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CHARACTERIZATION OF α-AMYLASE FROM *PENNISETUM TYPHOIDES* IMMOBILIZED INSIDE CALCIUM ALGINATE BEADS

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Abstract:

α-Amylase purified from *Pennisetum typhoides* (specific activity 578.8 U/mg protein) was immobilized within calcium alginate beads. The required hardness and consistency of the bead was achieved at 3% sodium alginate and 5% $CaCl_2$. The percent immobilization achieved was 69%. The pH optimum of α-amylase calcium alginate bead was 7.0 without any shift from the pH optimum of soluble enzyme (7.0). The value of K_m for soluble starch in calcium alginate beads is 1.57 mg/mL. The immobilized enzyme showed temperature optimum at 70°C. From the Arrhenius plot, activation energy of α-amylase calcium alginate bead was found to be 14.8 kJ/mol. The α-amylase calcium alginate bead retained 67.1% activity, when incubated for 10 min at 70°C. The calcium alginate α-amylase beads when stored at 4°C in 50 mM imidazole buffer, pH 7.0 (assay buffer), retained 95% activity when stored for 90 days. The same enzyme bead could be reused to up to 20 cycles with a loss of only 30% activity. Thus immobilized α-amylase may have potential applications in various industries.

Keywords: α-Amylase; *P. typhoides*, Immobilization; Calcium alginate beads; Storage stability; Reusability

Introduction

α-Amylase finds extensive applications in industries like food, brewing, detergents, paper, textile and dyeing. It is being used for liquefaction of starch, to reduce haze formation in juices, pre-treatment of animal feed to improve digestibility [1]. Enzymes in the soluble form catalyze the reaction in presence of substrates but cannot be recovered from the reaction mixture. Immobilized enzyme on the other hand can be separated from the reaction mixture. The major advantage of immobilization is its reuse for several cycles, better stability and economy with respect to cost of enzyme production. Enzymes can be immobilized on surface of an inert matrix by binding or can be entrapped inside gel beads [2]. More robust immobilized enzymes were developed that can work under hostile conditions [3,4].

α-Amylase (EC 3.2.1.1; 1,4-α-D-Glucanglucanohydrolase) acts on (α 1→4) linkages in polysaccharides (starch, glycogen) containing three or more 1,4-α-linked D-glucose units and catalyzes the hydrolysis in a random manner to generate reducing groups in the α-configuration [5].

α-Amylases have been immobilized on different matrices like silochrome [6], glass [6,7], gelatin [8,9], poly(methyl methacrylateacrylic acid) microspheres[10], polyanilines [11], cellulose fibers [12], alginate beads and capsules [13-22], celite [23], amberlite and chitosan beads [24, 25] and Fuller's earth [26]. There are reports on immobilization of α-amylase on gold nanorods [27], nano pore zeolite [28] and nanoporous polyacrylamide-graphene oxide nanocomposite [29]. α-Amylase from microbial sources have been extensively studied with respect to immobilization [6-7,12,15,17-19, 22-23, 30-31]. Limited information however, is available on immobilization of α-amylases from plants [8-9, 14, 21, 24-26].

Calcium alginate beads have been used to entrap enzyme inside the beads. Alginate is anionic linear copolymer composed of blocks of α-L-guluronic acid and β-D-mannuronic acid residues in different sequential arrangements [32]. Alginates have the ability to form gels where blocks of guluronic acid residues are primarily responsible. This is through cross linking reaction of carboxylic acid with calcium chloride, barium chloride, strontium chloride, or poly (L-lysine) [33-35]. There is exchange of sodium ions from guluronic acid with Ca^{2+}. The latter ions fit in as egg in an egg box. The porosity of the calcium alginate beads depend upon the alginate type and the gelling agent concentration [36]. The major advantage of use of calcium alginate for entrapment of enzymes is that it is cheap, non-toxic and method is rapid [37].

In the present paper we describe the entrapment of α-amylase from *P. typhoides* inside calcium alginate beads and compare its properties with soluble enzyme.

