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Immobilization of Haloalkalophilic Lipase from *Bacillus Cereus MS6* Bacteria and its Characterization

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Abstract

Lipases belong to the class of hydrolases which are able to catalyze the hydrolysis of long chain triglycerides and many other reactions including ester synthesis and transesterification in organic media containing minute concentration of water. The immobilization of enzyme is an important strategy to improve desirable features of enzymes besides enhancing the stability of the enzyme and to obtain additional beneficial properties via immobilization process. In addition, the immobilized enzyme could be used repeatedly or continuously. In our study, the isolated lipase produced by *Bacillus cereus* MS6 was immobilized on 3% sodium-alginate beads by the entrapment methods. The alginate bead was prepared as an aqueous mixture of sodium alginate, the enzyme and $CaCl_2$ to increase its reusability, and overall enzyme stability. Various parameters such as alginate and $CaCl_2$ concentration, lipase concentration loading and bead size were evaluated for the optimum immobilization yield. It was observed that with an increase in alginate concentration, the yield of immobilized enzyme was also increased up to a certain limit. A similar case was observed with $CaCl_2$ addition, the optimum concentrations of alginate and $CaCl_2$ were observed to be 3% and 2% (w/v), respectively. The maximum production and activity of immobilized lipase were 180 mg/ml and 550 U/ml, respectively with an observed yield of 88.70 %, while the maximum activity of free enzyme was 450 U/ml with an activity yield of 40%. An increased beads size resulted in the decrease of the yield of immobilization. These lipase immobilized beads could be reused for many cycles for the hydrolysis of triglycerides without any loss in the activity. The entrapped lipase was more stable over a wide range of temperatures, pH, and storage time as compared to free enzyme. The catalytic properties of the immobilized lipase were also compared with that of the free enzyme. The optimum pH of the free enzyme was found to be 9.0 at a temperature maximum of 40^0C, while that the immobilized of lipase was pH10.0 at a temperature maximum of 50^0C. The optimum substrate concentration for free and immobilized enzyme was found to be 2% and 3%, respectively. The overall characterization data reveals an increase in the stability of the immobilized lipase as compared to free lipase.

Keywords: *Bacillus cereusMS6*, immobilization of lipase, Sodium alginate beads, entrapment method

Introduction

Lipases (Triacylglycerol acylhydrolase EC. 3.1.1.3) catalyze the hydrolysis of long chain triglycerides into free fatty acids and glycerol at the interface of emulsified lipase substrates. These liberated fatty acids will further be oxidized endogenously to get energy while the glycerol moiety produces energy to some specific tissues through oxidation procedure [1]. Further, the lipases are able to catalyze many reactions with a preference to water-insoluble substrates and are also able to catalyze ester synthesis and transesterification in organic media containing minute water concentration [2]. The lipase is a ubiquitous enzyme of considerable not only of physiological significance and industrial potential but also the enzyme has a wide range of application in food technology, dairy, detergent, textile and pharmaceutical industries [3]. The lipase may be produced by various sources such as plants, animals and microorganisms. The microbial lipases are commercially important because of their unique properties and the ease of bulk extracellular production compared to lipase from other natural sources. Most of the lipase research focused on the production of extracellular enzyme though a wide variety of microorganisms such as fungi, bacteria and archea [4].

Thus, among the lipase producing bacteria, several species of *Bacillus* such as *Bacillus subtitles*, *Bacillus pummels*, *Bacillus licheniformis*, and *Bacillus sphaericus possess*are of prime importance [5]. Among these, the majority of bacterial lipases are glycoprotein but some bacterial lipases are lipoproteins and a few are thermo stable and these enzymes remained active at the interface of aqueous and non-aqueous phases which distinguished them from esterase [6].Therefore, most lipases are mesophilic enzymes which could not hydrolyze a substrate that existed in solid form at room temperature and thermophilic lipase showed higher thermo stability, higher activity at elevated temperature and often showed more resistance to chemical denaturation [7]. Lipase is suitable for biotechnological applications and in addition, alkalophilic and thermophilic microorganisms have been focused for many studies and new sources of lipase are found to be highly stable and function optimally at extreme alkaline pH values and high temperatures. Further, the lipase production from these sources depends upon a number of factors including, carbon and nitrogen sources, pH, temperature, aeration, substrate and inoculums size [8].This will consequently enhances the ability of the purified lipase produced from microorganisms to efficiently hydrolyze various substrates.

