodium phosphate buffer (pH 7.0, 0.2 M) for 1 h in the refrigerator. These cubes were washed thoroughly two to three times with sterile water [18].

2.8. Protein Assay

The amount of protein content before and after immobilization was determined by the method of Bradford using BSA as the standard [19].

III. Result and Discussion

3.1 Characterization and Identification of psychrotrophic lipase producing bacteria

The strain was identified as *Pseudomonas sp.ADT3* based on biochemical tests satisfying the Bergey's manual of Determinative Bacteriology and 16S rDNA Gene Amplification and Sequencing. The NCBI GenBank Accession number of the isolate was JX914667. SEM micrograph reveals that the cells

were rod shaped of length 4.5 µm and diameter 0.9 µm. The results of experiments on Antibiotic sensitivity assay showed that ADT3 strain is resistant to Oxycillin and ampicillin [12].The organism is capable of growth on lipid as the sole carbon source which was confirmed by Rhodamine B plate assay where orange fluorescence under UV illumination indicates the presence of lipolytic activity.

3.2 Partial purification of *Pseudomonas sp. ADT3 lipase*

The purification procedure of the cold-active lipase produced by *Pseudomonas* ADT3 was summarized in Table 1.The lipase was partially purified about 2.9 fold over the crude extract with 64.4%recovery using 60-80% ammonium sulphate for precipitation. The specific enzyme activity of the partially purified lipase increased to 1433.8 U/mg proteins **Table1.**

Table 1 Purification process of lipase from the psychrotrophic bacterium

Purification steps	Total enzyme (U/L)	Total protein (mg)	Specific activity (U/mg)	Purification Fold	Enzyme Yield (%)
Crude extract	4,45,000	900	494.4	1.0	100
Ammonium sulphate precipitation (60-80%) saturation	2,86,760	200	1433.8	2.9	64.4

3.3 Production of extracellular lipase from *Pseudomonas sp. ADT3*

Pseudomonas produced an extracellular lipase during growth on a medium containing olive oil as the sole carbon source. The results recorded in Figure 1 indicated that the production of lipase increased steadily with the cultivation time and the best enzyme production, about 88.0 U/mg was reached after 48 days of cultivation. Maximum lipase production was observed during the stationary phase of growth and then the activity declined. In our study, the secretion of lipase was induced by 1% olive oil, as no other organic nutrient or carbon substrate other than olive oil was supplemented in the growth medium for the production of lipase. However, the enzyme production decreased on increasing the incubation period showing lipase activity of 64.11 U/mg after 3 days of incubation. This may be attributed to the fact that lipase production has been frequently inhibited by the end products namely, glycerol and fatty acids [20, 21] **Figure 1**

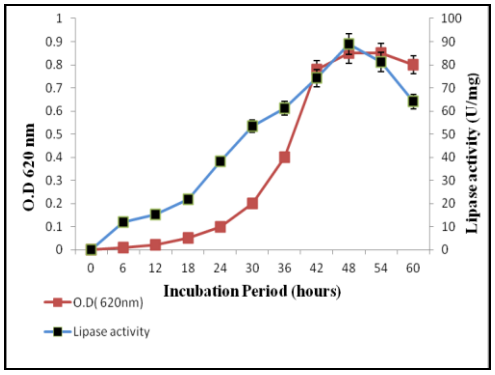

Fig 1: **Effect of different incubation period on lipase activity and growth by Pseudomonas ADT3** Lipase production was observed during the stationary phase of growth. Maximum enzyme production (88.0 U/mg) was observed after 48 hours of cultivation and then the activity declined. The bar denotes standard deviation (SD). When the error bar cannot be seen, the deviation is <5%.

