Volume 59(2):179-188, 2015
Acta Biologica Szegediensis
http://www2.sci.u-szeged.hu/ABS

ARTICLE

Statistical optimization of α-amylase production from *Penicillium notatum* NCIM 923 and kinetics study of the purified enzyme

Priyanka Ghosh[1], Arpan Das[2], Soumi Gayen[1], Keshab Chandra Mondal[2], Uma Ghosh[1]*

[1]Food Technology and Biochemical Engineering Department, Jadavpur University, Kolkata-700032, West Bengal, India
[2]Department of Microbiology, Vidyasagar University, Midnapore-721102, West Bengal, India

ABSTRACT In this study, response surface methodology (RSM) was employed to optimize the production of α-amylase by *Penicillium notatum* NCIM 923 through solid-state fermentation. The individual and combinational effects of the factors, *i.e.* substrate amount, initial moisture, fermentation time, temperature and size of inoculum were found to have significant effects on α-amylase production: the optimum values of the tested variables were 5 g, 70%, 94 h, 28 °C and 20%, respectively. The predicted amylase production (2819.24 U/g) was in good agreement with the value measured under optimized surrounding (2810.33 U/g). The molecular mass of purified α-amylase was about 52 kDa. The enzyme activity exhibited its pH optimum between pH 4.6 and 6.6, and it had maximal activity at 50 °C. The apparent K_m and V_{max} of α-amylase for starch were 4.1 mg/ml and 247.6 μmol/min, respectively. The activation energy (E_a) for starch hydrolysis was found to be 14.133 kJ/mol. The enzyme was thermostable with half-life ($t_{1/2}$) of 110 min at 80 °C and temperature coefficient (Q_{10}) value of 1.0. Purified enzyme was activated by Ca^{2+} and inhibited by Hg^{2+} ions. EDTA also inhibited the enzyme activity, indicating that the purified enzyme is a metalloenzyme.
Acta Biol Szeged 59(2):179-188 (2015)

KEY WORDS
α-amylase
activation energy
statistical optimization
temperature coefficient
thermostability

Introduction

In the past few decades, starch degrading enzymes like α-amylase have received a great deal of attention because of their perceived technological significance and economic benefit (Gupta et al. 2003). Amylases share about 25% of the industrial enzyme market of the world. Further, with the advancement of biotechnology, the applications of α-amylase have been expanded to various fields such as medicinal, clinical and analytical chemistry. It is also being widely used in starch saccharification, detergent, textile, food and brewing industries (Adeniran and Abiose 2009). Industrial bioconversion of starch with α-amylase has been reported to represent an economically superior alternative to the conventional process which uses chemically treated pregelatinised starch as substrates and consume high energy. α-Amylases are ubiquitous enzymes produced by plants, animals and microorganisms. The major advantages of using microorganisms for enzyme production are their high production capacity and easy genetic manipulation to obtain enzymes with desired characteristics (Kumar and Duhan 2011; Pandey et al. 2000). α-Amylases (1,4-α-D-glucan-glucanohydrolase; EC3.2.1.1) are endo-amylases catalyzing the hydrolysis of internal α-1-4-glucosidic linkages in polysaccharides (Petrova et al. 2000). Although several microorganisms are capable of α-amylase production, fungi (especially *Aspergillus* and *Penicillium* species) are frequently used for enzyme production as they have non-fastidious nutritional requirements and high product yield (Abu et al. 2005). The production of α-amylase in submerged fermentation (SmF) is quite expensive, due to its high energy consumption and operating costs. There is a need for reduction of production cost by alternative methods; among these, solid state fermentation (SSF) is rather promising. It has low energy requirement, high product concentration, and reduced levels of catabolite repression. Furthermore, it produces less wastewater, resolves the problem of solid waste disposal and requires a lower input of infrastructure and skill (Pandey et al. 2000; Ellaiah et al. 2002; Baysal et al. 2003).

The optimization of process parameters is an important prerequisite for improving the production of microbial amylases. Therefore, development of a cost-effective production medium requires proper selection of different media components followed by their optimization. However, media optimization by single dimensional search is laborious and

Submitted July 29, 2015; Accepted Oct 27, 2015
*Corresponding author. E-mail: ughoshftbe@yahoo.co.in

Table 1. Factors coded with different levels for Box-Behnken response surface methodology.

Independent variable	Code units	Coded variable level		
		-1	0	+1
Substrate amount (g)	A.	5	10	15
Initial moisture (%)	B.	50	60	70
Fermentation time (h)	C.	48	72	96
Fermentation temperature (°C)	D.	25	30	35
Inoculum (%)	E.	10	15	20

time-consuming, especially for a large number of variables. Due to aforementioned facts, statistical methodologies like response surface methodology (RSM) for media optimization are generally preferred over the strain improvement for enhancing the yield (Das et al. 2012; Das et al. 2013a). The methodology is based on the construction of balance designs which are rotatable enabling each factor level to be tested several times. Box-Behnken designs provide excellent predictability within the spherical design space and require fewer experiments compared to full factorial designs. RSM has already been effectively applied during optimization of different media and culture conditions for production of microbial metabolites (Boyaci 2005; Xiong et al. 2005), amino acid, ethanol and enzymes (Das et al. 2013b). Therefore, the objective of this study was statistical optimization of process parameters for enhancing the α-amylase yield of *Penicillium notatum* NCIM 923 through solid state fermentation using Box-Behnken response surface methodology; purification and partial characterization of the enzyme were also carried out.

