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CHARACTERIZATION OF IMMOBILIZED ENDO-1, 4-β-GLUCANASE FROM *THERMOTOGA PETROPHILA* TAKAHATA *ET AL.*

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ABSTRACT

Cellulases are the collection of the enzyme mainly produced by the microorganisms. Recombinant strain of *Thermotoga peterophila* was used to produce the enzyme through submerged fermentation at 37°C for two days in latent broth (LB) as fermentation medium. The enzyme was purified by Ion exchange chromatography and activity was determined according to the procedure of Bernfeld. The maximum activity in enzyme entrapped in gel slices was observed at 90^0C when the enzyme was incubated for 2 hours and 30 minutes. For the enzyme entrapped in calcium alginate beads, maximum activity of the enzyme was observed at 100^0C when the enzyme was incubated for 3 hours. No defined pH shift was observed in the enzyme entrapped in acrylamide gel slices whereas pH shift towards more acidic range for enzyme entrapped in calcium alginate beads. The shelf life of the enzyme, stored at 4^0C, 32 and 18 days enzyme entrapped in calcium alginate beads and acrylamide gel slices respectively.

Key Words: - Cellulases, Enzyme Immobilization, *Thermotoga peterophila*,

INTRODUCTION

Generally Cellulases are the collection of enzymes that are produced mainly by different kinds of fungi, bacteria and protozoans. Two main types of cellulases are Endo-cellulases and Exo-cellulases. Endocellulases mostly refers to the term "Endoglucanases". The main function of endoglucanases is the cleavage of internal bonds at amorphous sites that are directly involved in creating smaller fragments of cellulase. Exocellulase works on the product of endocellulases. Two to four units from exposed chains produced by endocellulase are cleaved by exocellulases that results in the production of tetrasaccharides or disaccharides. Exo-cellulaseare considered as highly active and specific in their action (Rajoka*et al*., 2007).

Endo-glucanases (β-1,4-D glucanglucano-hydrolase, E.C.3.2.1.4) are the only enzymes that cleave the glucose chains in an endo-fashion and open the molecule for the action of exo-glucanases (β-1,4-D glucancellobio-hydrolases, E.C.3.1.1.91) and glucosidases (cellobiases) leading to formation of two molecules of glucose for subsequent conversion of cellulose in abundantly available lignocellulosic biomass, to ethanol or other products of industrial applications (Beguin and Aubert, 1994).

The main problems of free enzymes are their high cost of production, difficulty in their recovery from reaction mixtures for eventual recycling, instability towards higher temperatures, pH, storage stability and biodegradability. Immobilization is an artificial and intentional containment, compartmentalization, packing, inclusion or entrapment of enzymes in polymeric matrices or binding and affixing onto surface of a carrier. The purpose of immobilization is to restrict mobility, leakage and diffusion of enzymes to make them reusable again and again (Tischer and Wedekind, 1999). This process also reduces the cost of their production. Immobilized enzymes have gained much value for a variety of applications in the fields including drug delivery systems and tumor identification, as well as in the sensors for managing of weight and diabetes (Worsfold, 1995). Immobilized enzymes are used as biocatalysts to convert electrochemically unreactive compounds into compounds which undergo redox reactions producing a detectable current (Trevan, 1980). The biosensors used in monitoring glucose levels in diabetic patients have been one of the most popular biosensors (Zabrosky, 1973).

There are a variety of methods used to immobilize enzymes. The most common methods of immobilization of enzymes are adsorption, entrapment and cross-linking or covalently binding to a support. There is a drawback of crosslinking or covalently binding the enzyme to the support material surface that is it typically decreases the degree of movement of the enzyme resulting in a dramatic decrease in the enzyme activity. (Hanefeld *et al.*, 2009)

Polyacrylamide is the most widely used matrix for entrapping enzymes. It has the advantage that it is non-ionic. The consequence is that the properties of the enzymes are only minimally modified in the presence of the gel matrix (Richardson *et al*., 2002). At the same time, the diffusion of the charged substrate and products is not affected, neither. However, dimethylamino-propionitrile, the polymerization initiator, is highly toxic and must be handled with great care (Raviyan,*et al.*, 2003). The requirement to purge the monomer solution with nitrogen is also troublesome, although not totally crippling (Aksoy *et al*., 1998).

Calcium alginate is just as widely used as polyacrylamide. Unlike polyacrylamide gels, gelation of calcium alginate does not depend on the formation of more permanent covalent bonds between polymer chains. Rather,

polymer molecules are cross-linked by calcium ions (Pandey *et al*., 2000). Because of this, calcium alginate beads can be formed in extremely mild conditions, which ensure that enzyme activity yields of over 80% can be routinely achieved. However, just as easily as calcium ions can be exchanged for sodium ions, they can also be displaced by other ions. This property can both be advantageous and disadvantageous (Aisina, 1992). If needed, enzymes or microbial cells can be easily recovered by dissolving the gel in a sodium solution. On the other hand, proper caution must be exercised to ensure that the substrate solution does not contain high concentrations of those ions that can disintegrate the gel. In this research work we report studies based on immobilization of a recombinant Endoglucanase from *Thermotoga petrophila* Takahata *et al*.

MATERIALS AND METHODS

Enzyme Production

Recombinant strain of *Thermotoga peterophila* Takahata *et al*. was used to produce the enzyme β-1, 4endoglucanase through submerged fermentation. The fermentation was carried out at 37℃ for two days in latent broth (LB) as fermentation medium. The enzyme was purified by Ion exchange chromatography.

