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Kinetics of fungal extracellular α-amylase from *Fusarium solani* immobilized in calcium alginate beads

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Abstract

Extracellular α-amylase mass produced by *Fusarium solani* using mango kernel as substrate was immobilized in calcium alginate beads through entrapment technique. Maximum enzyme immobilization efficiency was achieved in 2 mm size beads formed by 6.5 % (w/v) of sodium alginate in 2% (w/v) calcium chloride. The catalytic properties of the immobilized α-amylase were compared with that of free enzyme (soluble). The activity yield of the immobilized enzyme was 81% of the free enzyme. The immobilized enzyme showed optimum activity at pH 4.5-6.0 and temperature 40 ºC, in contrast to the free enzyme at 5.5 and 30ºC, respectively. Thermal stability of the immobilized enzyme was found to be more than the free enzyme over a longer time interval. The immobilized enzyme retained activity upto 20% of optimum even after 180 min. While the free enzyme lost its 80% activity after 60 min and lost total activity down to zero by 120 min. The kinetic constants, *viz*., K_M (Michaelis constant), V_{max} and activation energy were affected by immobilization. However, the immobilized α-amylase in calcium alginate beads supports its long term storage which has immense industrial applications.

Key words

Calcium alginate, Immobilization, α-amylase, *Fusarium solani*, Mango kernel

Introduction

Extracellular amylase s produced by several filamentous fungi have been used in baking, detergent, paper, textile and food industry (Selvakumar *et al*., 1996; Pandey *et al*., 2000; Mishra and Dadich, 2010). Moreover, mass production of extracellular α-amylases were reported by fungi like *Aspergillus*, *Bacillus*, *Trichoderma* and *Fusarium* (Saville *et al*., 2004; Kubrak and Luschchak, 2008; De Castro *et al*., 2010) using various substrates. *Fusarium solani* was used to mass produce α-amylase using mango kernel, a solid processing waste as substrate. Product recovery and long term storage of the mass produced and purified enzyme is cumbersome which hinders its commercial utilization. Immobilization of the enzymes onto solid supports that are either organic or inorganic is a very effective way to increase enzyme stability and operational life time as well as eases its downstream applications (Doaa *et al.,* 2009). Entrapping method of immobilization involving gelation into porous gel facilitates immobilized enzyme with high retention of specific activity and stability. Over decades, several matrices have been reported for entrapping, among which alginate in the form of beads, was found to be reasonably safe, simple and cheap offering good mechanical strength (Le-Tein *et al*., 2004). Calcium alginate beads are widely used in immobilization of enzymes like α-amylases, proteases, etc. (Dey *et al*., 2003; Le-Tein *et al*., 2004; Ahmed *et al*.,

2008). In this study extra cellular α-amylase from *Fusarium solani* was immobilized in calcium alginate beads for longer storage stability over a range of pH and temperature.

Materials and Methods

Production and purification of α-amylase: For production of enzyme using mango kernel as a substrate by *Fusarium solani* (NAIMCC-F-02956) the process described by Kumar *et al.* (2010) was followed. After fermentation, α-amylase being extracellular protein was isolated by centrifugation at 12000 rpm and was partially purified by ammonium sulphate precipitation and dialyzed using acetate buffer (50mM, pH 5.0). The specific activity of the enzyme was determined as per method described by Sadasivam and Manickam (1996). The partially purified enzyme was used for immobilization studies.

Immobilization of α-amylase and assay : The partially purified α-amylase was mixed with sodium alginate solution in 1:1 ratio by varying the final concentration of the latter between 3.5-7.5 % (w/v). The α-amylase-alginate mixture was added drop-by-drop into calcium chloride 2% (w/v) solution with continuous shaking at 4°C. The beads thus formed were washed 3-4 times with de-ionized water and finally with 50mM acetate buffer of pH 5.0. The beads were dried and stored at 4°C for further studies.

Assay reaction for the immobilized α-amylase was set up according to the protocols described by Dey *et al*. (2003) and Kumar *et al.* (2006) with minor modifications. For assay, 200 mg of calcium alginate beads was incubated with 400µl of 1% (w/v) starch solution in acetate buffers (50mM, pH5) at 30ºC for 60 min. Starch was digested by α-amylase and the products formed were assayed using DNS (Di-nitro salicylic acid) reagent. The reaction was stopped by incubating for 10 min at 100ºC is water bath. Enzyme activity was recorded in Units, wherein, one unit was defined as the amount of α-amylase that produced 1 µmole of reducing sugar under assay condition per gram of bead. Protein concentration was estimated by Lowry's method using with bovine serum albumin as a standard (Lowry *et al*.,1951). Immobilization efficiency was determined from the difference in enzyme activity in the solution before and after the immobilization (Konsoula *et al*., 2006).