3.4 Immobilization of *Pseudomonas ADT3* lipase using different carriers

The experimental results of immobilized *Pseudomonas ADT3* lipase using different entrapment techniques are shown in **Table 2**

Table 2: Immobilization of *Pseudomonas ADT3* lipase using

Carriers	Enzyme added (U/mg) P	Bound Enzyme (U/mg) R	Immobilization yield (%)
Ca-alginate	350	245	70.0
Polyacrylamide	350	140	40.0

$$\text{Immobilization yield (\%)} = \frac{R}{P} \times 100$$

The immobilization yield of lipase entrapped in calcium alginate (70.0%) was higher than that of enzyme entrapped in polyacrylamide (40.0%). The low yield with polyacrylamide blocks may be due to the diffusional resistance of nutrients and oxygen into the matrices. These results are in accordance with studies on *A. niger* [22, 18] and *Candida rugosa* [23].
Therefore, alginate was considered to be the best matrix for the production of lipase and the partially purified enzyme entrapped by sodium alginate was used for further investigation **Figure 2.**

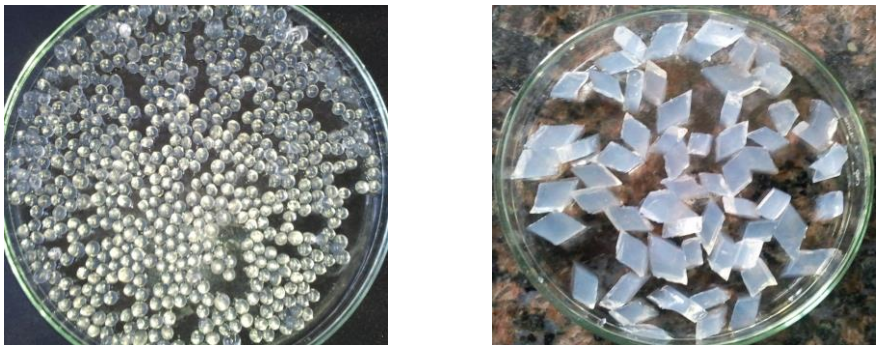

Fig 2 a. Calcium alginate beads containing immobilised lipase of *Pseudomonas ADT3. b. Polyacrylamide cubes containing immobilised lipase of Pseudomonas ADT3*

3.5 Effect of Sodium Alginate Concentration:

Due to the cross-linking between alginate and Ca^{2+} leads to gelation, alginate concentration is a major parameter for enzyme gel entrapment. Therefore effect of alginate concentration on immobilization yield was investigated. For the determination of suitable concentration of sodium alginate different percentage of sodium alginate (1%-5%) were used to acquire beads with greater stability maintaining the $CaCl_2$ concentration (0.12 M). The percent entrapped activity was found maximal at 2% (w/v) sodium alginate concentration. The percentage immobilization yield was 55.7%. Maximum leakage of enzyme from beads occurred at 1% (w/v) sodium alginate concentration owing to the larger pore sizes of the less tightly crossed linked fragile Ca-alginate beads. Immobilization yield decreased on increasing alginate concentration. At 4% and 5% (w/v) sodium alginate concentration the entrapped activity of the enzyme was found very low which might be due to the high viscosity of enzyme entrapped beads, which decreased the pores size and thus hindered the substrate transfer from the bulk phase into the alginate beads [24] **Figure 3**

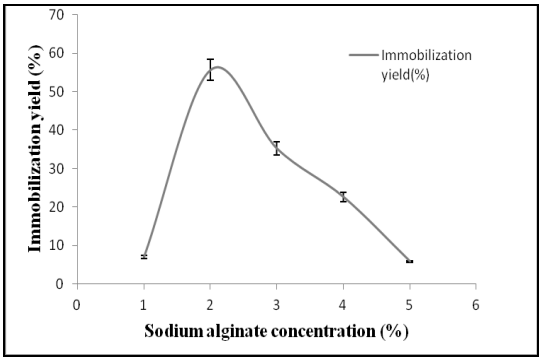

Fig 3: Effect of sodium alginate concentration on percent immobilization of lipase from *Pseudomonas ADT3* The Percentage immobilization yield peak shows maxima at 2% sodium alginate concentration. Immobilization yield decreases on increasing alginate concentration. The bar denotes standard deviation (SD). When the error bar cannot be seen, the deviation is <5%.

