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Immobilized bacterial α-amylase for effective hydrolysis of raw and soluble starch

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ABSTRACT

The major concern in an enzymatic process is the instability of the enzyme under repetitive or prolonged use and inhibition by high substrate and product concentration. Immobilization is a very effective alternative in overcoming problems of instability and repetitive use of enzymes. Entrapment method of immobilization is advantageous over other methods as they do not involve chemical modification of the enzyme. α-Amylase produced by *Bacillus amyloliquefaciens* ATCC 23842 was immobilized in calcium alginate beads and used for the effective hydrolysis of soluble and raw potato starch which was comparable to the free enzyme. The levels of parameters (sodium alginate, calcium chloride and curing time) that significantly influence the immobilization of α-amylase in calcium alginate were analyzed and optimized using response surface methodology. Reactor studies were performed to study the reusability and operational stability of the beads. The alginate beads retained more than 60% of their initial efficiency after five batches of successive use and 40% of efficiency was exhibited in the 6th and 7th batch run of 6 h duration.

1. Introduction

Amylase has a great deal of application in starch saccharification. The amylolytic enzymes find a wide spectrum of applications in food industry for production of glucose syrups, high fructose corn syrups, maltose syrups, reduction of viscosity of sugar syrups, reduction of haze formation in juices, solubilization and saccharification of starch for alcohol fermentation in brewing industries, and also find a wide range of application in baking, paper, textile and detergent industry (Sivaramakrishnan, Gangadharan, Nampoothiri, & Pandey, 2006). In most cases the enzymatic process is inhibited by high substrate and product concentration and also instability of the enzyme under repetitive or prolonged use. Immobilization is an important technique for continuous and repeated use of enzymes in industrial application and also rapid separation of the enzyme from the reaction medium. The above features would be important in the development of an economically feasible bioreactor for the starch hydrolysis industry thus immobilizing α-amylase would be of great importance. The general methods employed for immobilization are entrapment, microencapsulation, copolymerization, cross linking, physical adsorption, chemical attachment and covalent binding (Hasirci, Aksoy, & Tumturk, 2006; Markweghanke, Lang, & Wagner, 1995; Mozhaev et al., 1989; Rajagopalan & Krishnan, 2008; Reshmi, Sanjay, & Sugunan, 2006).

Immobilization by physical adsorption on inorganic materials such as porous silica (Cao, Bornscheuer, & Schmid, 1999) clay (Sanjay & Sugunan, 2005a, 2005b) and collagen (Groom, Meising, & White, 1988) has been reported. The immobilization of α-amylase adsorption on alumina has been reported for the hydrolysis of starch to low molecular weight carbohydrates (Reshmi et al., 2006). Functional glass beads were used as support for covalent attachment. Poly(dimer acid-co-alkyl polyamine) particles activated by using various chemicals such as carbodiimide, ethylene diamine, and hexamethylene diamine have been studied as support materials for covalent immobilization of α-amylase (Hasirci et al., 2006). Covalent immobilization of amylase has been carried out using UV-curable methacrylated/fumaric acid modified cycloaliphatic epoxide as a rigid support material. Even though immobilization of enzymes via covalent binding on solid supports have some advantages such as increased thermal and storage stability problems of diffusional resistance were overcome in the case of chemical crosslinked matrix by the use of super porous CELBEADS (Satish, Shewale, & Pandit, 2007). Immobilization techniques that involve chemical modification may cause detrimental effects or may be stressful to the enzyme which is overcome by the entrapment method. Among different immobilization techniques, entrapment in calcium alginate gel offers many advantages due to its simplicity and non-toxic character (Gombotz & Wee, 1998). The gelation characteristics can be altered easily thus capsule characteristics such as the thickness or permeability to different substrates of the gel membrane can be easily controlled (Bladino, Macias, & Cantero, 2002). Alginate beads have been successfully used for the entrapment of α-amylase of *Bacillus subtillus* and effectively used for starch hydrolysis (Rajagopalan & Krishnan, 2008). The present study has exploited the simple technique of

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entrapment using calcium alginate for the immobilization of α-amylase produced by *Bacillus amyloliquefaciens* ATCC 23842 and also performs reactor studies to confirm their operational stability and reusability.