Materials and Methods

Substrate collection

Wheat bran (≤5 mm mesh size) was used as a solid substrate for α-amylase production. The substrate was collected from local market of Uluberia (Howrah, India). The substrate was stored at room temperature without any pretreatment.

Microorganism and inoculum preparation

The *P. notatum* NCIM 923 was grown on modified Czapek-Dox agar (composition in %w/v: glucose 5, sodium nitrate 0.2, magnesium sulfate 0.05, potassium chloride 0.05, iron(III) sulfate 0.001, di-potassium hydrogen phosphate 0.1, agar 1.75; pH 5.0) slants at 30 °C for 5 days. Fully sporulated slants were used immediately or stored at 4 °C for further use. A conidial suspension was prepared in sterile distilled water with a spore count of 10^6-10^7 spores/ml.

Solid-state fermentation for α-amylase production

In a 250-ml Erlenmeyer flask, a mass of 10 g of wheat bran was mixed with distilled water to set the initial moisture of 60%. After sterilization by autoclaving at 121 °C for 30 min, the medium was inoculated with spore suspension and incubated at 30 °C temperature for 72 h. After fermentation, it was mixed with 60 ml of distilled water and was shaken for 3 h in a rotary shaker to extract the enzyme. Then the culture broth was centrifuged at 10 000 g for 10 min and total α-amylase yield was determined in the supernatant.

Box-Behnken response surface methodology

The effects of substrate amount (g), initial moisture (%), incubation time (h), incubation temperature (°C) and inoculum size (%) on α-amylase activity was evaluated. These factors were chosen as they showed influencing effects in OVAT (one variable at time) optimization (data not shown). Levels of these factors were optimized for maximum α-amylase production using Box-Behnken statistical design (Box and Behnken 1960). Table 1 represents different selected factors where each variable was tested in three different coded levels: low (-1), middle (0) and high (+1). Table 2 represents a 46-trial of the experimental design.

Enzyme assay

α-Amylase activity was assayed according to the method of Anto et al. (2006). The amount of reducing sugar liberated was estimated according to the method of Miller (1959) using dinitrosalicylic acid reagent. The absorbance was measured spectrophotometrically (Hitachi 2000, Japan) at 540 nm. One unit (U) of α-amylase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar per min under the assay conditions.

Protein assay

Protein content was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Enzyme purification

All purification steps were performed at 4 °C unless otherwise stated. Crude enzyme was mixed with 10% acetone (Oboh 2005) and was kept overnight at 4 °C to precipitate the protein. Resulting precipitate was collected by centrifugation at 10 000 g for 10 min, and dissolved in 50 mM acetate buffer (pH 5.0). Total proteins and α-amylase activity was determined before and after precipitation. The enzyme preparation was

Table 2. Box-Behnken experiments design matrix with experimental and predicted values for α-amylase production from *P. notatum* NCIM 923.

Run no.	A. Substrate amount (g)	B. Initial moisture (%)	C. Fermentation time (h)	D. Fermentation temperature (°C)	E. Inoculum (%)	Observed response (U/g)	Predicted response (U/g)
1	15	60	96	30	15	1840.6	1838.73
2	5	60	72	30	20	2130.3	2131.35
3	5	60	72	25	15	980.1	979.14
4	15	60	72	30	20	2030.6	2032.70
5	15	60	72	25	15	1750.6	1750.79
6	10	70	72	25	15	1860.8	1860.31
7	15	60	72	35	15	1890.1	1892.87
8	10	70	48	30	15	1400.4	1405.61
9	10	60	72	30	15	1739.7	1732.08
10	15	50	72	30	15	2030.3	2034.10
11	5	60	72	35	15	1970.4	1972.02
12	10	60	48	35	15	1200.6	1198.66
13	10	60	48	30	20	880.8	875.61
14	10	60	72	30	15	1730.5	1732.08
15	10	60	48	30	10	630.2	630.21
16	10	50	72	25	15	1220.8	1216.53
17	10	60	72	30	15	1730.6	1732.08
18	10	50	72	35	15	2200.9	2199.26
19	10	70	72	35	15	2010.4	2012.55
20	5	70	72	30	15	1920.2	1916.39
21	10	60	72	35	10	790.3	789.76
22	10	50	96	30	15	1880.5	1877.07
23	10	60	72	30	15	1730.1	1732.08
24	10	70	72	30	10	1140.2	1139.72
25	10	50	48	30	15	1060.5	1062.07
26	10	70	96	30	15	1990.4	1990.61
27	10	50	72	30	10	410.2	410.94
28	15	70	72	30	15	1960.3	1957.89
29	15	60	48	30	15	1330.1	1328.28
30	10	60	72	30	15	1730.9	1732.08
31	10	70	72	30	20	2120.0	2119.62
32	10	60	72	25	20	1700.0	1702.42
33	5	60	96	30	15	1680.1	1682.03
34	5	50	72	30	15	1380.7	1383.10
35	5	60	72	30	10	210.3	206.30
36	10	60	48	25	15	920.3	920.68
37	15	60	72	30	10	1000.4	997.45
38	10	50	72	30	20	2390.5	2391.34
39	10	60	96	30	20	2810.7	2810.36
40	10	60	72	30	15	1730.7	1732.08
41	5	60	48	30	15	790.7	792.48
42	10	60	96	25	15	1330.8	1331.18
43	10	60	72	35	20	2460.7	2460.21
44	10	60	72	25	10	410.2	412.57
45	10	60	96	30	10	90.6	95.46
46	10	60	96	35	15	2190.1	2188.16

