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Pullulan-complexed α-amylase and glucosidase in alginate beads: Enhanced entrapment and stability

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ABSTRACT

Enhanced entrapment of the enzymes, α-amylase and glucoamylase, was found in alginate beads on addition of pullulan in the enzyme mixture. Under optimized process conditions of entrapment, enzymes–pullulan complex showed an entrapment of 85% in the alginate beads as opposed to 25% for the free enzymes. Beads of enzymes–pullulan complex showed lower inactivation rate constant and higher half life than corresponding beads of free enzymes. Activation energy of beads of enzymes–pullulan was increased by 6.81 kJ/mole compared to beads of free enzymes. This implies better stability the enzymes in enzymes–pullulan beads along with increased immobilization yield. Moreover, enzymes–pullulan beads also showed pH stability at extreme acidic and alkaline pH. Addition of pullulan in the enzymes mixture lowered the K_m and increased the V_{max} as compared to beads of free enzymes. Hydrolysis of starch and reusability study showed better applicability of beads of enzymes–pullulan as compared to free enzymes.

1. Introduction

Enzymes can be used in synergistic action in the industry to increase the yield of the desired product. This process is more economical for multistep industrial processes to reduce cost and time (Minakshi & Pundir, 2008). Starch hydrolysis is one such multistep process. This can be carried out using amylase and glucoamylase either separately or simultaneously. Amylase and glucoamylase has shown better results for saccharification of cassava starch while using them simultaneously rather than separately (Ruiz, Sanchez, Torres, & Molina, 2011). Co-immobilization of α-amylase and glucoamylase on hydrophilic silica gel and DEAE-cellulose for starch hydrolysis has also been reported (Park, Haam, Jang, Ahn, & Kim, 2005). Co-conjugation and individual conjugation of α-amylase and glucoamylase with pectin has been successful wherein the individual conjugation with simultaneous use proved to be more advantageous (Jadhav & Singhal, 2013). Hydrolysis of starch has also been carried out using individually entrapped glucoamylase and pullulanase in alginate beads to make them reusable. Simultaneous use of these entrapped enzymes gave much higher amount of products than that of separate use (Roy & Gupta, 2004). Entrapment of these enzymes together can increase their reusability and ease product separation but the enzyme leakage is the major drawback.

Immobilization is one of the best techniques for industrial applications of enzymes. Immobilized enzymes are technically and economically advantageous as it is reusable, can be easily separated from the reaction mixture, and are stable under extreme conditions. Various methods of immobilization include adsorption on insoluble materials, entrapment in polymeric gels, encapsulation in membranes, cross linking with bifunctional agents, and covalent linking to an insoluble carrier. Among these, entrapment in alginate beads is the simplest method (Ortega, Busto, & Perez-Mateos, 1998) as alginate is safe, inexpensive, has good mechanical strength, and has porosity for substrate and product diffusion (Talekar & Chavare, 2012). Moreover, this method causes minimum disturbance to the enzyme structure and hence the activity.

A major concern for immobilization in alginate beads is the lower yields of immobilization due to the loss of enzymes in the gel forming solution (Tanriseven & Dogan, 2001). A significant loss of water soluble protein (about 30%) due to diffusion through the beads without denaturation has been reported (Dashevsky, 1998). Immobilization techniques also influence the extent of immobilization of enzymes. For instance, immobilization of lipase by cross linking through gluteraldehyde and entrapment in alginate–carrageenan hybrid matrix is reported to show 89% immobilization yield (Abdulla & Ravindra, 2013), while that immobilized in cellulose–biopolymer composite hydrogels using a biocompatible ionic liquid showed 52% immobilization yield of lipase (Kim, An, Won, Kim, & Lee, 2012). Coating of beads with

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chitosan and silicate showed good immobilization of lipase (Won, Kim, Kim, Park, & Moon, 2005). Though immobilization in alginate beads is the simplest technique, it shows low immobilization yield. Besides, leakage of enzymes from these beads makes it economically unsuitable for applications. Hence, there is need to develop a simple and cost effective method which can avoid the protein loss in the solution. This can be done by retention of water, cross linking of enzymes with some other polymers, or by increasing the size of enzyme molecules. These objectives can be achieved by the use of polysaccharide as it possesses all these characteristics.

Non-covalent interaction between enzymes and polysaccharide is well known in scientific literature. It is a simple method of stabilization with good retention of enzyme activity (Li, Zhou, Li, Huang, & Zhong, 2010). Polysaccharides have many characteristics including water absorption, film formation and cross linking with protein. These characteristics could be potentially helpful to increase the entrapment yield and stability of enzymes in the alginate beads. Although enzymes are found to be stable in beads, there is chance of enhancing their stability along with immobilization yield by the use of polysaccharide in the enzyme mixture during entrapment in beads.

This study was aimed to enhance the entrapment efficiency of enzymes in alginate beads and study of the effect of added polysaccharide on the activity and stability of enzymes. Polysaccharides were screened to form complex with enzyme to achieve the highest entrapment of enzyme within the alginate beads. Beads with the selected polysaccharide, pullulan, were evaluated for thermal and pH stability of enzymes as compared to beads without added pullulan. Kinetic constants and starch hydrolysis were studied to check the efficiency of beads. Reusability of beads was compared to find the best possible beads for further applications.