The immobilization of enzyme is an important strategy to improve desirable features of conventional heterogeneous catalysts on to biological catalysts besides enhancing stability of the enzyme and to obtain additional beneficial properties via immobilization process [9]. In addition, the immobilized enzyme could be used repeatedly or continuously in a variety of reactors and can be easily separated from soluble reaction products and unreacted substrate, thus, simplifying work-up and preventing protein contamination in final products. Immobilization process often stabilizes an enzyme thereby allowing its applications under harsher environmental conditions, such as pH, temperature and organic solvents. The basic concept for enzyme immobilization is either to covalently attach or capture the protein in support materials, thus preventing the enzyme from leaving while allowing substrates, products and co-factors to filter through to the enzyme [10].

The immobilization of lipase has earlier been carried out by entrapment method which has been more extensively used for the immobilization process and commonly used matrix for entrapment was polyacrylamide, sodium alginate, sol–gel powder, and cellulose acetate [11]. The lipase from *Candida Ragusa* was immobilized in the polymer of poly vinyl alcohol, alginate and boric acid [12]. This immobilized enzyme was reused for 10 cycles without any loss in the activity [12]. *Candida Ragusa* lipase was also immobilized in sodium-alginate beads and used in the hydrolysis of oil and grease originating from pet food industrial wastewater [13]. Different matrices, such as agar, alginate, and polyacrylamide, were also employed to immobilize whole enzyme producing organism such as a gram-negative, rod shaped beta proteobacteria found in moist environments such as soil, river, effluent industries and lakes, resulting in an optimal lipase activity in 4% alginate beads [14]. In the earlier studies, lipase produced by *Arthrobactersp* RRLJ-1/95, MTCC 5125 which was immobilized by entrapment in sodium-alginate beads has shown a wide substrate specificity and high enantioselectivity in comparison to commercially available lipases like *Candida Cylindracealipase*(CCL), *PseudomonasStutzerilipase*(PSL) and porcine pancreatic lipase (PPL) [15]. In addition, the immobilized lipases on various matrices have been used for kinetic resolution of many chiral drugs intermediates [16-18].

In the present investigation, we describe the immobilization and characterization of lipase produced by *Bacillus cereus* MS6 bacteria, obtained from a localindustry effluent waste. The *Bacillus cereus* MS6 lipase was immobilized by entrapment method in sodium alginate and beads prepared from 3% (w/v) Sodium alginate and 2% (w/v) $CaCl_2$has been found to the best support for enzyme immobilization, providing 40 folds higher lipase activity in comparison to the free enzyme [19]. The enzyme immobilization technique using entrapment method within sodium alginate beads offered many advantages due to its simplicity and non-toxic character [20, 21].The enhanced stability of the immobilized lipase enzyme has been studied in relation to optimum temperature, optimum pH, optimum substrate concentration and incubation period. The immobilized enzyme was reused for many reaction cycles for the hydrolysis of triglyceride using olive oil and tributyrin as the substrate and the enzyme immobilized beads have shown enhancement in pH, thermal and storage stability when compared with the free enzyme.

