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Immobilization of lipase from *Pseudomonas* sp. Lp1 by different techniques

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ABSTRACT

Psudomonas sp.Lp1 extracellular lipase was immobilized by three different techniques such as entrapment in alginate, physical adsorption using celite, and ionic binding to two different kinds of cation exchangers Amberlite IR120 (Na^+) and IRCSO (H^+). The objective of this study was to develop a suitable method for immobilizing lipase. The stability of the free and immobilized lipase at different pH, Temperature and Metal ions and its storage stability were also evaluated. Among the three techniques, the immobilization by ionic binding with the cation exchangers -Amberlite IR120 (Na^+) and IRCSO (H^+) showed greater efficiency of 73% and 78% respectively. The immobilized lipase showed improved stability when compared to free lipase. The stability of lipase in celite, Amberlite IR120 (Na^+) and IRCSO (H^+) at pH 8.0 was 87.3%, 94.6% and 96.7% respectively. The immobilizates -Amberlite IR120 (Na^+) and IRCSO (H^+) showed 65% and 68% of enzyme activity even at 60°C. The activity retained by immobilized lipase by ionic binding with Amberlite resin IR120 (Na^+) and Amberlite IRCSO (H^+) in the presence of Ca^{2+} ion was higher (152% and 140%) than any other methods of immobilization respectively. The ionic binding method of immobilization retained 70% of its original activity till 20 days of storage at 4°C.

KEYWORDS

Pseudomonas;
Immobilization;
Adsorption;
Entrapment;
Ionic binding.

INTRODUCTION

Lipases (triacyl glycerol ester hydrolase, EC 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. They are being employed for the synthesis of drug intermediates and pharmaceutically important molecules as well as for the resolution of racemic mixtures for absorbing physiologically active enantiomers[1] Lipases find promising applications in organic chemical processing, detergent formulations, and synthesis of biosurfactants, oleo chemical industry, dairy industry, agrochemical industry, paper manufacture, nutrition, cosmetics and pharmaceuticals. The lipases used are usually of fungal

or bacterial origin: *Pseudomonas* being the most important bacterial genus[2,3].

Immobilized means it has been confined or localized so that it can be reused continuously. The first attempt to immobilize a biocatalyst back to 1953, while in 1969 an immobilized enzyme was used for the first time in an industrial process, since then this technique has gained more and more importance, and now a wide variety of immobilized enzymes are employed in the food, pharmaceutical and chemical industries. Although lipases presently account for no more than 3% of all enzymes produced worldwide, the use of immobilized lipases for the modification of melted fats and oils is currently a subject of expanding interest[4]. There are several reasons for using an enzyme in an immobilized form. It provides convenient handling of the enzyme, it provides for its facile separation from the product, thereby minimizing or eliminating protein contamination of the product. Immobilization also facilitates the efficient recovery and reuse of costly enzymes, and enables their use in continuous, fixed-bed operation. A further benefit is often enhanced stability under storage and operational conditions[5].

The high cost of lipase, however, makes enzymatically driven processes economically unattractive. The use of immobilized lipase is a possible solution to this problem because the enzyme can be recovered from the product and reused[6]. The reuse of lipase provides cost advantages that are often an essential prerequisite for establishing a lipase-catalyzed process[7,8]. From an industrial point of view, immobilized lipases offer economic incentives of enhanced thermal and chemical stability, ease of handling, easy recovery and reuse relative to non immobilized forms[9]. Immobilized enzymes have received considerable attention because of their advantages over unimmobilized counter parts as they improve storage and operational, thermal and conformational stabilities. They are easily recovered for reuse[10]. Numerous methods for achieving the immobilization of lipases is available; each involves a different degree of complexity and efficiency. The various methods used to date are: adsorption, ionic bonding, covalent binding, cross-linking, entrapment, and encapsulation[11].