2. Experimental

2.1. Materials

Crude α-amylase from *Bacillus licheniformis* was obtained as gift sample from Sigma Chemical Industries, Mumbai, India. It was dialyzed against 20 mM of sodium citrate buffer of pH 5.4 at 4 °C overnight and then used for study. Glucoamylase from *Aspergillus niger* was obtained from Sigma–Aldrich, Mumbai, India. Glucose oxidase–peroxidase (GOD-POD) kit was purchased from Accurex Biomedical Pvt. Ltd., Mumbai, India. Pullulan was a commercial sample manufactured and gifted by Hayashibara, Okayama, Japan. All other polysaccharides were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India.

2.2. Preparation of alginate gel beads containing complex of α-amylase, glucoamylase and pullulan

Mixture of α-amylase (5 mg/ml) and glucoamylase (5 mg/ml) was prepared in 0.02 M sodium citrate buffer (pH 5.0). Pullulan (50 mg/ml) was added to the enzyme mixture and kept for 30 min at room temperature (~28 ± 2 °C). Sodium alginate (2%, w/v) was dissolved in 0.02 M sodium citrate buffer (pH 5.0). The prepared sodium alginate solution (9 ml) was mixed with enzymes–pullulan mixture (1 ml) and used for preparation of beads. The solution was dropped through a syringe (0.8 mm × 25 mm) into 2% $CaCl_2$ solution (in water) and incubated for 2 h at room temperature to entrap enzymes in calcium alginate beads. Beads were washed with distilled water till no enzyme activity was detected in the washings. Finally, the prepared beads were kept at 4 °C till further use. For all the subsequent experiments, these beads were considered as entrapped enzymes–pullulan complex. Another set of beads was prepared using α-amylase and glucoamylase complex without addition of pullulan as beads of free enzymes (Ertan, Yagar, & Balkan, 2007; Rajagopalan & Krishnan, 2008).

2.3. α-Amylase and glucoamylase combined activity assay

Prepared alginate beads (160 mg) containing free enzymes and enzymes–pullulan complex were added to 1% starch (500 μl) solution prepared in 0.02 M sodium-citrate buffer of pH 5.0. Mixture was incubated at 60 °C for 15 min after which it was kept on ice and the released glucose was detected by a glucose oxidase–peroxidase (GOD-POD) kit (Sathish, Madhavan, Vasanthi, & Amuthan, 2012). Beads were separated and sample containing released glucose (10 μl) was added to GOD-POD working enzyme solution (1 ml) and kept for 30 min at room temperature. The absorbance was measured at 505 nm using UV-vis spectrophotometer (Hitachi U2001, Japan). The combined enzyme activity of α-amylase and glucoamylase was calculated using a standard curve plotted using glucose in the range of 0.0–1.0 mg/ml. One unit of combined enzyme activity was defined as the micromole of glucose released per minute per ml per mg of enzyme solution at 60 °C.

2.4. Screening of polysaccharide to enhance the entrapment of α-amylase and glucoamylase in alginate beads

Four different polysaccharides including carboxymethyl cellulose (CMC), gum Arabic, pectin and pullulan were used. Each polysaccharide (50 mg/ml) was added to the mixture of α-amylase and glucoamylase. Different enzymes–polysaccharide complexes were kept for 30 min at room temperature to make stable complex. All the complexes were used for the preparation of alginate beads as per the protocol described in Section 2.2. Beads of free enzymes and enzymes–polysaccharide complex were checked for activity (as per the protocol in Section 2.3) to determine immobilization yield. Immobilization yield can be calculated as:

$$\text{immobilization yield}\,(\%) = \frac{\text{activity of immobilized enzyme}}{A - B} \times 100$$

where A is the activity of free enzyme added and B is the activity of remaining enzyme in washed water.

2.5. Optimization of process of entrapment of enzymes–pullulan complex in alginate gel beads

The process of entrapment of α-amylase and glucoamylase complex with pullulan in alginate gel beads were optimized for parameters which affects the immobilization yield. Ratio of enzymes to pullulan (1:1 to 1:20), different concentrations of sodium alginate (0.5–3%, w/v) and calcium chloride (0.5–4%), curing time (30–180 min) and bead size (0.8–2.1 mm) were used for the immobilization process to get highest possible immobilization yield. The beads were prepared and checked for activity as described in Sections 2.2 and 2.3. Immobilization yield was calculated as described above in Section 2.4.

2.6. Determination of optimum temperature and pH of free enzymes and enzymes–pullulan complex in the alginate beads

The optimum temperature of the beads of free enzymes and enzymes–pullulan complex was determined by carrying out activity assay at different temperatures (30–80 °C) at pH 5.0. Similarly, optimum pH was determined by carrying out activity assay at different pH (20 mM citric acid/sodium citrate buffer of pH 3.0–6.0, 20 mM KH_2PO_4/K_2HPO_4 buffer of pH 7.0–8.0, 20 mM glycine/NaOH buffer of pH 9.0–10.0) at temperature 60 °C. The reaction conditions were as described in Section 2.3.

2.7. Effect of entrapment in beads on combined kinetics of thermal inactivation and pH stability of α-amylase and glucoamylase

The beads (320 mg) of free enzymes (α-amylase and glucoamylase) and enzymes–pullulan complex were incubated at 45 °C, 50 °C, 55 °C and 60 °C in 20 mM sodium citrate buffer of pH 5.0. One set of beads was removed every 30 min for 180 min, and then assayed for combined residual enzyme activity. A semi-log plot of percent residual activity vs time was plotted from which the inactivation rate constant, k, was calculated as the slope, and $t_{1/2}$, the time required for the activity to decrease to half its original activity was calculated as 0.693/k.