Materials and Methods

Sampling and isolation of bacteria

The effluent sample was collected from Mysore Paper Mills (MPM) Bhadravathi, Shimoga district, Karnataka, India. The effluent was collected in different 30 ml sterile plastic tubes and stored in cold condition until use. The sample was serially diluted and each diluted sample was plated with the nutrient rich media. Liquid sample where serially diluted and plated on to olive oil agar base containing 1% peptone, 1% yeast extract, 2% olive oil emulsion and 2% agar, at pH9.0 by spread plate method [22]. Plates were incubated at37°C for 48 hours and the pure cultures of the isolates were maintained on minimal media agar slants (yeast extract, NaCl, peptone and 2% agar, pH9.0) and were sub cultured every 48 hours.

Optimization of production of lipase

The optimization of production of the enzyme from *Bacillus cereus* MS6 was carried out in liquid medium containing 2% olive oil emulsion, 1.0 %, yeast 1.0% extract peptone, 1.2% $Na_2HPO_4.7H_2O$, 0.5% KH_2PO_2, 0.5% NH_4Cl, 0.1% $MgSO_4.7H_2O$, 0.2 % $MgCl_2$, 1% NaCl, 1% glucose and 100 ml distilled water at pH9.0. For the optimization of *Bacillus cereus*, the inoculums was added to the conical flask culture medium and incubated in the rotary shaker at 37°C for 48 hours at 100 rpm. After specific intervals of time, sample was taken out and centrifuged at 9, 000 rpm for 15 minutes. The supernatant was subjected for ammonium sulphate precipitation and the precipitate was subjected to dialysis and lyophilized. The enzyme preparation was further purified by gel filtration using Sephadex G-100 column chromatography followed by ion exchange chromatography using DEAE cellulose and then analyzed for the enzymatic activity and protein concentration.

Lipase activity assay by titration method

The free lipase activity was measured in mechanically stirred emulsion of olive oil using pH stat equipment. The released free fatty acids were neutralized by adding NaOH in order to maintain a constant pH end point value. The reaction mixture contains 1ml purified lipase, 2 ml of 2% olive oil, 1ml 0.2 MTris-HCl buffer at pH8.0. The mixture was incubated at room temperature with constant agitation on a magnetic stirrer for 30 minutes. After 30 minutes, 3ml of ethanol was added to stop the reaction and 3-5 drops *Thymolphthalein* indicator was added and the released of fatty acids measured by the titration method using 0.05M NaOH. One unit of lipase activity was defined as the amount of lipase required to release 1µmol/min of fatty acid from triacylglycerols.

Immobilization of lipase on sodium-alginate beads

The immobilization of *Bacillus cereus*MS6 lipase was carried out by using entrapment method in 3% sodium alginate beads which was found to have highest activity as compared to the preparations at other concentrations. The activity of the immobilized lipase and the activity of free lipase were calculated based on the bound protein basis. Further, the optimal pH and optimum reaction temperature was also determined for both immobilized and free lipase as described earlier [23]. The process of immobilization was carried out under sterile conditions. The enzyme was dissolved in a minimum volume of 0.2 M Tris-HCl buffers, pH8.0 and mixed with Sodium alginate solution (3gm dissolved in 100 ml warm distilled water). About 2 ml enzyme was suspended in 10 ml of 3% (w/v) sodium alginate solution. The mixture obtained was extruded drop-wise through a burette into 15 ml of 2 % calcium chloride solution (2gm dissolved in 100 ml distilled water). Alginate drops become solidified upon contact with calcium chloride forming beads with an approximate diameter of 5-10 mm. The sodium alginate beads containing the immobilized enzyme were removed by thoroughly washing with citrate-phosphate buffer, pH8.0 and kept in the same buffer at 4°C for 72 hours to remove the unbound enzyme and used for further studies [24]. Immobilized beads were checked for lipase activity using olive oil as the substrate by using titration method as discussed above [25].