Activity Assay Method

B-1, 4Endoglucanase activity was determined Bernfeld Activity Assay Method. The diluted enzyme about 100 µl was added to 500 µl of 4% CMC and 400 µl of Tri-sodium citrate buffer. After incubation at 100ºC for 10 minutes, the reaction was stopped by the addition of 3ml of 3, 5 Di nitro salicylic acid (DNS). Then it was incubated at 100 °C for 10 min to develop colour. After cooling the absorbance was taken at 540 nm. To eliminate any reducing sugar already found in enzyme extract, blank was made which included all the components as in test sample except enzyme for incubation at 100˚C. Reducing sugar was measured using glucose standard curve. To determine the specific activity of enzyme, protein concentration was estimated by Bradford Dye Binding method (1976) with Bovine serum albumin (BSA) as a standard.

Protein Determination of Endoglucanase

The amount of endoglucanase enzyme used for activity assay was determined by Bradford protein assay method (Bradford, 1976). The 100 µl of enzyme was diluted in 900 µl of distilled water in which 5 ml of Bradford reagent was added. The reaction mixture was left for 2-5 min for the colour development due to the presence of protein. The absorbance was taken at 595 nm.

Entrapment of Endoglucanase

Entrapment in Alginate Beads

Enzyme was entrapped in Alginate by using the method described by Gupta *et al.*, (2005a). 3% (w/v) Sodium alginate was dissolved in double distilled water and enzyme dilution in equal ratio i.e. 1:1. The solution was mixed and was incubated at 37˚C for 30 min to allow even mixing of enzyme with alginate solution. Then the Alginate solution was poured drop wise with the help of 10 ml syringe by maintaining a height of 10 cm in calcium chloride solution that was kept at constant stirring in a beaker. The solution was again incubated at 37˚C for two hours. The beads formed were then filtered, washed two or three times with double distilled water and stored for furthers studies.

Entrapment in Native Gel

The enzyme β-1, 4-endoglucanase was also trapped in native PAGE gel. Tris-HCl of pH 7, Acrylamide/Bisacrylamide, distilled Water and enzyme were added in a tube containing APS and TEMED. The solution was mixed and then poured in the gel caster of 1cm dimension and allowed to polymerize. After the gel was set, it was carefully taken out of the caster, washed with double distilled water and cut into slices of $1cm^3$ dimension. The gel slices with entrapped β-1, 4-endoglucanase enzyme were stored in Tri-sodium citrate buffer at 4˚C for further use in different assays.

Enzyme Characterization

Enzyme β-1, 4Endoglucanase was characterized at different temperatures with varying incubation time intervals and at different pH. Furthermore shelf life and reusability characteristics were also determined and compared with that of the pH and temperature optima of crude untrapped enzyme.

Effect of Temperature

Optimum temperature for enzyme entrapped in gel slices and calcium-alginate beads was determined at various temperatures ranging from 70-100˚C under different time intervals starting from 15 minutes ending upto 7 hours.

Effect of pH

The entrapped enzyme in the beads as well as gel slices was also assayed using buffer of different pH ranging from 3 to 10. The buffers used were Tri-sodium citrate buffer having a pH range of 3-7, CAPS buffer having pH range of 9-11 and HEPES buffer with pH 8.

Reusability

Reusability of the immobilized enzyme was also checked by reusing the beads after washing them 2 or 3 times by double distilled water after each cycle of assay instead of discarding them. After incubation, the beads were removed from the reaction mixture and reused after washing thrice with double distilled water. Activity was determined in the same manner as described above. The decrease in activity after each cycle of assay was determined by assuming activity of immobilized enzyme in the first cycle as 100%.The beads were reused unless the activity was decreased to negligible values.

Shelf-Life

The shelf-life of immobilized enzyme was determined by keeping the entrapped enzyme in calcium-alginate beads and acrylamide gel slices at 4˚C. The activity was calculated at each passing day after the beads were made upto 21 days for acrylamide gel slices and 32 days for calcium-alginate beads.

RESULTS

Comparison of activity after immobilization in 2%, 2.5%, 3% and 3.5% calcium alginate beads at different temperatures and pH:

Temperature Characterization

The beads of different percentages (2%, 2.5%, 3% and 3.5%) with entrapped enzyme were incubated in tri-sodium citrate buffer (pH 6.0) and 4% CMC at different temperatures ranging from 70˚C to 100˚C under different time intervals starting from 1 hour and ending upto 6 hours showing the activity of enzyme entrapped in these beads after the incubation time. The Fig 1(a), 1(b), 1(c) and 1(d) illustrate the activity difference at various time intervals between different percentages of beads. The results show the maximum activity at 100˚C.

pH Characterization

2%, 2.5%, 3% and 3.5% beads were incubated in tri-sodium citrate buffer of different pH ranging from 3 to 7 separately. For pH 8, the beads were incubated in HEPES buffer and for pH 9, 10 and 11 the beads were incubated in CAPS buffer. The beads were found to have no activity at pH 9, 10 and 11 and at pH 8, negligible activity was found in the beads as indicated in Fig 2(a) and 2(b). Moreover the beads got shrink at pH 8, 9, 10 and 11.pH characterization was carried out at two different temperatures that are 100˚C and 90˚C and the incubation time given was 3 hours for each pH.

Comparison of Activity after immobilization in 6%, 9% & 12% Acrylaymide gel slices at Different Temperatures and pH

The gel slice of different percentages that are 6%, 9%, and 12.0% were incubated in 0.5 ml tri-sodium citrate buffer(pH 6.0) and 0.5 ml 4% CMC at different temperatures ranging from 70˚C to 100˚C at different time intervals starting from 30 minutes and ending upto 4 hours 30 minutes showing the activity of enzyme entrapped in these beads during this assay conditions. The Fig. 3a, 3b, 3c and 3d illustrate the activity difference at various time intervals between different percentages of gel slices with entrapped enzyme.

pH Characterization

Entrapped enzyme gel slices (6%, 9% and 12 %) were incubated in tri-sodium citrate buffer of different pH ranging from 3 to 7 separately. For pH 8, the gel slices were incubated in HEPES buffer and for pH 9, 10 and 11 the gel slices were incubated in CAPS buffer. The gel slices were found to have no activity at pH 9, 10 and 11 and at pH

8.0 negligible activity was found as shown in the fig 4a and 4b. pH characterization was carried out at two different temperatures that are 100˚C and 90˚C and the incubation time given was 2 hours and 30 minutes for each pH value.

