Malaysian Journal of Microbiology, Vol 14(3) 2018, pp. 215-228

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Production and application of α-amylase from indigenous fungal strain *Aspergillus luchuensis* bs1

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Received 23 May 2017; Received in revised form 27 July 2017; Accepted 8 August 2017

ABSTRACT

Aim: The study aimed at isolation, screening, optimization and partial purification of α-amylase and evaluating its desizing efficiency in textile industry.
Methodology and results: The AF01 showed the highest α-amylase activity of 128 KU. This isolate was identified as *Aspergillus luchuensis* strain bs1 using 18S rRNA gene sequencing. The process parameters were screened by employing Plackett-Burman Design (PBD) with seven variables and followed by Box-Behnken Design with three positively influencing factors. The investigation revealed that the maximum α-amylase production (192KU) at medium pH 5.6, starch 3% (w/v) and sodium nitrate 0.5% (w/v). The partial purification of α-amylase was done by acetone precipitation and it resulted in 6.1 fold purification. Partially purified α-amylase recorded optimum activity at pH 5.5, 60 min of contact time, temperature stability at 60 °C and 93% specificity to potato starch. The desized cotton fabric showed 9.5% weight loss, 5 sec of absorbency time and 8 rating in Tegewa analysis.
Conclusion, significance and impact of study: The study proposes a novel indigenous fungal strain having ability to produce alpha amylase and an enzyme preparation for desizing sized cotton fabric in minimal concentration.

Keywords: α-Amylase, *Aspergillus luchuensis*, Plackett-Burman design, Box Behnken Design, desizing

INTRODUCTION

Starch hydrolyzing enzymes such as amylase have attained a great deal of attention because of their usage in many industries and economic benefits. α-Amylases are extracellular endo-1-4-α-D-glucan glucohydrolase (EC 3.2.1.1). They catalyze the hydrolysis of internal 1, 4-α-D-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. The α-amylase family can be grouped by their mechanisms as the starch hydrolyzing enzymes and the starch modifying, or trans-glycosylating enzymes. The α-amylase is a primary metabolite produced by microorganism for their growth and its production is growth related process (Sudo *et al.*, 1994; Spohr *et al.*, 1998). α-Amylase have been reported from varies sources including bacterial and fungal. *Bacillus spp*. are widely used for thermostable α-amylase production to meet industrial needs. However, fungal α-amylases preferred due to its cost effectiveness, consistency, ease of process modification and optimization under industrial production (Gupta *et al.*, 2003). In comparison, filamentous fungi have been widely used for the commercial production of amylases, it includes *Aspergillus* and *Rhizopus* species because of the thermostablity and excessive quantities of enzyme production (Gupta *et al.*, 2008; Khan and Yadav, 2011; Kim *et al.*, 2011; Irfan *et al.*, 2012).

α-Amylases are used for variety of industrial applications including the conversion of starch to sugar syrups (Tonkova, 2006; Prakash and Jaiswal, 2009), production of cyclodextrins for the pharmaceutical industry (Pedersen *et al.*, 1995; Thompson, 1997), simultaneous saccharification (liquefaction) in bioethanol production (Shigechi *et al.*, 2004; Oner, 2006; Chi *et al.*, 2009), desizing in texitle industry (Gupta *et al.*, 2003; Feitkenhauer, 2003; Ahlawat *et al.*, 2009), food and brewing industry (Gavrilescu and Chisti, 2005; Couto and Sanroman, 2006; Ghorai *et al.*, 2009). Due to the great significance of applications amylases occupies about 25% world market demand and to meet this situation about 30 % of the world's enzyme production was targeted (Ali *et al.*, 2014: Cripwell *et al.*, 2015; Prajapati *et al.*, 2015).

The present study was designed to screen and isolate a potential α-amylase fungal producer from the soil samples. To optimize the process parameters for the maximum production of enzyme, a systematic statistical analysis was outlined to identify the significant factors and their interactions in development of fermentation process.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

The most influencing physiochemical factor predicted by performing Placket Burman Design and the response surface methodology was performed by employing Box – Behnken design. An attempt was also made to partially purify and characterize enzyme. Partially purified α-amylase was tested for desizing the cotton fabric that is used in textile industry.

MATERIALS AND METHODS

Sources of strains and media

Soil samples were collected in sterile plastic bags from different locations in the state of Tamil Nadu, India. The collected soil samples were added individually to sterile distilled water and 1% (w/v) were serially diluted in Phosphate Buffer Saline, spread plated on starch agar plate and incubated at 28±2 °C. After 3 days of incubation, the plates were flooded with potassium iodine solution (1% (w/v) iodine; 2% (w/v) potassium iodide) and plates were observed for amylolytic activity. Among morphologically different fungal isolates, 6 fungal strains (AF1 to AF6) were selected based on the prominent halo zone around their growth. Fungal isolates AF1 to AF6 were assayed quantitatively to determine actual amylase activities under submerged fermentation conditions using soluble starch as substrate. All the chemicals and reagents were used of analytical grade and were obtained from Hi-media, Mumbai, India.