Optimization of temperature and pH : The activity of free and immobilized α-amylase was assayed at various temperatures (20–80 ºC) at 10ºC interval by the method of Anwar *et al.* (2009) and by following assay conditions as per Dey *et al*. (2003) with minor modifications.

Free and immobilized enzymes were incubated at 60ºC in 50mM acetate buffer for 3 hr. Sample were taken at different intervals and activity were measured by method as described earlier. The change in activity as a function of time was measured as the temperature stability (Zeng *et al*., 2009).

Kinetics of the immobilized as well as free enzymes was analyzed using Arrhenius, Lineweaver-Burke and Michaelis-Menton plots. The enzyme kinetics experiment was performed by measuring the initial enzyme reaction velocity at different substrate concentration of starch in 50mM acetate buffer. The Lineweaver-Burke plot was used to establish the Michaelis constant (K_M) and maximum velocity (V_{max}) of the enzyme reaction.

Results and Discussion

α-amylase, an enzyme of hydrolase family, is produced extracellularly by many fungi. In our earlier study, α- amylase was mass produced using submerged fermentation of mango kernel as substrate by *Fusarium solani*. The crude enzyme was extracted, precipitated by ammonium sulphate upto 70% saturation and dialyzed twice in acetate buffer (50mM, pH 5.0) which resulted in purification of 4.083 fold. The specific activity before and after purification were 0.092 and 0.375 U mg^{-1}, respectively. This partially purified enzyme was immobilized in calcium alginate beads *via* entrapment technique and the enzymatic properties were characterized.

Alginate, a natural polysaccharide, is a copolymer of alternating sequences of β-D-mannuronic acid and α-L guluronic acid residues linked by 1-4 glycosidic bonds (Le-Tien *et al.*, 2004). Immobilization of α-amylase by entrapment technique into gel matrix under mild conditions using calcium alginate involves ionotropic gelation. When α-amylase-sodium alginate mixture was dropped into $CaCl_2$ solution, Na^{+2} ions of Na-alginate were replaced by the Ca^{+2} ions of $CaCl_2$ forming Ca-alginate beads and ionic cross linking of carboxylate group in uronate block of alginate occurs giving it a gel like character (Le-Tien *et al*., 2004). The α-amylase remains entrapped within the gel matrix of calcium alginate beads.

The immobilization yield is a key parameter which influences immobilization efficiency. The effect of sodium alginate concentration on immobilization yield was assessed to determine the per cent concentration forming uniform sized beads retaining maximum enzyme activity and greater stability. Different researchers have earlier reported that sodium alginate concentration ranging between 2–6% (w/v) was suitable for the immobilization of α-amylase (Konsoula *et al*., 2005; Kumar *et al*., 2006; Prabakaran and Pugalvendhan, 2009). The effect of sodium alginate concentration on immobilization efficiency of α-amylase is reflected in Table1. The percent entrapped activity of calcium alginate immobilized α-amylase was found maximum at 6.5% (w/v) sodium alginate. Enzyme beads having less than 6.5% (w/v) sodium alginate resulted in lower entrapped activity as well as immobilization efficiency because leakage of enzyme from beads occurred due to larger pore size of the less tightly crossed linked and fragile Ca-alginate beads. But at sodium alginate concentration of 7.5% (w/v), the entrapped enzyme activity and immobilization efficiency were found to be low. At higher sodium alginate concentration, decrease in immobilization yield and efficiency occur at which might be due to decreased gel porosity, high viscosity of enzyme entrapped beads and substrate diffusion limitation (Ahmed *et al.,* 2008). Reduced pore size reduces leakage

though; some initial leakage of the enzyme molecule is certain (Zaborsky *et al.,* 1973).

