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α-Amylase Production by *Streptomyces erumpens* MTCC 7317 in Solid State Fermentation Using Response Surface Methodology (RSM)

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Abstract

Production of á-amylase under solid state fermentation by *Streptomyces erumpens* MTCC 7317 has been investigated using different agro-industrial residues, *i.e.* cassava bagasse, sugarcane bagasse and wheat bran; wheat bran was found to be the best substrate. Among different nitrogen source supplemented to wheat bran, beef extract or peptone (1%) showed maximum enzyme production. Response surface methodology was used to evaluate the effect of main process parameters as incubation period (48 h), moisture holding capacity (70%), pH (7.0) and temperature (50°C) on enzyme production by applying a full factorial central composite design. The maximum hydrolysis of soluble starch (90%) and cassava starch (75%) was obtained with the application of 4 ml (~12096 U) of *S. erumpens* crude enzyme after 5 h of incubation.

K e y w o r d s: α-amylase, process optimization, response surface methodology, solid state fermentation, wheat bran

Introduction

α-Amylases (E.C. 3.2.1.1) are extracellular enzymes that randomly cleave the α-1, 4 linkages between adjacent glucose units in the linear amylose chain and generate glucose, maltose and maltotriose units. Enzymatic hydrolysis of starch has now replaced acid hydrolysis in over 75% of starch hydrolyzing processes due to many advantages, not least its highest yields (Tonkova, 2006). Spectrum of application of α-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharifaction, they also find applications in baking, brewing, detergent, textile, paper and distillery industries (Kandra, 2003). Approximately 90% of all industrial enzymes are produced in submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH. However, solid state fermentation (SSF) constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly (Gangadharan *et al.*, 2006). SSF is preferred to SmF because of simple technique, low capital investment, lower level of end product inhibition and low waste water output. Moreover, SSF has of late, emerged as an appropriate technology for the management of agro-industrial residues and their value addition (Pandey *et al.*, 2001). Among the agro-industrial residues wheat bran, cassava bagasse and sugarcane bagasse are considered as good substrates for enzyme production in SSF (Pandey *et al.*, 2000a; Anto *et al.*, 2006; Swain and Ray, 2007).

The optimization of fermentation parameters is an important problem in the development of economically feasible bioprocesses. Response surface methodology (RSM) consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental parameters (factors) and the measured responses, according to one or more selected criteria (Kunamneni and Singh, 2005). A prior knowledge and understanding of the process and the process parameters under investigation are necessary for achieving a more realistic model. RSM has already been successfully applied for optimization of the media and culture conditions in many cultivation processes for production of primary and secondary metabolites (Boyaci, 2005), amino acid (Xiong *et al.*, 2005), ethanol (Carvalho *et al.*, 2003) and enzymes (Rao and Satyanarayana, 2003).

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In our earlier study an actinomycetes, *Streptomyces erumpens* MTCC 7317 isolated from brick kiln soil, produced thermostable α-amylase at pH 6.0, temperature 50°C and had a molecular mass of 54.5 kDa (Kar and Ray, 2008). The present study was carried out to identify an effective agro-residue as the substrate as well as carbon source for production of α-amylase by SSF. Four physico-chemical fermentation parameters (incubation period, moisture holding capacity – MHC, pH and temperature) have been optimized by applying RSM.

Experimental

Materials and Methods

Microorganism. *S. erumpens*, isolated from a brick kiln soil (Kar and Ray, 2008) was used as the biological material and maintained at 4°C in soluble starch-beef extract (SB) (soluble starch, 1%; beef extract, 1%; yeast extract, 0.2%; $MgSO_4$, 0.02%; glycerol, 0.02%; agar, 2%; pH was adjusted to 7.0) agar slants. The inoculum preparation was carried out in SB liquid medium by transferring a loop full of organism from stock culture and incubating at 50°C and 120 rpm for 24 h in an orbital incubator shaker (Remi Pvt. Ltd, Bombay, India).

Preparation of substrate for SSF. Wheat bran was obtained commercially from a local flour mill and oven-dried. The dry material was composed of (g/100 g: crude fibre, 9.2; starch, 34.0; reducing sugar, 1.9; protein, 1.4 and ash, 3.5). Cassava bagasse was collected during starch extraction from cassava and oven-dried. The residue contains (g/100g: crude fibre, 10.8; starch, 63.0; reducing sugar, 1.45; protein, 0.88 and ash, 1.2). Sugarcane bagasse was obtained from sugarcane mill. The dry material was composed of (g/100 g: crude fibre, 75.0; reducing sugar, 3.53; protein, 0.8 and ash, 3.4).