The Czapek Dox media composition was modified as Soluble Starch (3%), Yeast Extract (0.15%), Peptone (0.15%), Sodium nitrate (0.25%), Potassium Dihydrogen Phosphate (0.2%), Di-potassium hydrogen phosphate (0.2%), 0.005% of the trace elements (Calcium chloride, Potassium chloride, Magnesium sulphate and Ferrous Sulphate). pH of the medium was adjusted to 5.6 and the flasks were autoclaved. The sterilized flasks were inoculated with a log phase fungal mycelia and the flasks were kept on an orbital shaker at 150 rpm and 28°C. After 3 days, the fermented broth was filtered and filtrate was centrifuged at 10000rpm and supernatant was collected. The supernatant thus obtained was used as crude source of enzyme and α-amylase activity was determined using 1% soluble starch. Reducing sugar liberated was estimated by DNSA method. Among 6 fungal isolates, AF01 showed maximum α-amylase activity and was used for the medium optimization studies.

Molecular characterization and Identification of fungal isolate

The molecular characterization of potent fungal isolate AF01 was performed to reveal the phylogenetic relationship with existing fungal species. The genomic DNA was isolated and PCR amplified with the 18S rRNA primers ITS1 and ITS4 as described by Bruns *et al.*, (1992) and Gargas and Taylor, (1992) The amplicon of size 700 bp DNA fragment was eluted from gel and sequencing was done by employing Sangar's method. The sequence obtained was subjected to sequence comparison with NCBI database. A non-redundant BLAST search was performed to identify the closest relatives. Phylogenetic tree was constructed using MOLE BLAST adopting neighbor-joining method. Pairwise evolutionary distances were calculated using the method of Jukes and Cantor, (1969).

Table 1: Plakett Burman Design Table for the seven factors.

Run No.	pH	Starch (% w/v)	Yeast extract (% w/v)	Peptone (% w/v)	$NaNO_3$ (%w/v)	K_2HPO_4 (% w/v)	KH_2PO_4 (% w/v)	α-amylase activity (KU/mL)	
								Observed	Predicted
1	5.5	3	0.1	0.2	0.5	0.1	0.3	1.08	1.07
2	4	3	0.2	0.1	0.5	0.1	0.1	0.74	0.64
3	5.5	1	0.2	0.2	0.25	0.2	0.1	1.79	1.68
4	4	3	0.1	0.1	0.25	0.2	0.3	0.95	1.04
5	4	1	0.1	0.2	0.5	0.2	0.1	0.98	1.14
6	4	1	0.2	0.2	0.5	0.1	0.3	1.5	1.25
7	5.5	3	0.1	0.2	0.25	0.1	0.1	1.49	1.18
8	5.5	1	0.2	0.1	0.25	0.1	0.3	0.91	0.98
9	5.5	3	0.2	0.1	0.5	0.2	0.1	1.21	1.18
10	4	1	0.1	0.1	0.25	0.1	0.1	1.47	1.49
11	5.5	1	0.1	0.1	0.5	0.2	0.3	1.06	1.25
12	4	3	0.2	0.2	0.25	0.2	0.3	0.88	0.98

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Enzyme assay

α-Amylaseactivity was determined by the addition of 50µL of crude enzyme to 1 mL of 1% (w/v) soluble starch made in 0.1 M acetate buffer of pH 5.6 at 40 °C for 15 min. The reducing sugar released were measured by 3, 5-dinitrosalicyclic acid method (Miller, 1959). A separate blank was set for each sample to correct non-enzymatic release of sugars.
One unit of α-amylase was defined as the amount of enzyme that released reducing sugars equivalent to 1 µmol glucose per minute under standard assay conditions.

Estimation of protein by Lowry's method

The total protein concentration in the enzyme extract was determined at 660 nm using BSA as standard and reported as mg/mL as described by Lowry *et al*. (1951).