The beads (320 mg) of free enzymes (α-amylase and glucoamylase) and enzymes–pullulan complex were incubated at room temperature in 50 mM citric acid/sodium citrate buffer of pH 3.0–6.0, 50 mM KH_2PO_4/K_2HPO_4 buffer of pH 7.0–8.0, 50 mM glycine/NaOH buffer of pH 9.0–10.0. The beads were incubated for 1 h and tested for residual activity to check the pH stability.

2.8. Kinetics of free enzymes and enzymes–pullulan complex in alginate beads

The beads of free enzymes (α-amylase and glucoamylase) and enzymes–pullulan complex were evaluated for determination of kinetic constants K_m and V_{max} using substrate concentration from 0.5 to 10 mg/ml. Lineweaver–Burk plot was used to calculate kinetic constants.

2.9. Hydrolysis of starch using beads of free enzymes and enzymes–pullulan complex

Beads of free enzymes and enzymes–pullulan complex (160 mg) were used to hydrolyse starch (5%) prepared in 0.02 M sodium citrate buffer of pH 5.0 at 60 °C. Samples were withdrawn at regular time interval of 30 min till 180 min, and checked for the amount of glucose released. Released glucose was detected using glucose oxidase–peroxidase (GOD-POD) kit. Appropriately diluted sample (10 μl) was mixed with GOD-POD working enzyme solution (1 ml), kept at room temperature for 30 min and absorbance was taken at 505 nm using UV-vis spectrophotometer (Hitachi U2001, Japan). The degree of starch hydrolysis (%) was calculated as [mg of glucose released/mg of starch] × 100.

2.10. Reusability of beads of free enzymes and enzymes–pullulan complex

Reusability of beads of free enzymes and enzymes–pullulan complex were investigated by checking enzyme activity after every reaction cycle of 30 min. Both types of beads were added to reaction mixture (5% starch prepared in 0.02 M sodium citrate buffer of pH 5.0) at 60 °C. After every cycle of 30 min, beads were removed and washed. Same beads were again added to fresh reaction mixture to measure enzyme activity. The residual activity was calculated by taking the enzyme activity of the first cycle as 100%.

3. Results and discussion

3.1. Screening of polysaccharide to enhance the entrapment of α-amylase and glucoamylase in alginate beads

Four different water soluble polysaccharides (CMC, gum Arabic, pectin and pullulan) were mixed with an enzyme mixture of α-amylase and glucoamylase to prepare a complex of enzyme and polysaccharide. These complexes were used to check the effect on the entrapment of enzymes in alginate beads with respect to increase immobilization yield. Gum Arabic and pullulan showed increased entrapment of enzymes (α-amylase and glucoamylase) in the alginate beads. Beads of enzymes–pullulan complex showed 57% of immobilization yield as compared to 24% yield shown by beads of free enzymes (Fig. 1). Pullulan is a linear homopolysaccharide of glucopyranose residues without any branching. It forms viscous solution in water without gelling and it also has ability to form water soluble film (Israilides, Smith, Scanlon, & Barnett, 1999). High viscosity and film forming capacity of pullulan may help in protein–protein association. Protein–protein association in highly concentrated and viscous solution of protein has been previously reported (Kozer, Kuttner, Haran, & Schreiber, 2007). Many polymers have been reported to create macromolecular crowding in the protein solution causing protein aggregation and compact folding (Berg, Ellis, & Dobson, 1999). Linear structure of pullulan did not affect the enzyme activity, while its viscosity and film forming capacity may have facilitated close association between enzyme molecules which may explain high entrapment of enzymes observed in alginate beads. Pullulan has been reported to form films with concentrated protein where the film strength has been increased by addition of propylene glycol alginate (Shih, 1996).

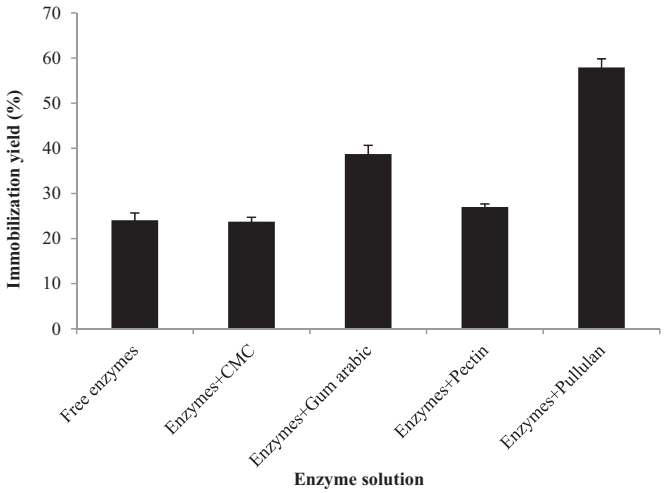

Fig. 1. Screening of polysaccharides to enhance entrapment of enzymes in alginate beads.