3.6 Effect of calcium chloride

Concentration of calcium chloride (0.05-0.3 M) was also varied keeping alginate concentration constant (2%) in order to acquire stable beads capable of securing maximum enzyme and it was found that $CaCl_2$ (0.12 M) retained highest activity of entrapped enzyme and as calcium chloride concentration increased beyond 0.12 M the activity decreased. It was also reported a decrease in the relative enzyme activity of alkaline protease when they increased the concentration

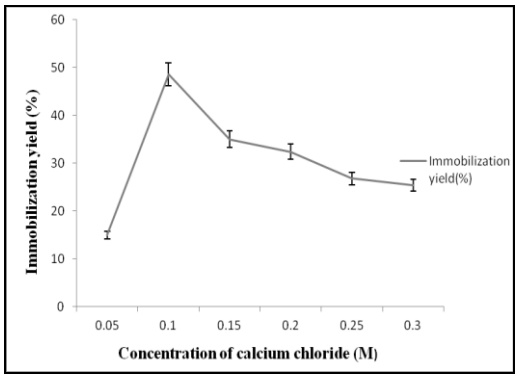

Fig 4: Effect of calcium chloride concentration on percent immobilization of lipase from *Pseudomonas ADT3* The Percentage immobilization yield peak shows maxima at 1.2mM calcium chloride concentration. Immobilization yield decreases on increasing calcium chloride salt concentration**.** The bar denotes standard deviation (SD). When the error bar cannot be seen, the deviation is <5%.

of $CaCl_2$ used to form capsule [25]. They observed that the pH of the $CaCl_2$ solution changes with its concentration which might be a factor to affects the activity of entrapped enzyme **Figure 4**

3.7 Effect of pH on activity of the free and immobilized *Pseudomonas ADT3* lipase

Effect of pH on the specific enzyme activity of both free and immobilized lipase of *Pseudomonas ADT3* was studied by varying the pH of the reaction medium from 2.0–9.0 with1.2 mM Pb as cofactor at 22°C and the pH profile is shown in **Figure 5**. The lipase was found to be nearly alkaline which showed higher specific enzyme activities for both free and immobilized enzyme at pH 8.5. In free lipase first, there is a sharp increase in enzyme activity at pH 3.5 which drops around neutral pH followed by a peak at pH 8.5 that gradually falls into a plateau. The two peaks for free enzyme were due to presence of two isoforms of the enzyme. The maximum activity of enzyme was found around pH 8.5 (alkaline) for free and immobilized lipase.

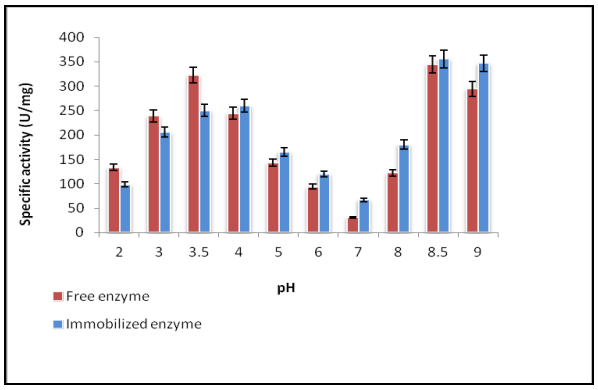

Fig 5 Effect of pH on the specific enzyme activity of free and immobilized lipase with 1.2 mM Pb at 22°C. Optimum activity could be detected for both free and immobilized lipase at pH 8.5. Error bars with 5% SEM are displayed.

It was reported that the surface of the beads in which the enzyme is localized has a cationic or anionic nature. This charged surface of beads and entrapped enzyme produces a charged microenvironment, which ultimately affects the nature of the active enzyme protein and alters the pH of the entrapped enzyme [26]. So, in the present case the surface of beads at acidic pH might produce some effect on the active lipase, thus the activity of enzyme is lowered than free enzyme. The surface of beads at neutral pH had produced no effect on the active lipase, thus the activity of free and immobilized enzyme remained almost the same. Residual activity was measured and found that immobilized enzyme was stable over a broad range of pH. This

indicated that immobilization appreciably improved the stability of lipase in alkaline region. Similar results were obtained on using immobilized *Candida rugosa* lipase on chitosan [27]