Experimental design and statistical analysis

A classical analysis, One Factor at a Time (OFAT) was employed to short out the positively influencing physiochemical parameters for maximum production of α-amylase enzyme. The process parameters considered for OFAT includes incubation time (in days), temperature (°C), pH, concentration of (% w/v) substrate, sodium nitrate, yeast extract, peptone, potassium dihydrogen phosphate and dipotassium hydrogen phosphate. From the OFAT analysis the promising 7 positive factors influencing amylase production were chosen to evaluate their effect by outlining Plackett-Burman Design (PBD). Plackett and Burman, (1946) design (N=12) [12 experiments, 7 factors] was investigated (Viswanathan and Surlikar, 2001; Francis *et al.*, 2003; Djekrif-Dakhmouche *et al.*, 2006). This fractional factorial design plan allows the investigation of up to N-1 variables with N experiments (N is multiple of 4 and less or equal to 100). Using Design expert software, the design was employed. Table 1 shows the PBD design outline. The amylase activity was considered as characteristic response of PBD. Analysis of variance (F test) was performed to determine the most significant factors influencing amylase production.

The Box-Behnken Design composite, with three replicates at the central points was employed to fit the quadratic model and to obtain experimental error. The design has limited capability for orthogonal blocking compared to the central composite design (Dey *et al.*, 2001; Vohra and Satyanarayana, 2002; Krishna Prasad *et al.*, 2005). The factors concluded by PBD were the medium pH, concentration of substrate and concentration of sodium nitrate and these parameters were analyzed in BBD for their interactions. The factors set in this statistical experiment are shown in Table 2. The central values chosen for the BBD are as follows: the pH 5.5; the substrate 3% (w/v) and the sodium nitrate concentration 0.5% (w/v). The substrate used was soluble starch. Amylase activity was considered as the dependent variable. The response variable was fitted to the second order model in the form of quadratic polynomial equation.

Table 2: Box-Behken Design plan for three variables.

Run No.	Factor 1 A:pH	Factor 2 B:Starch %(w/v)	Factor 3 C:Sodium nitrate %(w/v)	α-amylase activity (KU/mL)	
				Observed	Predicted
1	7	3	0.3	82.9	81.34
2	5.5	3	0.5	191.9	192.6
3	5.5	3	0.5	191.8	190.04
4	5.5	3	0.5	192.7	193.28
5	4	3	0.3	58.7	57.86
6	4	1	0.5	67.2	69.23
7	4	3	0.7	61.8	60.9
8	5.5	1	0.3	58.9	58.4
9	7	5	0.5	79.7	81.45
10	5.5	3	0.5	191.4	193.2
11	7	1	0.5	80.5	78.4
12	7	3	0.7	50.7	53.1
13	5.5	5	0.7	52.2	51.9
14	5.5	3	0.5	192.5	193.3
15	4	5	0.5	62.5	61.9
16	5.5	5	0.3	72.1	70.9
17	5.5	1	0.7	64.6	63.9

Partial purification of enzyme

The *Aspergillus luchuensis* was cultured under the optimum conditions for submerged fermentation in a standardized media for 3 days. The culture was filtered through filter paper to remove the biomass. The filtrate thus obtained was centrifuged at 10,000 rpm for 15 min at 4 °C. Enzyme in the supernatant was precipitated by the addition of ice cold acetone in 1:1.5 (v/v) with mild stirring at 4 °C and the solution was kept for overnight. The precipitates formed were centrifuged at 10000 rpm for 30 min at 4 °C. The precipitated was dissolved 0.1 M acetate buffer and centrifuged at 10,000 rpm for 20 min. The supernatant was estimated for total protein concentration and amylase activity.

Characterization of partially purified α- amylase

Effect of pH

One of the important criteria for the usage of an enzyme is its working pH. The effect of pH on partially purified α-amylase was done at different pH from 3.5 to 8.0. The buffers (acetate, phosphate and Tris-HCl) at 0.1M concentration were used. Enzyme activity was done for 15 min at 40 °C. The amylase activity was expressed as the relative activity.

Effect of temperature

The optimum temperature of purified α- amylase was determined by carrying out assays at different temperatures from 30 °C to 80 °C at its optimum pH. The

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

relative activity was expressed as percentage of the maximum α- amylase activity.

Effect of contact time

The effect of time on α- amylase activity was carried out for 15, 30, 45, 60, 75 and 90 min under optimized pH and temperature. The relative enzyme activity was calculated to express the purified amylase activity.

Substrate specificity

To determine the substrate specificity of the α-amylase, soluble starch, amylose, maltose, potato starch, rice starch and cassava starch were used as substrate at 1% for enzyme assay. The relative activity of amylase was expressed in percentage.

Application of α- amylase in fabric desizing

The sized cotton fabric was desized by performing conventional methods as per procedure given in literature (Shore 1995; Koushik and Josico 2003). The reaction mixture includes α-amylase at different concentration (0.3, 0.8, 1.3 and 1.8 % (v/v)), wetting agent 0.5 g/L and the fabric to water ratio was fixed at 1:30. The recipie was prepared and incubated at 60 °C for 1 h. The treated cotton fabric was analyzed for its weight loss percentage, Tegawa rating and absorbency after treatment. The weight loss percentage is add-on percentage of starch removed by the α- amylase. Absorbency of a fabric was analyzed by employing AATCC Test Method 79-2000. The sample (dimension 1×1cm) was excised and a drop of water (10 µL) was placed to fall from fixed height onto the test specimen surface. The time required for water drop to disappear in the fabric was measured and denoted as wetting time (Chehna *et al.*, 2007).