Water leakage from the beads is the main reason for loss of water soluble protein and enzyme leakage (Dashevsky, 1998). Pullulan is well known for its water absorption property and moisture retention (Li et al., 2011). Pullulan added to enzyme mixture may aid in holding water in the bead structure and subsequently retain the enzymes in the beads. Thus, high viscosity, film forming property and water retention property of added pullulan may be responsible for high immobilization yield in the beads of enzyme–pullulan complex as compared to bead of free enzymes and beads of enzyme with other polysaccharides. It is quite difficult to explain the reasons for this observation, but we propose a possible hypothesis for the same. CMC and pectin are anionic polysaccharides which may show repulsion with anionic alginate and may not influence the enzyme entrapment positively. However pullulan is neutral polysaccharide which may not repel anionic alginate and thereby help in enzyme entrapment. Although gum Arabic and pectin are both anionic in nature, the relatively higher entrapment in the presence of gum Arabic (as compared to pectin) may be attributed to the presence of glycoprotein therein which may show good protein–protein interaction with the enzyme and hence have shown good immobilization yield over the free enzymes in beads. Previously, other researchers have shown an increased

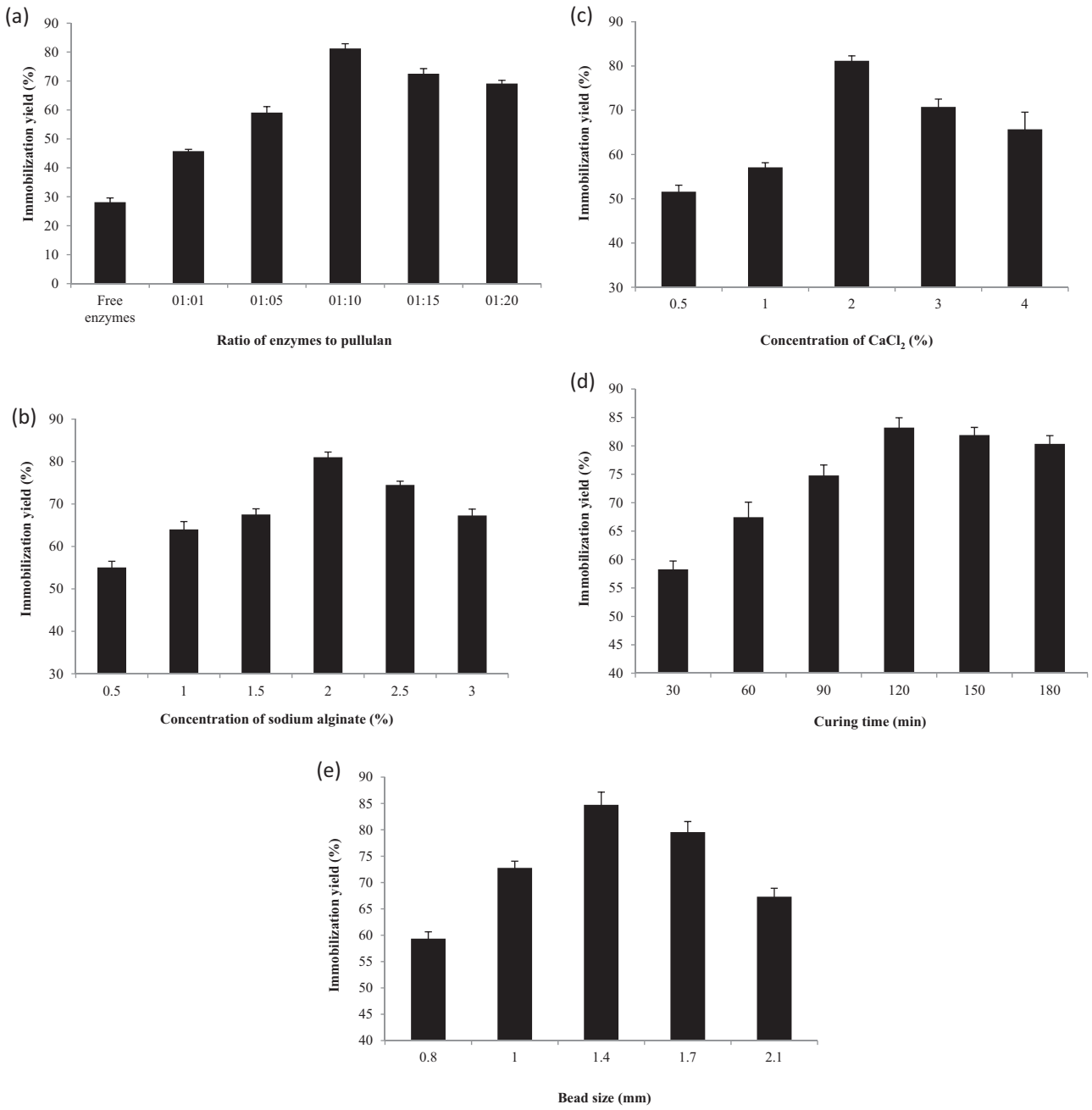

Fig. 2. Effect of different parameters of immobilization on the immobilization yield (a) ratio of enzyme to pullulan, (b) concentration of sodium alginate, (c) concentration of calcium chloride, (d) curing time and (e) bead size.

immobilization yield on addition of bentonite and xanthan gum in the protein solution. Significant reduction in protein loss (36–3%) from alginate beads was achieved by maintaining lower pH value of alginate solution and addition of bentonite in the solution (Dashevsky, 1998). Xanthan gum was successfully used to increase water uptake in beads (Pongjanyakul & Puttipipatkhachorn, 2007).