2. Materials and methods

2.1. Microorganism and enzyme production

B. amyloliquefaciens ATCC 23842 was used for the present study. The strain was grown on nutrient agar (Hi-media, Mumbai, India) slants at 37 °C for 24 h and sub-cultured every 2 weeks. The production medium was composed of 12.5% w/v of wheat bran and groundnut oil cake (1:1) supplemented with $MgSO_4$ 0.05 M, NH_4NO_3 0.2 M, KH_2PO_4 0.05 M, $CaCl_2$ 0.0275 M. The production was carried out in 250 ml Erlenmeyer flask inoculated with 10^6 CFU/ml of 18 h old culture and incubated at 37 °C with 180 rpm. The sample was withdrawn after 42 h fermentation, centrifuged at 2862*g* for 20 min and the clear supernatant collected was used as crude enzyme.

2.2. Immobilization of α-amylase

The entrapment of the enzyme in calcium alginate beads were carried out by thoroughly mixing the enzyme with sodium alginate (1:1 v/v) by mild shaking on a rotary shaker. The alginate–enzyme mixture was taken in a syringe (0.7 × 32 mm) fitted with a needle and the solution was added drop by drop from the syringe into the $CaCl_2$ solution. The beads were cured at 4 °C and were filtered and washed with distilled water thoroughly to remove any unbound protein. The unbound enzyme activity or enzyme leakage was determined in the curing and wash out solution.

The levels of parameters (sodium alginate, calcium chloride and curing time) that influence the immobilization of α-amylase in calcium alginate beads significantly were analyzed and optimized using response surface methodology. The Box–Behnken design (Box & Behnken, 1960) was used in the present study and the experimental plan (Table 1) consisted of 17 trials. The independent variables were studied at three different levels, low (−1), medium (0) and high (+1). All the experiments were done in triplicate and the reducing sugar produced and binding efficiency of the enzyme was determined and taken as the dependent variables or response (Y_1 and Y_2). The binding efficiency of the enzyme (Y_2) was calculated using the following equation

$$\text{Enzyme binding} = (E_A - E_B/E_A) \times 100,$$

Table 1
Box–Benken design for the optimization of variables for effective immobilization of α-amylase of *Bacillus amyloliquefaciens*.

Run	A: Sodium alginate (%)	B: Calcium chloride (M)	C: Curing time (h)
1	3	0.1	6
2	3	0.1	3
3	1	1	4.5
4	5	0.55	3
5	3	1	3
6	3	0.55	4.5
7	3	0.55	4.5
8	3	0.55	4.5
9	3	0.55	4.5
10	5	1	4.5
11	3	1	6
12	3	0.55	4.5
13	5	0.1	4.5
14	5	0.55	6
15	1	0.55	6
16	1	0.1	4.5
17	1	0.55	3

Table 2
Responses obtained for Box–Behnken design.

Run	Efficiency of bead formation Y_1 (reducing sugar mg/ml)	Binding efficiency Y_2 %
1	179	60
2	199	62
3	211	74
4	275	63
5	270	88
6	321	80
7	319	79
8	325	83
9	314	81
10	241	68
11	234	55
12	328	68
13	220	45
14	204	65
15	196	38
16	168	35
17	192	66

where E_A is enzyme added (U/ml) and E_B is the unbound enzyme (U/ml).

The second order polynomial coefficients were calculated and analyzed using the 'Design Expert' software (Version 6.0, Stat-Ease Inc., Minneapolis, USA) statistical package. The general form of the second degree polynomial equation (1) is:

$$Y_i = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y_i is the predicted response, X_iXj are input variables which influence the response variable Y; β_o is the offset term; β_i is the ith linear coefficient; β_{ii} the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient.

Table 3
Analysis of variance (ANOVA) for bead formation efficiency.