The problem of selecting the support material and the proper technique is very important and therefore the pursuit for suitable materials has not yet ceased. In the search for suitable and low cost materials in this study the lipase from *Pseudomonas* sp.Lp1 was immobilized in four different ways, *i.e.*, entrapment in alginate, physical adsorption using celite, and ionic binding to two different kinds of cation exchangers Amberlite IR120 (Na^+) and IRCSO (H^+). The activities of the immobilizates of *Pseudomonas* sp.Lp1 lipase was assessed in an aqueous medium by the hydrolysis of p- Nitrophenyl palmitate (pNPP) in the reaction system. The factors investigated n this study were the, the pH, temperature, effect of metal ions on the immobilizates, as well as the storage stability. The objective of this study was to develop a suitable method for immobilizing lipase from *Pseudomonas* sp.Lp1already reported by us[12]. Many investigators have studied lipase immobilization, but detailed stability of each immobilizate has been lacking. Therefore, present study was the focus on establishing these factors.

EXPERIMENTAL

Microorganism and crude lipase

Lipase producing *Pseudomonas* sp.Lp1 isolated from edible oil contaminated soil was maintained by repeated subcultures in the broth medium at 40°C containing olive oil 1% (v/v) as the carbon source and inducer of lipase. The medium contained Peptone-5 g; Yeast extract-5 g; NaCl-0.5 g/l; $CaCl_2$-0.05 g/l; Olive oil-10/l ml (emulsified with gum acacia- 0.5%) at pH-8.0. About 100 ml of this sterile production broth was prepared in 250 ml conical flask and 3% inoculum was transferred aseptically in to the production medium. The flasks were incubated overnight at shaker incubator at 40°C, pH 8.0 and 150 rpm. Culture broth was harvested by centrifugation (10,000 *g*, 10min, and 4 °C) and referred as the crude extract of the enzyme. The enzyme activity was determined in the cell-free broth thereof and immobilized.

Lipase assay

Extracellular lipase activity was estimated by the colorimetric method[13] by measuring the micromoles of 4-nitrophenol released from 4-nitrophenyl palmitate. A stock solution (20mM) of 4-nitrophenyl palmitate (4-NPP) was prepared in isopropanol. The reaction mixture contained 150 µl of 4-nitrophenyl palmitate, 100 µl enzyme solution and 0.1M Tris buffer (pH 8.5) was

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made a final volume of 3 ml was incubated at 55ºC for 10 min in water bath. Then it was chilled at -20ºC for 10 min to stop the enzyme reaction. The absorbance was measured at 410 nm (Elico). The reaction mixture contained heat inactivated enzyme was used as control. The standard was prepared using (2µg-40µg) concentrations of 4-nitrophenol. One unit (U) was defined as the amount of enzyme catalyzing the liberation of 1 µmol *p*-nitrophenol/min under the assay conditions.

Determination of protein concentration

Protein analysis of the different supernatants was determined spectrophotometrically[14]. The different protein concentrations were derived from Bovine serum Albumin standard curve.

Methods of lipase immobilization

Method-1 immobilization by entrapment

Immobilization with calcium alginate is an entrapment method in which the enzyme solution was mixed with 2% sodium alginate in the ratio of 1:1(v/v). The mixture was slowly dropped into 10 ml 0.1M $CaCl_2$ under continuous stirring using a Pasteur pipette. This resulted in the polymerization of the alginate to form calcium alginate gel beads of about 3-5 mm diameter were formed within 3 to 5 min. Gel beads with entrapped enzyme were collected by filtration through a sieve. The total weight of the gel beads obtained was measured, and this was taken as the amount of beads having the total activity of the 1 ml enzyme used for immobilization (if 1ml enzyme solution was added with 1 ml of sodium alginate). The beads were kept in $CaCl_2$ solution until used. Only the amount of gel beads that contained approximately 150 µl enzyme was used for each lipolytic assay. The activity of bound enzyme and unbound lipase remaining in the calcium chloride solution used for immobilization was measured.

Method-2 immobilization by physical adsorption

Lipase adsorption was carried out at 4ºC by mixing enzyme solution and celite (10:1 ratio) under low stirring by using a magnetic stirrer. At different time intervals, 5 ml of sample was removed and centrifuged for 10 min at 15000 rpm. The supernatant was then analyzed for determining the remaining lipase activity[15]. The unbound enzyme solution was removed and the immobilizate was vacuum filtered[16].