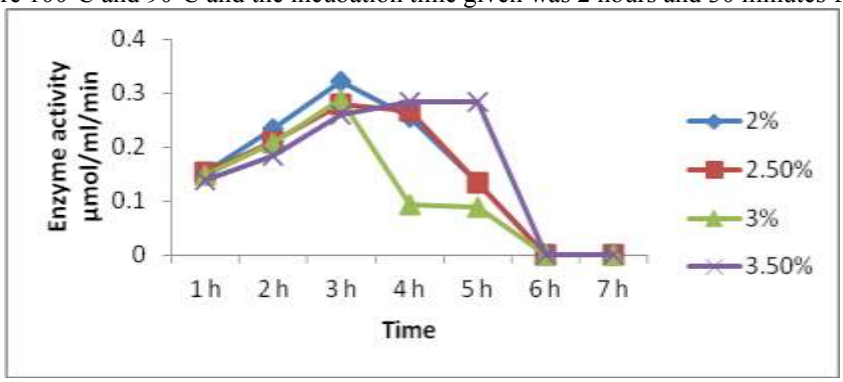

Fig. 1(a). Activity of Enzyme Entrapped in Calcium-alginate beads at 100 ˚C.

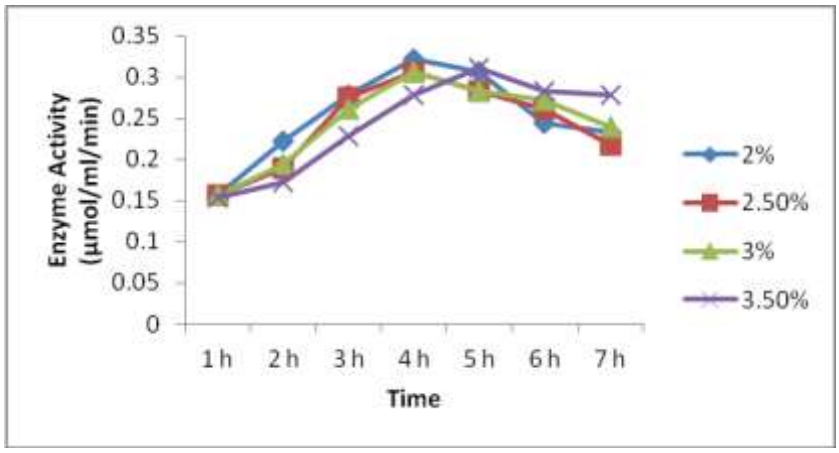

Fig. 1(b). Activity of Enzyme Entrapped in Calcium-alginate beads at 90 ˚C.

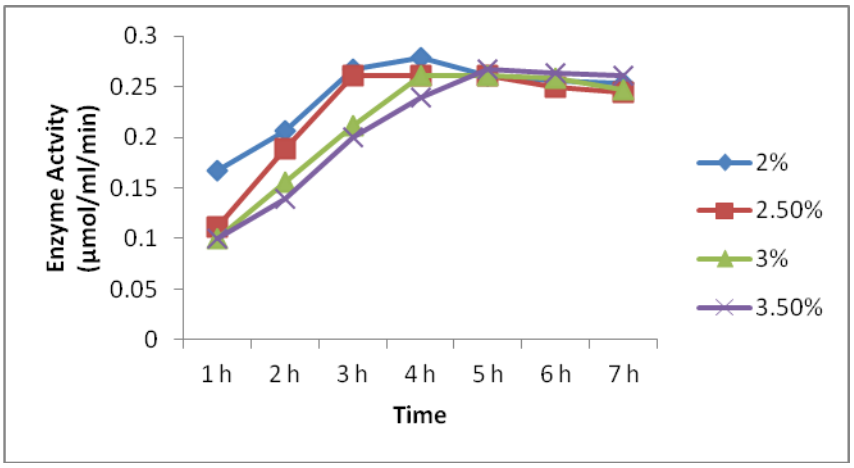

Fig. 1(c). Activity of Enzyme Entrapped in Calcium-alginate beads at 80 ˚C.

Shelf-Life of Immobilized enzyme at 4^{o}C

The shelf-life of entrapped enzyme was also analyzed under the optimum assay conditions by a method described earlier. The Fig 5(a) and 5(b) showed no decrease in activity upto 29 days. After 29 days the enzyme

activity was found to be decreasing. The first day activity was calculated to be 0.15 µmol/ml/min and remained same upto 29 days, the 30th day activity was found to be 0.138µmol/ml/min. For native gel slices, the activity was found to be 0.072µmol/ml/min for the first 19 days after enzyme entrapment in gel slices. The activity started decreasing from 20th day onwards with negligible difference.