Results and Discussion

The total number of enzyme beads obtained from 6.67 mL of sodium alginate enzyme solution was 62. The percent immobilization of α-amylase inside calcium alginate beads was determined and was found to be 69%, with specific activity 402.4 U/mg protein and 0.0795 U per bead. Similar result (65%) was obtained when *Bacillus subtilis* and *B. thuringienesis*α-amylase were entrapped inside calcium alginate, respectively [17, 22]. It suggests that there is little leaching of enzyme from calcium alginate beads. For entrapment of α-amylase inside calcium alginate beads, relatively lower concentrations of sodium alginate (3%) and $CaCl_2$ (5%) were used. Attempts were made to immobilize enzyme using higher concentrations of sodium alginate solution posed difficulties due to increased viscosity and consistency of beads. Sodium alginate (3%) concentration was also used in another report [38]. A reduced sodium alginate concentration however is not the best choice since it has a high risk of enzyme leaching. At higher concentrations of $CaCl_2$ (6-8%), hardness of bead increased resulted in difficulty for soluble starch (substrate) to get inside the bead, giving lower activity. Calcium chloride concentration was kept relatively low (2%) for entrapment of α-amylase inside calcium alginate bead from *B. circulans* GRS 313 [13]. In case of *B. subtilis* and *B. amyloliquifaciens* α-amylase, optimum sodium alginate and $CaCl_2$

concentrations were 2 and 5% (w/v), respectively [15,19]. It has been reported that the porosity of the calcium alginate beads depend upon the gelling agent concentration [36]. For immobilization of urease, a higher concentration of sodium alginate (5.5%) and $CaCl_2$ (8%) have been used [39]. Some other plant α-amylases immobilized on other matrices also showed % immobilization close to 70% [24-25]. The magnified photograph (12.5x) of α-amylase entrapped inside calcium alginate bead is shown in Fig. 1.

The effect of pH on % relative rates of enzyme calcium alginate bead and soluble enzyme catalyzed reaction is shown in Fig. 2. The optimum pH is observed to be 7.0 like the soluble enzyme suggesting that entrapment of enzyme inside the bead did not change the property of the enzyme. The method also does not involve any cross-linking agent and therefore the enzyme property is less likely to change. No deviation in pH optimum has also been observed when compared to soluble α-amylase in celite [23, 40]. At pH 6.5, soluble enzyme and immobilized enzyme show similar % relative activities. At pH 7.5, immobilized enzyme showed a higher % relative activity in comparison to soluble enzyme. α-Amylase from *P. typhoides* immobilized inside gelatin beads also showed no deviation in optimum pH [9]. Some other α-amylases immobilized inside alginate beads, however, have shown deviation of 0.5 pH units towards alkaline side [14, 16-17].

The immobilized α-amylase inside calcium alginate beads were used to study effect of [starch] on rates of reaction catalyzed by them at pH 7.0. The Line weaver Burk plot of the data is shown in Fig. 3. The K_m value for soluble starch was 1.57 mg/mL for α-amylase calcium alginate bead catalyzed reactions. The K_m value is higher with respect to the soluble α-amylase (0.5 mg/mL) determined at pH 7.0 [41]. Increase in K_m value has been observed in results obtained with other immobilized enzymes [13, 16, 24-25]. In one case, almost 15 times increase in K_m value was observed in comparison to soluble enzyme [10]. Increase in K_m value has been observed with the size of the bead [13]. The increase in the value of K_m is due to diffusional limitation of substrate [42]. A higher concentration of substrate will therefore be required to saturate the immobilized enzyme and leads to increase in K_m [42-43].

The effect of temperature (30 to 70°C) on rate of reaction catalyzed by α-amylase immobilized inside calcium alginate bead was studied at pH 7.0 and the plot % relative activity versus temperature is shown in Fig. 4a. It is evident that temperature optimum for immobilized enzyme is 70°C, whereas that of soluble enzyme 60°C. Some other α-amylases immobilized inside alginate bead also showed a higher temperature optima [14, 17]. From the above data Arrhenius plot was made and is shown in Fig. 4b. The energy of activation (E_a) was determined and was found to be 3.54 kcal/mol (14.8 kJ/mol). The value is lower than that of soluble enzyme (6.9 kcal mol^{-1}) determined at the same pH 7 [41]. A fall in the value of E_a suggests that immobilized enzyme will

catalyze the reaction more efficiently. Further, from the plot, it is evident that rate of reaction catalyzed will be zero at 2.86°C.