Bead size is another major factor that influences the immobilization efficiency. The substrate has to diffuse into the beads for the enzymatic reaction to take place in the immobilized enzyme system. The rate of hydrolysis is thus affected by the size of the final lattice formed by the bead. In such situations, both the intra-particular diffusion and the external mass transfer should be taken into consideration for assessing immobilization efficiency. However, in the present study, the external transport has not been considered on the assumption that greater contribution is from the intra-particle mass transfer. Using an enzyme loading concentration 0.054 U and bead (diameter 2mm) amount of 33 mg, maximum enzyme activity (0.54 U ml^{-1}) in terms of highest rate of starch hydrolysis was observed the activity yield reduce (81.0±2%) of the free enzyme. Contrarily, the larger bead sizes (diameter 3 mm and above) resulted in lower enzyme activity. Moreover, the immobilization efficiency was drastically influenced by bead size as shown in Table 2. It evinced that the beads of 2mm diameter offered lesser diffusion resistance compared to the larger beads which is in accordance to the earlier reports (Dey *et al*., 2003; Ertan *et al*., 2007**).** It was reported that larger alginate bead size resulted in structural deformation or denaturation of enzyme during immobilization, eventually altering the catalytic site leading to loss of activity or even deactivation of enzyme. Other factor probably related to lower reaction rate of immobilization is the steric hindrance of the alginate matrix which limits accessibility of the substrate to the enzyme active site; influenced by bead size.

In immobilized enzyme system, the temperature and pH affect relative activity because the behavior of an enzyme molecule gets modified by its immediate microenvironment. Based on the relative activity, the pH optimum of the free enzyme was 5.5. In contrast, pH optimum of the entrapped enzyme shifted towards acidic by 0.5 units to 5.0. The variation of activity with pH, within a range of 2–3 units each side of the pI (isoelectric point), is normally a reversible process (Bayramoglu *et al*., 2004). The conformation of the enzyme will be more favorable in the higher pH range so that maximum activity is achieved. Extremes of pH will, however, cause a time and temperature-dependant, essentially irreversible denaturation. A change in pH affects the intramolecular hydrogen bonding leading to a distorted enzyme conformation that reduces its relative activity (Reshmi *et al*., 2006). However, the relative activity of immobilized α-amylase was wider in the pH range of 4.5 - 6.0, It was also noted that it was stable upto 77% of the optimum. Similarly, immobilized α-amylase from *Bacillus subtilis* retaining 77% activity was earlier reported by Prabakaran and Pulgalvendhan (2009).

Immobilization of α-amylase results in the formation of less polymerized products resulting in an apparent decrease in the number of transglycosylation reactions. It was demonstrated that diffusional resistances were in direct relation to the apparent modification of the enzyme action pattern after immobilization (Siso *et al*., 1990). Immobilized enzyme was more active also because of surface interaction between enzyme and substrate affected by alginate beads entrapment. This broader pH profile of the immobilized enzyme than that of the free enzyme indicated that the calcium alginate immobilization method retain the enzyme activity in a wider pH range. This may also be due to diffusion limitations or secondary interactions between the enzyme and the matrix (Reshmi *et al*., 2006).

The immobilized enzyme was active at a higher temperature than free enzyme with respect to relative activity. The temperature optimum shifted from 30ºC (free enzyme) to 40ºC for the entrapped enzyme. The relative activity of free α-amylase reduced sharply at 50ºC to 80% of optimum while immobilized α- amylase retained 80% of optimum. This result indicated that as the temperature increases, relative activity of the free enzyme reduces rapidly compared to immobilized form. Similar result was also reported in immobilized α-amylase by Kahraman *et al.* (2007). The temperature optimum of immobilized α- amylase was also wider in the range between 30 to 60 ºC with the relative activity

Table- 1: Effect of sodium alginate concentration on immobilization efficiency

Sodium alginate (g %)	Immobilization efficiency[a] (%)
3.5	26.88
4.5	57.43
5.5	66.83
6.5	90.33
7.5	3.38

Values are mean of three replicates ; [a]Immobilization efficiency % was determined from the difference in enzyme activity in the solution before and after the immobilization .