applied on a DEAE cellulose column (50×1 cm; Sigma, St. Louis, MO) pre-equilibrated with elution buffer (50 mM sodium acetate buffer, pH 5.0). Elution was achieved with a linear gradient of the same buffer (50-200 mM) at a flow rate of 0.3 ml/min. Five ml fractions were collected and studied at 280 nm for the presence of protein. α-Amylase activity was determined in each fraction, and the active fractions were pooled, dialyzed and concentrated. The concentrate was loaded on Sephadex G-100 (80×2 cm; Sigma, St. Louis, MO) column. The enzyme was eluted with 50 mM acetate buffer (pH 5.0) at a flow rate of 1 ml/min. Eluted protein fractions were collected through fraction collector and the active part

Table 3. Analysis of variance (ANOVA) for α-amylase production in second-order polynomial model.

Source	Sum of squares	Degree of freedom	F value	Prob>F
Model	1.742E+007	20	71537.49	<0.0001
A	4.796E+005	1	39397.66	<0.0001
B	2.089E+005	1	17163.50	<0.0001
C	1.960E+006	1	1.610E+005	<0.0001
D	1.288E+006	1	1.058E+005	<0.0001
E	8.763E+006	1	7.200E+005	0.0001
AB	92872.56	1	7629.89	<0.0001
AC	35929.20	1	2951.74	0.0001
AD	1.810E+005	1	14867.09	<0.0001
AE	1.979E+005	1	16261.32	<0.0001
BC	13225.00	1	1086.49	0.0001
BD	1.724E+005	1	14166.10	<0.0001
BE	2.503E+005	1	20559.15	<0.0001
CD	83810.25	1	6885.38	<0.0001
CE	1.525E+006	1	1.253E+005	<0.0001
DE	36214.09	1	2975.15	<0.0001
A^2	14911.56	1	1225.05	0.0001
B^2	1.523E+005	1	12516.01	<0.0001
C^2	6.860E+005	1	56359.70	<0.0001
D^2	15427.00	1	1267.40	0.0001
E^2	1.062E+006	1	87230.35	<0.0001
Residual	304.30	25		
Lack of fit	234.34	20	0.84	0.6527
Cor total	1.742E+007	45		

R^2 value - 0.995; Adj R^2 value - 0.987; Predicted R^2 value - 0.999; Adequate precision value - 1151.698; Predicted sum of square (PRESS) value - 1038.10

was pooled and concentrated.

SDS-PAGE analysis

Molecular mass and the purity of the purified α-amylase was determined by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (10% SDS-PAGE), using a MiniProtean (Bio-Rad) gel electrophoresis apparatus. After electrophoresis, gels were stained by Coomassie Brilliant Blue R-250. Molecular mass was determined against standard markers (Sigma, St. Louis, MO).

Zymogram analysis

For zymogram analysis, purified α-amylase was subjected to 10% polyacrylamide gel supplemented with 0.5% soluble starch. After electrophoresis, gel was incubated in 50 mM acetate buffer (pH 5.0) for 30 min. The clear starch hydrolysis zone that corresponded to enzyme activity was visualized using iodine solution.

Determination of optimum pH, temperature and thermostability

The optimum pH was determined by measuring the activity at 50 °C using the following buffers: 50 mM sodium acetate buffer (pH 4.0-5.0), 50 mM phosphate buffer (pH 6.0-7.0) and 50 mM Tris buffer (pH 8.0). The pH stability of the enzyme was determined by incubating the enzyme in the range from pH 4.0 to 8.0 at 50 °C for 2 h, and then residual enzyme activity was measured.

Optimum temperature was determined by assaying the enzyme activity at pH 5.0 in the range from 30 to 80 °C. The activation energy (E_a) for substrate hydrolysis was determined by plotting the data as an Arrhenius plot (Das et al. 2012). Thermostability was determined by pre-incubation of the enzyme in temperature range from 30 to 80 °C at pH 5.0 in the absence of substrates. Aliquots were removed at different time intervals between 0 and 2 h, and immediately cooled on ice. The treated enzyme solutions were then assayed for residual enzymatic activities using standard assay conditions. From the slope of the plot of residual activity vs. time, the first order rate constant of thermal inactivation of enzyme activity (k_d) was calculated and apparent half-lives ($t_{1/2}$) at different temperatures were estimated using the following formula (Griffin et al. 1984):

$$T_{1/2} = \ln 2/k_d = 0.693/ k_d$$

Temperature coefficient (Q_{10})

The Q_{10} value, the rate of an enzymatic catalysis reaction changes for every 10 °C rise in temperature, was calculated