Method-3 immobilization by ionic binding

For the ionic binding, cation exchangers on polystyrene such as Amberlite IR120 (Na^+) and IRCSO (H^+) were used. The resins, both types were first equilibrated in phosphate buffer 50 mM ; KH_2PO_4-0.68 g; Na_2HPO_4-0.709 g; Distilled water-100 ml, pH 7.0, at room temperature over night. After vacuum filtration, the resins were incubated with the crude lipase in phosphate buffer at 4°C for 1 h. Resin was mixed with enzyme solution in the ratio of 1:1 was (w/v). The mixture was continuously stirred in 10 ml 0.05 M phosphate buffer, at 37°C for 30 min. Resin immobilized enzyme was collected using vacuum filtration and weighed. The activity of unbound lipase remaining in the buffer solution used for immobilization was measured. The vacuum filtered immobilizates were used as obtained[17].

Evaluation of the immobilization techniques[16]

The efficiency of the immobilization techiuque η was estimated by calculating the lipolytic activity of the lipase solution before (E0) and after immobilization (Ef), and the volumes of the solution before (V0) and after (Vf), using the relation,

$$\eta = \frac{(E0V0 - EfVf)}{V0E0 \times 100}$$

The activities are given in U/ml, and the volumes are in mL.

The availability activities of the immobilized *Pseudomonas* sp.Lp1 was estimated as the activity retention,

$$\text{Activity retention}(\%) = \frac{\text{Immobilized lipase activity}}{\text{Free lipase activity} \times 100}$$

pH stability of free and immobilized lipase

The pH stability of both the free and immobilized lipase was determined by keeping the enzyme solution 4ºC at for up to 24 h in various buffers in equal volumes at pH (4, 5, 6, 7, 8, 9, 10 and 11). The enzyme activity retention of both free and immobilized enzyme was evaluated and at time zero it was considered as 100%.

Thermal stability free and immobilized lipase

The thermal stability of both the free and immobilized lipase was determined by measuring the residual enzymatic activity by preincubating them at different temperatures such as 30ºC, 40ºC, 50ºC,

60ºC, 70ºC, and 80ºC. The activity retention was determined after one hour.

Effect of metal ions and EDTA on lipase stability

The effect of metal ions on both the free and immobilized lipase was determined after preincubating the enzyme with various metal ions (1.0mM) viz., Fe^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, K^{+}, Ca^{2+} and EDTA separately in equal volumes and incubated at 40°C. The activity retention was determined after one hour.

Storage stability immobilized lipase

Immobilized lipase was stored at 4°C in a refrigerator for one month and the residual lipase activity was analyzed for every two days using 4-Nitrophenyl palmitate substrate.

RESULTS AND DISCUSSION

The production of extracellular lipase from *Pseudomonas* sp.Lp1 isolated from edible oil contaminated soil was carried out in the production medium with olive oil (1%) as the carbon and inducer for the enzyme production. The hydrolysis of 4-Nitrophenyl palmitate into 4-Nitrophenol by extracellular lipase produced was measured to evaluate the lipase activity and the lipase activity of culture supernatant was found to be 78.0 U/ml.

Lipase immobilization

The lipase from *Pseudomonas* sp.Lp1was immobilized with different carriers using different methods of immobilization. The efficiency of the enzyme immobilization was evaluated and the stability of the free and immobilized lipase at different pH, temperatures, metal ions and the storage stability of free and immobilized lipase was evaluated.

Efficiency of immobilization

Entrapment

Physical entrapment in a polymeric gel microsphere is by far the most commonly used technique for enzyme immobilization and true successes may be limited to the problems associated to the mass transfer resistance imposed by the fact that the substrate has to diffuse to the reaction site and mechanical stability of the carriers in bioreactors[18]. Lipase immobilization by entrapment is based on low porosity of matrix which at the same time retains enzyme within the carrier and provides substrate/or products diffusion[19]. In the present study the entrapment of lipase was established in calcium alginate beads of 3- 5 mm diameter size. The activity of bound enzyme and unbound lipase remaining in the calcium chloride solution used for immobilization was measured to give the efficiency of immobilization which was found to be 48 % (Figure 1).