The effect of temperature on % residual activity of calcium alginate α-amylase bead and soluble α-amylase was studied at different temperatures for fixed time (10 min). The results are shown in Fig. 5. It is evident that at any temperature, there is relatively higher activity retention in calcium alginate enzyme bead in comparison to soluble enzyme. The immobilization of enzyme by entrapment within calcium alginate provides a kind of protection or shield to the enzyme, and hence the enzyme could withstand the adverse effects under higher temperatures making it more thermal stable.

The storage stability of α-amylase entrapped inside calcium alginate bead was studied, when beads were stored at 4°C. The results are shown in Fig. 6. More than 95% activity is retained in case of calcium alginate enzyme bead after 90 days. These results suggest that enzyme is more stable when entrapped inside calcium alginate beads. Soluble enzyme however, retained only 50% activity when stored at 4°C after 90 days [41]. Under similar conditions more activity loss has been reported in other immobilized α-amylases [30, 7, 24-25]. In one case there was no loss in enzyme activity up to 180 days [6]. At higher temperatures also immobilized enzymes have been stored without loss in activity [16, 44].

The number of cycles of the reuse of same bead was tested for calcium alginate enzyme bead. The results are shown in Fig. 7. It is evident that calcium alginate bead retained more than 95% activity after 5th time reuse, 90% activity after 10th time reuse. The same bead retained more than 70% activity even after 20th time reuse. Similar results were obtained in α-amylase immobilized inside calcium alginate capsules from *B. subtilis* [15]. Further, when silica gel was introduced inside calcium alginate capsule formation process, retained 90% activity after 20th time reuse [15]. The above observations suggest that α-amylase entrapped inside calcium alginate shows lesser leaching on reuse. Significant loss in activity was however observed in α-amylase from *B. amyloliquefaciens* entrapped inside calcium alginate [19]. Losses have been observed in some other immobilized α-amylases [14, 24-25, 45-46]. The loss in activity may be due to weakening in the strength of binding between matrix and enzyme and on further repeated use, the enzyme may leach out from the matrix.

Experimental

Materials

Calcium chloride, soluble starch, sodium potassium tartrate, 3,5- dinitrosalicylic acid, sodium hydroxide, orthophosphoric acid (HiMedia, India); sodium alginate, (Sigma Aldrich, USA); dehydrated alcohol (Bengal Chemicals and Pharmaceuticals Ltd., India); imidazole, maltose, bovine serum albumin (BSA), Coomassie Brilliant Blue G-250 (Sisco Research Laboratory, India). α-Amylase isolated from *P. typhoides*

(specific activity 578.8 U/mg protein) was used for the present immobilization studies as has been described earlier [41]. All solutions were prepared in de-ionized water from Milli-Q system (Millipore, USA).

Soluble α-amylase assay

The α-amylase activity was assayed by discontinuous procedure using 3,5-dinitrosalicylate (DNS) reagent [47]. The 1 mL assay solution contained 0.5 mL of 1% soluble starch and 0.4 mL assay buffer (50 mM imidazole buffer, pH 7.0), maintained at 37°C in a water bath. The reaction was initiated by addition of an aliquot of enzyme (0.1 mL). After 3 min, DNS reagent (1 mL) was added. The tube was incubated for 5 min in boiling water bath, and then brought to room temperature (30°C). The volume was raised to 10 mL with de-ionized water. The absorbance was recorded at 540 nm using a Varian 50 spectrophotometer [41]. One unit of α-amylase was defined as the amount of enzyme releasing one µmole maltose equivalent per min under the assay conditions.

Protein quantification

Protein content of soluble α-amylase and in the washings of immobilized α-amylase entrapped inside calcium alginate beads was done according to Bradford's method [48] with little modifications.