3.8 Effect of temperature on activity and stability of the free and immobilized *Pseudomonas ADT3* lipase

The temperature dependence of the hydrolytic activity of free and immobilized lipase is shown in **Figure 6**. The optimum reaction temperature (22°C) of the lipase was not altered by immobilization at pH 8.5. The immobilized lipase showed higher specific enzyme activities above 22 °C as compared to the free lipase. However enzyme activity starts decreasing at 40 °C for immobilized lipase and at 30 °C for the free enzyme. These results indicate that lipase is more stable when immobilized in a matrix with higher hydrophobicity [28].

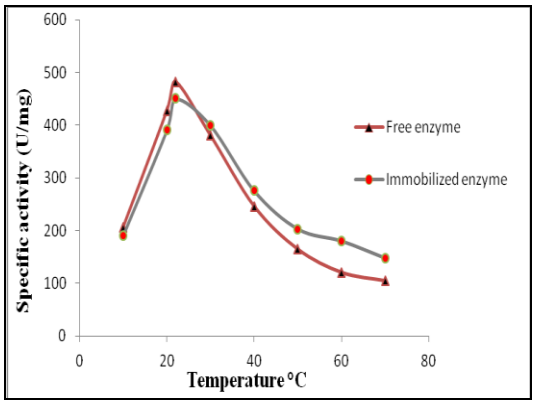

Fig 6 Effect of reaction temperature on lipase activity of immobilized and free lipase from *Pseudomonas ADT3* The lipase activity peaks shows maxima at 22°C for both free and immobilized lipase. With increase in temperature the activity decreases.

Heat stability of the lipase entrapped in alginate matrices is much better than that of the corresponding free enzyme **Figure 7**. Immobilized enzyme remained active up to 50 °C while the activity of the free enzyme started to decrease from 40 °C. At 60 °C the residual activity of the immobilized lipase was 46% compared to 10% for free enzyme. Even at 70°C immobilized enzyme retained 25% of residual activity but free enzyme totally loses enzyme activity.

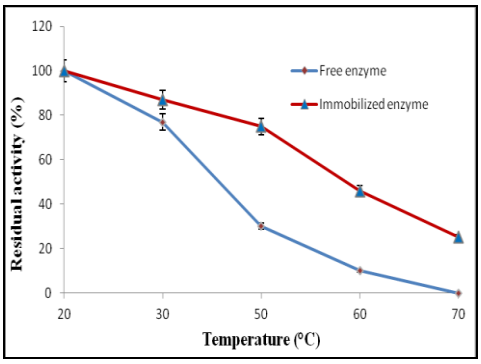

Fig 7 Thermal stability of free and immobilized lipase Residual activity of the lipase was measured at different temperature at pH 8.5. The activity of the free enzyme started to decrease from 40 °C but immobilized enzyme remained active up to 50 °. At 60 °C the residual activity of the free enzyme was only 10% compared to 46% for immobilized enzyme. Free enzyme totally loses enzyme activity at 70°C but immobilized enzyme retained 25% of residual activity. The bar denotes standard deviation (SD). When the error bar cannot be seen, the deviation is <5%.

3.9 Reusability of Immobilized lipases

One of the important characteristics of an immobilized enzyme is its stability and reusability over an extended period of time. The residual activity of the immobilized enzyme for the repeated use is shown in **Figure 8**. The enzyme showed 83% activity during the second reuse, 75% activity on its third use and 62% activity of entrapped enzyme were

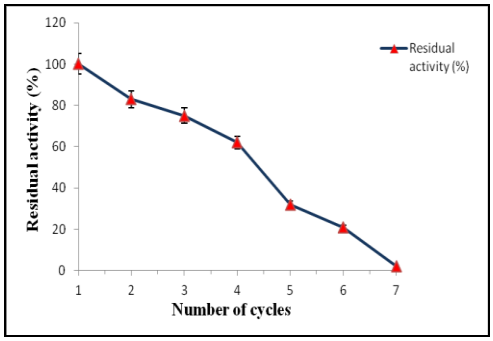

Fig 8 Effect of repeated use of immobilized *Pseudomonas* ADT3 lipase on residual activity at pH 8.5 and 22°C The immobilized enzyme can be reused up to 5cycles with residual activity of 32%. The enzyme showed 83% activity during the second reuse, 75% activity on its third use and 62% activity of entrapped enzyme were observed during the fourth cycle. The bar denotes standard deviation (SD). When the error bar cannot be seen, the deviation is <5%.