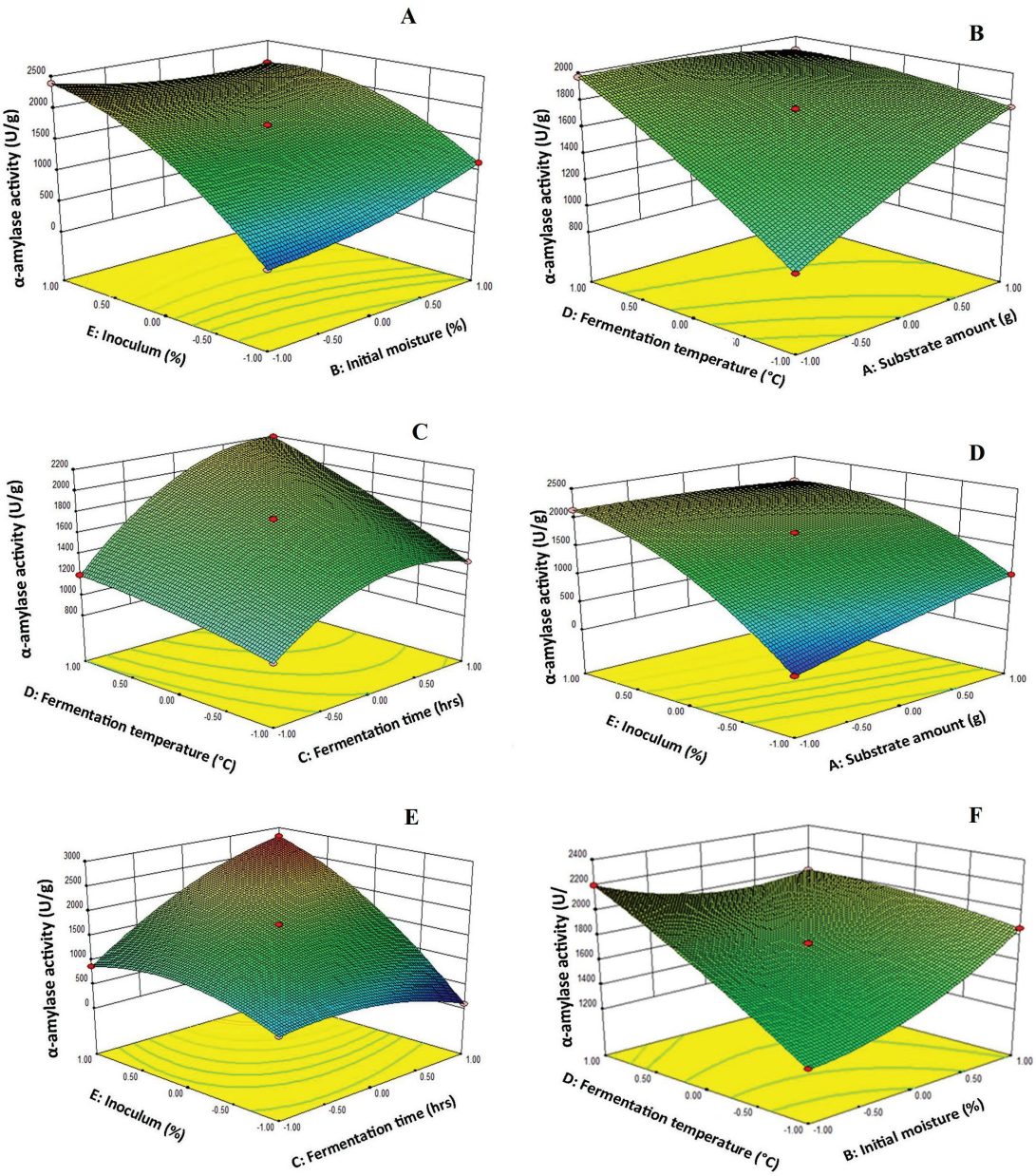

Figure 1. The response surface plots showing the effects of different factors on α-amylase production.

by the Dixon and Webb equation (1979):

$$\ln Q_{10} = (E_a \times 10)/RT^2$$

where E_a is the activation energy of the enzyme (J/mol).

Effect of different ions on the activity

The effects of various metal ions and additives on purified amylase activity were determined by pre-incubating the enzyme with 1 mM of NaCl, KCl, NH_4Cl, $CoCl_2$, $MnCl_2$, $MgCl_2$, $HgCl_2$, $CuCl_2$, $ZnCl_2$, $CaCl_2$, $FeCl_3$ and EDTA for 30 min at 25 °C (pH 5.0). Residual enzyme activity was measured. The activity assayed in the absence of metal ions was taken as a control and recorded as 100%.

Statistical analysis

Statistical analyses were performed using statistical software Design Expert (version 8.2; STATEASE Inc., Minneapolis, MN, USA).

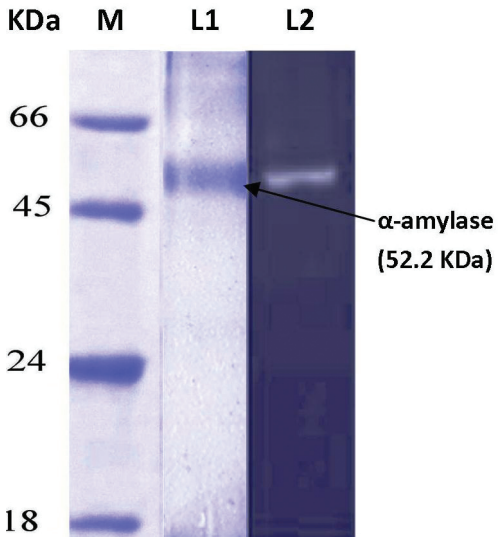

Figure 2. SDS-PAGE profile of α-amylase purified from *P. notatum* NCIM 923. Lane M: molecular marker. Lane L1: Sephadex G100 column fraction; L2: zymogram analysis of purified α-amylase after staining with iodine solution.