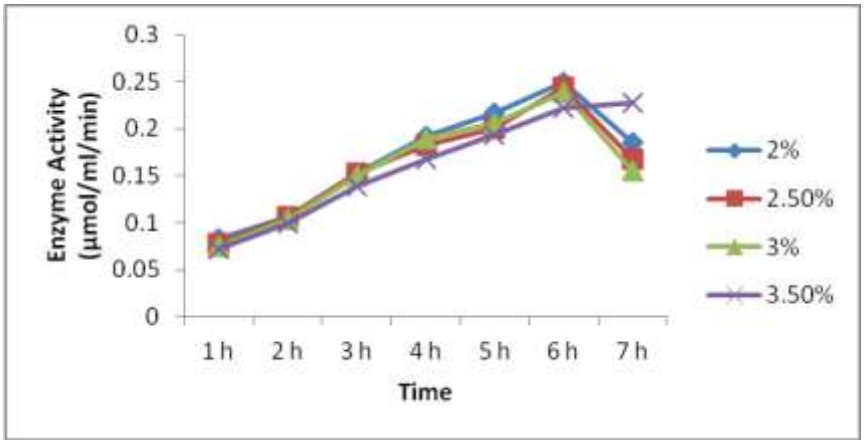

Fig. 1(d). Activity of Enzyme Entrapped in Calcium-alginate beads at 70 ˚C.

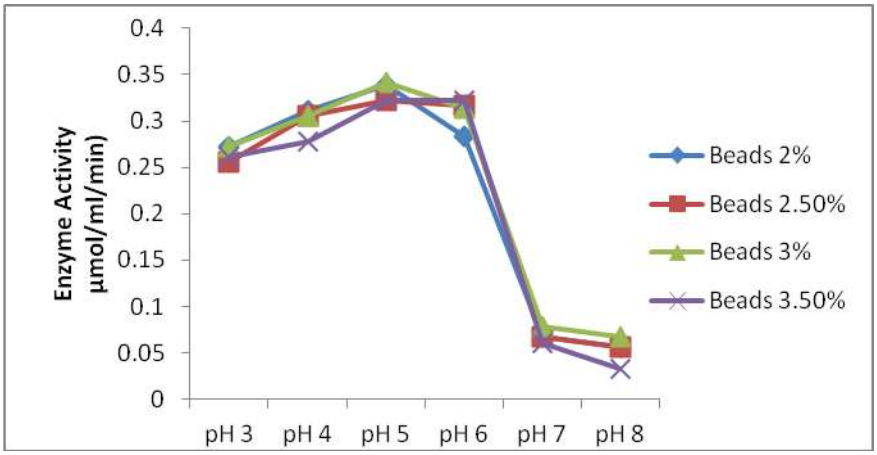

Fig. 2(a).Activity of Enzyme Entrapped in Calcium-alginate Beads (µmol/ml/min) at100 °C and 3 hrs incubation.

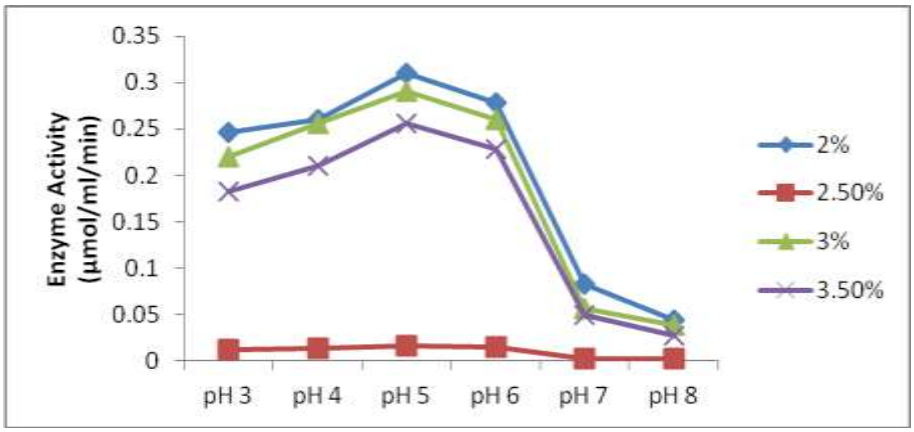

Fig. 2(b).Activity of Enzyme Entrapped in Calcium-alginate Beads (µmol/ml/min) at 90 °C and 3 hrs incubation.

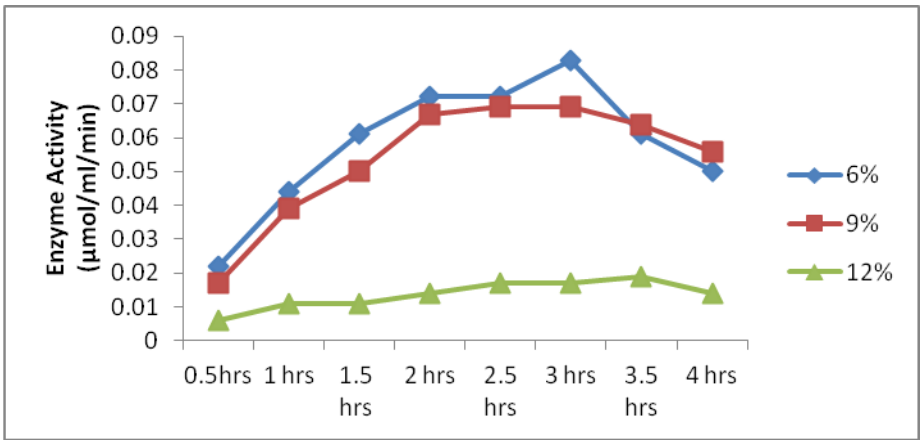

Fig. 3(a).Activity of Enzyme Entrapped in Acrylamide gel slices (µmol/ml/min) at 100 °C.