Source	Sum of squares	DF	Mean square	*F*-value	Prob > *F*
Model	51357.52	9	5706.391	64.122	<0.0001
A	3741.125	1	3741.125	42.038	0.0003
B	4512.5	1	4512.5	50.706	0.0002
C	1891.125	1	1891.125	21.250	0.0025
A2	13957.39	1	13957.39	156.837	<0.0001
B2	12198.44	1	12198.44	137.072	<0.0001
C2	9330.761	1	9330.761	104.848	<0.0001
AB	121	1	121	1.360	0.2818
AC	1406.25	1	1406.25	15.802	0.0054
BC	64	1	64	0.719	0.4245
Residual	622.95	7	88.99286		
Lack of fit	505.75	3	168.5833	5.754	0.0620

CV – 3.82; R^2 – 0.99.

Table 4
Analysis of variance (ANOVA) for binding efficiency.

Source	Sum of squares	DF	Mean square	*F*-value	Prob > *F*
Model	3386.479	9	376.2755	6.439	0.0113
A	98	1	98	1.677	0.2364
B	861.125	1	861.125	14.736	0.0064
C	465.125	1	465.125	7.960	0.0257
A2	1008.318	1	1008.318	17.255	0.0043
B2	219.7921	1	219.7921	3.761	0.0936
C2	94.00263	1	94.00263	1.609	0.2452
AB	64	1	64	1.095	0.3301
AC	225	1	225	3.850	0.0905
BC	240.25	1	240.25	4.111	0.0822
Residual	409.05	7	58.43571		
Lack of fit	270.25	3	90.08333	2.596061	0.1897

CV – 11.71; R^2 – 0.892.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the Fisher's *F*-test (overall model significance), its associated probability $p(F)$, correlation coefficient R, determination coefficient R^2 which measures the goodness of fit of regression model. For each variable, the quadratic models were represented as contour plots (2D) and response surface curves were generated using STATISTICA (StatSoft Inc., Tulsa, USA).

2.3. Starch hydrolysis using immobilized enzyme

The experiments were conducted in 100 ml Erlenmeyer flasks with a working volume of 20 ml. 100 U of the immobilized enzyme or free enzyme was used for the hydrolysis 2% of soluble starch and raw potato starch. The hydrolysis was carried out at 50 °C for 6 h.

2.4. Effect of immobilization on the temperature stability of α-amylase

The starch hydrolysis was carried out at various temperatures (50, 60, 70 and 80 °C) for 3 h with the immobilized enzyme and free enzyme. The reducing sugars produced were determined in each case to compare the efficiency of the immobilization on enhancing temperature stability.

2.5. Reactor studies using immobilized beads

The immobilized beads were loaded into a glass column (30 × 10 cm) with a headspace of 3 cm. Starch solution (2%) was passed through the column using a peristaltic pump with a flow rate of 0.5 ml/min for 6 h (Fig. 6). The efficiency of the beads in starch hydrolysis was analyzed by collecting the hydro-