Results and Discussion

Optimization of α-amylase production through Box-Behnken Response Surface design

The optimal level of the key factors and the effect of their interactions on α-amylase production were explored by the Box-Behnken response surface methodology. Experimental design and results are shown in Table 2. By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was established to explain the α-amylase production:

$Y=1731.50+173.13A+114.37B+353.44C+283.75D+736.56E-152.50AB-95.00AC-212.50AD-222.50AE-57.50BC-207.50BD-250.00BE+145.00CD+603.75CE+ 95.00DE-41.31A^2+131.02B^2-276.90C^2-43.15D^2-345.23E^2$

where, Y is the predicted yield of α-amylase; A, B, C, D and E are the coded values of substrate amount, initial moisture, fermentation time, fermentation temperature and inoculum percent, respectively. The analysis of variance (ANOVA) was conducted to test the significance of the fit of the second-order polynomial equation for the experimental data as shown in Table 3. The Model F-value of 71537.49 implies the model is significant. There is only a 0.01% chance that a "Model F-value" could occur due to noise. Model P-value in this study was <0.0001 which also indicates that the model was significant. The coefficient of variation (R^2= 0.995) indicates a good agreement between experimental and predicted values, which implies that the model was reliable for α-amylase production and statistically sound. The response surface plots and the contour plots are shown in Figure 1. Shapes of response surfaces and contour plots indicate the nature and extent of the interaction between different factors (Prakash et al. 2008). Circular contour plots generally indicate a less prominent or negligible interactions, while elliptical contour plots indicates comparatively prominent interactions. Ferreira et al. (2009) showed that the maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram. Previous studies also reported that elliptical contours are obtained when there is a perfect interaction between the independent variables (Li et al. 2007; Xiao et al. 2007). On the basis of medium optimization, the quadratic model predicted that the maximum production of α-amylase was 2819.24 U/g when the substrate amount, initial moisture, fermentation time, fermentation temperature and amount of inoculum were 5 g, 70%, 94 h, 28 °C and 20%, respectively.

Validation of the optimized condition

To verify the predicted result, validation experiment was performed in triplicate tests. Under the optimized condition, the observed experimental titer of α-amylase was 2810.33 U/g, suggesting that experimental and predicted values of α-amylase yield were in good agreement. This result therefore corroborated the predicted values and the effectiveness of the model, indicating that the optimized medium favors the production of α-amylase through solid state fermentation.

Purification of P. notatum NCIM 923 α-amylase

The enzyme was successfully purified to homogeneity through

Table 4. Stepwise purification of α-amylase from solid state fermentation of *P. notatum* NCIM 923.

Purification steps	Total activity U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude enzyme	14206	248	57.28	1	100
Acetone precipitation	11638	182	63.95	1.12	81.92
DEAE-cellulose	7820	39.8	196.48	3.43	55.05
Sephadex G-100	1314	2.9	453.10	7.91	9.2

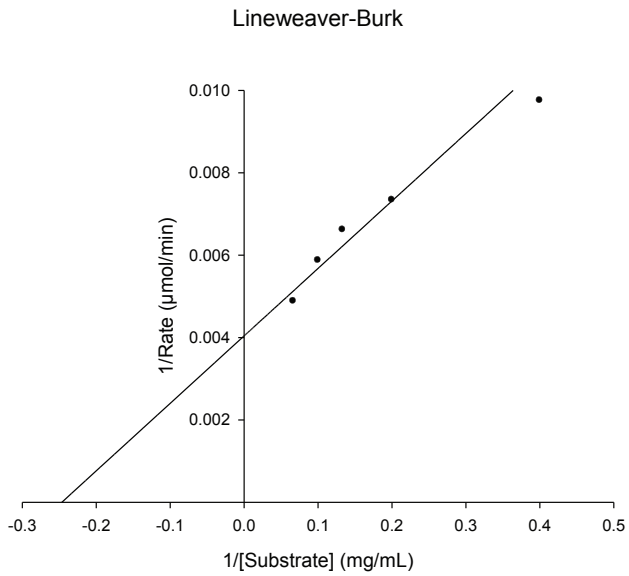

Figure 3. Lineweaver-Burk plot determination of K_m and V_{max} of purified α-amylase from *P. notatum* NCIM 923.

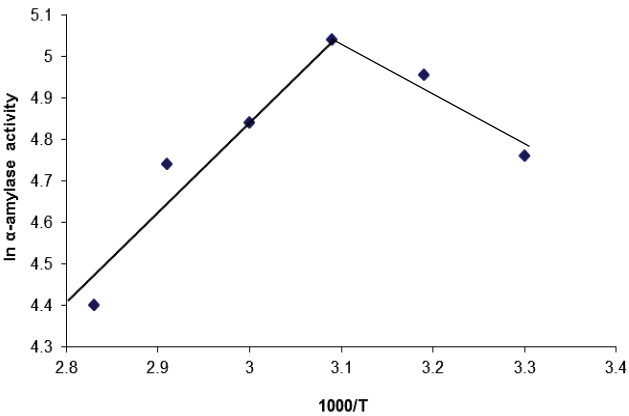

Figure 5. Arrhenius plot for the effect of temperature on the of the purified α-amylase activity. Activation energy (Ea) for the substrate hydrolysis was calculated from the slope.