Entrapment of α-amylase inside calcium alginate beads

The α-amylase (171.8 U/mL) was diluted 40-fold (4.295 U/mL) for the entrapment experiment. A 4% sodium alginate solution was prepared by dissolving 0.4 g sodium alginate in 10 mL de-ionized water. To the above sodium alginate solution (5 mL), 1.665 mL enzyme (7.15 U) was added so that concentration of sodium alginate was reduced to 3%. A 5% $CaCl_2$ solution was prepared in de-ionized water and stored under refrigeration. The enzyme-sodium alginate mixture was allowed to fall drop-wise into cold 5% $CaCl_2$ solution with continuous stirring using magnetic stirrer. Beads of enzyme-calcium alginate were formed once the drop came in contact with the $CaCl_2$. The enzyme-calcium alginate beads were kept in the $CaCl_2$ solution for 2 h till the beads attained the required consistency of hardness. The beads were retrieved from the above solution and then washed with de-ionized water, and stored in assay buffer at 4°C for further experimental uses.

Activity assay of immobilized α-amylase

The 1 mL test solution consisted of 0.5 mL of 1% soluble starch and 0.5 mL assay buffer kept in a water bath maintained at 37°C. Two calcium alginate-enzyme beads were added to the test solution and the reaction was allowed to take place for 10

min with continuous shaking. After 10 min, the beads were taken out and 1 mL of DNS reagent was added. The further steps were as described earlier.

Photo image

The photograph of calcium alginate α-amylase beads was recorded by Kodak C43 Digital Camera with 12.5x magnification.

Steady state kinetics

The effect of variation of pH on soluble and immobilized enzyme activity has been studied using 50 mM imidazole buffers (pH 5.0- 9.0). From the data, a plot of % relative activity versus pH was made to determine pH optima.

A stock solution of 2% soluble starch was prepared in 50 mM imidazole buffer, pH 7. The rate of immobilized α-amylase catalyzed reaction was monitored at different concentrations of starch (0.1 mg to 6 mg) in the test solution at 37°C. From this data, Lineweaver Burk plot (1/v versus 1/[starch]) was made to determine value of K_m.

Effect of temperature on immobilized α-amylase catalyzed reaction has been studied in the range of 30 to 70°C.The reaction mixture was maintained at the particular temperature. Concentration of soluble starch was the same (5 mg/mL) as in the activity assay. From the data, a plot of relative % activity versus temperature was made to determine optimum temperature and was compared with that of soluble enzyme. Further, from the data, Arrhenius plot was made and energy of activation was determined.

Thermal denaturation

The thermal denaturation of soluble enzyme and immobilized enzyme was studied. The enzyme solution was transferred in different tubes and were quickly brought to and maintained at different temperatures 40-70°C, for 10 min, chilled immediately and were tested for residual enzyme activity. The % residual activity was computed. In case of immobilized enzyme, α-amylase entrapped inside calcium alginate beads suspended in assay buffer were maintained at different temperatures as above and were used to test for residual enzyme activity. A plot of % relative residual activity versus temperature was made and compared with that of soluble enzyme.

Storage and reusability of immobilized α-amylase

α-Amylase immobilized inside calcium alginate beads was routinely stored in assay buffer at 4°C. In one set of experiment, enzyme activity was routinely tested on different days using different beads. A plot of % residual activity versus days was made. In another set of experiment, activity assay of a particular bead was performed for several cycles and a plot of % residual activity versus cycle of reuse was made.

The results reported in the present study represent an average of data generated from experiments carried out in triplicate.

Conclusion

The present work on immobilization of α-amylase inside calcium alginate beads implicates important role in industries. The properties of enzyme do not change as a result of immobilization (pH optima 7.0). The immobilized enzyme is thermally more stable, shows little loss in activity when stored at 4°C and can be used for several cycles without significant loss in activity.

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Conflict of interest

The authors do not have any conflict of interest.