3.2. Optimization of process of entrapment of enzymes–pullulan complex in alginate gel beads

Process of entrapment of enzymes–pullulan complex in the calcium alginate beads were optimized for the ratio of enzymes to pullulan, concentration of sodium alginate and calcium chloride, curing time and bead size. Since pullulan enhanced the entrapment of enzymes in the alginate beads, the ratio of enzymes to pullulan would be an important parameter which was therefore evaluated. The effect of different ratios of enzyme to pullulan (1:1–1:20) on immobilization yield were studied by using 2% sodium alginate, 2% $CaCl_2$, curing time of 120 min and bead size of 1.4 mm. A 1:10 ratio of enzymes to pullulan gave the highest immobilization yield (Fig. 2a). Lower amount of pullulan may not be sufficient to give desirable effect and the higher amount of pullulan may affect on enzyme activity. In our previous study, we found high concentration of dextran to affect enzyme activity after covalent binding to amylase (Jadhav & Singhal, 2012).

The porosity of the calcium alginate bead depends on the concentration of sodium alginate which affects the immobilization yield. The sodium alginate concentrations were varied from 0.5 to 3% (w/v) with 1:10 ratio of enzyme to pullulan, 2% $CaCl_2$, curing time of 120 min and bead size of 1.4 mm. Highest immobilization

yield (81%) was observed at 2% (w/v) sodium alginate (Fig. 2b). Lower concentration of sodium alginate showed lower immobilization yield which may be attributed to larger pore size and leakage of enzymes from bead matrix. However, higher concentration of the alginate decreased the porosity of beads which might cause diffusion limitation of substrate (Talekar & Chavare, 2012). Further, $CaCl_2$ concentrations were varied from 0.5 to 4% using a 1:10 ratio of enzyme to pullulan, 2% sodium alginate, curing time of 120 min and bead size of 1.4 mm. Stable calcium alginate beads were formed at 2% (w/v) calcium chloride with high immobilization yield of 81%. Beads were fragile at low concentration of calcium chloride, while high concentration decreased the immobilization yield (Fig. 2c). The pH of $CaCl_2$ solution change (pH change from 5.4 to 4.1 as concentration increased from 0.5% to 4%) with concentration which may explain the lower immobilization yield at high concentration of $CaCl_2$ (Roig, Rashid, & Kenndy, 1995). Similar observation has been reported by other investigators (Anwar, Qader, Raiz, Iqbal, & Azhar, 2009; Talekar & Chavare, 2012). Hardness of the beads depends upon the time of exposure to calcium chloride solution and hard beads are stable. Various curing time from 30 to 180 min were studied to achieve the highest immobilization yield by using 1:10 ratio of enzyme to pullulan, 2% sodium alginate, 2% $CaCl_2$ and bead size of 1.4 mm. Curing time of 120 min was found to be necessary to get stable beads with maximum immobilization yield (Fig. 2d). After 120 min of curing time immobilization yield was not found to be altered significantly. At low curing time, beads were softer and fragile causing more enzyme leakage.

Bead size is the critical parameter as it limits the mass transfer of the substrate. Bead sizes were varied from 0.8 to 2.1 mm by keeping other parameters constant which were 1:10 ratio of enzyme to pullulan, 2% sodium alginate, 2% $CaCl_2$ and a curing time of 120 min. Different sizes of beads of enzymes–pullulan complex showed different immobilization yield (Fig. 2e). It was found that the optimum size of bead was 1.4 mm to get highest entrapment (85%). A bead size higher than 1.4 mm may show substrate transfers resistance and hence lower the enzyme activity.

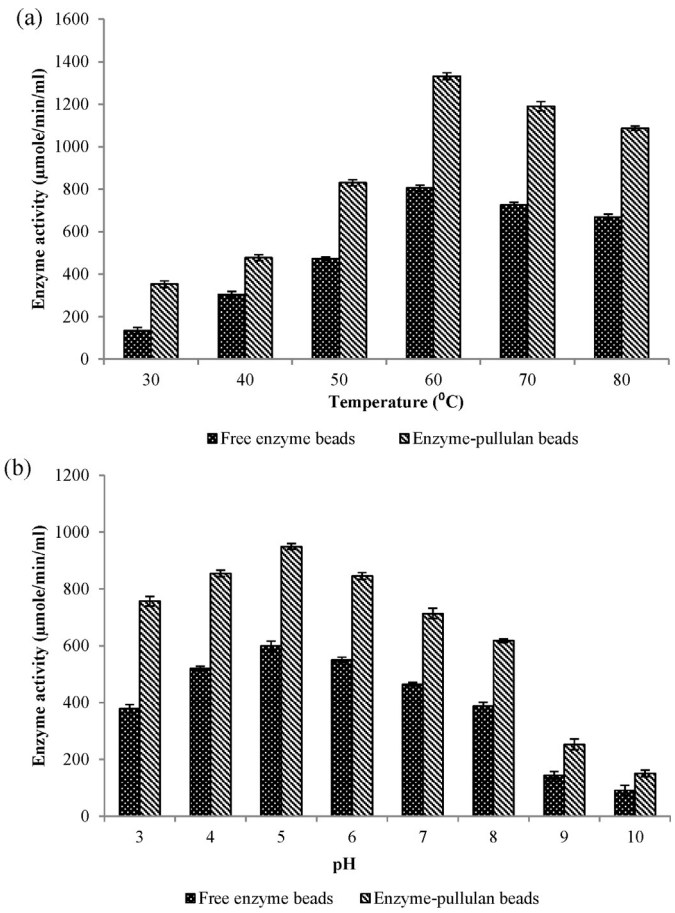

Fig. 3. Study of (a) optimum temperature (b) optimum pH for the free enzyme beads and enzyme–pullulan beads.