Immobilized enzyme assay

The activity of immobilized lipase was assayed by mixing10-15 Sodium alginate beads (3%) with 2 ml of olive oil/emulsion as substrate and 1ml of 0.2 M Tris-HCl buffer (pH8.0) by incubating the mixture at40^{0}C for 30 minutes. After incubation, 3 ml of ethanol was added and 3-5 drops of *Thymolphthalein* indicator was added also into the mixture reaction, subsequently the amount of free fatty acids was determined by titration method against with 0.05M NaOH. The activity of immobilized lipase was determined by measuring the fatty acid released under the assay conditions and unimmobilized lipase was washed with distilled water before re-measuring its activity [26]. On the another hand, the activity of unimmobilized enzyme was determined by using 1 ml of free enzyme with 2 ml of soluble olive oil/emulsion and 1 ml 0.2 M Tris-HCl buffer at pH8.0. The mixture reaction was incubated with constant shaking at 37^0C for 30 minutes. After that 3 ml of ethanol was added and 3-5 drops of *thymolphthalein* indicator and the unimmobilized lipase activity was measured by the titration method using 0.05 M NaOH.

Determination of activity yield

The immobilized activity yield for lipase was calculate using *Arrhenius equation and* expressed by the following formula [27]

$$\text{Immobilized yield (\%)} = (I/A\text{-}B)\ X100 \qquad (1)$$

Where

I = immobilized enzyme (U/g of bead).

A = added enzyme (U/g of purified free enzyme).

B= unbound enzyme (U/g of bead).

OR

The percentage conversion was calculated based on the following equation [28]:

$$\text{Conversion \%} = (V_{\text{control}} - V_{\text{sample}})\ /V_{\text{control}}\ X\ 100 \qquad (2)$$

Where V_{control} = average volume of NaOHwas used for blank, while V_{sample} = average volume of NaOH used for the sample.

Results and discussion

Results

The lipase produced by *Bacillus cereus* MS6under optimal growth conditions was subjected to purification. The crude enzyme preparation was purified using 80% ammonium sulfate precipitation, centrifuged at 9, 000 rpm and dialyzed. The lipase preparation was subjected to gel filtration chromatography using Sephadex G-100 column (2.5X50 cm) followed by DEAE-Cellulose column chromatography. In addition the purified lipase was subjected to immobilization process using entrapment method using various concentrations of sodium alginate (1-6%) solution. The results indicated that 3% Sodium alginate has highest immobilization activity yield and the catalytic property of the immobilized lipase was compared with that of the free lipase. The immobilized lipase was characterized further for its various biochemical parameters in comparison with that of free lipase. The experiments were carried out using immobilized lipase beads which were stored at 4^0C and there was no significant decrease in the activity of immobilized lipase for up to 2 weeks [29].

Effect of temperature on the activity of immobilized lipase

The effect of temperature on activity of immobilized lipase was carried out by measuring the residual enzymatic activity through pre-incubating at various temperatures ranging from 10 to 90^{0}C (Figure 1) for 1 hour. As shown in the figure, maximum immobilized lipase activity was at a temperature of 50°C with stability up to 60^{o}C. Above the temperature of 60°C, there is a sharp decrease in the activity of enzyme with an increase in the temperature reaching zero activity at 90°C. In comparison with this, the free lipase showed a temperature maximum of 40°C with stability up to 50^{0}C [30].