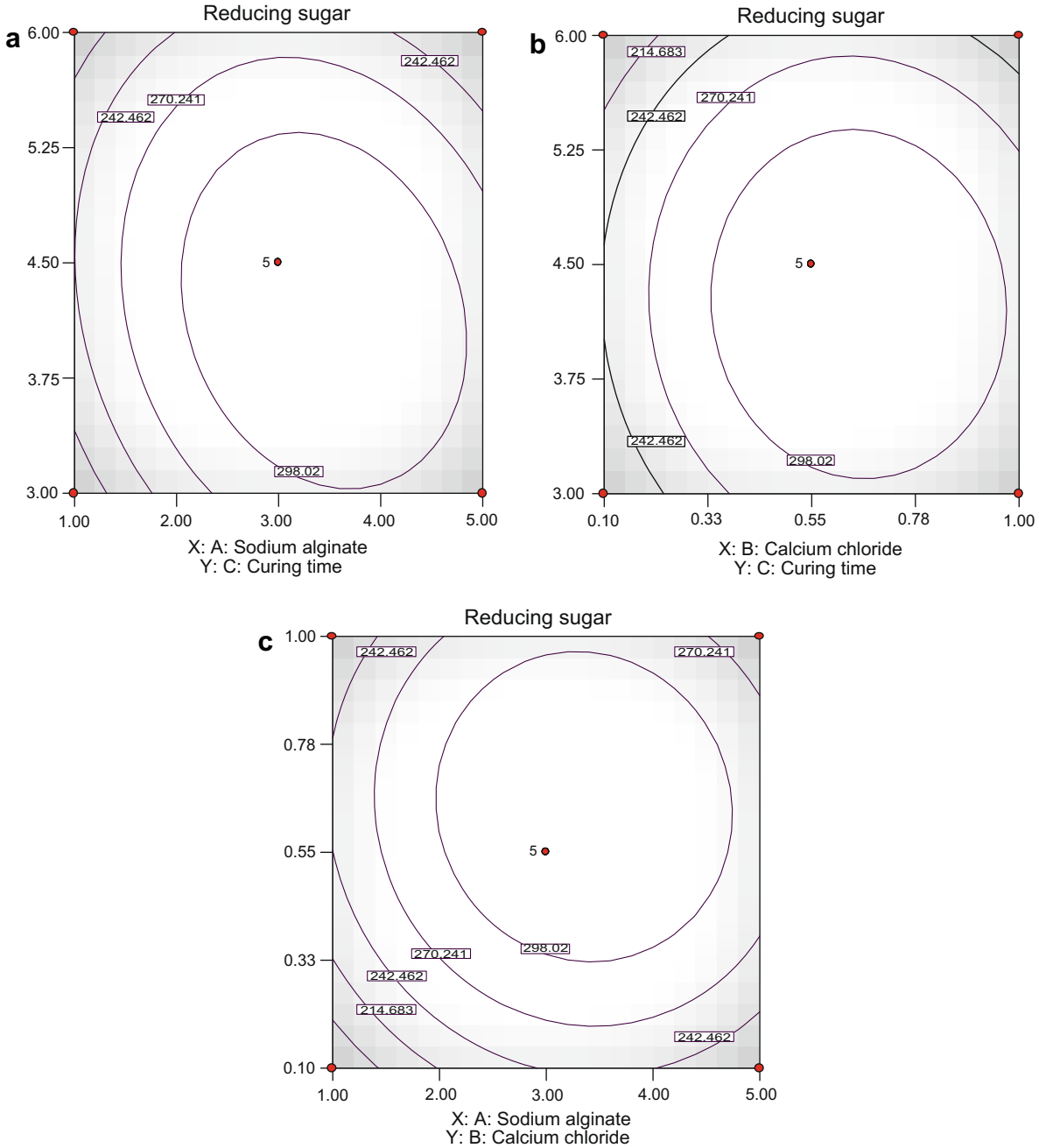

Fig. 1. Contour graphs showing interaction between variables. (a) Calcium chloride and curing time. (b) Sodium alginate and curing time. (c) Sodium alginate and calcium chloride.

lyzed product at regular intervals and estimating the reducing sugar.

The operational stability of the immobilized enzyme was determined by reusing the beads for 7 cycles. The beads were washed with distilled water after each 6 h duration cycle.

3. Results and discussion

3.1. Immobilization of α-amylase

The α-amylase produced by *B. amyloliquefaciens* ATCC 23842 was immobilized using sodium alginate. The factors for efficient bead formation and enzyme binding efficiency were optimized using response surface methodology (Table 3). The Box–Behnken design was employed to study the interactions among the factors and also determine their optimal levels. The results were analyzed using analysis of variance (ANOVA). Multiple regression analysis was used to analyse the data and thus a polynomial equation was derived from regression analysis as follows for efficiency of bead formation (Eq. (2)) and enzyme binding efficiency (Eq. (3)):

$$Y_1 = 321.4 + 21.625X_1 + 23.75X_2 - 15.375X_3 - 57.575X_1^2 - 53.825X_2^2 - 47.075X_3^2 - 5.5X_1X_2 - 18.75X_1X_3 - 4X_2X_3 \quad (2)$$

$$Y_2 = 78.2 + 3.5X_1 + 10.375X_2 - 7.625X_3 - 15.475X_1^2 - 7.225X_2^2 - 4.725X_3^2 - 4X_1X_2 + 7.5X_1X_3 - 7.75X_2X_3 \quad (3)$$

