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Statistical Optimization of α-Amylase Production by *Streptomyces erumpens* MTCC 7317 Cells in Calcium Alginate Beads Using Response Surface Methodology

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Abstract

α-Amylase has a wide range of applications in starch industries, *i.e*. baking, brewing, distillery, *etc*. The α-amylase production from *Streptomyces erumpens* MTCC 7317 immobilized cells was compared with that of free cells. The immobilized cells of *S. erumpens* in calcium alginate beads were more effective for production of α-amylase (12.2% more yield) than free cells. Response surface methodology (RSM) was used to evaluate the effect of main variables, *i.e.* incubation period, pH and temperature on enzyme production with immobilized cells. A full factorial Central Composite Design (CCD) was applied to study these main factors that affected α-amylase production. The experimental results showed that the optimum incubation period, pH and temperature were 36 h, 6.0 and 50°C, respectively for immobilized cells. Repeated batch fermentation of immobilized cells in shake flasks carried out in starch-beef extract medium showed that *S. erumpens* cells were physiologically active on the support even after four cycles of fermentation.

Key words: *Streptomyces erumpens*, α-amylase, cell immobilization, response surface methodology

Introduction

Amylases constitute one of the important groups of enzymes that are used in a wide range of starch industries *i.e*. baking, brewing, starch liquefaction and distillery (Haki and Rakshit, 2003; Tonkova, 2006). Microbial amylases are usually produced either by free or immobilized cells. The immobilization of whole microbial cells and their application in bioprocessing has been of interest for nearly thirty years (Carvalho *et al*., 2002). The technique has been used extensively in fermentation industries for producing amino acids (Bodalo *et al*., 1996), enzymes (Dey *et al*., 2003), organic acids (John *et al*., 2007) and ethanol (Sanchez *et al*., 1996; Swain *et al*., 2007).

Immobilization of whole cells for extracellular enzyme production offers several advantages, *i.e.* the ease to separate cellmass from the bulk liquid for possible reuse, facilitates continuous operation over a prolonged period, enhances reactor productivity, ensures higher efficiency of catalysis (Selvakumar *et al*., 1994). A number of carrier materials have been used for entrapping microbial cells for bio-product production (Selvakumar *et al*., 1994). One of the most suitable carriers for cell immobilization is entrapment in calcium alginate as bead (Bashay, 2003; Adinarayana *et al*., 2005) because this technique is simple and cost effective (Kourkoutas *et al*., 2004). Sodium alginate, the precursor of calcium alginate is readily available and is a non-toxic chemical. Therefore, it is most suitable as an immobilization matrix for entrapping biomolecules and microorganisms (Bashay, 2003).

Response Surface Methodology (RSM) is an experimental strategy for seeking the optimum conditions for a multivariable system (He *et al*., 2004) and is used for optimization of culture conditions (Rao *et al*., 1993). RSM consist of a group of mathematical and statistical procedures that can be used to study relationships between one or more responses and a number of independent variables. In addition to analyzing the effect of independent variables, this experimental methodology generates a mathematical model that accurately describes the over all process (Senanayake and Shahidi, 2002; He *et al*., 2004). Statistical optimization not only allows quick screening of large experimental domain, but also reflects the

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role of each of the components. RSM has already been successfully applied for optimization of the media and culture conditions in many cultivation process for the production of primary and secondary metabolites (Boyaci, 2005) including amino acid (Xiong *et al*., 2005), ethanol (Carvalho *et al*., 2003) and enzymes (Rao and Satyanarayana, 2003).

The present study has been carried out to explore the possibility of production of thermostable α-amylase in submerged fermentation (SmF) by using immobilized *Streptomyces erumpens* MTCC 7317 cells in calcium alginate as beads and optimization of the fermentation parameters (incubation period, pH and temperature) by applying RSM. Further, the application of *S. erumpens* crude enzyme on starch liquefaction has been studied.

Experimental

Materials and Methods

Microorganism. An α-amylase producing strain of *S. erumpens* used in the present study was isolated earlier from a brick kiln soil near Bhubaneswar (Kar and Ray, 2006) and was subsequently identified at the Institute of Microbial Technology, Chandigarh, India and given the code number MTCC 7317. The culture was maintained on starch-beef extract (SB) agar (soluble starch, 1%; beef extract, 1%; $MgSO_4$, 0.01%; glycerol, 0.1%; agar, 2%; distilled water, 1000ml; pH, 7.0) slants at 4^0C.

Inoculum preparation for free and immobilized cells. The inoculum was prepared in SB broth by transferring a loop full of microorganism from a fresh culture and incubating for 24 h at 50°C at 120 rpm in an orbital shaker incubator (Remi Pvt, Ltd, Bombay, India). The inoculum contained 1×10^5 cfu/ml. This served as the inoculum for α-amylase production.