Tegewa rating was performed by employing iodine drop test to check the desizing efficiency. The principle for the same is the violet colouration of starch in the sized/desized fabric with iodine. A sample (desized fabric) dimension 2×2 cm was cut and placed a drop of potassium iodide and iodine solution for 1 min. The fabric was then rinsed under tap water, drained with filter paper and immediately compared with the Tegewa scale or Violet scale of 1–9 rating. (Wurster, 1987).

RESULTS AND DISCUSSION

Isolation of fungal strain

In the present study, several fungal isolates showing extracellular α-amylase activity were obtained from soil samples collected from 16 distinct locations in Tamil Nadu. The amylolytic properties of the selected 6 fungal strains were screened by performing submerged fermentation using starch as a carbon source. Initially potassium iodine staining was done using 3 days grown culture in starch plate. Among the isolates AF01 showed a maximum zone of clearance about 4 cm diameters (Figure 1C) and also the highest amylase activity of 128 KU in submerged fermentation. The microscopic views of conidiospore are represented in Figure 1A and single colony of AF01 was represented in Figure 1B. Conidia, globose, smooth, 3.5-4.5 mm, mahogany brown. The colony colour is white to gray, stipes have a width 8-30 mm and length up to 1.5 mm; conidial heads are predominantly biseriate, radiate and spherical, walls thick, smooth and hyaline. Vesicles are nearly spherical, 20-50 mm; metulae 5.0-26.1 mm; ampulliform phialides are 5.4-12.5 mm; conidia are 3.0-4.5 mm, smooth, finely rough, or rarely rough. Table 3 represents the total enzyme activity and protein concentration of fungal isolates AF01 to AF06. From the results, it is evident that maximum amylase activity (127 KU) and protein concentration (78 mg/mL) was recorded in isolate AFO1.

Table 3: α-amylase activity of different fungal isolates

Fungal isolates	Amylase activity (KU/mL)	Total Protein concentration (mg/mL)
AF01	127.81±0.002	78±0.42
AF02	59.72±0.023	87±0.23
AF03	41.60±0.004	67±0.14
AF04	85.29±0.006	73±0.23
AF05	19.42±0.009	78±0.33
AF06	36.28±0.005	66±0.21

Identification and molecular characterization

To analyze the phylogenetic position of AF01, the 18S rRNA sequence was determined and phylogenetic tree based on 604 base pairs was constructed (Figure 1D). Phylogenetic analysis showed that AF01 belong to the member of genus *Aspergillus*. Dendrogram analysis from the MOLEBLAST revealed that AF01 formed coherent clusters and closely related to the *Aspergillus luchuensis* with the 99% similarity between the 18S rRNA Internally Transcribed Spacer (ITS) sequences. The sequence was submitted in NCBI GenBank under the Accession number KY780508 as *Aspergilllus luchuensis* strain bs1.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