The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using Fisher's statistical analysis

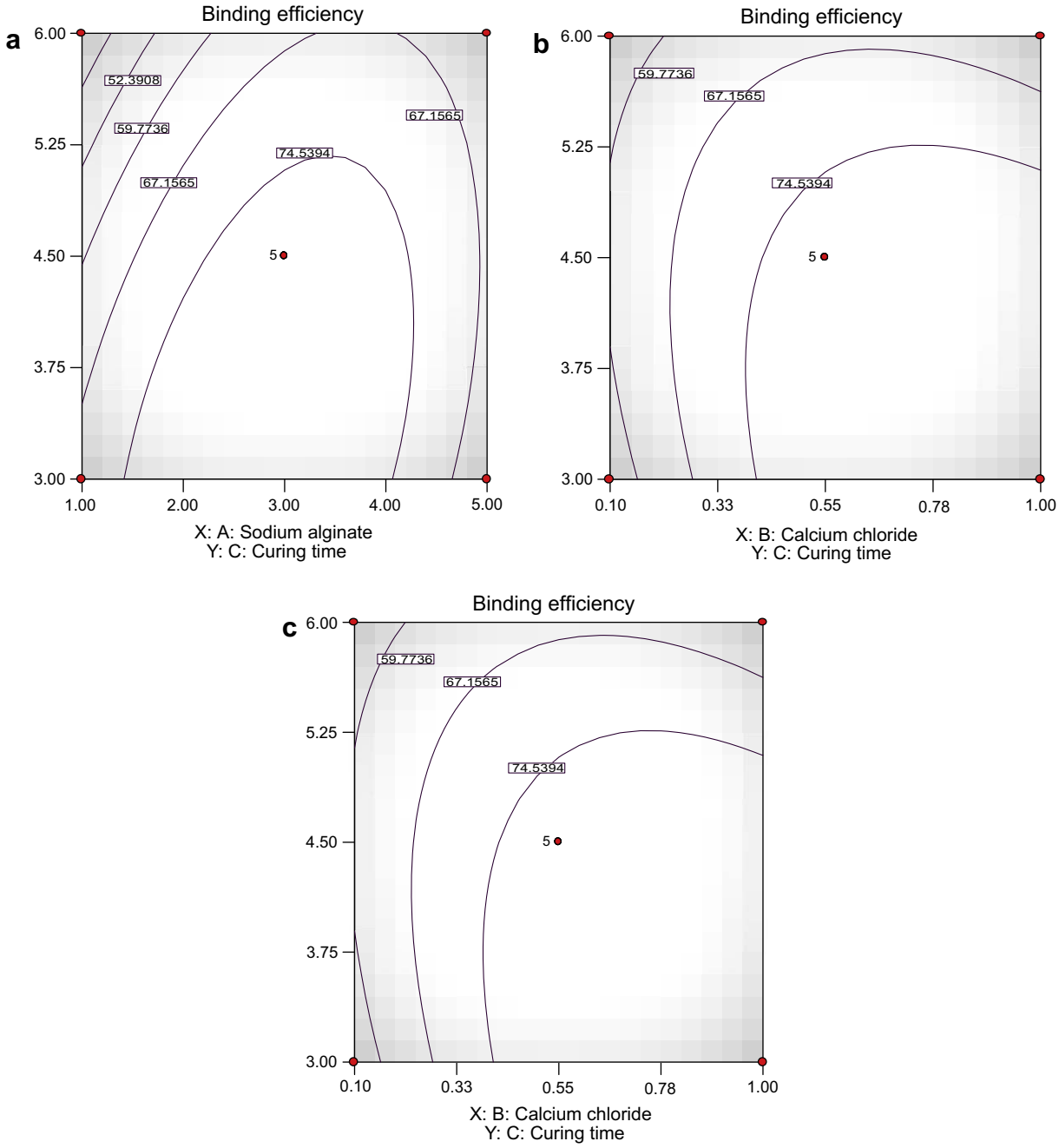

Fig. 2. Contour graphs showing interaction between variables. (a) Sodium alginate and calcium chloride. (b) Sodium alginate and curing time. (c) Calcium chloride and curing time.

and the results are showed in Tables 3 and 4. The model *F*-value for the efficiency of bead formation and enzyme binding efficiency were found to be 64.12 and 6.44, respectively, which implies the model is significant. There is only a 0.01% and 1.13% chance that the model *F-value* could occur due to noise. The R^2 value (multiple correlation coefficient) closer to one denotes better correlation between the observed and predicted values. In both the cases the value of R^2 (0.988 and 0.892) indicates good correlation between the experimental and predicted values. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case a low CV (3.8 and 11.7) denotes that the experiments performed are highly reliable. The *P* values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables. The efficiency of the bead formation was determined by level of reducing sugar formation The *P* values suggest that among the three variables studied, X_1 (sodium alginate concentration) and X_3 (curing time) shows maximum interaction for efficient bead formation. Maximum interaction between X_1 (sodium alginate concentration) and X_3 (curing time), X_2 ($CaCl_2$ concentration) and X_3 (curing time) for enzyme binding efficiency.

The results (Table 2) show maximum reducing sugar yield and maximum binding efficiency at 3% sodium alginate, 0.55 M $CaCl_2$ and 4.5 h curing time. The formation and the mechanical and structural properties of alginate beads depend upon different parameters such as the alginate concentration, nature of the cations and concentration of the cations (Ouwerx, Velings, Mestdagh, & Axelos, 1998). In the case of immobilized laccase the binding efficiency was found to be maximum at 2.5% alginate concentration (Niladevi & Prema, 2007). Lower concentration