Fig. : 1. Magnified photograph (12.5 x) of α-amylase entrapped inside calcium alginate beads using Kodak digital camera

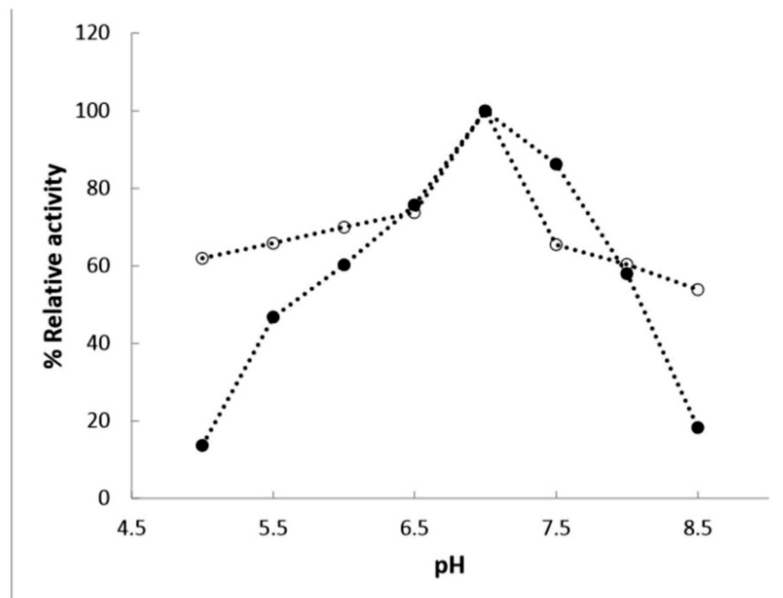

Fig. : 2. Effect of pH on relative activities of soluble (○) and calcium alginate immobilized α-amylase (●) bead catalyzed reaction. The effect has been studied using 50 mM imidazole buffers, (pH 5.0- 9.0). The assay procedure has been described in methods.

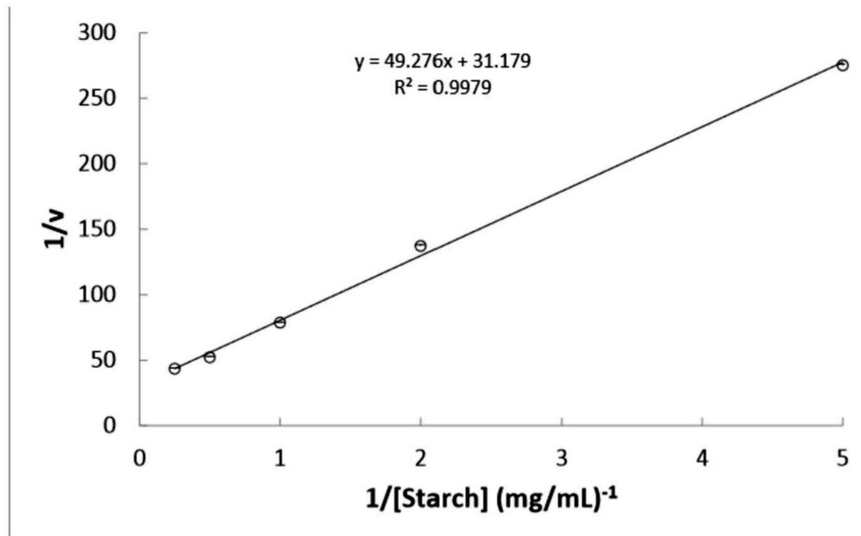

Fig. : 3. Lineweaver Burk plot of calcium alginate immobilized α-amylase bead catalyzed reaction rate at different starch concentrations (0.1 mg to 6.0 mg). The assay procedure has been described in methods.