The 25 ml of culture broth (prepared as above) was centrifuged at 8000 × g for 20 min in a refrigerated centrifuge (Model C-24, Remi Pvt. Ltd, Bombay, India), washed and then suspended in 25 ml of deionized water. The cell suspension was used for cell immobilization. The *S. erumpens* cell suspension (1×10^5 cfu/ml) was added to 4% (w/v) sodium alginate in a 1:1 volume ratio and mixed thoroughly. The cell-alginate mixture was then cast into beads by dropping from a hypodermic syringe into cold sterile 0.1 M $CaCl_2$ solution. These beads had a diameter of approximately 3.0 mm and were hardened by keeping in dilute (0.1 M) $CaCl_2$ solution for 24 h at 4°C with gentle agitation (Swain *et al*., 2007). Finally, these beads were washed with sterile distilled water to remove excess Ca^{2+} ions and unentrapped cells before being used for the fermentation process. The flow-chart for *S. erumpens* cells immobilization method in calcium alginate beads is given in Fig. 1. In order to obtain a high cell density, gel beads containing immobilized *S. erumpens* cell were immersed in SB broth for 24 h at 32–34°C and the cell population in the immobilization had increased from 10^5 to 10^8 cell/g of gel.

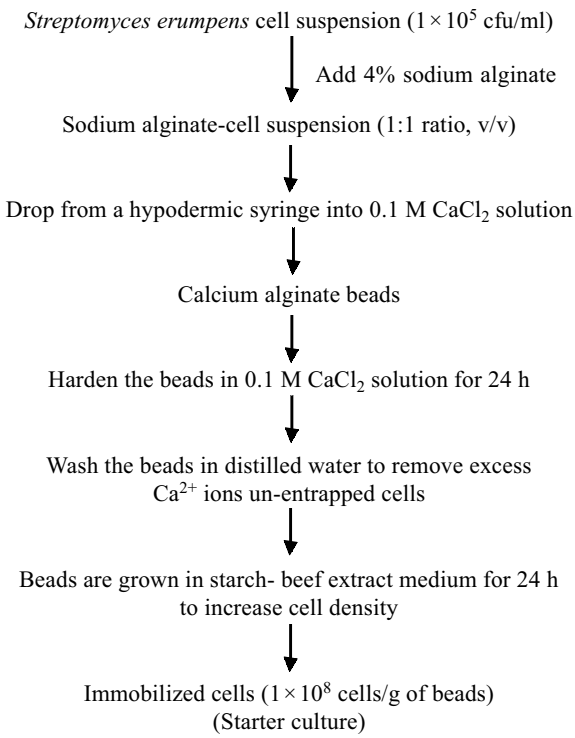

Fig. 1. Process of immobilization of *S. erumpens* MTCC 7317 cells in calcium alginate beads.

α-Amylase production. The amylase production (by free and immobilized cell) was carried out in SB liquid medium. Fifty ml of sterile SB medium was taken in 250 ml Erlenmeyer flasks and inoculated with 2% inoculum [free cell and equivalent amount of immobilized cell (dry weight basis)]. The flasks (n = 3) were incubated for 60 h at 50°C in an incubator shaker at 120 rpm. At 12 h interval, the culture broths from triplicate flasks were centrifuged at 8000 × g for 20 min at 4°C in a refrigerated centrifuge. The clear supernatant was used for α-amylase assay. The immobilized cells, separated after fermentation, were re-used for successive four batches and the enzyme yield was compared with the free cells in each batch.

Cell growth and cell leakage. The biomass of immobilized cells was determined by dissolving the gel beads in 4% EDTA solution and reading absorbance at 550 nm against a suitable blank in a UV-Vis Spectrophotometer (Model No. CE 7250, Cecil Instrument, UK). The corresponding dry weight was obtained from a standard curve of absorbance verses dry weight (Swain *et al*., 2007). The cells leaked from the gel matrix were collected by centrifugation at 3000 × g for 10 min and the biomass was determined likewise. The biomass of free cells was also determined.

Optimization of α-amylase production by applying RSM of immobilized cells. The characterization of different factors for α-amylase production by immobilized cells was optimized by applying RSM. The statistical model was obtained using Central Composite Design (CCD) with three independent variables [incubation period (A), pH (B) and temperature (C)]. Each factor in this design was studied at five different levels (Table I). A set of 20 experiments was performed. All variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables were used and the full experimental plan with respect to their values in coded form is listed in Table II. Upon completion of the experiments, the average of α-amylase production was taken as the dependent variable or response.