Twenty gram substrate taken in Roux bottles (132 mm×275 mm) were moistened with distilled water containing 1% beef extract and 0.02% glycerol to provide 70% MHC and mixed thoroughly. The initial pH of the substrate was adjusted to 7.0 by using 0.1 N NaOH. The bottles were autoclaved at 15 lb pressure for 30 min and then cooled at room temperature, 30± 2°C and were inoculated with 15% (w/v) inoculum (determined by pre-experiments). The inoculated substrates were incubated at 50°C for 72 h in an incubator. The contents in the bottle were periodically mixed by gentle tapping.

Beef extract (1%) present in the basal medium was substituted with equal amount of different organic and inorganic nitrogen source for enzyme production using wheat bran as the substrate (pH adjusted to 7.0) and incubated at 50°C for 48 h.

Optimization of fermentation parameters using wheat bran. RSM was a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors and searching for the optimum conditions of factors for desirable responses (Liew *et al.*, 2005). In order to maximize amylase production the role of interacting factors under SSF were optimized by employing RSM using wheat bran as substrate.

RSM was carried out using statistical software package Design Export 7.1 (Stat-Ease, Inc, Minneapolis, USA). The levels of independent factors (incubation period, MHC, pH and temperature) were optimized by studying each factor in the design at five different levels ($-\alpha$, -1, 0, $+1$ and $+\alpha$) (Table I). The optimum value of each factor was taken at a central coded value considered as zero. The minimum [coded as (–1)] and maximum [coded as (+1)] range of experimental values of each factor used and the full experimental plan for RSM performed with 30 experiments were listed in Table II.

Table I
Range of the values for the response surface methodology

Independent factors	Coded Factor Levels				
	$-\alpha$	–1	0	+1	$+\alpha$
Incubation period (h)	0	24	48	72	96
Moisture holding capacity (%)	30	50	70	90	110
pH	3	5	7	9	11
Temperature (°C)	10	30	50	70	90

Statistical analysis and modeling. The data obtained from RSM on α-amylase production was subjected to the analysis of variance (ANOVA). The quadratic models for predicting the optimal points were expressed according to the quadratic equation;

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad (1)$$

Where Y was response variable, β_0 was intercept, β_1, β_2, β_3 and β_4 were linear coefficients, β_{11}, β_{22}, β_{33} and β_{44} were squared coefficient, β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} were interaction coefficient and A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD were level of independent factors. The significance of the quadratic model equation was expressed by the coefficient of determination (R^2) and its statistical significance was checked by Fischer's test value (F-value).

Effect of MHC and initial medium pH. The influence of MHC on enzyme titre was evaluated by varying the moisture content of the substrate from 50 to 90% MHC and initial medium pHs were adjusted to 5.0–9.0 by using 0.1 N HCl or NaOH. The samples (n = 3) were incubated for 48 h at 50°C. The moisture content of the substrate was analyzed by a Mettler LP16 Infra-Red analyzer.

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

Observation	A: Incubation period (h)	B: Moisture holding capacity (%)	C: pH	D: Temperature (°C)	Enzyme production (U/gds)	
					Predicted	Experimental
1	–1	–1	–1	–1	2987	3117
2	1	–1	–1	–1	3198	3308
3	–1	1	–1	–1	2802	2937
4	1	1	–1	–1	3013	3128
5	–1	–1	1	–1	2318	2422
6	1	–1	1	–1	2529	2613
7	–1	1	1	–1	2133	2242
8	1	1	1	–1	2344	2432
9	–1	–1	–1	1	2921	2923
10	1	–1	–1	1	3132	3114
11	–1	1	–1	1	2736	2743
12	1	1	–1	1	2947	2934
13	–1	–1	1	1	2252	2228
14	1	–1	1	1	2463	2419
15	–1	1	1	1	2067	2048
16	1	1	1	1	2278	2239
17	– α	0	0	0	3157	3025
18	+α	0	0	0	3580	3530
19	0	– α	0	0	2906	2825
20	0	+ α	0	0	2536	2435
21	0	0	– α	0	3003	2860
22	0	0	+ α	0	1664	1625
23	0	0	0	– α	2172	1825
24	0	0	0	+ α	2040	2205
25	0	0	0	0	3747	3831
26	0	0	0	0	3747	3731
27	0	0	0	0	3747	3775
28	0	0	0	0	3747	3650
29	0	0	0	0	3747	3685
30	0	0	0	0	3747	3815

Effect of temperature on enzyme production. The effect of temperature was studied by evaluating the solid substrate at different incubation temperatures (30–70°C) maintained in an incubator and the samples were incubated for 48 h.