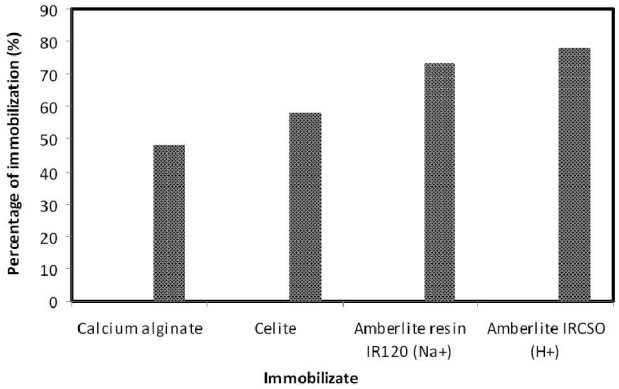

Figure 1 : Efficiency of immobilization of lipase from *Pseudomonas* sp.Lp1 by different techniques

When compared with other methods of immobilization in the present study the efficiency is less which might be due to the reasons as described in earlier studies. This method has the inevitable disadvantage that the support will act as a barrier to mass transfer and restricted movement of enzyme in the lattice structure of gel[4]. In contrast to our results the purified lipase from *Pseudomonas aeroginosa* BTS- 2 was 80% efficiently immobilized in calcium alginate than carrageenan beads[9], the soluble bioconjugate of *Pseudomonas cepacia* lipase exhibited increased enzyme activity in terms of high effectiveness factor[20] and entrapment of lipase from *Rhizopus recemosus* NRRL 3631 in a 4% calcium aiginate gel beads with the yield of 82 %[21]. Similar to our results the studies on immobilization of lipase producing *Arthrobacter* sp. (RRLJ-1/95) after optimization of all the parameters, the entrapped lipase cells yielded 40% of lipase activity[22]. Alginate as an immobilization medium has been extensively studied, mainly due to its ease of formulation, mild gelation, biocompatibility and acceptability as food additive and as oral drug delivery systems. However there is limitation, alginate gels have a wide pore distribution due to the open lattice structure of the matrix. Larger pores may result in enzyme leakage or release, or access to undesired reactants[23].

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Physical adsorption

Celite is an inexpensive solid support obtained from the fossilized silica remains of diatoms and is commercially available in different forms, which vary in particle size, shape and porosity widely, employed for the adsorption or deposition of biocatalysts[24]. Lipase immobilization by adsorption is the simplest method involving reversible hydrophobic surface interactions between enzymes and support material with the advantages of cheap, fast and simple process, no chemical changes to support or enzymes are necessary and a reversible immobilization. Disadvantages are the leakage of the enzyme from the support, the possible steric hindrance by the support and the nonspecific binding[4]. In the present study of physical adsorption, the enzyme solution was bound to celite powder by mixing the enzyme solution with celite in the ratio of 10:1 (v/w). The efficiency of immobilization was 58% (Figure 1). Our results coincides with the earlier reports[25] in which the The highest specific hydrolytic activity (0.71 U mg-1) and esterification activity (26%) were obtained from immobilized lipase on celite *Aspergillus oryzae* and *Aspergillus niger* respectively. But the addition of salts enhanced the efficiency of immobilization in the lipase from *Acinetobacter radioresistens* adsorption on Celite 535 where the highest specific activity of 167% of the support was found when the enzyme solution contains 100 mM NH_4Cl[15].

Ionic binding

The immobilization by ionic binding is based on electrostatic interactions between differently charged ionic groups of the matrix and of the enzymes. The advantages and disadvantages are the same of the adsorption process, but through ionic binding the enzyme conformation is influenced more than through adsorption and less than through covalent binding[4]. In the present investigation in the ionic binding, cation exchangers on polystyrene such as Amberlite IR120 (Na^+) and IRCSO (H^+) were used in which the immobilization efficiency of 73% and 78% respectively (Figure 1). Among the other methods of immobilizates ionic binding, cation exchangers such as Amberlite IR120 (Na^+) and IRCSO (H^+) showed efficient immobilization. Similar to our results many researchers obtained good percentage of immobilization on resins. *Rhizopus deleniar* showed good a growth on Amberlite resins and synthesized lipase[26]. Lipase (Lip094) from *Aeromonas sobria* immobilized by ionic binding with Amberlite resins IR120 (Na+)- and IRCSO (H+)- in which the resins were stable, and could be used to immobilize the enzyme repeatedly[27]. Lipase was from *Candida rugosa* Amberlite XAD-7 adsorbed most (65%) whereas celite adsorbed the least at about 46%[28]. The immobilized form *Pseudomonas* sp. with Amberlite XAD-7 resin was found to be a better support retaining 92.85% of the initial activity in the formation of 1,4:3,6-dianhydro-D-glucitol 2-acetate and its isomeric 5-actetate derivatives[29]. Among various immobilizates tested with lipase from *Candida rugosa* many exhibited either a low enzyme activity or difficulties during the hydrolytic reaction except those prepared by ionic adsorption on Amberlite IRC-50 showed better activity with 2000 U/g support[16].