Statistical analysis and modeling. The data obtained from RSM on α-amylase production was subjected to the analysis of variance (ANOVA). The results of RSM were used to fit a second order polynomial equation (1) as it represents the behaviour of such a system more appropriately.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1 \beta_1 A^2 + \\ + \beta_2 \beta_2 B^2 + \beta_3 \beta_3 C^2 + \beta_1 \beta_2 AB + \\ + \beta_1 \beta_3 AC + \beta_2 \beta_3 BC \quad (1)$$

Where Y is response variable, β_0 is intercept, β_1, β_2 and β_3 are linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$ and $\beta_{3,3}$ are squared coefficient, $\beta_{1,2}$, $\beta_{1,3}$ and $\beta_{2,3}$ are interaction coefficient and A, B, C, A^2, B^2, C^2, AB, AC and BC are level of independent variables. Statistical significance of the model equation was determined by Fisher's test value, and the production of variance explained by the model was given by the multiple coefficient of determination, R squired (R^2) value. Design Expert (ver, 7.1; STATEASE INC; Minneapolis, MN, USA) was used in this investigation.

The effects of pH and temperature were studied by evaluating the enzyme activity at various pH-s (4.0–8.0) and incubation temperatures (30–70°C) of the medium. The pH measurements were carried out with a pH meter (Systronic, Ahamadabad, India) using a glass electrode. The pHs of 4.0–6.0 were maintained with acetate buffer (0.2 M) while pH 6.0–8.0 were achieved with phosphate buffer (0.1 M).

Analytical methods. α-Amylase assay was based on the reduction in blue colour intensity resulting from enzymatic hydrolysis of starch and formation of starch-iodine complex (Swain *et al*., 2006). The reaction mixture consisted of 0.2 ml enzyme (cell free supernatant), 0.25 ml of 0.1% starch solution and 0.5 ml of phosphate buffer (0.1 M, pH 6.0) incubated at 50°C for 10 min. The reaction was stopped by adding 0.25 ml of 0.1 N HCl and the colour was developed by adding 0.25 ml of I/KI solution (2% KI in 0.2% I). The optical density (OD) of the blue colour solution was determined using a UV-Vis Spectrophotometer at 690 nm. One unit of enzyme activity is defined as the quantity of enzyme that causes 0.01% reduction of blue colour intensity of starch iodine solution at 50°C in one minute per ml (Swain *et al*., 2006).

Rate of hydrolysis of starch. A 2% (w/v) solution of soluble starch and cassava starch were incubated with 2–4 ml of *S. erumpens* crude enzyme (obtained from immobilized cell culture medium from 36 h incubation) at 50°C in an incubator. The degradation of starch was evaluated at one hour interval up to 5 h.

Table I
Range of the values for the response surface methodology

Independent factors	Coded Factor Levels				
	–α	–1	0	+1	+α
Incubation period (h)	–4.36303	12	36	60	76.363
pH	2.63641	4.0	6.0	8.0	9.36359
Temperature (°C)	16.3641	30	50	70	83.6359

Table II
Experimental design and result of CCD of response surface methodology

Std	A: Incubation period (h)	B: pH	C: Temperature (°C)	Enzyme production (Units)	
				Predicted	Experimental
1	–1	–1	–1	1983	1960
2	1	–1	–1	2561	2485
3	–1	1	–1	2138	2070
4	1	1	–1	2715	2595
5	–1	–1	1	1316	1167
6	1	–1	1	1893	1692
7	–1	1	1	1470	1277
8	1	1	1	2047	1802
9	–α	0	0	2425	2575
10	+α	0	0	3690	3670
11	0	–α	0	1920	1815
12	0	+α	0	2130	2180
13	0	0	–α	2140	2250
14	0	0	+α	1510	1425
15	0	0	0	3988	4143
16	0	0	0	3988	4015
17	0	0	0	3988	3950
18	0	0	0	3988	3825
19	0	0	0	3988	4050
20	0	0	0	3988	3880

Results

Amylase production by *S. erumprns* MTCC 7317 free and immobilized cells started in the log phase of growth and maximum enzyme production was achieved during the stationary phase (36 h) of the