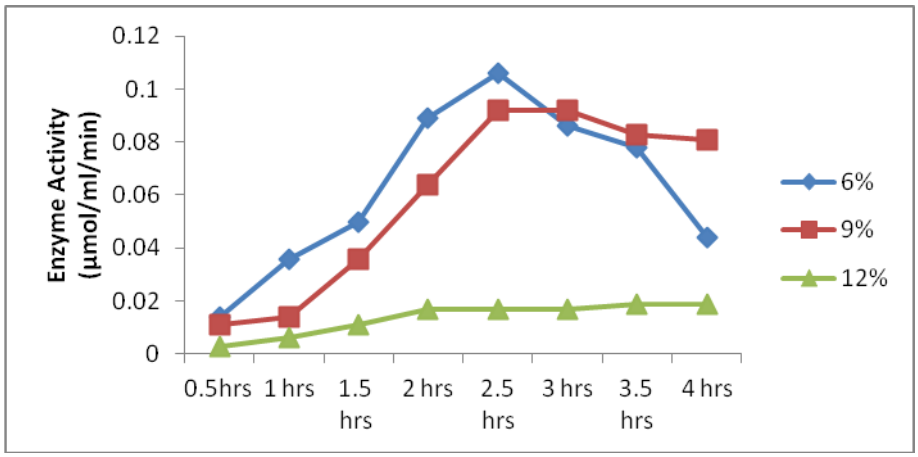

Fig. 3(b).Activity of Enzyme Entrapped in Acrylamide gel slices (µmol/ml/min) at 90 °C.

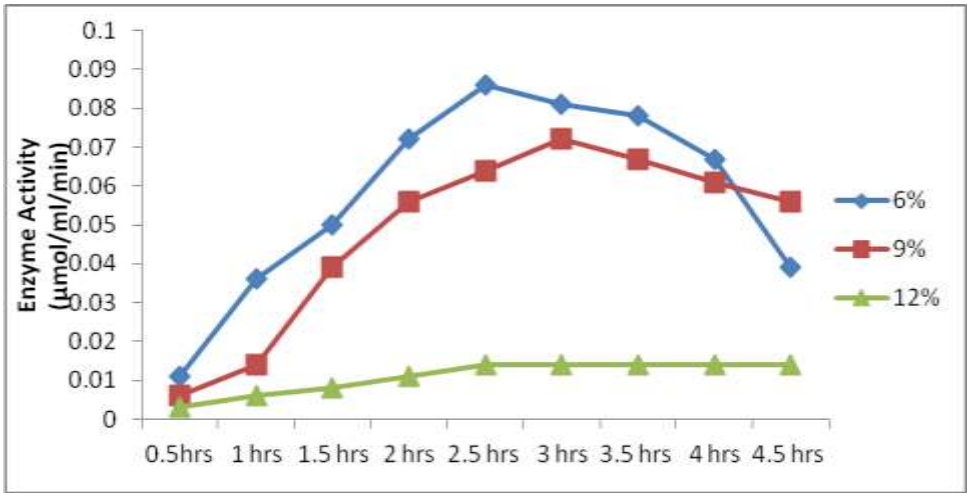

Fig. 3(c).Activity of Enzyme Entrapped in Acrylamide gel slices (µmol/ml/min) at 80 °C.

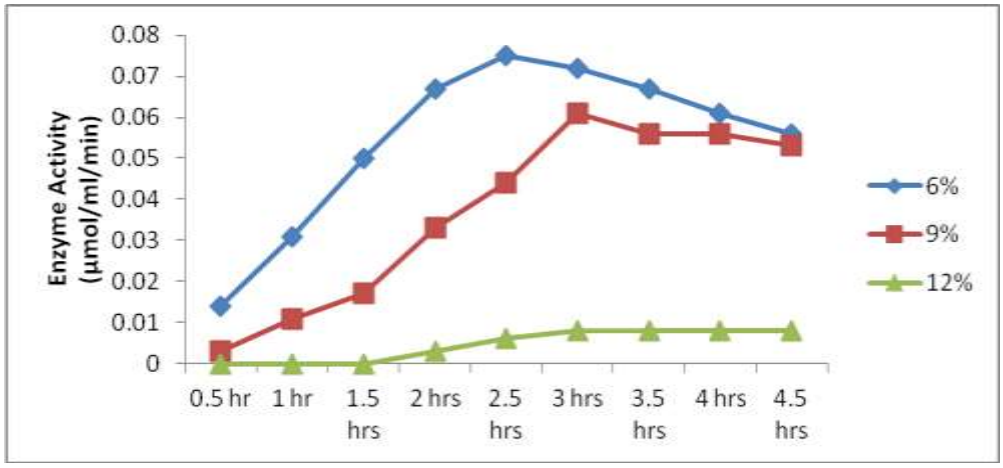

Fig. 3(d).Activity of Enzyme Entrapped in Acrylamide gel slices (µmol/ml/min) at 70 °C.

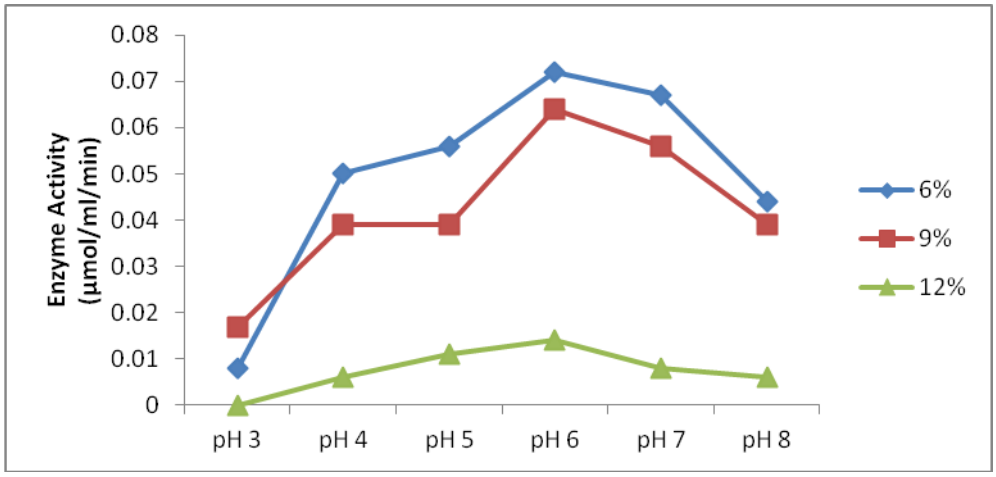

Fig. 4(a).Activity of Enzyme Entrapped in Acrylamide gel slices at 100 ˚C.