pH stability

Both free and immobilized enzyme showed their optimum stability at pH 8.0. The free enzyme showed optimum stability at pH 8.0 by retaining its activity of 85%. The enzyme retained 55% and 30% of its activity at pH 10.0 and pH 11.0 respectively at 12 hours of incubation. The immobilized enzyme by calcium alginate showed its maximum stability at the pH of 8.0 with 86.2% when incubated for 24 hours. The stability of enzyme was retained for increased incubation periods in all methods of immobilization when compared to free enzyme. The stability of lipase in celite, Amberlite IR120 (Na^+) and IRCSO (H^+) at pH was 87.3%, 94.6% and 96.7% respectively (Figure 2).

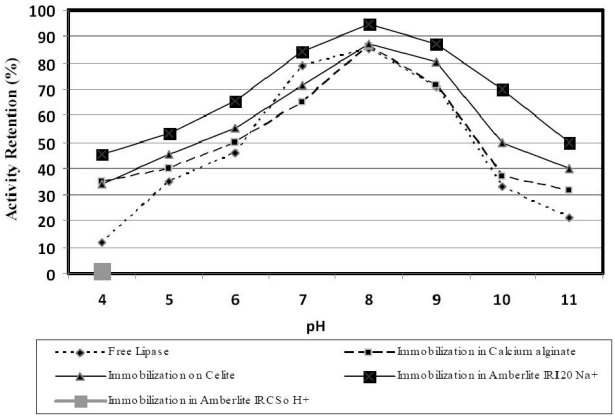

Figure 2 : pH stability of free and immobilized lipase from *Pseudomonas* sp.Lp1

The immobilized lipase in beads of calcium alginate and carrageenan beads showed the maximum lipase activity at pH 7.5[9]. The immobilized lipase from *Rhizo-*

pus recemosus NRRL 3631 remained nearly stable at a pH range of 7.0-5.0 whereas the free enzyme showed a sharp decrease from 77.11- 46.73% of lipase activity[21]. *Arthrobacter* sp. (RRLJ-1/95) immobilized in alginate beads was stable at pH 7; however, at pH 6, 8, 9, and 5, the residual activity obtained after 96 h of incubation was 85, 85, 40, and 20%, respectively whereas the free enzyme, became completely inactive within 24 h when incubated at pH 4 and 5[22]. Both the free and immobilized preparations of *Aspergillus niger* lipase on celite showed similar biochemical properties, with maximum activity at pH 6.0[25]. The immobilized *Candida rugosa* preparation with Amberlite IRC-50 was stable and active in the whole range of pH from 4 to 9[16].

Temperature stability

The thermal stability was studied by The thermal stability of the lipase in Ca-alginate was markedly increased after immobilization in all the immobilization methods when pre-incubating both the free and immobilized enzyme at various temperatures ranging from 30°C to 80°C for 1 h.. The free enzyme showed only 48% of activity at 40°C when incubated for 1h whereas the immobilized enzyme in Ca-alginate retained 62.5% of its activity, 65.3% in physical adsorption by celite, 76.2% in Amberlite resin IR120 (Na^+) and 80.5% in Amberlite IRCSO (H^+) when incubated for 1h . The immobilizates -Amberlite IR120 (Na^+) and IRCSO (H^+) showed 65% and 68% of enzyme activity at even at 60°C. The immobilized lipase exhibited 50% and 54% of its activity in the temperatures of 30°C and 50°C. In both the ionic binding agents the enzyme retained 53.2% and 51.5% of its activity when incubated for beyond 1h (90 minutes) whereas in all the other methods the enzyme inactivated (Figure 3).