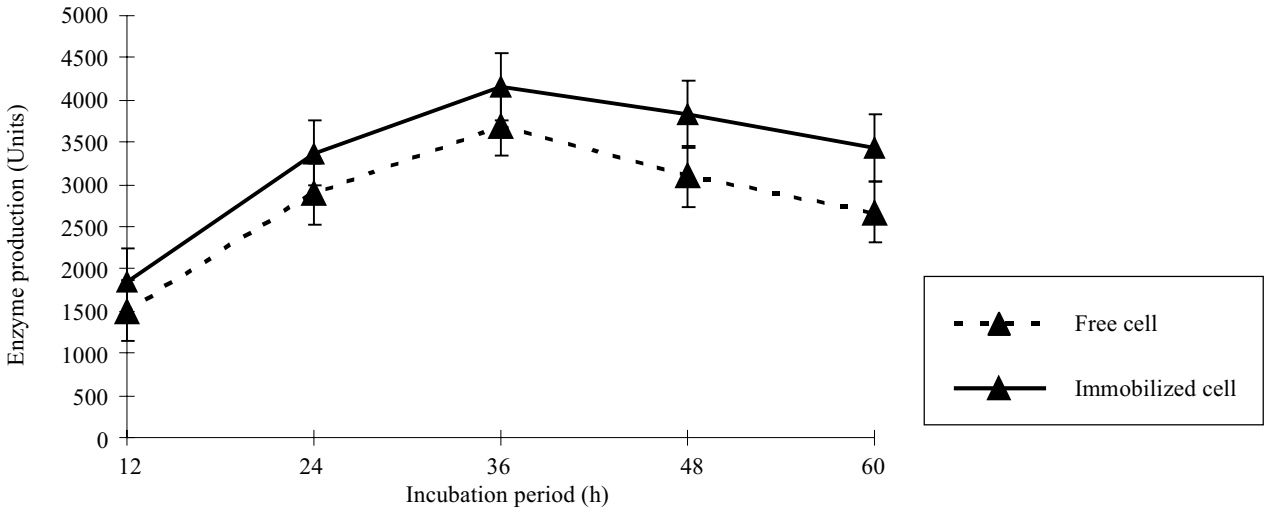

Fig. 2. Effect of incubation period on enzyme production by free and immobilized cells.

growth of the organism (Fig. 2). Further at 36 h, the enzyme production was 12.2% more in immobilized cell than in free (whole) cell system. Further experiments were conducted only with immobilized cells.

Optimization of α-amylase production by applying RSM. The results of CCD experiments for studying the effect of three independent fermentation variables (incubation period, pH and temperature) for α-amylase production by immobilized cells are presented along with the mean predicted and observed responses in Table II. The regression equations obtained after the ANOVA gave the level of α-amylase production as a function of the initial values of incubation period, pH and temperature. The final response equation that represented a suitable model for α-amylase production is given below:

$$Y = 63.18 + 3.02 \times A + 0.88 \times B - 3.91 \times C - \\ - 3.39 \times A^2 - 7.29 \times B^2 - 8.02 \times C^2 - 0.049 \times \\ \times AB + 0.33 \times AC + 0.072 \times BC$$

Where Y is enzyme production, A is incubation period (h), B is pH and C is temperature (°C). The coefficient of determination (R^2) was calculated as 0.9705 for α-amylase production (Table III), indicating that the statistical model can explain 97.05% of variability in the response. The R^2 value is always between 0 and 1.

Table III
ANOVA for α-amylase production
in submerged fermentation

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Model	1943.77	9	82.29	36.51	0.0001
Pure Error	4.24	5	0.43		
Total	2002.93	19			

R^2 = 0.9705; adjusted R^2 = 0.9439; predicted R^2 = 0.7898; adequate precision = 17.0; lack of fit F- value = 12.94

The closer the value of R^2 is to 1.0, the stronger the model and the better it predicts the response (Rao and Satyanarayana, 2003). An adequate precision of 17.0 for α-amylase production was recorded. The predicted R^2 of 0.7898 is in reasonable agreement with the adjusted R^2 of 0.9439. This indicated a good agreement between the experimental and predicted value for α-amylase production.

The model F-value of 36.51 and values of prob > F (< 0.05) indicated that the model terms are significant. For α-amylase production A, C, A^2, B^2 and C^2 are significant model. The "lack of fit F-value" of 12.94 implied that the "lack of fit" is significant.

Response surface was generated by plotting the response (α-amylase production) on the z-axis against any two independent variables while keeping the other independent variable at zero level. Therefore, three response surfaces were obtained by considering all the possible combinations. Fig. 3A depicts three dimensional diagram and a contour plot of calculated response surface from the interaction between incubation period and pH while keeping the other variable (temperature) at "0" level. A linear increase in α-amylase production was observed when incubation period was increased up to 36 h, and there after, it declined. When the level of pH was increased from 4.0 to 6.0, a linear increase in α-amylase production was recorded. At the "0" level of pH, the response between incubation period and temperature indicated that a temperature of 50°C was optimum (Fig. 3B) with 36 h incubation period for α-amylase production. The response surface was mainly used to find out the optima of the variables for which the response was maximized. An interaction between the remaining two parameters (pH and temperature) (Fig. 3C) suggested a little difference with the earlier responses. The three contour plots proved the significance of earlier response *i.e.* incubation period with pH, incubation