Enzyme extraction and assay. After 24 h of incubation (determined by pre-treatments), samples (n = 3) from each treatment were taken out at 12 h intervals up to 72 h and enzyme was extracted by mixing the substrate with 40 ml of distilled water [1 : 2 (substrate:water) ratio] and squeezed through a wet cheese cloth. The pooled enzyme extract was centrifuged at 8000 × g for 20 min in refrigerated centrifuge (Remi Pvt. Ltd, Bombay, India) and the clear supernatant was used for enzyme assay.

The 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 7.0) 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 (Model no CE 7250, Cecil Instrument, UK) at 690 nm. One unit of enzyme activity was 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). In SSF, units of enzyme activity were calculated as units (U) per gram of dry substrate (gds) (*i.e.* U/gds).

Application. A 2% (w/v) of soluble starch and cassava starch were incubated with 2 ml (~6048 U), 3 ml (~9072 U), 4 ml (~12096 U) and 5 ml (~15120 U) of *S. erumpens* crude enzyme obtained through SSF (50°C) using wheat bran. The degradation of starch was evaluated at 1 h interval up to 5 h.

Results

In this study, all the substrates supplemented with 1% beef extract and 0.02% glycerol supported thermostable (50°C) α-amylase production by *S. erumpens*; however, using wheat bran gave the highest enzyme production (3781 U/gds) at 48 h followed by cassava bagasse (3437 U/gds) at 60 h (Table III). Maximum α-amylase production was obtained when either beef extract or peptone was supplemented as the nitrogen source in comparison to other nitrogen sources (Table IV). Further studies were carried out, therefore, using wheat bran as the substrate and carbon source, supplemented with beef extract (1%) as the nitrogen source.

Table III
Screening of agro-residues (pH adjusted to 7.0) incubated at 50°Cfor the production of α-amylase using *S. erumpens*

	α-amylase production (U/gds)				
Incubation time (h)	24	36	48	60	72
Wheat bran	2562	3356	3781	3562	3325
Cassava bagasse	2300	3168	3306	3437	3215
Sugarcane bagasse	1781	2419	2821	2531	2280

Table IV
Effect of nitrogen source on α-amylase production in SSF using wheat bran as the substrate (pH adjusted to 7.0) and incubated at 50°C for 48 h

Nitrogen sources	Enzyme production (U/gds)
Ammonium acetate	1512 ± 105.0
Ammonium chloride	1120 ± 95.0
Ammonium nitrate	1980 ± 110.0
Ammonium sulphate	1435± 110.0
Potassium nitrate	1850 ± 120.0
Urea	937 ± 115.0
Beef extract (control)	3775± 105.0
Casein	2985 ± 121.0
Peptone	3850 ± 101.0
Yeast extract	3437 ± 105.0

±: Standard deviation

Optimization of fermentation parameters. The effect of four independent factors (incubation period, MHC, pH and temperature) for α-amylase production by *S. erumpens* in wheat bran were presented along with predicted and observed responses in Table II. Regression analysis was performed to fit the response function with the experimental data. The results obtained after CCD were then analyzed by standard analysis of variance (ANOVA), which gave the second order polynomial regression equation.

$$Y = 61.22 + 0.99\,A - 0.90\,B - 3.34\,C - 0.28\,D - 0.74\,A^2 - 2.23\,B^2 - 3.32\,C^2 - 3.83\,D^2 + 0.015\,AB + 0.060\,AC + 0.018\,AD - 0.058\,BC - 0.016\,BD - 0.061\,CD$$

Where Y was enzyme production, A was incubation period (h), B was MHC (%), C was pH and D was temperature (°C).