of sodium alginate and calcium chloride resulted in low bead formation and binding efficiency. The lower immobilization efficiency in case of lower percentage sodium alginate solution might be due to larger pore size and consequently greater leakage of the enzyme from the matrix (Dey, Singh, & Banerjee, 2003).

The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. The response surface curves for factors showing maximum interaction are represented in Figs. 1 and 2. Response surface methodology was used for the immobilization of *Erwinia rhapontici* cells for the production of palatinose (Mundra, Desai, & Lele, 2007).

3.2. Starch hydrolysis using immobilized enzyme

Fig. 3 clearly compares the hydrolysis efficiency of free and immobilized enzyme on soluble starch. The amount of reducing sugars produced by the immobilized enzyme was highly comparable to the free enzyme. The thermostable α-amylase produced extracellularly from *B. subtilis* immobilized in calcium alginate gel capsules or in calcium alginate gel capsules impregnated with silica gel have been used for the hydrolysis of starch which resulted in 70% degradation. Lower rates of hydrolysis were attributed to the interference of the gel matrix of capsules in the diffusion of starch molecules to the enzymically active core (Konsoula & Kyriakides, 2006).

Immobilized enzyme was equally efficient in the hydrolysis of raw starch (Fig. 4). High energy input is required for the gelatinization of starch resulting in increased production cost of starch-based products. Thus in view of economy, effective utilization of natural resources and overcome viscosity problems hydrolysis of raw starch below gelatinization temperatures has gained importance (Goyal, Gupta, & Soni, 2005). The α-amylase of *B. amyloliquefaciens* have been reported to effectively hydrolyse potato, corn, wheat and rice starch. The free enzyme exhibited 85% hydrolysis of raw potato starch. Konsoula & Kyriakides (2006) reported maximum hydrolysis of potato starch by immobilized α-amylase when compared to rice and corn starch.