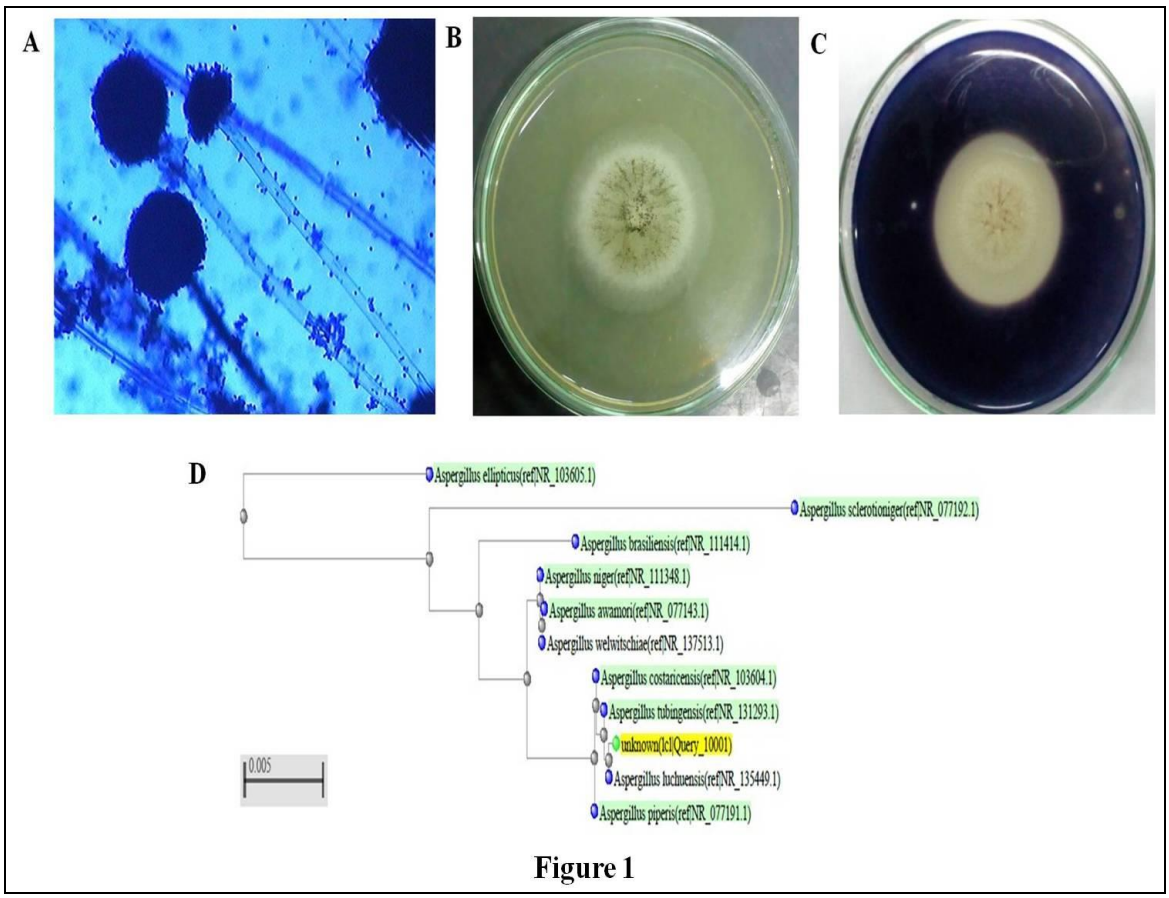

Figure 1: Microscopic view of conidiospore (A), single colony of AFO1 (B), Potassium Iodine staining of *Aspergillus luchuensis* strain bs1 showed 4 cm diameter zone of clearance (C) and Phylogenetic dendrogram based on 604 unambiguous base pairs of the 18s rRNA sequence indicating the position of *Aspergillus luchensis* strain bs1 and its closet relative sequences validated at species level in the genus *Aspergillus* (D).

Statistical analysis to optimize physiochemical factors

The study was initiated to screen the possible factors that influenced the production of α-amylase. From the one factor at time analysis (OFAT) out of nine factors, only seven factors were found to be most influencing parameters on the production of α-amylase by *A. luchensis* strain bs1. The incubation period of 3 days at 30 °C showed the maximum α-amylase production.The factors pH at 5.5, soluble starch (3%), sodium nitrate (0.5%), yeast extract (0.2%), peptone (0.15%), potassium dihydrogen phosphate (0.2%) and dipotassium hydrogen phosphate (0.3%) showed maximum enzyme production which was denoted as high value for PBD. The factors pH at 4, soluble starch (1%), sodium nitrate (0.25%), yeast extract (0.1%), peptone (0.1%), potassium dihydrogen phosphate (0.1%) and dipotassium hydrogen phosphate (0.1%) showed minimum enzyme production which was denoted as low value for PBD.The predicted high and low values of the significant factors were taken for the PBD analysis are represented in Table 1. The PBD experiment was conducted in 12 runs to study the selected variables. Table 1 also represents the observed and predicted values for PBD. Statistical analysis of response was performed which is represented in Table 4. The model F value 11.83 indicates that the model is significant. The Probability values < 0.005 indicated the model terms were significant. Regression analysis was performed on the results and a first order polynomial equation was derived representing α- amylase production as a function of the independent variables

α-amylase activity (KU/mL) = (3.12)+ (8.32)*starch + (0.43)*yeast extract +(3.77)*sodium nitrate + (0.17)*pH – (0.50)*K_2HPO_4+ (0.92)*KH_2PO_4 – (0.43)*peptone

The magnitude of the effects indicated the level of the significance of the variables on α-amylase production. Among the variables screened, starch, sodium nitrate and medium pH were identified as the most significant

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

variables influencing α-amylase production. Starch has been reported as a potential substrate for the production for α-amylase under SSF (Mulimani *et al.*, 2000; Ramachandran *et al.*, 2004; Gangadharan *et al.*, 2006). It is well documented that yeast extract and peptone are rich sources of carbon and nitrogen, thus supplementation of other nitrogen sources in the medium did not show significant rise in enzyme yield. The supplementation of metal ions such as $CaCl_2$ has been reported to provide good growth and also enhance enzyme production (Sivaramakrishnan *et al.*, 2006). The tested levels of K_2HPO_4, KH_2PO_4, peptone and yeast extract did not result in any significant variations onα-amylase production. Thus, the three variables such as initial pH, concentration of soluble starch and sodium nitrate were selected and optimal levels and interaction among these variables were determined by response surface methodology.