The regression equation obtained indicated R^2 (coefficient of determination) values of 0.9643 for α-amylase production and thus the model could explain more than 96.43% of the variability in the response (Table V). Moreover, the predicted R^2 value (0.8017) was in reasonable agreement with adjusted R^2 value of 0.9310. Further, a high similarity was observed between the predicted and experimental result (Fig. 1). An adequate precision of 18.112 for α-amylase production was recorded. The model F-value of 28.95 and Value of "prob> F" (<0.05) indicated that model terms were significant. For α-amylase production, the coefficients of A, B, C, A^2, B^2, C^2 and D^2 were significant at 1% level.

Table V
ANOVA for α-amylase production in solid state fermentation

Source	Sum of squares	Degree of freedom	Mean Square	F-Value	p-value
Model	984.92	14	70.35	28.95	0.0001
Pure Error	1.74	5	0.35		
Total	1021.38	29			

$R^2 = 0.9643$; adjusted $R^2 = 0.9310$;
predicted $R^2 = 0.8017$; adequate precision = 18.112

Response surface estimation for maximum enzyme production. To investigate the interactive effect of factors on the amylase production, the response surface graphs were employed by plotting the effect of independent factors (incubation period, MHC, pH and temperature). Out of four factors, two were fixed at zero level while other two were varied.