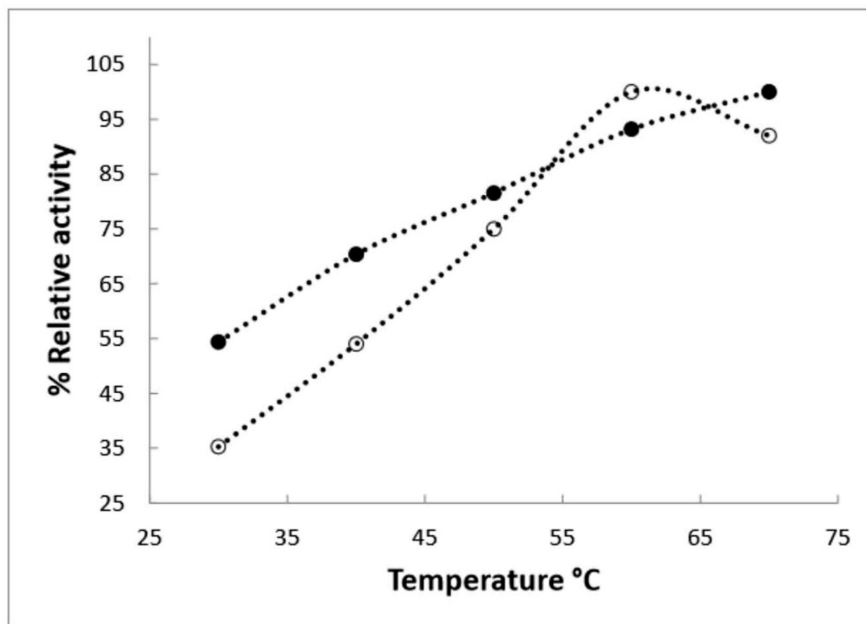

Fig. : 4a. Effect of temperature (30 to 70°C) on soluble α-amylase (○) and calcium alginate immobilized α-amylase (●) bead catalyzed reactions. The reaction mixture was maintained at the particular temperature.

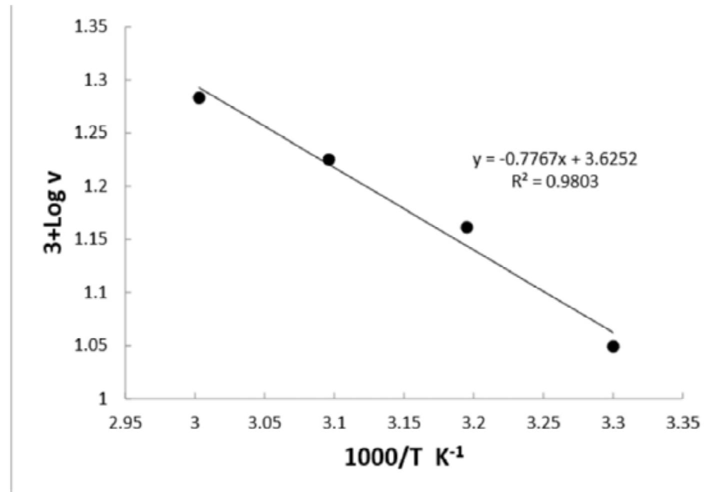

Fig. : 4b. Arrhenius plot of calcium alginate immobilized α-amylase bead catalyzed reaction at different temperatures. The plot was made from above data.

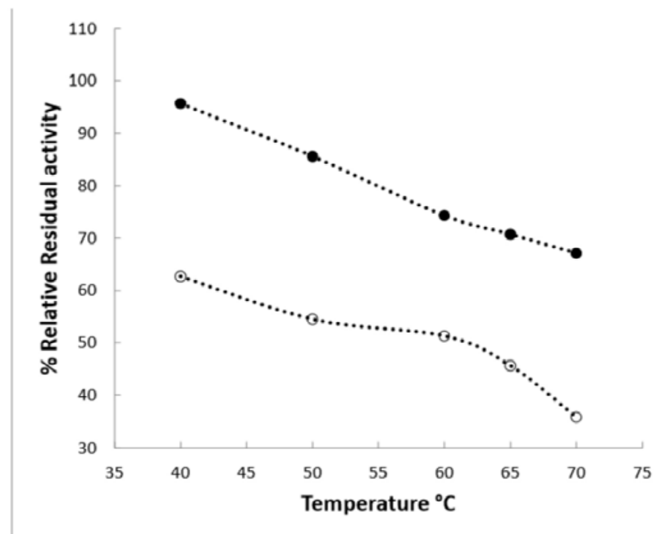

Fig. : 5. Effect of temperature on soluble α-amylase (○) and calcium alginate immobilized α-amylase (●) bead. The enzyme solution was transferred in different tubes and were quickly brought to and maintained at different temperatures 40-70°C, for 10 min, chilled immediately and were tested for residual enzyme activity as described in methods. In case of immobilized enzyme, α- amylase entrapped inside calcium alginate beads suspended in assay buffer were maintained at different temperatures as above and were used to test for residual enzyme activity.