Table 4: Statistical analysis of PBD.

Source	Sum of square	Degree of freedom	Mean square	F value	Prob>F
Model value	4.71 E+005	8	1.008E +005	11.83	0.009
Starch	1261.01	1	1261.01	3.92	0.014
Sodium nitrate	633.41	1	633.41	3.77	0.003
Yeast extract	287.14	1	287.14	1.83	0.143
Peptone	1.41	1	1.41	1.11	0.335
KH_2PO_4	33.45	1	33.45	1.66	0.247
K_2HPO_2	2.52	1	2.52	1.22	0.156
pH	965.23	1	965.23	2.57	0.008
Residual	500.68	3			
Correlatio n	4.69 E+005				

CV-9.3; R^2-0.973

The Box-Behnken Design was employed to study the interactions among the significant factors and also determine their optimal levels. The constant level for other variables in the experiment was maintained to give maximal yield as determined from Plackett–Burman experiments. The BBD outline and its response (observed and predicted values) for the experiment are represented in Table 2.2. Multiple regression analysis was performed to analyse the data and thus a polynomial equation was derived from regression analysis as follows:

α-amylase activity (KU/mL) = 977.38 + 305.89A + 98.97 B + 1920 C – 26.2 A^2 – 15.2 B^2 – 1738 C^2 + 32.51AB + 16.23 BC – 28.4 AC

The magnitude of the effect indicates the level of significance on α- amylase production. The Fischer's statistical analysis was employed for performing the analysis of variance and the results were represented in Table 5. The Model F-value of 163.79 implies that the model is significant. There is only a 0.01% chance that a F-value this large could occur due to noise. Values of "Prob > F" less than 0.05 indicates that model terms are significant. In this case A, C, AC, BC, A^2, B^2 and C^2 are significant model terms.

Table 5: Analysis of variance of BBD.

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	Prob > F
Model	3.633E+ 005	9	40364.2 4	163.7 9	0.000 1
A-pH	1758.84	1	1758.84	7.14	0.031 9
B-Starch	20.90	1	20.90	0.085	0.779 3
C-Sodium nitrate	1734.90	1	1734.90	7.04	0.032 8
AB	27.04	1	27.04	0.11	0.750 2
AC	2327.10	1	2327.10	9.44	0.018 0
BC	1216.27	1	1216.27	4.94	0.061 7
A^2	91557.7 6	1	91557.7 6	371.5 3	0.000 1
B^2	97023.2 1	1	97023.2 1	393.7 1	0.000 1
C^2	1.304E+ 005	1	1.304E+ 005	529.2 1	0.000 1
Residual	1725.05	7	246.44		
Lack of Fit	515.80	3	171.93	0.57	0.664 6
Pure Error	1209.26	4	302.31		
Cor Total	3.650E+ 005	16			

CV-3.33; R^2-0.9988

The Predicted R-Squared of 0.9803 is in reasonable agreement with the Adjusted R-Squared of 0.9971 which implies that only 1.97% of the total variations are not explained by the model. A higher value f correlation coefficient (R^2=0.9988), justifies an excellent correlation between the independent variables (Khuri and Cornell, 1987). Adequacy Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The signal to noise ratio of 58.062 indicates an adequate signal. The coefficient of variation (CV) value 3.33 indicates the greater degree of precision and reliability of the experiments carried out (Khuri and Cornell,1987). The P value suggests that among the three values studied, the maximum interaction was observed between the medium pH and concentration of starch and relatively significant interaction between concentration of starch and sodium nitrate.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