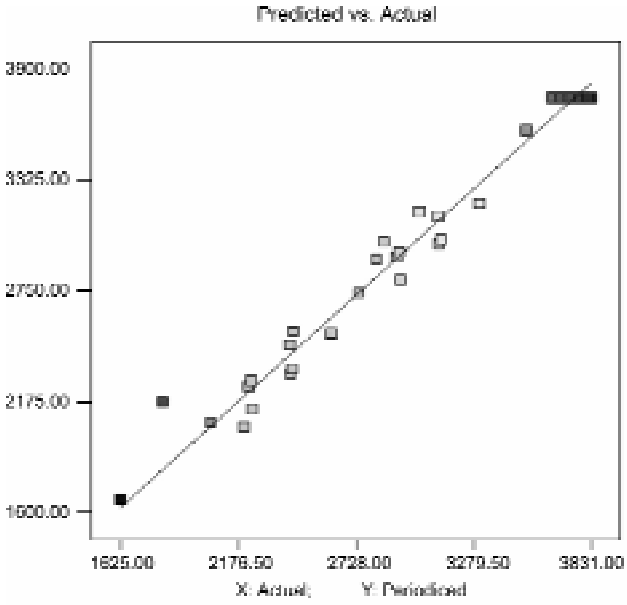

Fig. 1. Plot of predicted versus actual amylase production

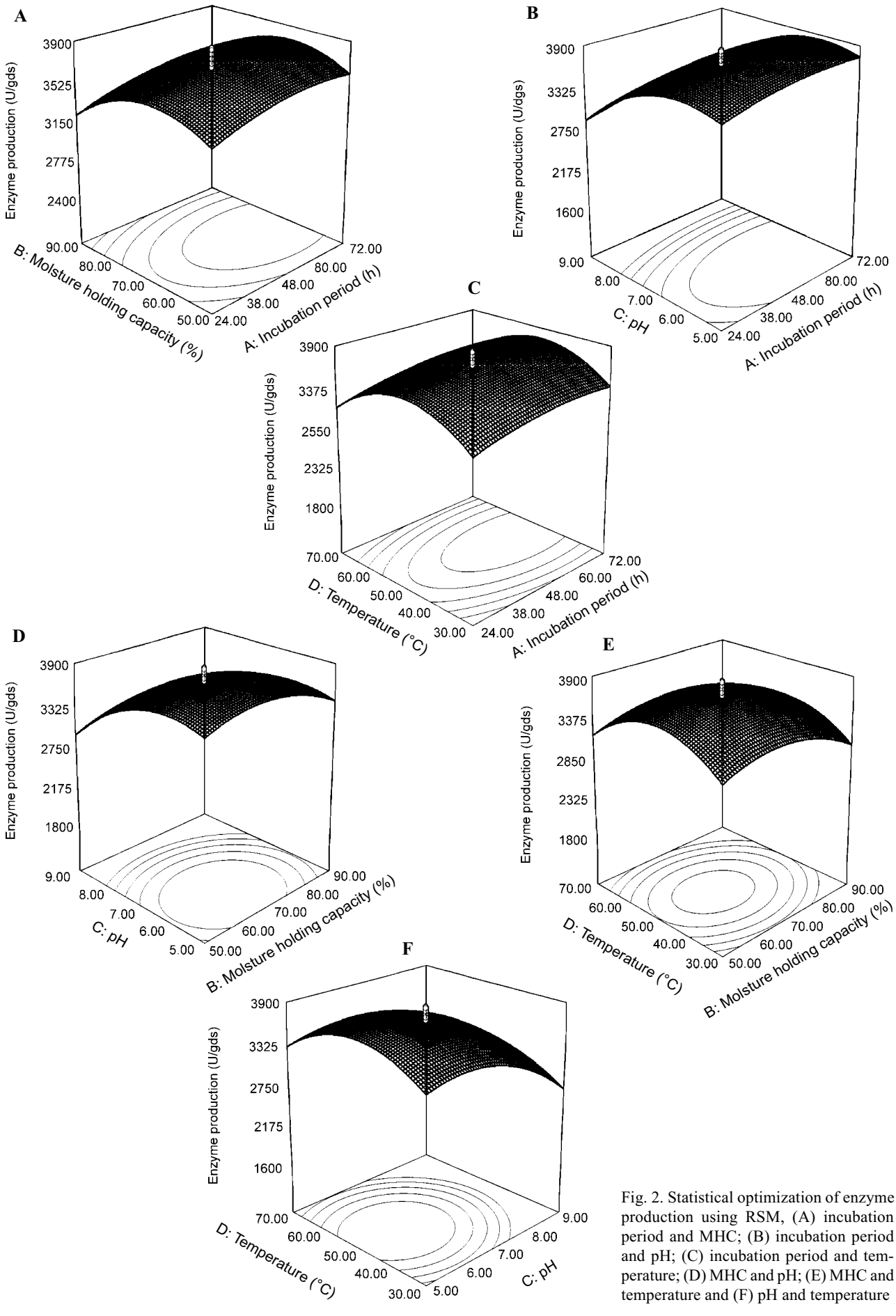

Fig. 2. Statistical optimization of enzyme production using RSM, (A) incubation period and MHC; (B) incubation period and pH; (C) incubation period and temperature; (D) MHC and pH; (E) MHC and temperature and (F) pH and temperature

Figure 2A depicts three dimensional diagram and contour plot of calculated response surface from the interaction between incubation period and MHC while keeping the other factors (pH and temperature) at zero level. The result demonstrated that with increase in incubation period and MHC up to 48 h and 70%, respectively, the enzyme production had increased up to 3745.55 U/gds and thereafter it declined.

Figure 2B shows the effect of incubation period and pH on enzyme production, keeping temperature and MHC at zero level. The graph shows that the maximum amylase production (3764 U/gds) occurred at pH of 7.0 and incubation period of 48 h, which was in conformity with the model. At '0' level of MHC and pH the response between incubation period and temperature indicated that a higher temperature (50°C) was optimum with 48 h incubation period for α-amylase production (3750 U/gds) (Fig. 2C). The response between MHC and pH (keeping incubation period and temperature at 0 level) indicated that pH 7.0 with 70% MHC showed the optimum enzyme production (3757 U/gds) (Fig. 2D). Fig. 2E represented interaction between MHC and temperature while keeping incubation period and pH at 0 level. An interaction between the remaining two factors (pH and temperature) (Fig. 2F) suggested a little difference with the earlier responses.

Optimization. To find out optimum level of process parameters for maximizing the response, the criteria were set, as given in Table VI. The optimization criteria were used to get maximum yield of amylase by minimizing incubation period (48 h) and maximizing pH (7.0), temperature (50°C) and MHC (70%).

Table VI
Optimization criteria used in this study

Parameter or Response	Limits		Importance	Criterion
	Lower	Upper		
Incubation period	24	72	3	Minimize
MHC	50	90	5	Maximize
pH	5.0	9.0	3	Maximize
Temperature	30	70	3	Maximize
Enzyme production	1625	3831	5	Maximize

Testing of model adequacy. Usually it was necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit, processing with investigation and optimization of the fitted response surface likely give poor or misleading results. By constructing a normal probability plot of the residuals, a check was made for the normality assumption, as given in Fig. 3. The normality assumption was satisfied as the residuals were approximated along a straight line.