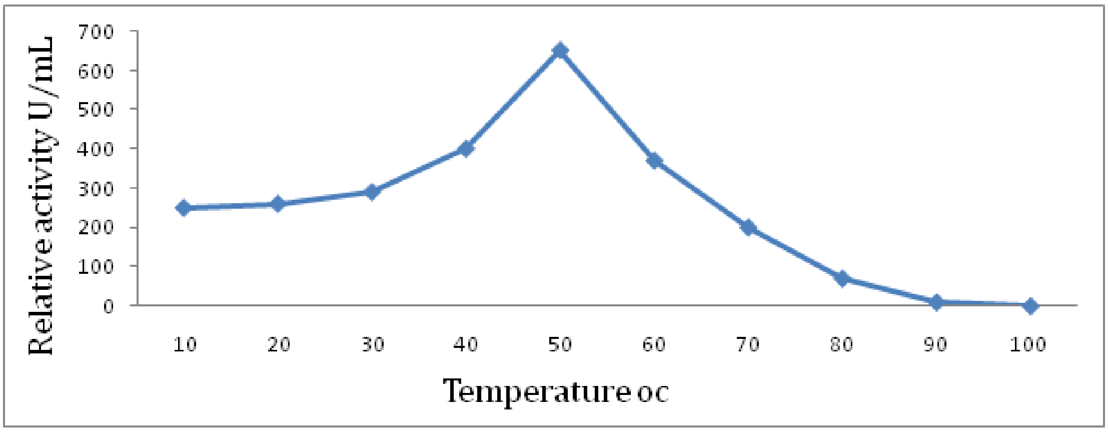

Fig.1. Effect of temperature on the activity of immobilized lipase. The effect of temperature on the activity of immobilized lipase was carried out at pH10.0 using 3% olive oil emulsion as substrate in the temperature range of 10-100°C. The relative activity values were plotted versus temperature.

Effect of pH on the activity of immobilized lipase

In Figure 2 is shown the effect of pH on the activity of immobilized lipase which was determined at different pH values in the range of 6.0-12.0 for free and immobilized lipase. Further, activity of immobilized of lipase was estimated by comparing the activity with that of free lipase. The results showed that immobilized lipase has the highest activity at pH10.0 which is stable up to pH11.0. On another hand, earlier results showed that the maximum activity of free lipase was at pH9.0 [30]. Hence, the enzymatic activity at pH10.0 for the immobilized enzyme additionally reveals that the immobilized enzyme held pH stability when compared to free lipase.

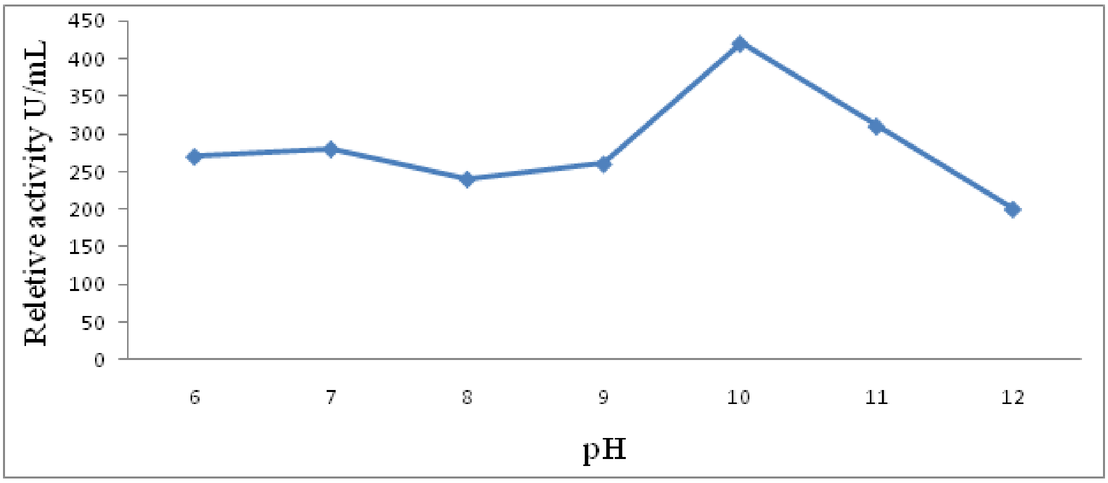

Fig.2. Effect of pH on the activity of immobilized lipase. The effect of pH on the activity of immobilized lipase was determined at 50°C using 3% olive oil emulsion as substrate at various pH values from 6-12. The relative activity values were plotted versus pH.