observed during the fourth cycle. This decrease in activity was due to the leakage of enzyme from the beads, occurred due to the washing of beads at the end of each cycle [17, 27].

3.10 Storage stability of enzyme

Immobilized enzyme was stored at two temperatures, viz. 4ºC and 30ºC. The activity was noted up to 10 days **Figure 9.** It was found that beads which were stored at 4ºC showed 30% loss of activity after 48 hours, 50% activity loss after 5 days and 95% loss of activity after 10 days. On the contrary beads which are stored at 30ºC showed 60% loss of activity after 48 hours, 95% loss of activity after 4 days and complete loss of activity after 5 days. These results clearly revealed the fact that the enzyme remained considerably stable at refrigeration temperature of 4ºC. It was reported 36% loss in the activity of immobilized dextransucrase at 30°C after 3 hours and 86% loss in activity at 40°C within two hours [29], also reported 96% loss of activity of dextransucrase at 40°C within two hours [30]. It was also reported the stability of alkaline proteases up to nine days after entrapment of cells of *Bacillus subtilis* PE-11 [31].

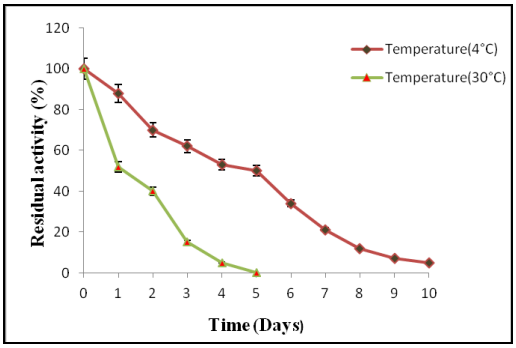

Fig 9 Effect of temperature on storage stability of immobilized lipase from *Pseudomonas ADT3* At 4°C 70% of residual activity was found after 2 days and 50 % activity after 5 days. On contrary beads stored at 30ºC showed 40% of residual activity after 2 days and 5% activity after 4 days and complete loss of activity after 5 days. The bar denotes standard deviation (SD). When the error bar cannot be seen, the deviation is <5%.

IV. Conclusion

The main objective of the present study was to increase the efficiency of the newly isolated strain of *Pseudomonas sp ADT3* from Arctic region via immobilization to produce lipase. The enzyme was partially purified approx.2.9-fold with an overall yield of 64.4 % and specific activity of 1433.8 U/mg with 80% ammonium sulphate precipitation. The immobilization yield of lipase entrapped in calcium alginate was found 70.0%. In order to acquire stable beads capable of securing maximum enzyme 2% sodium alginate and 0.12 M $CaCl_2$ was found optimum retaining highest activity of entrapped enzyme. The immobilized enzyme was found to be stable in alkaline pH. The maximal activity for immobilized enzyme was found at pH 8.5.The temperature optima for both free and immobilized remained same i.e. 22°C. But the immobilized enzyme remained active up to 50 °C while the activity of the free enzyme started to decrease from 40 °C. Even at 70°C immobilized enzyme retained 25% of residual activity but free enzyme totally loses enzyme activity. The enzyme can be reused up to 5 cycles which is a promising technique for large-scale preparation of immobilized lipase for industrial applications. Enhancement of stability of lipase from a

psychrotrophic *Pseudomonas* via immobilization leads to multiple industrial applications in food and flavour making, pharmaceuticals, biodetergents, cosmetics and perfumery. Further work needs to elucidate the immobilization of enzymes in combination with protein engineering techniques to improve enzymes of industrial significance.