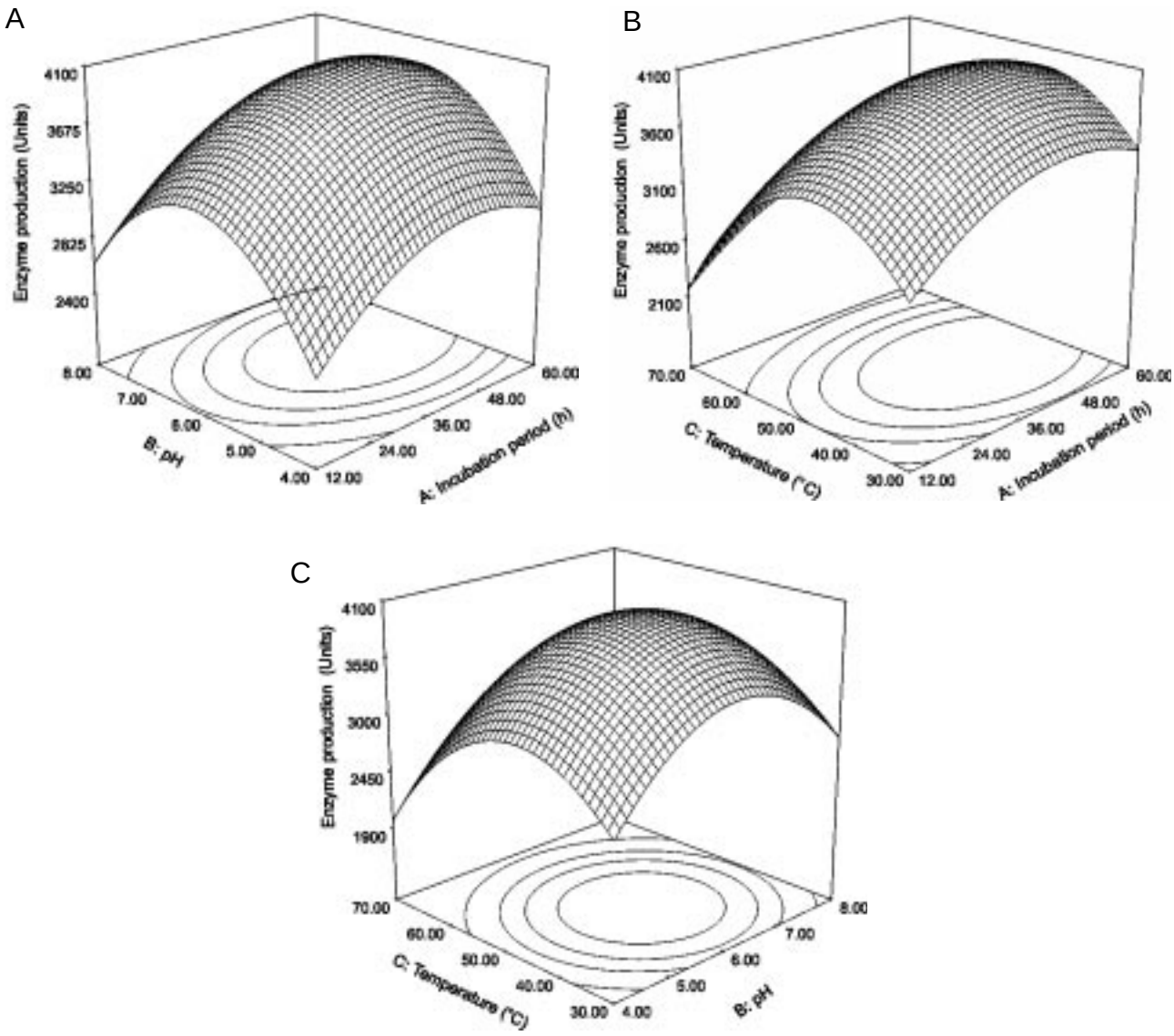

Fig. 3. Statistical optimization of enzyme production using RSM, (A) incubation period and pH; (B) incubation period and temperature and (C) temperature and pH.

period with temperature and pH with temperature (Fig. 4A, B and C). Thus incubation period (36 h), pH (6.0) and temperature (50°C) were adequate parameters for attaining maximum enzyme titre (4143 Units) as shown in Table II.

Validation of the model. Validation was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated. Validation of the statistical model and regression equation was performed by taking A (36 h), B (6.0) and C (50°C) in the experiment. The predicted response for α-amylase production was 3988 Units, while the actual (experimental) response was 4143 Units, thus proving the validity.

Rate of hydrolysis of starch. The rate of hydrolysis of 2% (w/v) soluble starch and cassava starch by *S. erumpens* α-amylase is shown in Fig. 5. There was a gradual hydrolysis of starches with increase in incubation period from 1 to 5 h and the rate of hydrolysis also increased with the increase in enzyme concentration. With application of 4 ml crude enzyme there was 94 and 82% hydrolysis of soluble starch and cassava starch, respectively.