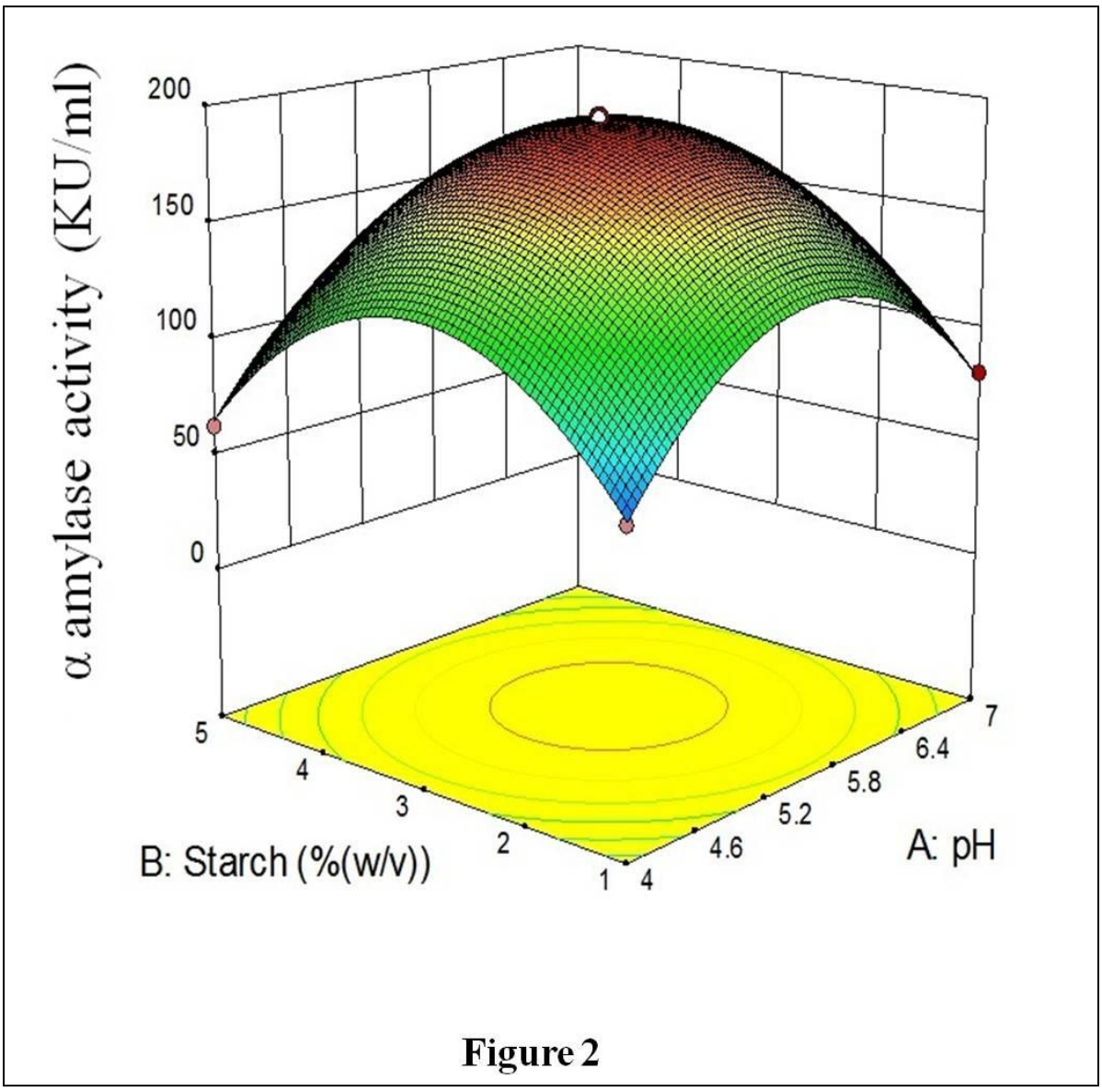

Figure 2: α-amylase production (KU/mL) observed as a response to the interaction of variables pH and concentration of starch (% w/v) and concentration of sodium nitrate (% w/v) at central point.

The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. The 3D response surfaces are the graphical representation of the regression equation of α-amylase yield by *A. luchensis*. The curves are represented in Figures 2-4. Figure 2 represents the interaction between medium pH and starch concentration. Lower and higher levels of both the pH and substrate concentration did not result in higher enzyme yield. The shape of the response surface curves showed a significant interaction between these tested variables. The response surface curve for the interaction of concentration of sodium nitrate and starch concentration is represented in Figure 3. The shape of the contour shows a positive interaction between the two variables. The α-amylase yield was found to increase with simultaneous increase with both the factors. This indicates the positive regulation of carbon source and nitrogen source influencing the growth of the fungi and production of α-amylase (Ghobadi *et al.*, 2017 and Li *et al.*, 2017). As observed from Figure 4, the pH and the concentration of sodium nitrate can significantly affect the α-amylase production. The 3D contour graph shows the highest α-amylase production at center point of both the factors.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