three steps: acetone precipitation, ion exchange chromatography through DEAE cellulose column and size exclusion chromatography through Sephadex G-100 column (Table 4). The purification process allowed an increase of 7.91 fold in the specific activity with a yield of about 9.2%. The purified α-amylase appeared as a single protein band with molecular weight about 52 kDa on SDS-PAGE (Fig. 2) which was further confirmed by zymogram analysis (Fig. 2). The molecular mass of the purified α-amylase was different from

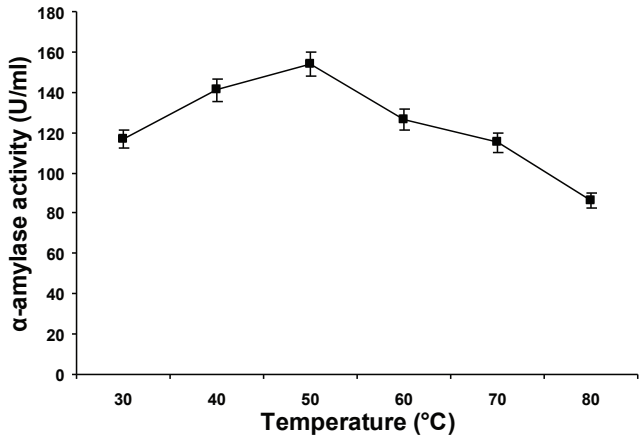

Figure 4. Optimum temperature for α-amylase activity purified from *P. notatum* NCIM 923. Data presented are average value +/- SD of n= 3 experiments.

other enzymes reported for *Penicillium* fungi (Nouadri et al. 2010; Sindhu et al. 2011).

Enzyme kinetic studies

The estimated K_m value for starch was 4.1 mg/ml and the maximum reaction velocity (V_{max}) was 247.6 µmol/min (Fig. 3). Other fungal and yeast α-amylases also have their K_m between 0.13-5 mg/ml (Gupta et al. 2003; Pandey et al. 2000). Additionally, the observed V_{max} was higher than those reported for other fungal strains on starch substrate (Petrova et al. 2000; Nouadri et al. 2010). Results indicate that the α-amylase from *P. notatum* NCIM 923 can be a good candidate for the bio-saccharification of starchy materials during industrial applications (Negi and Banerjee 2009).

Temperature optimum and stability

Optimum temperature of amylase was determined by varying the reaction temperature from 30 to 80 °C. The enzyme exhibited its highest activity (154 U/ml) at 50 °C, which was similar to that reported for *P. janthinellum* (NCIM 4960) (Sindhu et al. 2011). The purified α-amylase preserved 82-74.7% of its initial activity at 60-70 °C (Fig. 4). The activation energy (E_a) of α-amylase for starch hydrolysis was 14.133 kJ/mol. Biphasic pattern of the plot (Fig. 5) also indicated that the enzyme faces a lower energy barrier while hydrolyzing its substrate. The activation energy gave rise to another important parameter, the temperature coefficient, Q_{10}. The Q_{10} value at 50 °C was found to be 1.0. The Q_{10} for enzymes generally ranges between 1 and 2 (Singh and Chhatpar 2011). A lower

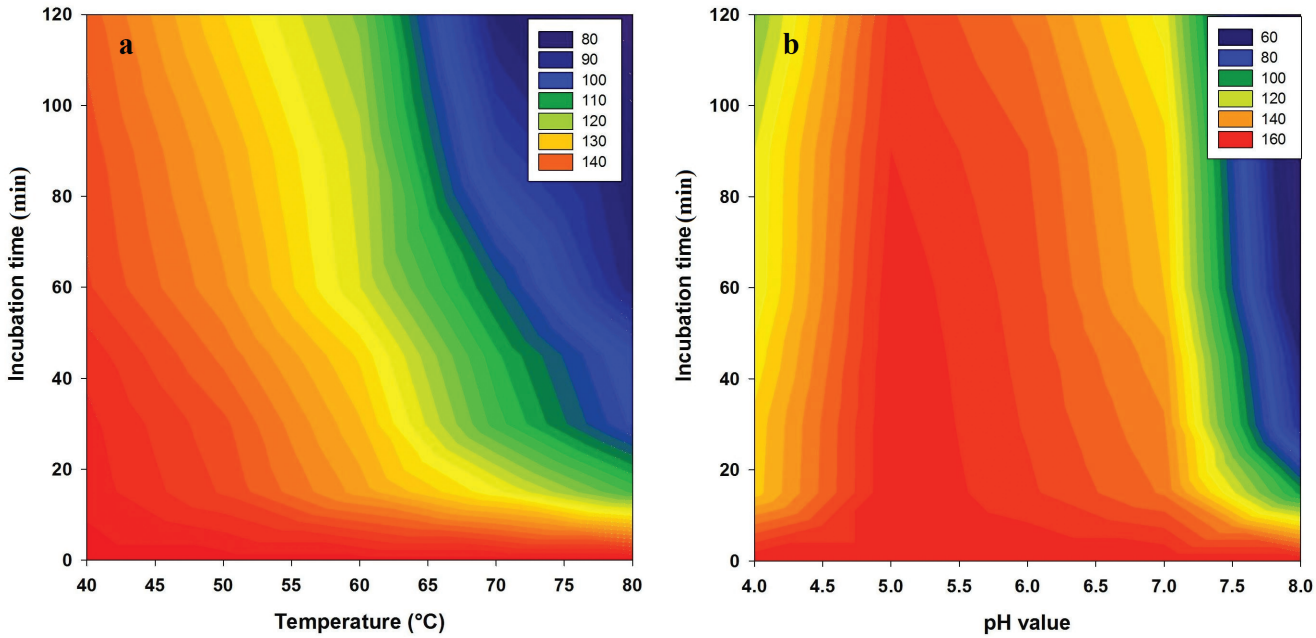

Figure 6. Thermostability (a) and pH stability (b) of α-amylase purified from *P. notatum* NCIM 923. Activity values (U/ml) are depicted with different colors (80-140 U/ml and 60-160 U/ml for temperature and pH, respectively).