Discussion

The advantages claimed for the use of immobilized cell for enzyme production compared to free cells are higher reaction velocity due to higher cell density, enhanced yield and cell viability for several cycles of operations (Selvakumar *et al*., 1994; Kourkoutas *et al*., 2004). Immobilization of whole cells may allow repeated operations (Dey *et al*., 2003) and is a strategy for protecting cells from shear forces during

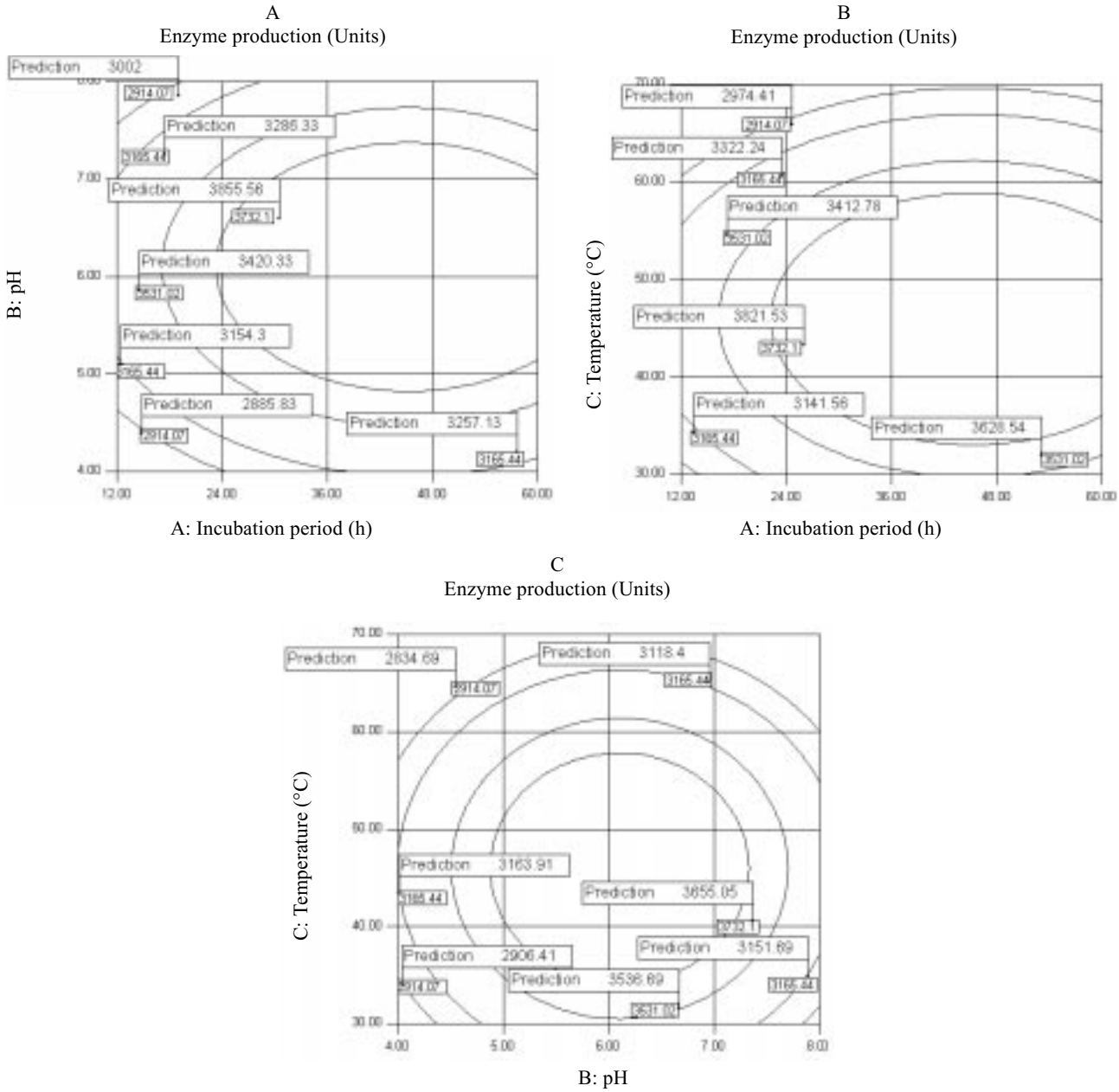

Fig. 4. Contour plot of the effect of, (A) incubation period and pH; (B) incubation period and temperature and (C) temperature and pH.