The role of immobilization on temperature stability was investigated by comparing the rate of starch hydrolysis at various temperatures (Fig. 5). The results clearly indicated an approximate increase of 5–10% enzyme activity as the temperature increased to 60 °C and up to 80 °C. Higher activity may be due to the ability of alginate to form gels stable over the temperature range of 0–100 °C (Gombotz & Wee, 1998). The catalytic activity of free and immobilized enzyme in a covalent bound system was compared at different temperatures and the immobilized enzyme had higher

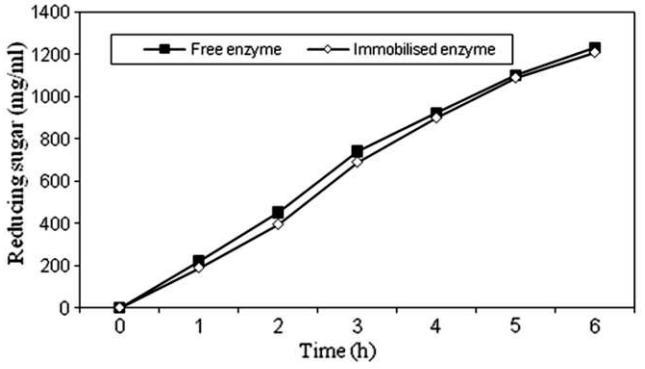

Fig. 3. Starch hydrolysis using α-amylase produced by *Bacillus amyloliquefaciens* immobilized in calcium alginate beads. (■) Free enzyme and (◇) immobilized enzyme.

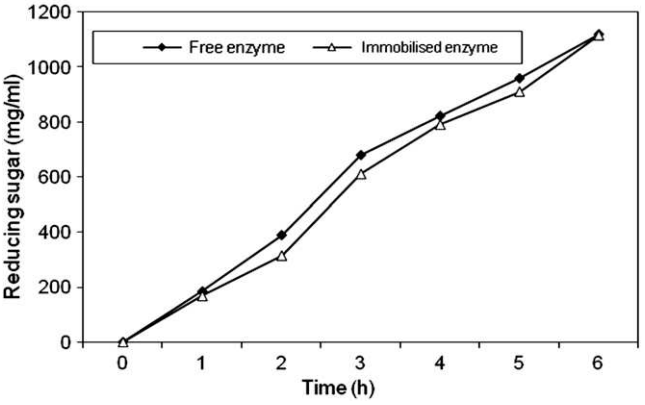

Fig. 4. Hydrolysis of raw potato starch by α-amylase produced by *Bacillus amyloliquefaciens* immobilized in calcium alginate beads. (♦) Free enzyme and (Δ) immobilized enzyme.

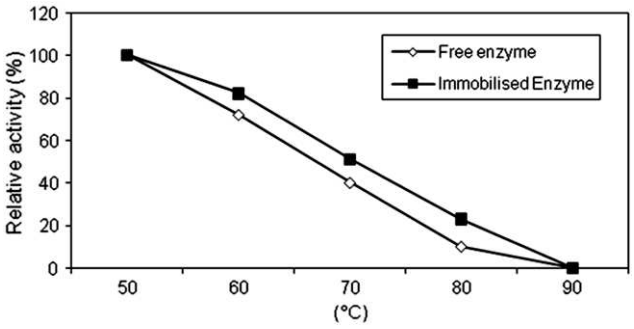

Fig. 5. Temperature stability studies of immobilized α-amylase of *Bacillus amyloliquefaciens* during starch hydrolysis. (◇) Free enzyme and (■) immobilized enzyme.

activity due to their higher resistance to heat than the free form (Hasirci et al., 2006; Kahraman, Bayramoglu, Kayaman-Apohan, & Gungor, 2007a). Previous studies have shown the presence of 5 mM $CaCl_2$ had increased the thermostability of the purified enzyme (Gangadharan, Nampoothiri, Sivaramakrishnan, & Pandey, 2008). Calcium has been shown to regulate the stability and reactivity of a wide variety of biological proteins. The binding of calcium ions to α-amylase has been reported to play an essential role in activating and stabilizing the enzyme (Tanaka & Hoshino, 2002).