Effect of sodium alginate concentration on the activity of immobilized lipase

The effect of sodium alginate concentration on the activity of immobilization lipase was carried out by using various concentrations of sodium alginate in the range 1-6% to achieve the highest immobilization activity. The immobilization activity was found to be highest with 3% Sodium alginate solution as shown in Figure 3. Lower or higher concentrations of sodium alginate solution, as compared to 3%, lead to a decrease in immobilized activity (Table 1). This is due to the fact that, greater the pore size of the beads there is a possibility of increase in the leakage of enzyme from the beads. Similarly, when the pore size of beads decreased, with an increase in concentration of sodium alginate solution, the entry of substrate into the beads was impeded that lead to the lower immobilization efficiency [29]. The results also indicate that the pore size of the beads is optimum for the passage of substrate into the beads at 3% sodium alginate concentration.

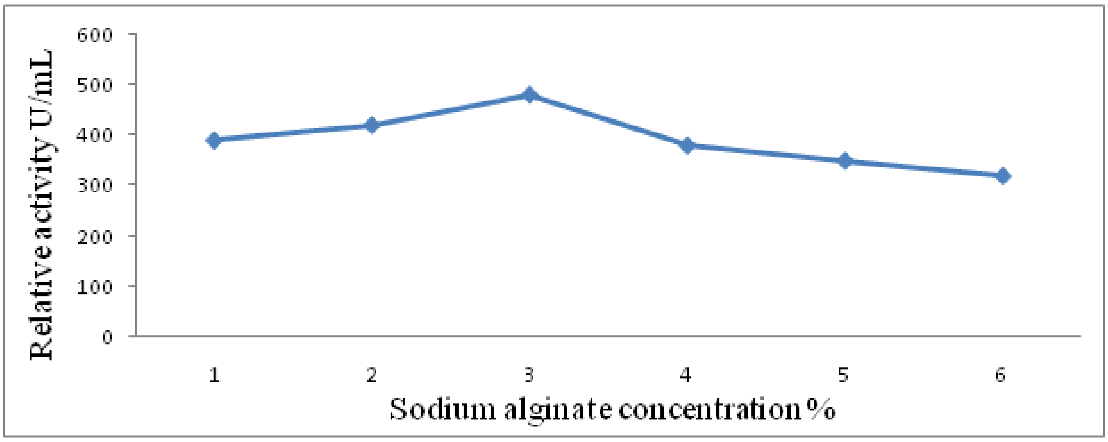

Fig.3. Effect of sodium alginate concentration on the activity of immobilized lipase. The effect of sodium alginate concentration on activity of immobilized lipase was determined at 50°C and at pH10.0 using various concentrations of sodium alginate (1-6%). The relative activity values were plotted versus concentration of sodium alginate.

Table 1. Summary of immobilization yield activity of *Bacillus Cereus* MS6 lipase by entrapment method.

Carrier Sodium Alginate Concentration (%)	Added enzyme (U/g Carrier) A	Un-immobilized enzyme (U/g Carrier) B	Immobilized enzyme I	Immobilization Yield (%) = I/A-B ×100
1	1000	300	400	57.14
2	1000	350	470	72.70
3	1000	380	550	88.70
4	1000	320	440	61.11
5	1000	280	390	54.16
6	1000	250	360	45.00

Effect of incubation period on the activity of immobilized lipase

The effect of incubation period on the activity of immobilized lipase was determined at various time periods in the range 5-60 minutes. The results show that the maximum activity of immobilized lipase is for 30 minutes incubation as shown in Figure 4. The activity measurements of immobilized lipase indicated decrease with an increase in the incubation period above 30 minutes. In comparison with this, the optimum incubation time of 30 minutes was also observed in the case of free lipase [30].