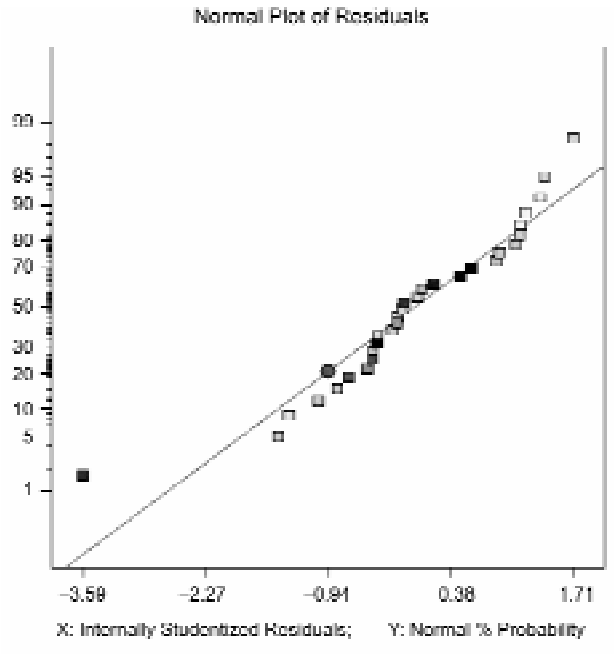

Fig. 3. Normal probability plot of studentized residuals

Practical verification of theoretical results. Further to support the optimized data as given by statistical modeling under optimized condition, the confirmatory experiments were conducted with the parameters as suggested by the model (incubation period, 48 h; MHC, 70%; pH, 7.0 and temperature, 50°C). The optimized process condition yielded amylase production (3830 U/gds) which was closer to the predicted amylase production (3755 U/gds) at same optimal point.

Application. The rate of hydrolysis of 2% (w/v) soluble starch and cassava starch by *S. erumpens* α-amylase is shown in Fig. 4. 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 (~12096 U) crude enzyme there was 90 and 75% hydrolysis of soluble starch and cassava starch, respectively.

Discussion

In recent years, the application of the agro-industrial residues (*i.e.* wheat bran, cassava bagasse, sugarcane bagasse, sugar beet pulp, apple pomace, *etc.*) provide an alternative way to replace the refined and costly raw materials and the use of such materials will help to solve many environmental hazards (John *et al.*, 2006). Several processes have been developed that utilize these as raw materials for the production of value added products such as ethanol, enzymes, organic acids and others (Pandey *et al.*, 2000a). Wheat bran has been widely reported to be the ideal substrate for production of several enzymes in SSF: α-amylase (Anto *et al.*, 2006), pectinase (Kashyap *et al.*, 2003),

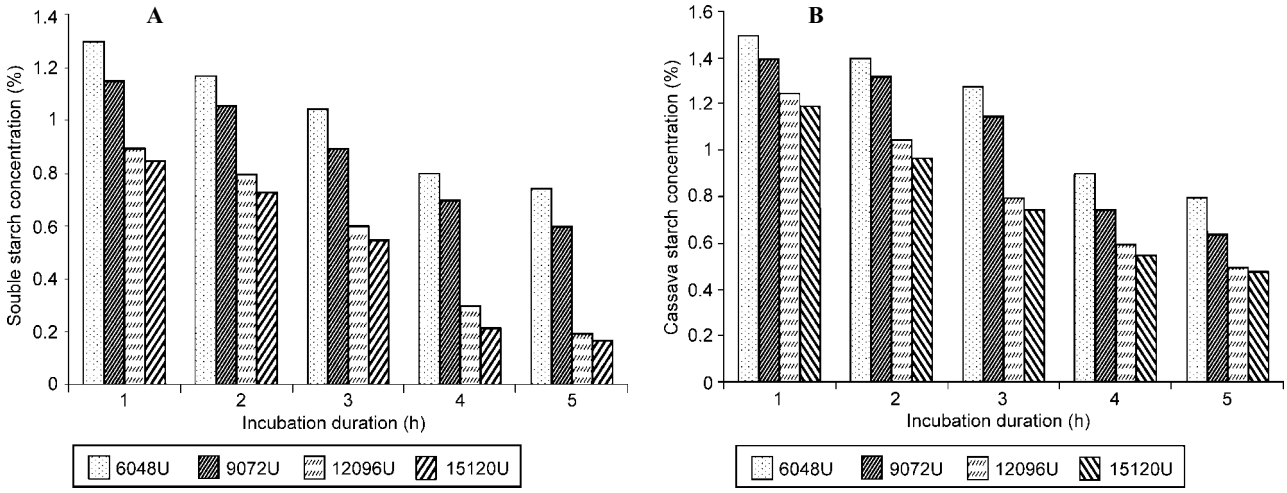

Fig. 4. Hydrolysis of (A) soluble starch and (B) cassava starch by application of crude α-amylase (6048- 15120 U) from *S. erumpens*