3.3. Reactor studies using immobilized beads

The alginate beads retained more than 60% of their initial efficiency after five batches of successive use and 40% of efficiency was exhibited in the 6th and 7th batch run of 6 h duration (Fig. 7). In view of an economical industrial bioreactor for starch hydrolysis the reusability and stability of the enzyme are the two important factors. In batch reaction with immobilized α-amylase of *B. subtilis* KCC 103 the rate and degree of starch hydrolysis were about 10–20% less compared to free enzyme. They reported the reduction in performance could be due to the interference of the gel matrix of beads with the diffusion of starch molecules to the active core of the enzyme. But the immobilized enzyme offered the advantage of using the enzyme in repeated batch reactions. Immobilized beads prepared from 3% (w/v) alginate and 4% (w/v) $CaCl_2$ were suitable for up to 10 repeated uses, losing only 25% of their efficiency (Rajagopalan & Krishnan, 2008). It was observed that α-amylase covalently immobilized onto phthaloyl chloride-containing amino group functionalized glass beads demonstrated more than 98% activity after six runs (Kahraman, Bayramoglu, Kayaman-Apohan, & Gungor, 2007b).

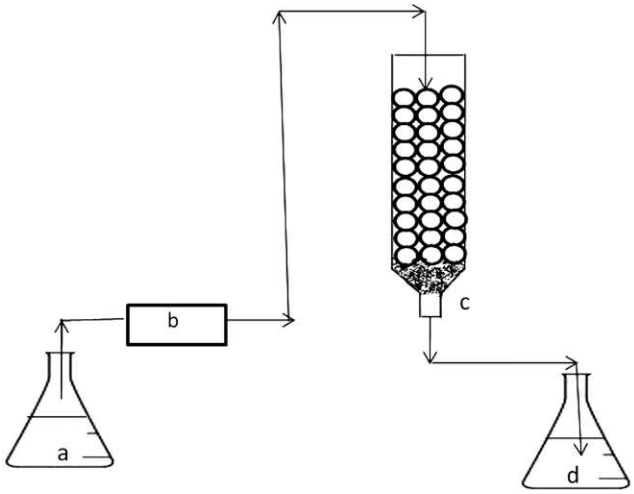

Fig. 6. Schematic diagram of packed bed bioreactor study of alginate immobilized α-amylase: (a) 2% starch, (b) peristaltic pump, (c) packed bed column reactor with alginate immobilized α-amylase and (d) hydrolysed starch.

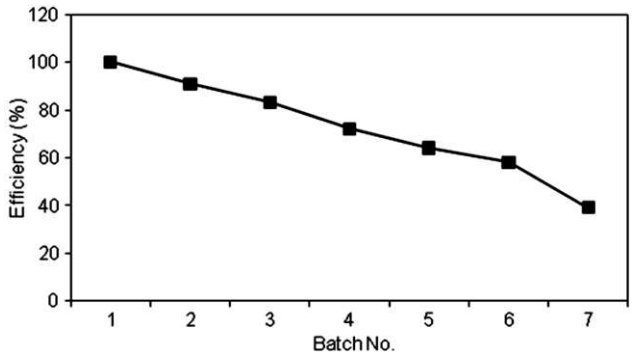

Fig. 7. Reusability and stability studies of immobilized α-amylase using packed bed bioreactor.

4. Conclusions

The present study has successfully demonstrated the efficiency of calcium alginate beads as an effective mechanism for immobilization of α-amylase for starch hydrolysis. The study has also proved immobilization as an important technique for continuous and repeated use of enzymes in industrial application and also rapid separation of the enzyme from the reaction medium thus improving their economic feasibility. Compared to the free enzyme, the higher activity of the immobilized enzyme at higher temperatures and the ability to hydrolyse raw starch such as that of potato would help overcome problems related to gelatinization of starch during hydrolysis.

Acknowledgement

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