agitation of the microbial cultures (Adinarayana *et al*., 2005). In literature, 10–60% increase in enzyme production has been reported with immobilized cells over free cells, *i.e.* protease (Bashay, 2003; Adinarayana *et al*., 2004; 2005), α-amylase (Stefanova *et al*., 1998; Dobreva *et al*., 1998), pectinase (Dinnella *et al*., 1996; Demir *et al*., 2001), *etc*. Above all, the separation of the product from immobilized cell system is comparatively easier than the free cell system (Kourkoutas *et al*., 2004). Among the different matrices used (*i.e.* calcium alginate, agar agar, gelatine, polyacrylamide, carrageenan, *etc*) for whole cell immobilization (Abd-El-Haleem *et al*., 2003), entrapment in form of calcium alginate beads is the common technique used successfully for enzyme production (Adinarayana *et al*., 2005). Calcium alginate gel forms rapidly in very mild conditions and provides suitable medium for immobilization by entrapment of whole microbial cells. Only a single type of sodium alginate (E. Merck, Bombay, India) used in this work was flexible enough in its properties to be suitable for successful immobilization of *S. erumpens* cells. Although, calcium alginate gel does not rank among highly mechanically persistent matrices, the mechanical stability of this gel beads (prepared in 0.1 M $CaCl_2$ and hardened for 24 h) was good enough since no fragments of alginate beads were found in culture broth up to 4th cycle in our experiment. In a separate study, repeated batch

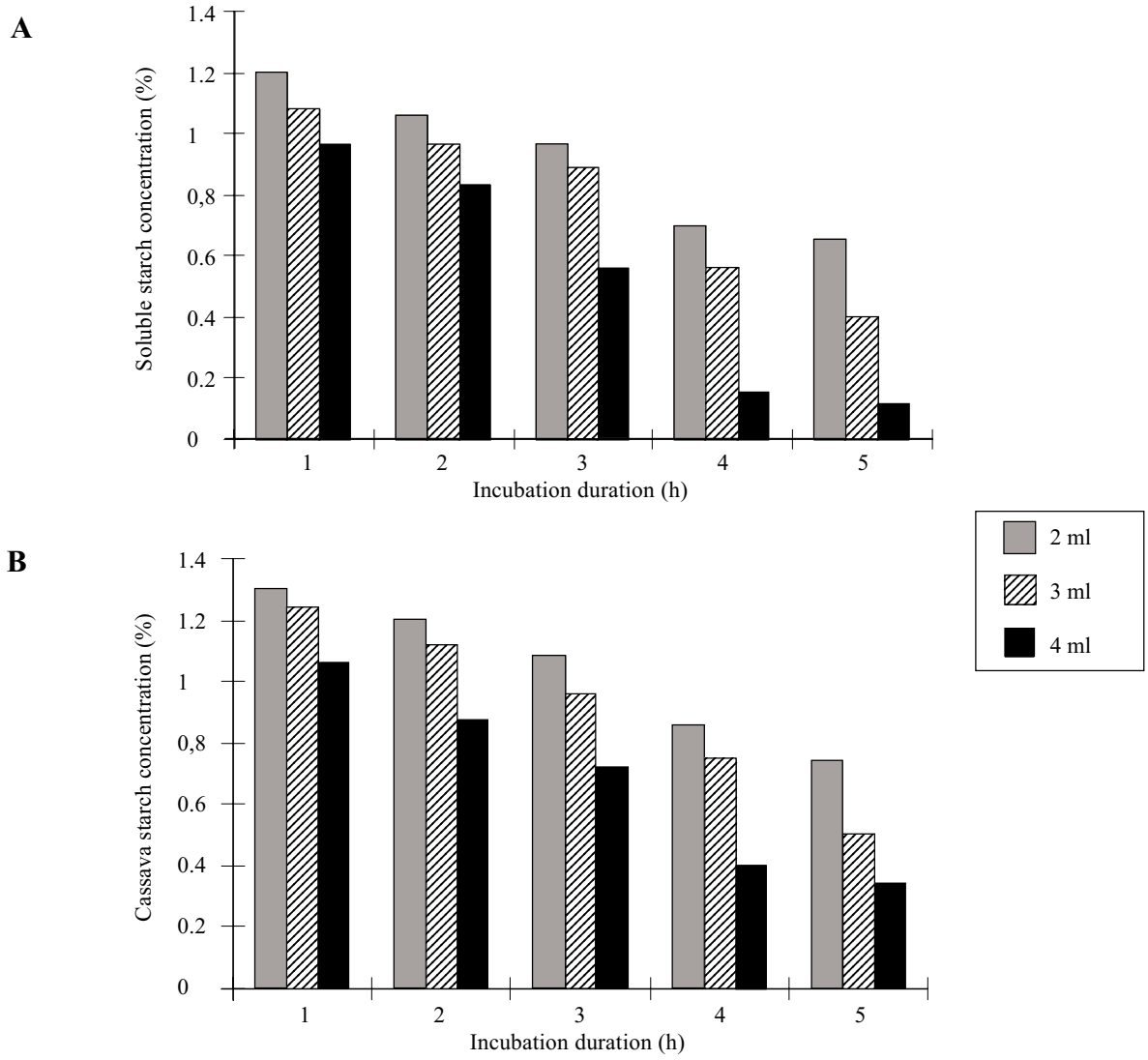

Fig. 5. Hydrolysis of commercial starch (A) and cassava starch (B) by different concentration (2–4 ml) of α-amylase from *S. erumpens.*