ACKNOWLEDGEMENT

The authors thank the Department of Science and Technology (DST), New Delhi, Govt of India, for the grant supporting this research work. Prof P. Roy acknowledges National Centre for Antarctic & Ocean Research, Goa for facilitating the expedition to Ny-Alesund, Norway where the samples were collected.

CONFLICT OF INTERESTS

There is no conflict of interests between the authors or with any other person regarding any of the works reported or software used in this paper.

REFERENCES

[1] Sharma R., Chisti Y. and Banerjee U. C. (2001) Production, purification, characterization and applications of lipases. Biotechnology Advance 19: 627-662.

[2] Rodrigues D. S., Mendes A., Adriano W. S., and Gonçalves L. R. B. (2008) Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. Journal of Molecular Catalysis B: Enzymatic 51: 100-109.

[3] Balcão V. M., Paiva A. L. and Malcato F. X. (1996) Bioreactors with immobilized lipases. Enzyme Microbial Technology 18: 392-416.

[4] Pandey A., Benjamin S., Soccol C. R., Nigam P., Krieger N. and Soccol V. T. (1999) The realm of microbial lipases in biotechnology. Biotechnology and Applied Biochemistry 29: 119–131.

[5] Tang Y. J., Li Y. (2007) China Bio. 27: 110.

[6] Guzmán F., Barberis S., Illanes A. (2007) Electron. J. Biotechnology 10: 279.

[7] Ken D., Johnstone M., Dieckelmann, Michael P., Jennings J. T., Blanchfield I. T. (2006) Understanding Biology Using Peptides American Peptide Symposia 9: 511.

[8] Hung T. C., Giridhar R., Chiou S. H. and Wu W. T. (2003). Binary immobilization of Candida rugosa lipase on chitosan. Journal of Molecular Catalysis B: Enzymatic 26: 69-78.

[9] Gemeiner P. (1992) Enzyme Engineering: Immobilized Biosystems. Chichester UK, Ellis Horwood, Ltd.

[10] Ruckenstein E. and Wang X. (1993). Lipase immobilized on hydrophobic porous polymer supports prepared by concentrated emulsion polymerization and their activity in the hydrolysis of triacylglycerides. Biotechnology and Bioengineering 42: 821-828.

[11] Fraser J.E. and Bickerstaff G.F. (1997). Entrapment of enzymes and cells in calcium alginate. In Immobilisation of Enzymes and Cells, Humana Press, pp: 61-66.

[12] Dey A., Chattopadhyay Amarnath, Mukhopadhyay Subhra Kanti, Saha Pradipta, Chatterjee Sabyasachi , Maiti Tushar Kanti and Roy Pranab (2014) An Approach to the Identification and Characterisation of a Psychrotrophic Lipase Producing Pseudomonas sp ADT3from Arctic Region. Advances in Bioscience and Biotechnology 5: 322-332

[13] Choo D.W., Kurihara T., Suzuki T., et al. (1998) A Cold-Adapted Lipase of an Alaskan Psychrotroph. Pseudomonas sp. Strain B11-1: Gene Cloning and Enzyme Purification and Characterization. Applied and Environmental Microbiology, 64: 486-491. http://dx.doi.org/10.1007/s11947-008-0104-8

[14] Coligan J. E., Dunn B. M., Speicher D.W., Wingfield P. T., and Ploegh H. L., Eds.(1998) Current Protocols in Protein Science, JohnWiley & Sons, Chichester, UK.

[15] Amada K, Haruki M, Imanaka T, Morikawa M, Kanaya S (2000) Overproduction in Escherichia coli, purification and characterization of a family I.3 lipase from Pseudomonas sp. MIS38. Biochem Biophys Acta 1478: 201-210.

[16] Watnabe N, Ota Y, Minoda Y, Yamada K (1977) Isolation and identification of alkaline lipase producing micororganisms, cultural conditions and some properties of crude enzymes. Agric. Biol. Chem. 41: 1353-135.

[17] Won K., Kim S., Kim K. J., Park H. W. and Moon S. J. (2005). Optimization of lipase entrapment in Ca-alginate gel beads. Process Biochemistry. 40: 2149- 2154.