3.3. Determination of optimum temperature and pH of free enzymes and enzymes–pullulan complex in the alginate beads

Immobilization of enzymes in alginate beads often affects the optimum temperature and pH of the enzyme system, as it causes mass transfer limitations for substrate. Optimum temperature of free enzymes and enzymes–pullulan complex within the beads was found to be 60 °C (Fig. 3a) whereas it was 50 °C for free enzymes without entrapment in alginate beads as shown in our previous study (data not shown). The optimum pH for the both types of beads was 5.0 (Fig. 3b) and it was same as for free enzymes without entrapment in alginate beads. Addition of pullulan to the enzyme mixture during preparation of beads was found to enhance the entrapment but did not affect the optimum temperature and pH of the enzyme system. At each operational temperature and pH, beads of enzymes–pullulan complex showed higher enzyme activity as compared to beads of free enzymes. This is due to the high entrapment of enzymes in beads containing pullulan.

3.4. Effect of entrapment in beads on combined kinetics of thermal inactivation and pH stability of α-amylase and glucoamylase

Pullulan showed increased immobilization yield of enzymes entrapped in alginate beads. Thermal and pH stability of both the type of beads (with and without pullulan) was determined to study the effect of presence of pullulan on the enzyme stability. Thermal inactivation of beads of free enzymes and enzymes–pullulan complex showed the activity of both the samples to decrease with time at each temperature. Kinetics of enzyme inactivation was evaluated by a semi-log plot of percent residual activity vs time (Fig. 4) in terms of inactivation rate constant, k and half life $t_{1/2}$. The k values of the beads of free enzymes at 45 °C, 50 °C, 55 °C and 60 °C were 0.0072 min^{-1}, 0.0075 min^{-1}, 0.0101 min^{-1} and 0.0135 min^{-1}, respectively, corresponding to $t_{1/2}$ of 96.25, 92.40, 68.61 and 51.33 min at their respective temperatures. The beads of enzymes–pullulan complex had k values of 0.0051 min^{-1}, 0.0058 min^{-1}, 0.0070 min^{-1} and 0.0113 min^{-1} at 45 °C, 50 °C, 55 °C and 60 °C corresponding to their $t_{1/2}$ of 135.88, 119.48, 99 and 61.32 min, respectively. It can be seen that the inactivation rate constant of beads of enzymes–pullulan was lower than the beads of free enzymes at each temperature indicating beads containing pullulan along with enzymes to be more stable against denaturation as compared to beads of free enzymes mixture (Amini, Sorouraddin, & Rashidi, 2011). The beads of enzymes–pullulan complex also showed higher half life as compared to beads of free enzymes, which also implies more stability of beads of enzymes–pullulan complex.

Further, activation energy of both the enzyme systems was calculated from Arrhenius plot (Fig. 5) as product of slope and R, where R is the universal gas constant (8.314 J mol^{-1} K^{-1}). The activation energy of beads of free enzymes and beads of enzymes–pullulan complex was found to be 38.30 kJ/mole and 45.11 kJ/mole, respectively. The activation energy is an index of the thermal stability of protein. Higher activation energy for denaturation of enzyme implies a more stable enzyme (Sundaram & Venkatesh, 1998). Higher activation energy of beads of enzymes–pullulan complex

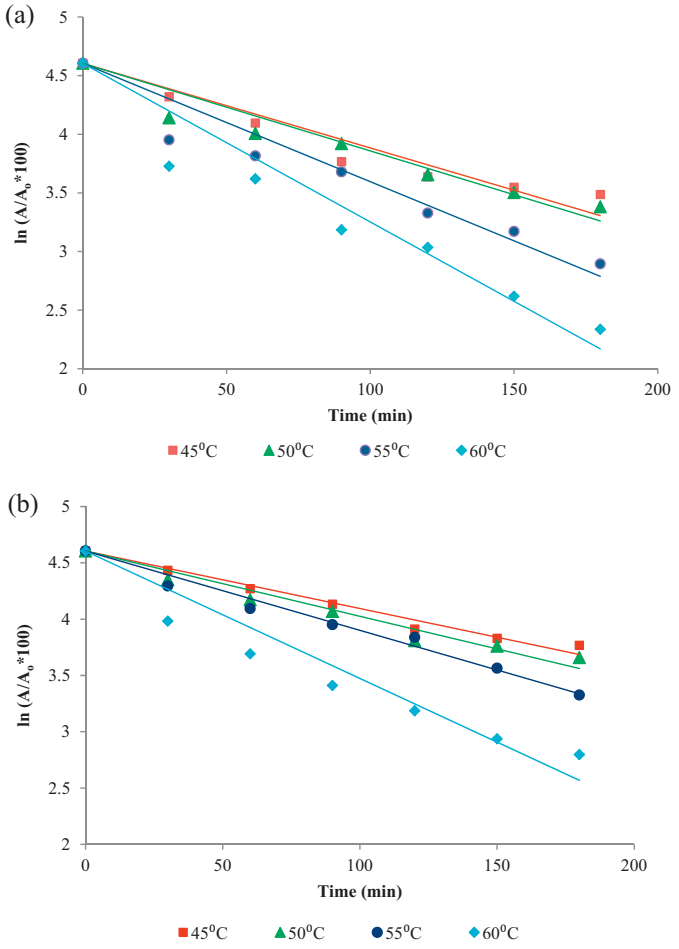

Fig. 4. Kinetics of thermal inactivation at different temperatures (a) free enzymes beads and (b) enzyme–pullulan beads.

than the beads of free enzymes suggested better thermal stability of beads of enzymes–pullulan complex.