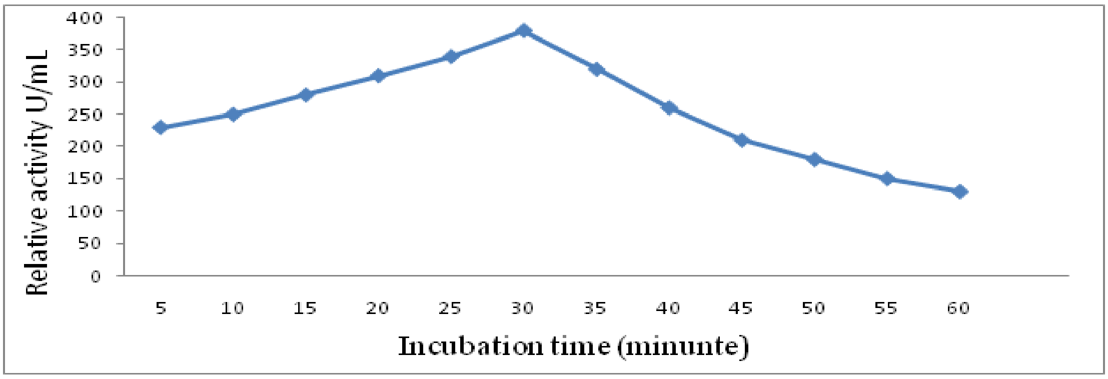

Fig.4. Effect of incubation time on activity of immobilized lipase. The effect of incubation time on the activity of immobilized lipase was determined at a temperature of 50^{0}C and pH10.0 by using various incubation periods of up to one hour using 3% olive oil emulsion as substrate. The relative activity values were plotted versus incubation period.

Effect of substrate concentration on the activity of immobilized lipase

Various concentrations of the substrate (1-6%) were used to determine the optimum concentration for the lipase catalyzed reaction. As shown in Figure 5, the olive oil concentration of 3% showed the maximum activity for the immobilized lipase. The activity of immobilized lipase was found to decline with either increase or decrease in substrate concentration from maximum value of 3% which was consistent with the earlier reports [32]. This suggests that the reaction rate of the enzyme and substrate is independent of immobilization as the diffusion limitation for enzyme has not been seen after the entrapment of enzyme. However, in comparison with this, the optimum substrate concentration of 2% was observed in the case of free lipase [30].

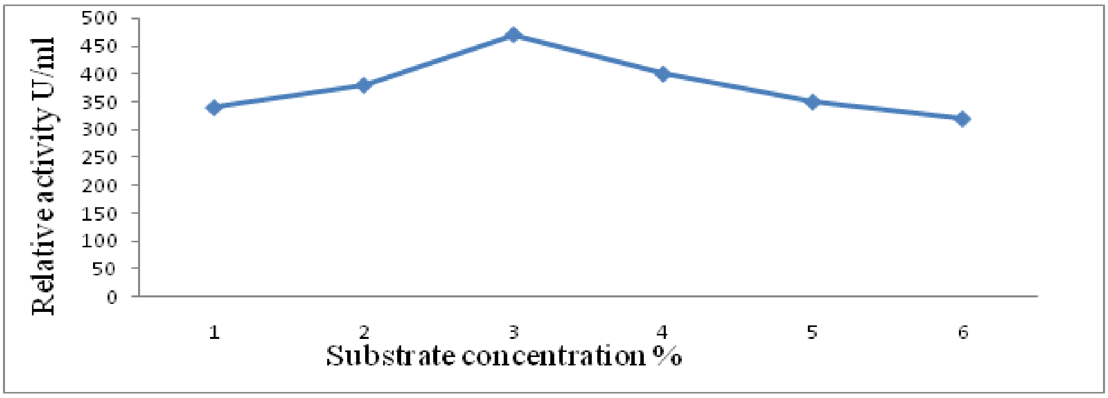

Fig.5. Effect of substrate concentration on the activity of immobilized lipase. The effect of substrate concentration on the activity of immobilized lipase was determined at 50°C and at pH10.0 at various olive oil substrate concentrations in the range of 1-6%. The relative activity values were plotted versus substrate concentration.