[18] Ellaiah P., Prabhakar T., Ramakrishna B., Thaer Taleb A. and Adinarayana K. (2004). Production of lipase by immobilized cells of Aspergillus niger. Process Biochemistry 39: 525-528.

[19] Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

[20] Rio Del, Serra J. L., Valero D. , Poch F. M. and Sola C. (1990). Reaction scheme of lipase production by Candida rugosa growing on olive oil. Biotechnology Letters 12: 335-338.

[21] Kanwar L. and Goswami P. (2002). Isolation of Pseudomonas lipase produced in pure hydrocarbon substrate and its application in the synthesis of isoamyl acetate using membrane immobilized lipase. Enzyme and Microbial Technology 31: 727-735.

[22] Jamil N. I. and Omar I. C. (1992). Lipase production using Asperiglus niger. Journal of Biosciences 3: 31- 41.

[23] Ferrer P. and Carles S. (1992). Lipase production by immobilized Candida rugosa cells. Applied Microbiology and Biotechnology 37:737-741.

[24] Knezevic Z., Bobic S., Milutinovic A., Obradovic,B., Mojovic L., Bugarski B. (2002). Alginate immobilized lipase by electrostatic extrusion for the purpose of palm oil hydrolysis in lecithin/isooctane system. Process Biochemistry 38: 313-318.

[25] Roig M.G., Rashid D.H. and Kenndy J.F. (1995). High-alkaline protease from Bacillus PB92 entrapped in calcium alginate gel: Physicochemical and microscopic studies. Appl. Biochem. Biotechnol. 55: 95-121.

[26] Norouzian D. (2003) Review, Enzyme immobilization,the state of art in Biotechnology, Iranian J. Biotechnol. 1: 197-206.

[27] Hung T. C., Giridhar R., Chiou S. H. and Wu W. T. (2003). Binary immobilization of Candida rugosa lipase on chitosan. Journal of Molecular Catalysis B: Enzymatic 26, 69-78.

[28] Shaw J. F., Chang R. C., Wang F. F. and Wang Y. J. (1990). Lipolytic activities of a lipase immobilized on six selected supporting materials. Biotechnology and Bioengineering 35: 132-137.

[29] Qader S.A.U.l., Aman A., Syed N., Bano S. and Azhar A. (2007). Characterization of dextransucrase immobilized on calcium alginate beads from Leuconostoc mesenteroides PCSIR-4. Ita. J. Biochem., 56: 158-162.

[30] Chellapandian M., Larios C., Sanchez-Gonzalez M., and Lopez-Munguia A. (1998). Production and properties of a dextransucrase from Leuconostoc mesenteroides IBT-PQ isolated from' pulque', a traditional Aztec alcoholic beverage. J. Ind. Microbiol. Biotechnol. 21: 51-56.

[31] Adinarayana K., Bapi Raju K.V.V.S.N. and Ellaiah P. (2004). Investigations on alkaline protease production with B. subtilis PE-11 immobilized in calcium alginate gel beads. Process Biochem. 39: 1331-1339.

AUTHORS

First Author – Arpita Dey, Msc. in Biotechnology, Department of Biotechnology (Recognized by DBT-Govt. Of India), The University of Burdwan, West Bengal-713104, India., e-mail address: arpitadey086@gmail.com
Second Author – Dr. Tuhar Kanti Maiti, Doctorate in Botany, Department of Botany, The University of Burdwan, West Bengal-713104, India., e-mail address: tkmbu@yahoo.co.in

Third Author – Prof. Pranab Roy, Post Doctorate, Department of Biotechnology, Haldia Institute of Technology, West Bengal, India., e-mail address: pranabroy@rediffmail.com

Correspondence Author – Arpita Dey, Msc. in Biotechnology, Department of Biotechnology (Recognized by DBT-Govt. Of India), The University of Burdwan, West Bengal-713104, India., e-mail address: arpitadey086@gmail.com, Mobile number: 09474787058, 09775270675, Fax number: 091342-2657231