Here, pullulan was responsible for increasing thermal stability of enzymes in the beads over the beads of free enzymes. Horseradish peroxidase and glucose oxidase binds to dextran by

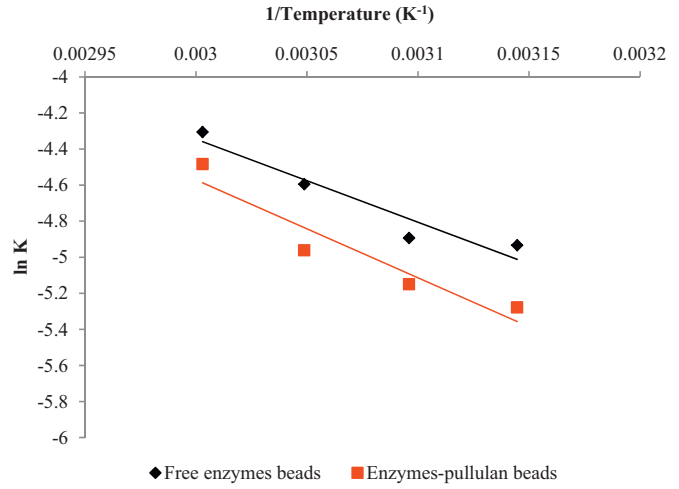

Fig. 5. Arrhenius plot for inactivation of free enzymes beads and enzymes–pullulan beads.

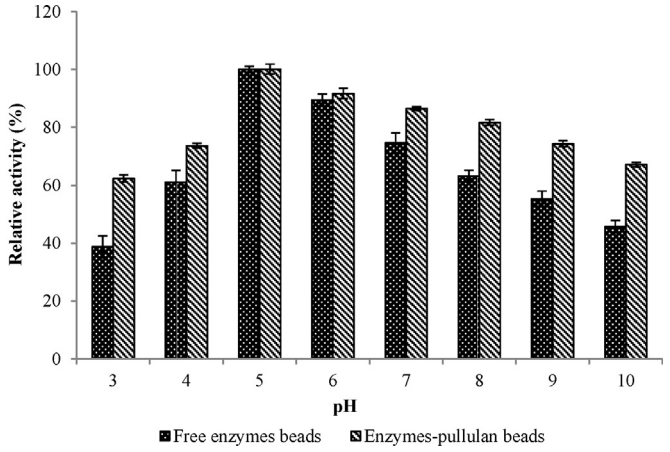

Fig. 6. The pH stability of beads of free enzymes and beads of enzymes–pullulan complex.

non-covalent linkages (Altikatoglu & Kuzu, 2010) and have shown improved thermal and pH stability. Molecular crowding created by polysaccharides is responsible for increasing stability of protein in a mixture of protein and polysaccharide. Dextran has been reported to play a significant role in protein folding and aggregation. Inert dextran decreases the volume available for the protein and hence induces more compact molten globule state (Sasahara, Mc Phie, & Minton, 2003). Enzymes show increased thermal stability after immobilization within calcium alginate beads (Milovanovic, Bozic, & Vujcic, 2007). In this study pullulan showed added advantage of further increase in stability of enzymes after immobilization within alginate beads along with increasing immobilization yield. Non-covalent interactions with enzymes and molecular crowding created by pullulan may be the reasons for the observed thermal stability of beads containing pullulan with enzymes.

The pH stability study showed beads of enzymes–pullulan complex to be more stable in acidic as well as in alkaline conditions as compared to beads of free enzymes (Fig. 6). The residual activity of beads of free enzymes at acidic pH of 3 and alkaline pH of 10 were 38.74% ± 3.78 and 45.68% ± 2.13, respectively, whereas beads of enzymes–pullulan complex showed 62.31% ± 1.18 and 67.13% ± 1.88 at pH 3 and pH 10, respectively. Change in the pH of enzyme system affects the enzyme activity by breaking ionic bonds which holds tertiary structure of the enzyme. It also cause changes in stereo configuration at or in the neighbourhood of active site of the enzyme molecule (Seyhan, Tijskens, & Evranuz, 2002). Immobilization of lipase in alginate and κ-carrageenan hybrid matrix had been shown to improve pH stability (Abdulla & Ravindra, 2013). Beads of enzymes with pullulan showed better stability over the beads without pullulan. Hence, pullulan might helps in holding the enzyme molecules in the tertiary structure by giving rigidity to the molecule as well as by providing molecular crowding. Molecular crowding created by pullulan may be responsible for maintaining compact tertiary structure of the enzyme in the extreme acidic and alkaline conditions.