glucoamylase (Bhatti *et al.*, 2007), protease (Aikat and Bhattacharyya, 2000) and xylanase (Poorna and Prema, 2007). Following the evaluation of nitrogen sources, it was observed that organic nitrogen supported higher amylase production than inorganic nitrogen sources. Similar results were obtained for SSF in case of *Bacillus* spp., *i.e. Bacillus amyloliquefaciens* (Gangadharan *et al.*, 2006), *Bacillus coagulans* (Babu and Satyanarayana, 1995) and for *Aspergillus niger* in wheat bran containing solid substrate medium (Ellaiah *et al.*, 2002).

The characterization of different factors for α-amylase production was optimized by applying RSM. A high similarity was observed between the predicted and experimental results (Fig. 1), which reflected the accuracy and applicability of RSM to optimize the process for enzyme production. In this study, an incubation period (48 h), MHC (70%), pH (7.0) and temperature (50°C) were major factors that influenced the enzyme titre. The production of α-amylase reached a peak at 48 h (3781 U/gds) using wheat bran and there after, it declined. This could be due to loss of moisture with prolonged incubation at 50°C and denaturation or decomposition of a-amylase due to interaction with other components in the culture medium (Gangadharan *et al.*, 2006). Moreover, the incubation time is governed by characteristics of the culture and is based on enzyme production (Baysal *et al.*, 2003). In most cases, the optimum incubation period for α-amylase production in SSF using *Bacillus* sp. culture varied from 24 to 74 h, depending on environmental conditions (Baysal *et al.*, 2003; Sivaramakrishnan *et al.*, 2006). In contrast, α-amylase production from *Streptomyces rimosus* was reported at 180 h incubation using sweet potato residue as the substrate (Yang and Wang, 1999).

Moisture is one of the most important parameter in SSF that influences the growth of the organism and thereby enzyme production (Baysal *et al.*, 2003). In the present study, 70% moisture content gave maximum enzyme production when compared to 50, 60, 80 and 90% MHC. A reduction in enzyme production at high initial moisture content might be due to porosity, lower oxygen transfer (Pandey *et al.*, 2000a), pore aeration and adsorption of enzyme to the substrate particle (Swain and Ray, 2007). The optimum amylase production for *S. rimosus* on a mixture of sweet potato and peanut meal residue was found to be at 65% MHC in SSF (Yang and Wang, 1999). In case of *Bacillus* spp, optimal moisture content was found to be at 60–85% (Pandey *et al.*, 2000b; Gangadharan *et al.*, 2006).

Among physicochemical parameters, the pH of the medium plays an important role including morphological changes in the organism and in enzyme production. It is evident from the study that α-amylase yield was significant over a range of pH 6.0–7.0 with an optimum at pH 7.0 (3837 U/gds). Further increase in pH resulted in a drastic reduction in enzyme production. α-Amylase of *S. rimosus* was reported to have pH optimum at 6.0 in SSF (Yang and Wang, 1999). Anto *et al.* (2006) reported pH 5.0 to be the best for the production of α-amylase by *Bacillus cereus* in SSF.

The influence of temperature on amylase production is related to the growth of the organism. However, the optimum temperature depends on whether the culture is mesophilic or thermophilic. The isolate, *S. erumpens* showed maximum α-amylase production at 50°C. Further increase the temperature led to decrease in enzyme production. The optimum α-amylase production for other actinomycetes, *i.e. Thermoactonomyces vulgaris* and *S. rimosus* were found to be 62.5 and 45°C, respectively (Heese *et al.*, 1991; Yang and Wang, 1999).

In conclusion, the parametric optimization of α-amylase production by *S. erumpens* in SSF using wheat bran differed to some extent from SmF (Kar and Ray,

2008). The optimum incubation period, pH and temperature in SmF were 36 h, 6.0 and 50°C, where as in the present study, these parameters were 48 h, 7.0 and 50°C, respectively. The variations in pH and incubation period optima between two forms of fermentation were because of cultural conditions. Further, the enzyme yield was somewhat similar in SmF (3500 U/ml) and SSF (3781 U/gds). Nevertheless, utilization of agro-waste such as wheat bran has multitude of advantages as discussed in the earlier section.

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