Q_{10} for the α-amylase from *P. notatum* NCIM 923 reflected that the change in temperature would not have significant effect on the tertiary protein structure of the enzyme at up to 50 °C. The study of the thermostability reveals that after 2 h of incubation enzyme was highly stable up to 65 °C, above this temperature activity was partially lost (Fig. 6a). $T_{1/2}$ of the enzyme was 110 min at 80 °C which indicates that the purified α-amylase was highly thermostable.

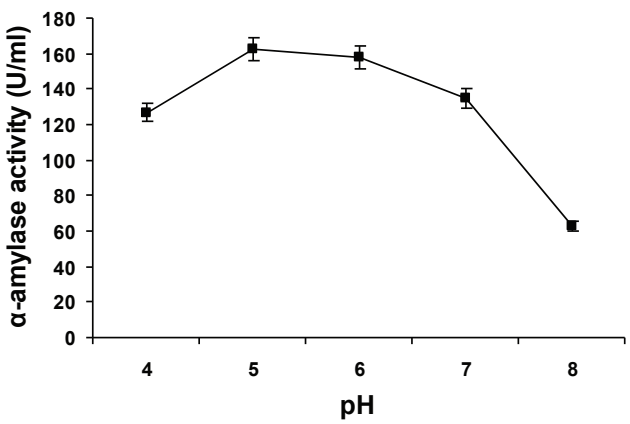

Figure 7. Optimum pH for α-amylase activity purified from *P. notatum* NCIM 923.

Effect of pH on enzyme activity and stability

The results in Figure 7 showed that the enzyme was mostly active between pH 5.0-7.0 with a maximum enzyme activity of 162.1 U/ml at pH 5.0. This range is similar to those of the most microbial α-amylases (De Silva et al. 2009). The pH stability of the purified α-amylase was also examined. It was found that the enzyme retained about 95-75% of its original activity in the pH range from 5.0 to pH 7.0 after 2 h incubation (Fig. 6b). Additionally, the enzyme was more tolerant to 50 °C under acidic pH, which is significant for its potential industrial application (Pandey et al. 2000).

Effect of metal ions and EDTA on α-amylase activity

The enzyme activity of α-amylase from *P. notatum* NCIM 923 was strongly enhanced by Ca^{2+}, Mn^{2+}, Co^{2+} and Fe^{3+} (Table 5). However, the enzyme was slightly stimulated by NH_4^+. The purified α-amylase was poorly affected by the other metal ions tested but partially inhibited by Hg^{2+}. α-Amylases generally contain at least one activating Ca^{2+} ion and the affinity of Ca^{2+} to the enzyme is much stronger than that of other ions (Gupta et al. 2003). Enhancement of amylase activity in the presence of Ca^{2+}, Mn^{2+}, Co^{2+} and Fe^{3+} ions could be based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acid (Linden et al. 2003). This could stabilize the enzyme retaining its tertiary

Table 5. Effect of metal ions and EDTA on purified α-amylase activity. Relative activities were presented with respect to control (100%) where experiment was performed without metal ions or EDTA.

Metal salts and reagent (1 mM)	Relative activity (%)
NaCl	95
KCl	91
NH_4Cl	101
$CoCl_2$	115
$MnCl_2$	118
$MgCl_2$	96
$HgCl_2$	65
$CuCl_2$	97
$ZnCl_2$	79
$CaCl_2$	127
$FeCl_3$	108
EDTA	72

structure. In previous reports, Ag^+, Hg^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Fe^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+} inhibited the activity of amylases (Sun et al. 2010; Pandey et al. 2000). The effect of mercuric ions indicates that indole amino acid residues may have important role in the enzymatic function (Ramirez-Zavala et al. 2004). EDTA inhibited the enzyme activity indicating that the purified enzyme is a metalloenzyme (Sun et al. 2010).

Conclusion

The Box-Behnken response surface methodology proved to be effective for optimizing the α-amylase production of *P. notatum* NCIM 923 in solid-state cultures, and the results are a good basis for the further large-scale fermentation studies. The low activation energy, and its pH and thermotolerance make the *P. notatum* NCIM 923 α-amylase a good candidate for the industry.

Acknowledgments

This work was financially supported by University Grant Commission.

References

Abu EA, Ado SA, James DB (2005) Raw starch degrading amylase production by mixed culture of *Aspergillus niger* and *Saccharomyces cerevisiae* grown on *Sorghum* pomace. Afr J Biotechnol 4:785-790.

Adeniran AH, Abiose SH (2009) Amylolytic potentiality of fungi isolated from some Nigerian agricultural wastes. Afr J Biotechnol 8:667-672.

Anto H, Trivedi U, Patel K (2006) Alpha amylase production by *Bacillus cereus* MTCC 1305 using solid-state fermentation. Food Technol Biotechnol 44:241-245.

Baysal Z, Uyar F, Aytekin C (2003) Solid-state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water. Proc. Biochem 38:1665-1668.