3.5. Kinetics of free enzymes and enzyme–pullulan complex in alginate beads

Kinetic constants of free enzymes beads and enzymes–pullulan beads were calculated using Lineweaver–Burk plot. The K_m of free enzymes beads and enzymes–pullulan beads were 3.831 mg/ml and 2.513 mg/ml, respectively. The V_{max} values of beads of free enzymes and enzymes–pullulan were found to be 1320 U/ml and 1615 U/ml, respectively. Enzymes–pullulan beads showed lower K_m and higher V_{max} value as compared to free enzymes beads. Many

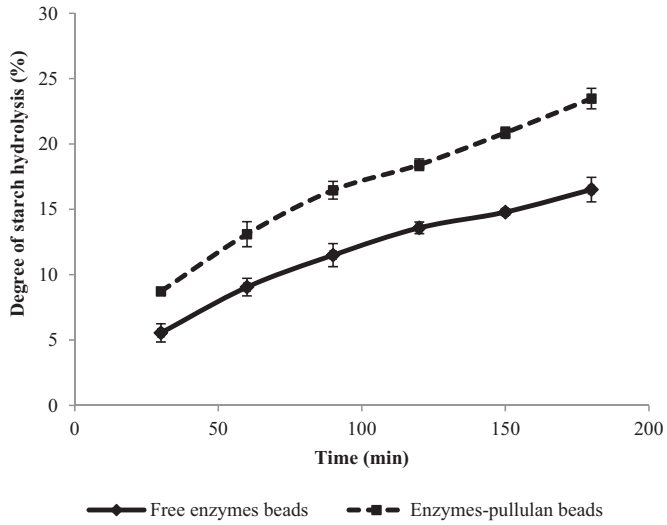

Fig. 7. Study of hydrolysis of starch using free enzymes beads and enzymes–pullulan beads.

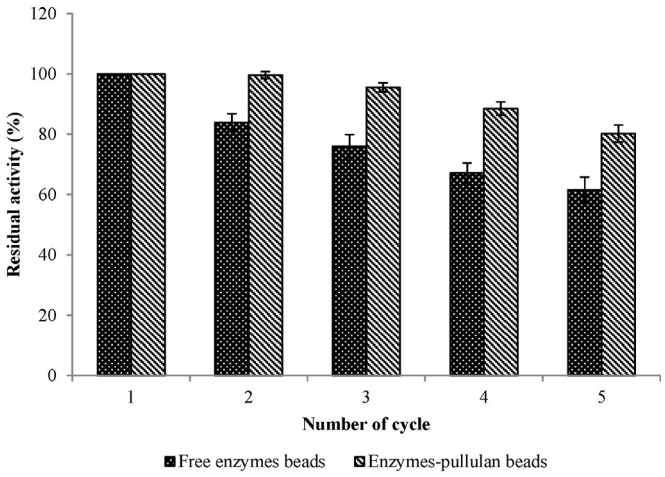

Fig. 8. Study of reusability of free enzymes beads and enzymes–pullulan beads.

reports have shown higher K_m value (lower affinity to substrate) of enzyme after immobilization (Milovanovic et al., 2007; Ortega et al., 1998). Alginate beads restrict the internal diffusion of substrate which is responsible to lower the K_m value of immobilized enzymes. Pullulan enhances the entrapment of enzyme within the alginate beads which increases the accessibility of enzymes for the substrate. This may be responsible for decreasing K_m and increasing V_{max} value of enzymes–pullulan beads than free enzymes beads.

3.6. Hydrolysis of starch using beads of free enzymes and enzymes–pullulan complex

α-Amylase and glucoamylase enzymes have been entrapped together in alginate beads. Both the enzymes work simultaneously for the production of glucose by starch hydrolysis. Free enzymes beads and enzymes–pullulan beads were analyzed for their efficiency to hydrolyze starch under optimum conditions of enzymes. According to the results obtained, pullulan added with the enzymes in the alginate beads showed enhanced entrapment of enzymes with increased thermal and pH stability. Enzymes–pullulan beads showed high amount of glucose released by starch hydrolysis than that of free enzymes beads (Fig. 7). After 180 min of incubation, 23.47% starch hydrolysis was obtained using enzymes–pullulan beads as against 16.5% using free enzymes beads. High amount of glucose release was observed due to large number of enzyme molecules entrapped in enzymes–pullulan beads.

3.7. Reusability of beads of free enzyme and enzyme–pullulan complex

Reusability of enzymes is one of the major advantages of immobilization. Reusability of both types of beads was determined after each 30 min of reaction time at 60 °C for 5 consecutive cycles of reaction. After fifth cycle, remaining enzyme activities of free enzymes beads and enzymes–pullulan beads were 61% and 80%, respectively (Fig. 8). Pullulan was found to increase immobilization yield as well as thermal stability of the enzymes. These attributes may be responsible for the high residual activity of enzymes–pullulan beads compared to free enzymes bead that was observed after repeated use of enzymes.

4. Conclusion

α-Amylase and glucoamylase mixture with pullulan showed a very high immobilization yield (85%) than the free enzymes (25%) in alginate beads. Inclusion of pullulan with enzyme in the alginate beads did not alter the temperature and pH optima of the enzymes. Beads of enzymes–pullulan complex showed lower inactivation rate constant with higher half life which implies their better thermal stability. They also showed more pH stability over beads of free enzymes. Beads of enzymes–pullulan complex released glucose more efficiently from starch than beads of free enzymes. Moreover, addition of pullulan also enhanced the reusability of enzymes entrapped in alginate beads.

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