Table- 2: Effect of bead size on immobilization efficiency

Bead size (mm)	Immobilization efficiency[a] (%)
1.5	85.16
2	100
2.5	90.46
3	85.75
3.5	45.6

Values are mean of three replicates ; [a]Immobilization efficiency % was determined from the difference in enzyme activity in the solution before and after the immobilization

Table- 3: Properties distinguishing immobilized and free α-amylase

Kinetics property	Free enzyme	Immobilized enzyme
Optimum pH	5.5	4.5-6.0
Temperature (ºC)	30ºC	40ºC
Thermal stability (min)	120	> 180
K_M (mg ml^{-1})	27.47	18.52
V_{max} (mole min^{-1} ml^{-1})	5.28	1.23
Activation energy, E_a (Kcal mol^{-1})	29.43	20.95

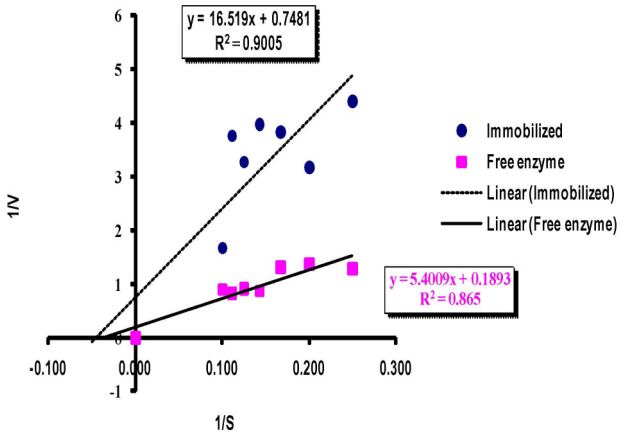

Fig. 1: Lineweaver-Burke plot to determine apparent Km for immobilized and free enzymes. The plot shows relationship between inverse of substrate concentration and reaction velocity for both immobilization and free enzyme where in the raised slope of immobilization enzyme indicates higher apparent K_m value reflecting lower substrate affinity over free enzyme

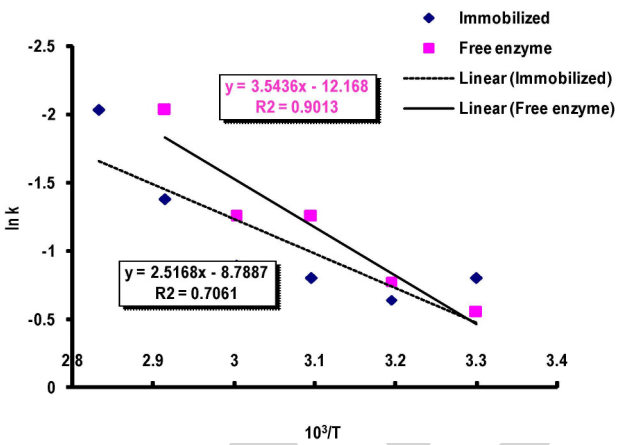

Fig. 2: Arrhenius plot showing activation energy levels of immobilized and free enzyme. Immobilized enzyme resulted in activation energy (20.95 K cal mol^{-1}) that has lower affinity towards the substrate as compared to the free enzyme with activation energy (29.43 K cal mol^{-1})

reduced by only 10-40% of optimum. Entrapped enzyme presented larger activity profile than the free enzyme which was in accordance with earlier report of Le-Tien *et al.* (2004).

Thermal stability of the immobilized α-amylase is a most important factor for industrial applications. The immobilized α-amylase was found to be more thermostable than the free enzyme over a long time interval after heat inactivation at 60ºC. The immobilized enzyme also retained activity upto 20% of optimum even after 180 min. However, as the temperature increases, the stability of free enzyme reduces rapidly compared to immobilized form. The free enzyme showed less than 20% activity after 60 min and reached zero level after120 min. Thus the immobilized α-amylase presented better thermo stability than the free enzyme similar to earlier reports of immobilized endo-β-gluconase (Busto *et al*., 1998**)**. Increased thermal stability has also been reported for a number of immobilized enzymes, and the support material is supposed to preserve the tertiary structure of the enzyme. The thermal stability of enzymes might be drastically increased if they are attached to a complementary surface of a relatively rigid support in a multipoint (Zeng *et al*., 2009).