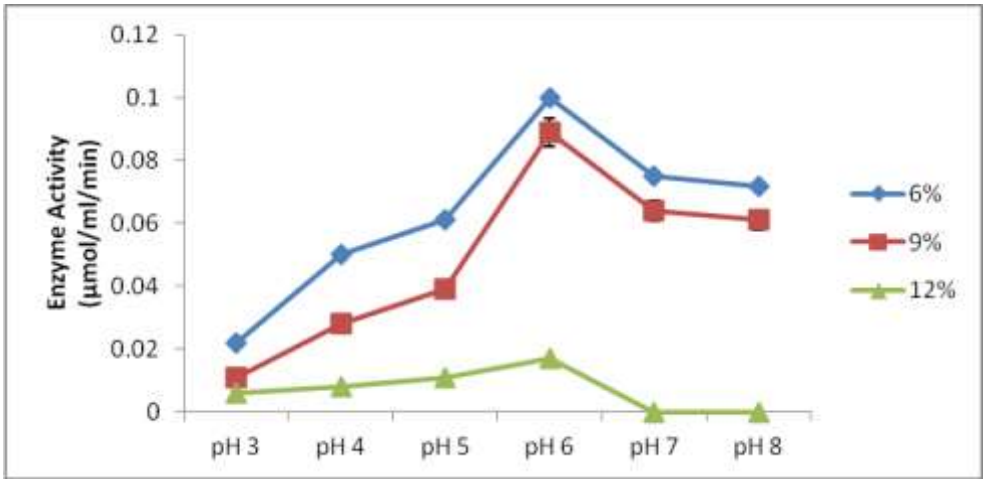

Fig. 4(b).Activity of Enzyme Entrapped in Acrylamide gel slices at 90 ˚C.

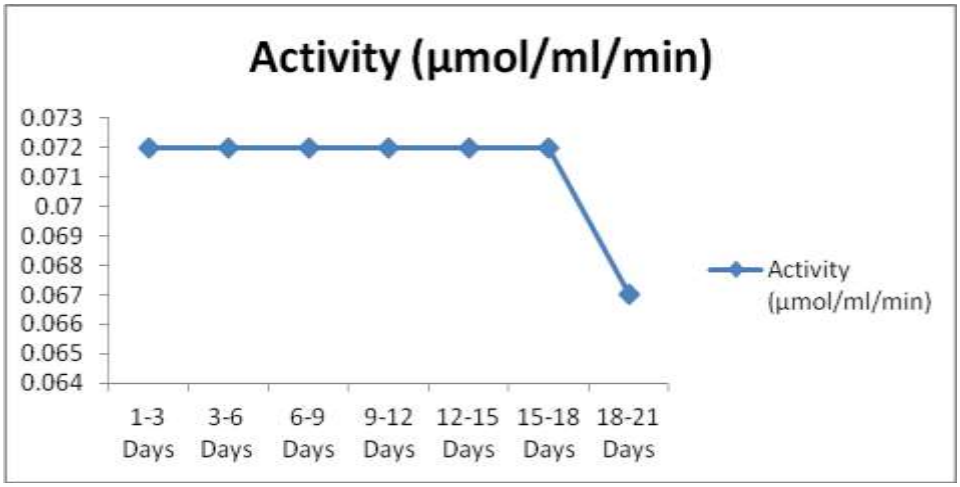

Fig. 5(a).Shelf-life of Endoglucanase entrapped in Calcium alginate gel.

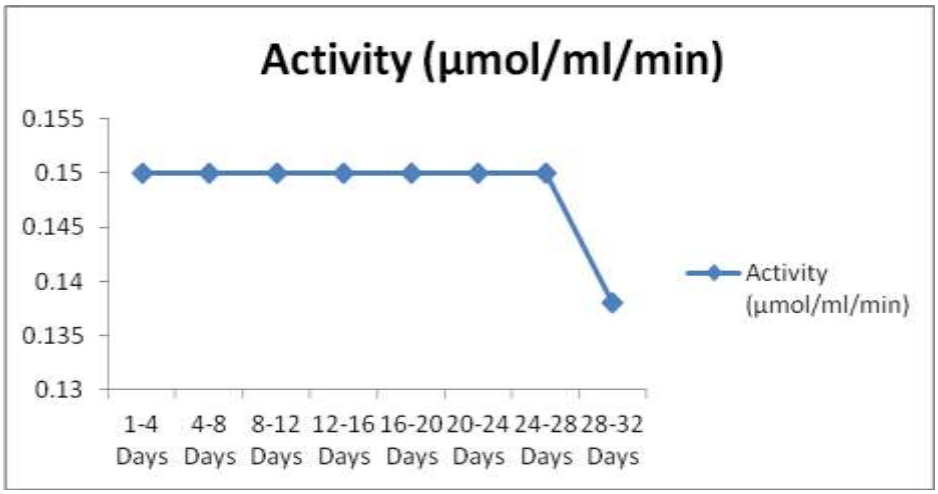

Fig. 5 (b). Shelf-life of Endoglucanase entrapped in Acrylamide gel slices.