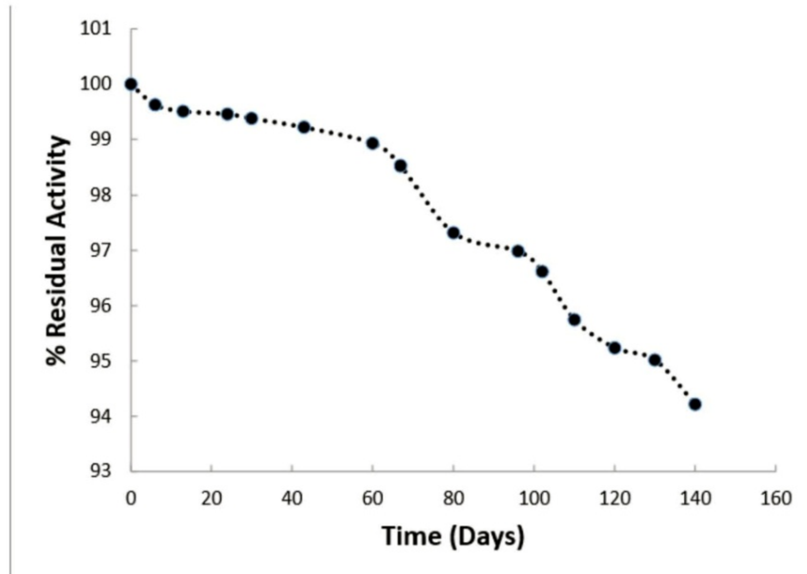

Fig. : 6. Storage stability of calcium alginate immobilized α-amylase beads at 4°C

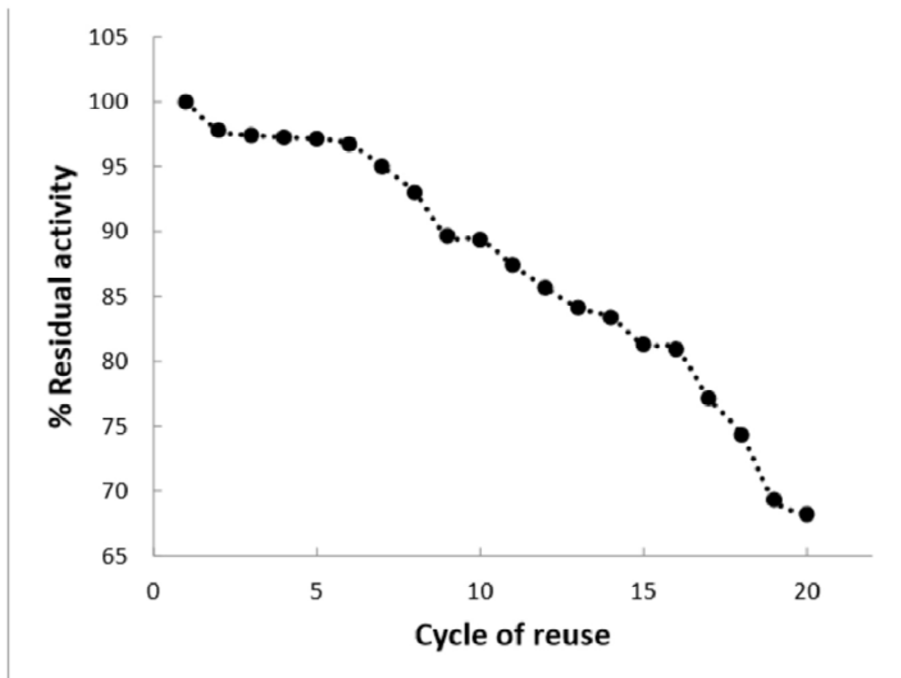

Fig.. : 7. Reusability of the beads calcium alginate immobilized α-amylase beads at 4°C.

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