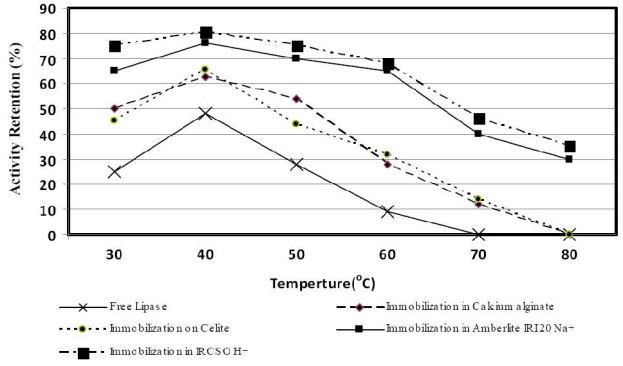

Figure 3 : Temperature stability of free and immobilized lipase from *Pseudomonas* sp.Lp1

The bioconjugate of *Pseudomonas cepacia* lipase immobilized with calcium alginate was more stable at 55°C as compared to the free enzyme[20]. Thermal stability studies of *Candida rugosa* immobilized lipase in calcium alginate beads and chitosan coated calcium alginate beads were stable throughout the temperature range of 30-60°C, however, free lipase became less stable at temperatures higher than 50°C[23]. At 60°C the immobilized lipase of *Rhizopus recemosus* in calcium alginate 4%, preserved about 40.74% of its original activity[21]. A significant improvement in the thermal stability of immobilized lipase on celite from *Aspergillus niger* compared to the native form was observed. The immobilization on Celite seems to play an important role in the stabilization of the enzymatic protein conformation and its resistance to thermal denaturation[18]. Both the free and immobilized preparations of *Aspergillus niger* lipase on celite showed similar biochemical properties, with maximum activity at a temperature of 30-40°C. The most important effects observed when the lipase was immobilized were thermal stability and an improved esterification activity[18]. The immobilized preparation of lipase from *Candida rugosa* with Amberlite IRC-50 was stable and active in the whole range of pH (4 to 9) and temperature (20 to 50 °C), demonstrating a 99% degree hydrolysis[16].

Stability in the presence of metal ions and EDTA

The addition of metal ions such as Ca^{2+} and K^+ enhanced the activity of both free and immobilized enzyme from *Pseudomonas* sp.Lp1 when incubated at 40°C for 30 minutes. The metal ions such as Na^{2+}, Mg^{2+} and Mn^{2+} did not alter the activity of the enzyme in which the enzyme was stable and retained its original activity. The metal ions Cu^{2+}, Fe^{2+} and Zn^{2+} inhibited the stability of the enzyme. The presence of EDTA deactivated the enzyme activity. In all the methods of immobilization the activity retention was higher than the free soluble form of enzyme. The activity retained by lipase immobilized by ionic binding with Amberlite resin IR120 (Na^+) and Amberlite IRCSO (H^+) in the presence of Ca^{2+} ion was higher (152% and 140%) than any other methods of immobilization respectively. The activity retained by Amberlite resin IR120 (Na^+) and Amberlite IRCSO (H^+) immobilized enzyme was greater than free enzyme such as 115.5% and 120%

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respectively in Mn^{2+} respectively and 128% and 125% in K^+ respectively (Figure 4).

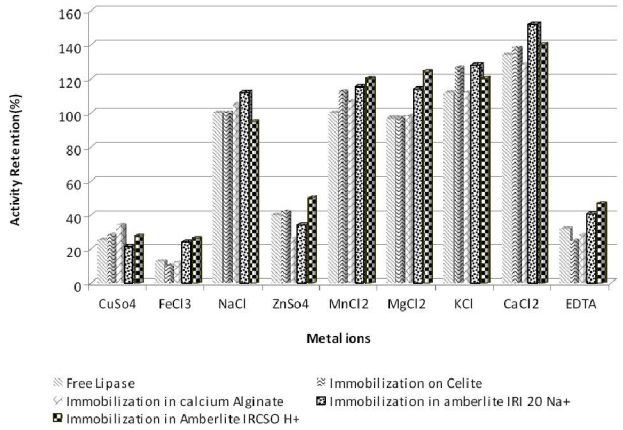

Figure 4 : Stability of free and immobilized lipase from *Pseudomonas* sp.Lp1 on metal ions and EDTA