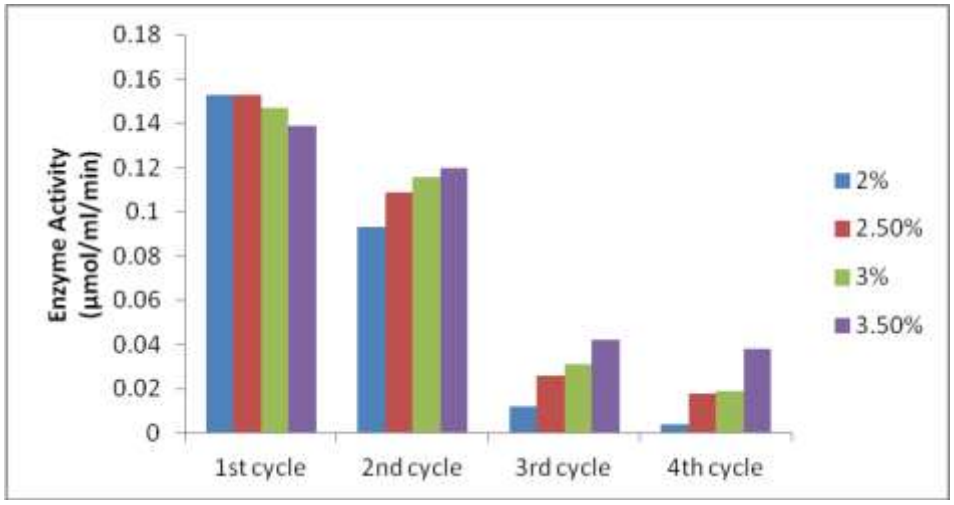

Fig. 6(a). Reusability of enzyme entrapped in calcium alginate gel.

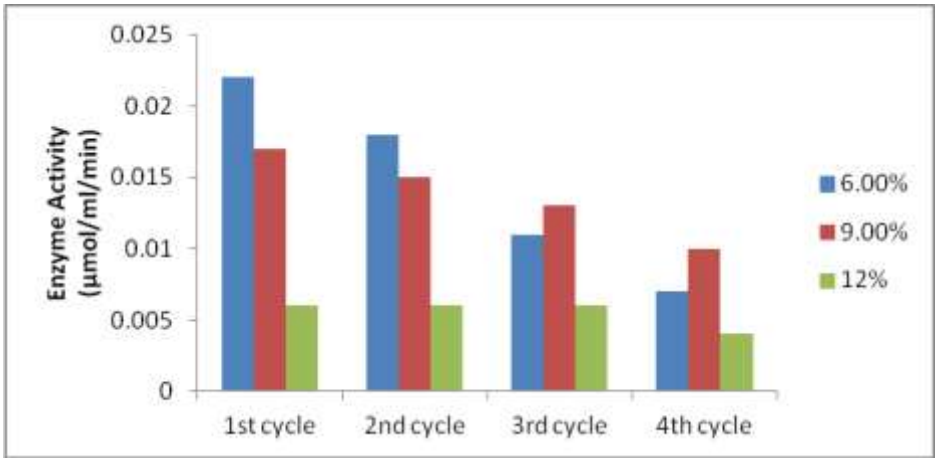

Fig. 6(b). Reusability of enzyme entrapped in Acrylamide gel.

Reusability

Reusability of enzyme entrapped calcium-alginate beads (2%, 2.5%, 3% and 3.5%) and acrylamide gel slices (6%, 9% and 12%) at 100˚C with incubation time of 1 hour was checked by reusing the beads again and again after washing them twice or thrice before being used in next cycle and then determining their activity after each cycle. The results of the reusability are shown in the Fig 6(a) and 6(b).

DISCUSSION

Immobilization of enzymes is usually carried out by solid matrix through adsorption on a solid support by ionic or covalent binding. Entrapment is taken as the most preferable method because it prevents excessive loss of enzyme activity after immobilization, increases enzyme stability, protects enzyme from microbial contamination. Physical entrapment of enzyme in calcium alginate beads has shown to a relatively easy, rapid and safe technique in comparison with other immobilization methods. In this study various concentrations of Sodium alginate (2%-3.5%) beads were made to acquire greater stability. The beads percentage was varied by varying the amount of sodium alginate. The percent entrapped activity was found to be approximately equal in all the percentages of beads (2%, 2.5%, 3%, and 3.5%). No significant difference was found in these percentages. Different researchers reported that sodium alginate concentration range from 2-3% was mostly suited for keratinase, lipase and protease immobilization (Farag and Hassan, 2004). It was also reported in a study that maximum leakage of enzyme occurred at 1% sodium alginate beads because of larger pore size and less tightly cross linked fragile calcium-alginate beads. On the other hand 3% and 4% calcium-alginate beads concentration entrapped activity of the enzyme was found to be very low in comparison which might be due to the high viscosity of beads which decreased the pore size and thus hindered the penetration of substrate into the beads (Anwar *et al*., 2009).

Polyacryl amide gels with 6%, 9% and 12% was also prepared with entrapped enzyme. The percent entrapped activity was found maximal in 6% as well as 9% gel slices. However 12% gel slices did not show significant activity most probably because of decreased pore size that may hinder the penetration of substrate into the gel slices to react with the enzyme.

Crude Endoglucanase from *Thermotoga petrophila* showed temperature maxima of 100˚C. After immobilization, the temperature maxima remained the same that is 100˚C for the enzyme entrapped in beads. There was no change in optimum temperature of enzyme's activity entrapped in beads however, in the enzyme entrapped in gel slices, maximum activity was observed at 90˚C instead of 100˚C. In the same way the optimum conditions of galactosidase was also not affected by its immobilization in gelatin alginate (Naganagouda and Mulimani, 2006). Ates and Mehmetoglu (1997) reported increased temperature optima and increased temperature stability of immobilized enzyme as compared to free enzyme. Abdel-Fattah *et al* in 1997 reported that thermal stability of cellobiase was improved by entrapping it in polyacylamide gel. Rajoka*et al.* (2007) immobilized endoglucanase in Eudragit L-100 in order to make it more stable and thus reported that immobilized endoglucanase exhibited maximum activity 2˚C higher as compared to free endoglucanase.