fermentations were conducted (every 36 h) using optimized calcium alginate beads employing SB medium (Kar and Ray, 2008). The fermentation was continued for 8th cycle; only then the beads disintegrated. RSM used in this investigation suggested the importance of various fermentation parameters at different levels. The methodology employed will be successful to any process, where an analysis of the effects and interaction of many experimental factors are required. CCD maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments (Kunamneni and Singh, 2005). Thus smaller and less time consuming experimental designs could generally suffice for the optimization of many fermentation processes. The result of this study endorses this point of view. In this study, a high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the process for enzyme production in SmF. In this study, an incubation period (36 h), pH (6.0) and temperature (50°C) were the major factors that influenced the enzyme titre. The incubation period is governed by characteristics of the culture, growth of microorganism and type of enzyme production (Selvakumar *et al*., 1994). In most cases, the average time employed was 36–48 h for α-amylase production in SmF (Dhanasekaran *et al*., 2006). Hence, the optimum incubation period (36 h) as found in our study is in agreement with majority reports (Pandey *et al*., 2000; Gupta *et al*., 2003; Tonkova, 2006). Further in the first cycle of operation the leakage of cells from immobilized support was negligible (<5%); hence the observed enzyme activity was presumed mainly because of the action of immobilized cells. Also, the re-usability of *S. erumpens* cells immobilized in alginate gel was examined. The cells not only survived but also were more or less equally active physiologically on the support even after four

cycles of fermentation (4143, 4050, 3985 and 3835 Units of enzyme production in cycle 1, 2, 3, and 4, respectively), which could save considerable time and energy. Gradual cell leakage (0.11–0.16 mg/ml) was also observed with each cycle of operation. Similar finding were observed by Adinarayana *et al.* (2004).

Among the physico-chemical parameters, the pH of the growth medium plays an important role on production of α-amylase (Pandey *et al*., 2000). In this study, for α-amylase production optimum at pH 6.0 was found for immobilized cells; below and above this pH there was a gradual decline in enzyme production which was probably due to poor microbial growth in acidic and alkaline medium (Heese *et al*., 1991; Yang and Wang, 1999). Similar findings were obtained for *S. erumpens* free cells (Kar and Ray, 2006). A pH range of 6.0–7.0 was reported to be optimum for growth and enzyme production by several *Streptomyces* spp. (Stefanova *et al*., 1998; Dobreva *et al*., 1998; Dey and Agarwal, 1999). Dey *et al*. (2003) reported that the optimum pH for amylase production by free and immobilized cells of *Bacillus circulans* GRS 313 was around 4.9. Similarly, the optimum pH for amylase production by *Bacillus* sp. isolate DPT 1 and 2 (free and immobilized cells) was in the range of 6.5–7.5 (Dhanasekaran *et al*., 2006).

Liquefaction of starch is usually carried out at higher temperature (50–80°C) depending on the type of product to be manufactured (Tonkava, 2006); therefore, the thermostability of α-amylase is of considerable significance. The optimum temperature for α-amylase production by *S. erumpens* immobilized cells was found at 50°C, which is concomitant with our earlier study with free cells (Kar and Ray, 2006). When the applicability of the *S. erumpens* amylase in liquefying starch was studied at its optimum temperature of 50°C, the crude enzyme (4 ml) could hydrolyze starch (82–94%) after 5 h of incubation. Similar results were obtained for free and immobilized *Bacillus* spp. cells for α-amylase production (Stefanova *et al*., 1998; Dobreva *et al*., 1998; Dey *et al*., 2003).

Moderately thermostable α-amylases have a number of commercial applications in baking, brewing and alcohol industries because of their inherent stability at temperature range of 50–60°C (Haki and Rakshit, 2003). Hence, α-amylase produced by *S. erumpens* as in this study, will be useful in these starch bioprocessing sectors, particularly for starch liquefaction for bio-ethanol production. Further, the result shows that calcium alginate entrapment is a promising method of *S. erumpens* immobilization for α-amylase production. α-Amylase production by immobilized cells is superior to that of free cells because it leads to higher production level with in the same time of fermentation. Further advantages of this technique is the reusability of the entrapped cells for four to five cycles without affecting the enzyme yield, as evidenced from this study. Further study is in progress in our laboratory on the application of *S. erumpens* α-amylase in saccharification of cassava starch for production of ethanol and in wine making.

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