Kinetics of the immobilized α-amylase was determined at pH and temperature of 5.0 and 40 ºC while for free enzyme at 5.5 and 30ºC. Michaelis-Menten constant (K_M) of immobilized α-amylase was determined by hydrolysis of starch at varying concentrations (0.25-10 mg ml^{-1}) from Lineweaver-Burke plot drawn between the inverse of starch concentration and reaction (Fig.1). The K_M and V_{max} value for immobilized α-amylase was found 27.47 mg ml^{-1} and 5.28 5 mole min^{-1} ml^{-1}, respectively while for free enzyme, 18.52 mg ml^{-1} and 1.23 mole min^{-1} ml^{-1}, respectively. K_M values of α-amylase were found to be significantly larger upon immobilization, indicating decreased affinity of the enzyme for its substrate. Unlike K_M values, V_{max} was smaller for immobilized amylase and results are in accordance with that reported by Saville *et al*. (2004). Earlier, the maximum reaction rate (V_{max}) of immobilized and free amylase of 10.4 and 25.7 mg starch degraded ml^{-1} min^{-1} mg protein, respectively was reported (Tee and Kaletunc., 2009). The K_M value was known as the criterion for the affinity of enzymes to substrates, and the lower value of K_M represented the higher affinity between enzymes and substrates (Shuler and Kargi., 2002). The decreasing K_M values might be due to conformational changes of enzyme during immobilization and inability of high molecular substrate (starch) to diffuse rapidly into the Ca-alginate matrix resulting in reduced substrate access to the active site of entrapped enzyme (Abdel-Naby *et al*., 1998 and Norouzian *et al*., 2003). The same reason could be accounted for V_{max} of immobilized α-amylase in comparison with the free enzyme or higher values indicated that the enzyme converted more substrate to product per unit of time upon saturated with substrate. At the same time, the diffusion resistance encountered by the product molecules might have caused the product to accumulate near the center of the gel to undesirable levels, leading to product inhibition (Le-Tien *et al.,* 2004).

The activation energy (E_a) of the immobilized and free enzymes were estimated from Arrhenius plot (Fig. 2) by plotting the log of the reaction rate against 1/T. The E_a of immobilized and free α-amylase was 20.95 and 29.43 Kcal mol^{-1}, respectively. Similar result was found in immobilized α-amylase obtained from *Bacillus circulans* GRS 313 using the method of immobilization on coconut fiber support (Dey, 2002). However, there was slight decline in activity enzyme which might due to reduced conformational flexibility of the immobilized enzyme as a result of the covalent immobilization (Table 3).

It may, therefore, be concluded that calcium alginate immobilization of α-amylase from *Fusarium solani* enhances the enzyme stability over wider pH and temperature range resulting in

longer storability of the enzyme.

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References

Abdel-Naby, M.A., A.M.S. Ismail, S.A. Ahmed and A.F. Abdel-Fatah: Production and immobilization of alkaline protease from *Bacillus mycoides. Bioresour. Technol.,* **64**, 205-210 (1998)

Ahmed, S.A., A. Ramadan, Al-Domany, M.A. Nefisa, El-Shayeb, H.R. Hesham and S.A. Saleh: Optimization, immobilization of extracellular alkaline protease and characterization of its enzymatic properties. *Res. J. Agri. Bio. Sci.,* **4**, 434-446 (2008).

Anwar, A., S.A. Qader, A. Raiz, S. Iqbal and A. Azhar: Calcium Alginate: A support material for immobilization of proteases from newly isolated strain of *Bacillus subtilis* KIBGE-HAS. *World Appl. Sci. J.,* **7**, 1281-1286 (2009).

Bayramoglu, G., M. Yilmaz and M.Y. Arica: Immobilization of thermostable α-amylase onto reactive membrane: Kinetics characterization and application to continuous starch hydrolysis. *Food Chem*., **84**, 591-599 (2004).

Busto, M.D., N. Ortega and M. Perez-Mateos: Characterization of microbial endo-β-gluconase immobilization in alginate. *Acta Biotechnol*., **18**, 189-200 (1998)

De Castro, A.M., D.F. Carvalho, D.M.G. Freire and L.R. Castilho: Economic analysis of the production of amylases and other hydrolases by *Aspergillus awamori* in solid-state fermentation of babassu cake enzyme. *Enzyme Res*., 1-9 (2010)

Dey, G., S. Palit, R. Banerjee and B.R. Maiti: Purification and characterization of maltooligosaccharide-forming amylase from *Bacillus circulans* GRS 313. *J. Ind. Microbiol. Biotechnol.,* **28**, 193-200 (2002).

Dey, G., B. Singh and R. Banerjee: Immobilization of α**-**amylase produced by *Bacillus circulans GRS 313. Braz. Arch. Biol. Technol.,* **46**, 167-176 (2003).