Effect of metal ions could be attributed to the change in the solubility and the behavior of the ionized fatty acids at the interface and from a change in the catalytic property of the enzyme itself[30]. The immobilized lipase of *Bacillus* sp.Mg^{2+}, Na^+ and Li^+ salts increased activity by 63%, 63% and 23% respectively whereas Hg^{2+} and Cd^{2+} had a strong inhibitory effect. Lipase fully retained its activity in Ca^{2+}, Fe^{2+} and K^+ salts. The lipase lost 40% of its activity with 10 mM EDTA (in absence of additional metal ion[31]. Ca^{2+}, K^+, Na^+ ions and EDTA activated the lipase of the immobilized *Pseudomonas* sp. KLB1 lipase Entrapment in sol-gel from rice husk ash and the free enzyme whereas Mg^{2+}, Fe^{2+} and ascorbic acid inhibited enzyme activity. All of the lipases immobilized by entrapment played higher activity than soluble form[32].

Storage stability

Immobilized lipase was stored at 4°C in a refrigerator for one month and the Activity retention by lipase was analyzed for every two days. The free enzyme lost its 50% of its activity within 15 days of storage. The results showed that the stability of immobilized enzyme was greater than the free enzyme in all methods of immobilization. The stability was higher in ionic bonding method of immobilization in which they retained more than 70% of its original activity till 20 days of storage after that the enzyme activity was ceased. The calcium alginate immobilizate retained only 50% activity till 17 days where as the celite retained nearly half if it's original activity after one month (Figure 5).

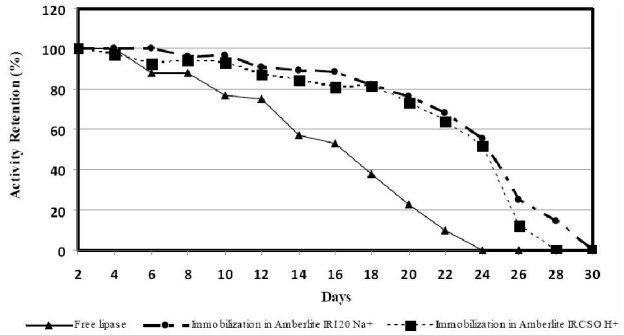

Figure 5 : Storage stability of free and immobilized lipase from *Pseudomonas* sp.Lp1

In contrast the lipase-producing strain, *Arthrobacter* sp. was immobilized in calcium alginate beads by entrapment stored at 4–20°C for 1 month where no loss in activity was observed during this duration[22]. The immobilized *Aspergillus niger* lipase on celite maintained an esterification activity of at least 73% after 5 days of storage at 40°C[25]. The storage stability of Amberlite IRC-50 immobilized lipase from *Candida rugosa* was too remarkable too after 9 months it had lost only 25 % of the initial activity. The immobilizate with Al_2O_3 was less stable and less active[16]. Immobilized lipase from *Aeromonas sobria* in Amberlite resins was found to retain more than 90% activity after 15 days storage at room temperature[27] and at 4°celite adsorption immobilized lipase from the yeast *Candida rugosa* DSM 70761showed 73% residual activity after more than 1 month, by comparison with only 48% residual activity for the immobilized lipase from the yeast *Yarrowia lipolytica* ATCC 8661[33].

The stability of the immobilized lipase was improved when compared with that of the free one. The efficiency of immobilization was greater in the method of ionic binding with Amberlite resin IR120 (Na^+) and Amberlite IRCSO (H^+) when compared to immobilization in calcium alginate and celite. The lipase immobilized with ionic resin showed more stability for longer duration at pH 8.0 and temperature at 60°C. The stability of the immobilized lipase was greater than free lipase. The results revealed that the lipase from *Pseudomonas* sp.Lp1 could be used for production of certain high-value compounds in immobilized state.

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