Box GEP, Behnken DW (1960) Some new three level designs for the study of quantitative variables. Technometrics 2:455-475.

Boyaci IH (2005) A new approach of determination of enzyme kinetic constants using response surface methodology. Biochem Eng J 25:55-62.

Das A, Ghosh, U, Das Mohapatra PK, Pat BR, Mondal KC (2012) Study on thermodynamics and adsorption kinetics of purified endoglucanase (Cmcase) From *Penicillium notatum* NCIM No-923 produced under mixed solid-state fermentation of waste cabbage and bagasse. Braz J Microbiol 43:1103-1111.

Das A, Paul T, Halder SK, Jana, A, Maity C, Das Mohapatra PK, Pati BR, Mondal, KC (2013a) Production of cellulolytic enzymes by *Aspergillus fumigatus* ABK9 in wheat bran-rice straw mixed substrate and use of cocktail enzymes for deinking of waste office paper pulp. Bioresour Technol 128:290-296.

Das A, Paul T, Jana A, Halder SK, Ghosh K, Maity C, Das Mohapatra PK, Pati BR, Mondal KC (2013b) Bioconversion of rice straw to sugar using multizyme complex of fungal origin and subsequent production of bioethanol by mixed fermentation of *Saccharomyces cerevisiae* MTCC 173 and *Zymomonas mobilis* MTCC 2428. Ind Crops Prod 46:217-225.

Dixon M, Webb EC (1979) Enzyme kinetics. In Enzymes, 3rd ed., Academic Press, New York, 47-206.

Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P, Srinivasulu B (2002) Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus* species. Proc Biochem 38:615-620.

Ferreira S, Duarte AP, Ribeiro MHL, Queiroz JA, Domingues FC (2009) Response surface optimization of enzymatic hydrolysis of *Cistus ladanifer* and *Cytisus striatus* for bioethanol production. Biochem Eng J 45:192-200.

Griffin H, Dintzis FR, Krull L, Baker FL (1984) A microfibril generating factor from the enzyme complex of *Trichoderma reesei*. Biotechnol Bioeng 26:269-300.

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial α amylases: a biotechnological perspective. Proc Biochem 38:1599-1616.

Kumar A, Duhan JS (2011) Production and characteriza-

tion of amylase enzyme isolated from *Aspergillus niger* MTCC-104 employing solid state fermentation. Int J Pharma Bio Sci 2:250-258.

Li J, Ma C, Ma Y, Li Y, Zhou W, Xu P (2007) Medium optimization by combination of response surface methodology and desirability function: an application in glutamine production. Appl Microbiol Biotechnol 74:563-571.

Linden A, Mayans O, Meyer-Klaucke W, Antranikian G, Wilmanns M (2003) Differential regulation of a hyperthermophilic amylase with a novel (Ca, Zn) two-metal center by zinc. J Biol Chem 278:9875-9884.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurements with the Folin phenol reagent. J Biol Chem 193:265-272.

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31:426-428.

Negi S, Banerjee R (2009) Characterization of amylase and protease produced by *Aspergillus awamori* in a single bioreactor. Food Res Int 42:443-448.

Nouadri T, Meraihi Z, Shahrazed DD, Leila B (2010) Purification and characterization of the α-amylase isolated from *Penicillium camemberti* PL21. Afr J Biochem Res 4:155-162.

Oboh G (2005) Isolation and characterization of amylase from fermented cassava (*Manihot esculenta* Crantz) wastewater. Afr J Biotechnol 4:1117-1123.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R (2000) Advances in microbial amylases. Biotechnol Appl Biochem 31:135-152.

Petrova SD, Ilieva SZ, Bakalova NG, Atev AP, Bhat MK, Kolev DN (2000) Production and characterization of extracellular α-amylases from the thermophilic fungus *Thermomyces lanuginosus* (wild and mutant strains). Biotechnol Lett 22:1619-1624.

Prakash O, Talat M, Hasan SH, Pandey RK (2008) Factorial design for the optimization of enzymatic detection of cadmium in aqueous solution using immobilized urease from vegetable waste. Bioresour Technol 99:7565-7572.

Ramirez-Zavala B, Mercado-Flores Y, Hernadez-Rodriguez C, Villa-Tanaca L (2004) Purification and characterization of lysine aminopeptidase from *Kluyveromyces marxiamus*. FEMS Microbiol Lett 235:369-375.

Sindhu R, Suprabha GN, Shashidhar S (2011) Purification and characterization of α-amylase from *Penicillium janthinellum* (NCIM 4960) and its application in detergent industry. Biotechnol Bioinf Bioeng 1:25-32.

Singh AK, Chhatpar HS (2011) Purification and characterization of chitinase from *Paenibacillus* sp. D1. Appl Biochem Biotechnol 164:77-88.

Sun H, Zhao P, Ge X, Xia Y, Hao Z, Liu J, Peng M (2010) Recent advances in microbial raw starch degrading enzymes. Appl Biochem Biotechnol 160:988-1003.

Xiao ZJ, Liu PH, Qin JY, Xu P (2007) Statistical optimization of medium components for enhanced acetoin production from molasses and soybean meal hydrolysate. Appl Microbiol Biotechnol 74:61-68.

Xiong C, Shouwen C, Ming S, Ziniu Y (2005) Medium optimization by response surface methodology for poly-Y glutamic acid production using dairy manure as the basis of a solid substrate. Appl Microbiol Biotechnol 69:390-396.