Discussion

The immobilized enzymes are known to be used for many applications including organic synthesis and the other applications of most common immobilized enzymes are in etherification and transesterificationreactions in organic media [32]. The immobilized enzymes have received considerable attention because of their advantages over the unimmobilized counter parts, as they improve storage and equipped thermal and conformational stabilities and they are easily recovered for reuse [33]. Most carriers for immobilization have been granular particles including celite or ion exchange resins [35] which are known for their high costs. The granular immobilized enzymes could be reused by filtration, but highly vulnerable as they are easily broken down by agitation in two-phase systems [35].

In the present investigation, immobilized lipase produced by *Bacillus cereus* MS6 bacteria which was isolated from the local effluent industry has been described. The *Bacillus cereus* MS6 strain was cultured in liquid media under optimum conditions to produce maximum lipase which was further subjected to characterization. Lipase purification was carried out by using 80% ammonium sulfate precipitation followed by ion exchange and gel filtration chromatography. The SDS-PAGE results showed a molecular weight of 35kDa for the purified enzyme [30]. Further, the purified lipase was also characterized for the enzymatic pH optimum which was at 9.0 and temperature optimum at 40°C [36].

The purified lipase obtained from *Bacillus cereus* MS6 was subjected to immobilization which was carried out in various concentrations of sodium alginate solution (1- 6%). The results indicated that 3% Sodium alginate gave the highest immobilization activity yield which was found to be 88.70 %, while at lower and higher carrier concentration, the immobilization yield decreased. The immobilized lipase was characterized for its optimum pH and temperature stability. The maximum activity of free lipase was found to be at pH9.0 whereas the peak activity of immobilized lipase was found to be at pH10.0. The data indicates that the optimal pH for the immobilized lipase was found to shift towards higher pH values as compared to that for the free lipase. The thermal stability and optimum reaction temperature was determined for the immobilized lipase which was found to be at 50°C and the stability in the temperature range of 40-60^{0}C for 2 hours which was 40°C with stability in the temperature range 40-50°C for 2 hours in the case of free enzyme. These values clearly indicate an increase in the temperature stability after immobilization. In general, the immobilized lipase was more thermo stable than the free lipase and it retained 80 % of activity while, the free enzyme retained 50 % of the activity [26].

In the literature, there are several studies on the immobilization of lipase from various sources leading to enhancement of structural and enzymatic stability. The studies on lipase from *Candida Ragusa* has shown the enhancement in the stability of the immobilized lipase onto palm-based polyurethane bubbles through the physical adsorption method, in which the polymer was presoaked in immobilized agent [37]. The activities of immobilized lipase were tested by the esterification reaction of oleic acid and Oley alcohol in hexane [37]. The immobilized lipase was then characterized in term of its thermal, operational and storage stability. The optimum temperature for native and PU-immobilized lipase was at temperature 40^{o}C and pH9.0. This showed that the immobilization did not alter the general character of the lipase. The PU-immobilized lipase indicated different enzymatic characteristics such as incubation time, enzyme concentration, solvent stability and temperature stability compared to lipozyme so that the reusability of PU-immobilized lipase was for at least four cycles. The conversion of above 80% was still achieved for the enzyme which was stored at 4^{o}C for 9 days [38].

Conclusions

In conclusion, we isolated and purified of lipase from Haloalkalophilic *Bacillus cereus*MS6 bacteria. The purified lipase was subjected to immobilization process by using entrapment method with 3% Sodium alginate beads and its characteristics discussed. The characterization data of the immobilized lipase as compared to the free lipase showed that the immobilized lipase was more thermo stable and could be used as a source for various applications. Additionally, immobilization of lipase provided a higher activity and stability of the lipase as compared with the free lipase. Thus the features of immobilized lipase were appropriate for various applications in industries and pharmacy. Further, the biochemical characteristics features of the immobilized *Bacillus cereus* MS6 lipase, as determined here, could establish the novel and uniqueness of this enzyme as compared to other lipases.

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