The effect of pH on free and immobilized endoglucanase was examined over pH 3-12 in buffer solutions. Maximum activities for both free and immobilized enzyme in acrylamide gel were obtained at pH 6. However for enzyme entrapped in calcium-alginate beads, maximum activity was observed at pH 5. So a pH shift towards acidic pH was observed in enzyme entrapped in calcium-alginate beads. Different researchers reported no change in activity of endoglucanase at different pH for free as well as immobilized enzyme. Ates and Mehmetoglu (1997) reported that immobilized and free enzyme activities were affected at alkaline pH indicating that immobilized endoglucanase was more stable at acidic pH. Moreover, after incubation in basic buffers of pH 8, 9, 10, 11 and 12, the beads got shrink and therefore no activity of the enzyme was observed. It might be due to the fact that high pH caused the less tightly fragile interactions of the molecule to be converted into strongly bound molecules that caused hindrance in substrate and enzyme interaction resulting in no activity of the enzyme. Rajoka *et al.* (2007) reported a shift in pH optimum of endoglucanase upon immobilization on Eudragit L-100 being shiftef from 6.4 to 7 pH because of some ionizable amino acids located on the periphery of the active site residues which are usually involved in substrate binding and catalysis that is the hydrolysis of CMC to Glucose. Abdel-Naby*et al*. (1999) reported that optimal pH of dextranase immobilized in polyacrylamide was shifted to a higher value 6.0 from 5.5 which was optimum for soluble enzyme.

Effect of incubation time on the activity of immobilized enzyme was assayed and was compared to that of the free enzyme. The free enzyme gave maximum activity when incubated for 10 minutes whereas enzyme entrapped in calcium-alginate beads showed maximum activity when incubated for 3 hours and enzyme entrapped in acrylamide gel gave maximum activity when incubated for 2 hours and 30 minutes. *Thermotoga petrophila*is already a thermostable organism. The increase in incubation is due to fact that after immobilization more time is required by the substrate in order to reach the active site of the immobilized enzyme as compared to the free enzyme as the enzyme is entrapped in cross linked linkages. Anwar *et al.* (2009) reported an increase in time for the activity of immobilized protease by 5 minutes compared to that of the free enzyme. Similarly, calcium-alginate entrapped dextransucrase took 60 min to achieve maximum enzyme activity which was 4 times higher than that of the free enzyme.

Shelf-life of the enzyme entrapped in beads as well as the gel slices was determined. The beads and gel slices with enzyme being entrapped in them were kept at 4˚C when formed. The activity was taken for enzyme entrapped in beads for 32 consecutive days. The first day activity was calculated to be 0.15µmol/ml/min and it remained the same for 28 consecutive days. At 29th day of beads formation, the activity was calculated to be 0.138µmol/ml/min determining a minor change in activity even after 28 days. It remained 0.138 µmol/ml/min upto 32nd day. For enzyme entrapped in gel slices, the activity calculated at first day of beads formation was 0.072µmol/ml/min. It remained the same for 18 days showing a minor decrease in activity at 19th day of gel formation to be 0.067µmol/ml/min. The results showed that enzyme became stable for a longer duration when immobilized as compared to the free enzyme that showed reduction in activity earlier. Anwar *et al* in 2009 found the storage stability of entrapped protease at 4˚C upto 10 days while at 30˚C the enzyme lost its activity within three days.

Entrapped endoglucanase remained in calcium-alginate beads as well as acrylamide gel slices even after 4 operational cycles. The activity dropped after each cycle as the enzyme was heated for 1 hour each time. The beads were reused by washing them twice or thrice with distilled water. For 2% calcium alginate beads, the activity was decreased by 0.06, 0.081, and 0.008µmol/ml/min, respectively after each cycle giving negligible activity at the fourth cycle. Similarly for 2.5%, 3% and 3.5% beads with entrapped enzyme the activity in first cycle were being 0.153, 0.147 and 0.139µmol/ml/min, respectively and for the fourth cycle being 0.018, 0.019 and 0.038µmol/ml/min respectively. Similarly for gel slices, the activity calculated for first cycle was 0.022, 0.017 and 0.006µmol/ml/min, respectively and for the fourth cycle 0.007, 0.010 and 0.004 µmol/ml/min respectively with activity which being decreased negligibly after each cycle. Saleem *et al.* (2005) reported that endoglucanase entrapped in polyacryamide gel exhibited the same activity even after six cycles of reuse. Rajoka *et al.* (2007) reused endoglucanase from C. *biazotea* mutant 51SMon Eudragit L-100 for six cycles with 2 hours of incubation time for each cycle and did not notice any change in the activity of the enzyme. Anwar *et al* in2009 found that protease can retain its activity for longer time and can be reused upto three times.

CONCLUSION

Endoglucanase was successfully immobilized in calcium-alginate beads as well as acrylamide gel slices. The maximum activity in enzyme entrapped in gel slices was observed at 90^{0}C when the enzyme was incubated for 2 hours and 30 min. So temperature shift of 10^{0}C was observed for gel slices. For the enzyme entrapped in calcium-alginate beads, maximum activity of the enzyme was observed at 100^{0}C when the enzyme was incubated for 3 hours showing that the enzyme became stable for longer duration of time. No defined pH shift was observed in the enzyme entrapped in acrylamide gel slices whereas for that of the enzyme entrapped in calcium alginate beads, pH shift

towards more acidic range was observed. The functional stability of the enzyme was improved as it gave activity when reused for four times. The shelf life of the enzyme, stored at 4^0C, entrapped in calcium-alginate beads was found to be 32 days with enzyme giving activity till the last day and the shelf-life of the enzyme entrapped in acrylamide gel slices was found to be 18 days. These results confirmed the utilization of immobilized enzyme for any industrial application.

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