Doaa, A., R. Mahmoud and W.A Helmy: Potential application of immobilization technology in enzyme and biomass production. *J. Appl. Sci. Res*., **5**, 2466-2476 (2009).

Ertan, F., H. Yagar and B. Balkan: Optimization of α-amylase immobilization in calcium alginate beads. *Prep. Biochem. Biotechnol*., **37**, 195-204 (2007).

Kahraman, M.V., G. Bayramoglu, N. Kayaman-Apohan and A. Gungor: α-amylase immobilization on functionalized glass beads by covalent attachment. *Food Chem*., **104**, 1385–1392 (2007).

Konsoula, Z. and M. Liakopoulou-Kyriakides: Thermostable α-amylase production by *Bacillus subtilis* entrapped in calcium alginate gel capsules. *Enz. Microbiol. Technol.,* **39**, 690–696 (2006).

Kubrak, O. and M. Lushchak: Optimization of conditions for immobilization of alpha amylase from *Bacillus* sp BKL20 in Ca^{2+} alginate beads. *Ukr. Biokhim Zh*., **80,** 32- 41(2008).

Kumar, R.S.S., R.K.S Vishwanath, S.A Singh and A.G. Appu Rao: Entrapment of α-amylase in alginate in beads: Single step protocol for purification and thermal stabilization. *Process Biochem*., **41**, 2282-2288 (2006).

Le-Tien, C., M. Millette, M. Lacroix and M.A. Mateecus: Modified alginate matrices for the immobilization of bioactive agents. *Biotechnol. Appl. Biochem.,* **38**, 189-198 (2004).

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall: Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., **193**, 265-275 (1951).

Mishra, B.K. and Dadhich: Production of amylase and xylanase enzymes from soil fungi of Rajasthan. *J. Adv. Dev. Res*., **1**, 21-23 (2010).

Norouzian, D.: Enzyme immobilization, the state of art in Biotechnology. *Iranian J. Biotechnol*., **1**, 197-206 (2003).

Pandey, A., P. Nigan, C.R. Soccol, V.T. Soccol, D. Singh and R. Mohan: Advances in microbial amylases. *Appl. Biochem. Biotechnol.,* **31**, 135-152 (2000).

Prabakaran, G. and R. Pugalvendhan: Production and immobilization of alpha amylase by using *Bacillus subtilis*. *Recent Res. Sci. Technol*., **1,** 189–194 (2009).

Reshmi, R., G. Sanjay and S. Sugunan: Enhanced activity and stability of α-amylase immobilized on alumina. *Catalysis Commun.,* **7**, 460–465 (2006).

Sadasivam, S. and A. Manickam: Biochemical Methods. New Age, International Publishers (P) Ltd., New Delhi, India (1996).

Saville, B.A., M. Khavkine, S. Gayathri, B. Marandi and Z. Yong-Li: Characterization and performance of immobilized amylase and cellulase. *Appl. Biochem. Biotechnol*., **113**, 251-259 (2004).

Selvakumar, P., L. Ashakumary and A. Pandey: Microbial synthesis of starch saccharifying enzyme in solid state fermentation. *J. Sci. Ind. Res*., **55**, 443-449 (1996).

Shuler, M. L. and F. Kargi: Bioprocess Engineering: Basic Concepts. 2nd Edn., Prentice Hall (2002).

Siso, M.I.G., M. Graber, J.S. Condoret and D. Combes: Effect of diffusional resistances on the action pattern of immobilized alpha-amylase. *J. Chem. Technol. Biotechnol*., **48**, 185–200 (1990).

Tee, B.L. and G. Kaletunç: Immobilization of a thermostable α-amylase by covalent binding to an alginate matrix increases high temperature usability. *Biotechnol. Progress,* **25**, 436–445 (2009).

Zaborsky: Entrapment with in cross linked polymers. In: Immobilized enzymes. CRC Press. Cleveland, Ohio, pp. 83-91 (1973).

Zeng, Y., Y. Hung, M. Chen, C. Peng, J.T. Tzen and J. Liu: Simultaneous refolding, purification, and immobilization of recombinant *Fibrobacter succinogenes* 1,3-1,4-β-D-glucanase on artificial oil bodies. *J. Chemical Technol. Biotechnol*., **84**, 1480–1485 (2009).