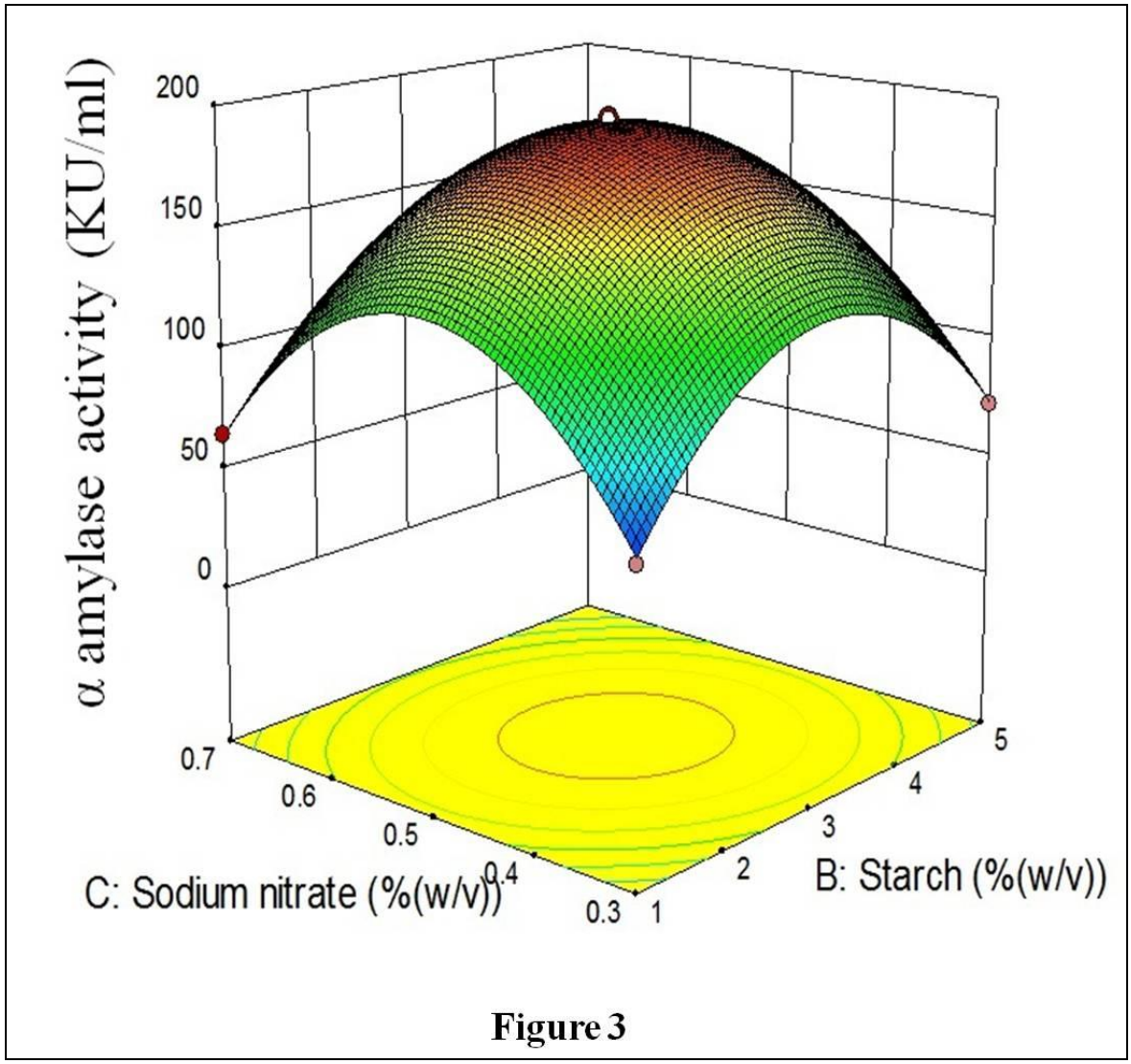

Figure 3: α-amylase production (KU/mL) observed as a response to the interaction of variables (% w/v) concentration of starch and sodium nitrate and medium pH at central point.

Validation of the model

The optimum level of the variables was found to be pH 5.6, % (w/v) 3 % starch, 0.5 % sodium nitrate, 0.25 % yeast extract, 0.3 % K_2HPO_4, 0.2 % KH_2PO_4 and 0.005 g/L of $CaCl_2$ and $MgSO_4$. The validation of model was carried out experimentally by performing fermentation for the production of α-amylase under the above mentioned conditions. The comparisons between the original and optimized media are represented in Table 6. The obtained α-amylase production yield (192 KU) is very close to the expected yield (195KU).

Partial purification of α- amylase

The partial purification of α- amylase was performed by acetone precipitation method. In the present study of partial purification process, the enzyme fold purification and yield were found to be higher with single step of purification. The specific activity of α-amylase was recorded as 15516.67 IU/mg with the purification fold of 6.1 and 12.1% yield. The investigated results were on par with results reported by Singh *et al.* (2014) by employing ammonium sulphate purification and higher than the results reported by Bhanja and Banerjee (2015) using acetone precipitation. The idea was to evaluate the enzyme for the textile industrial application so as to decrease the cost of textile processing.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

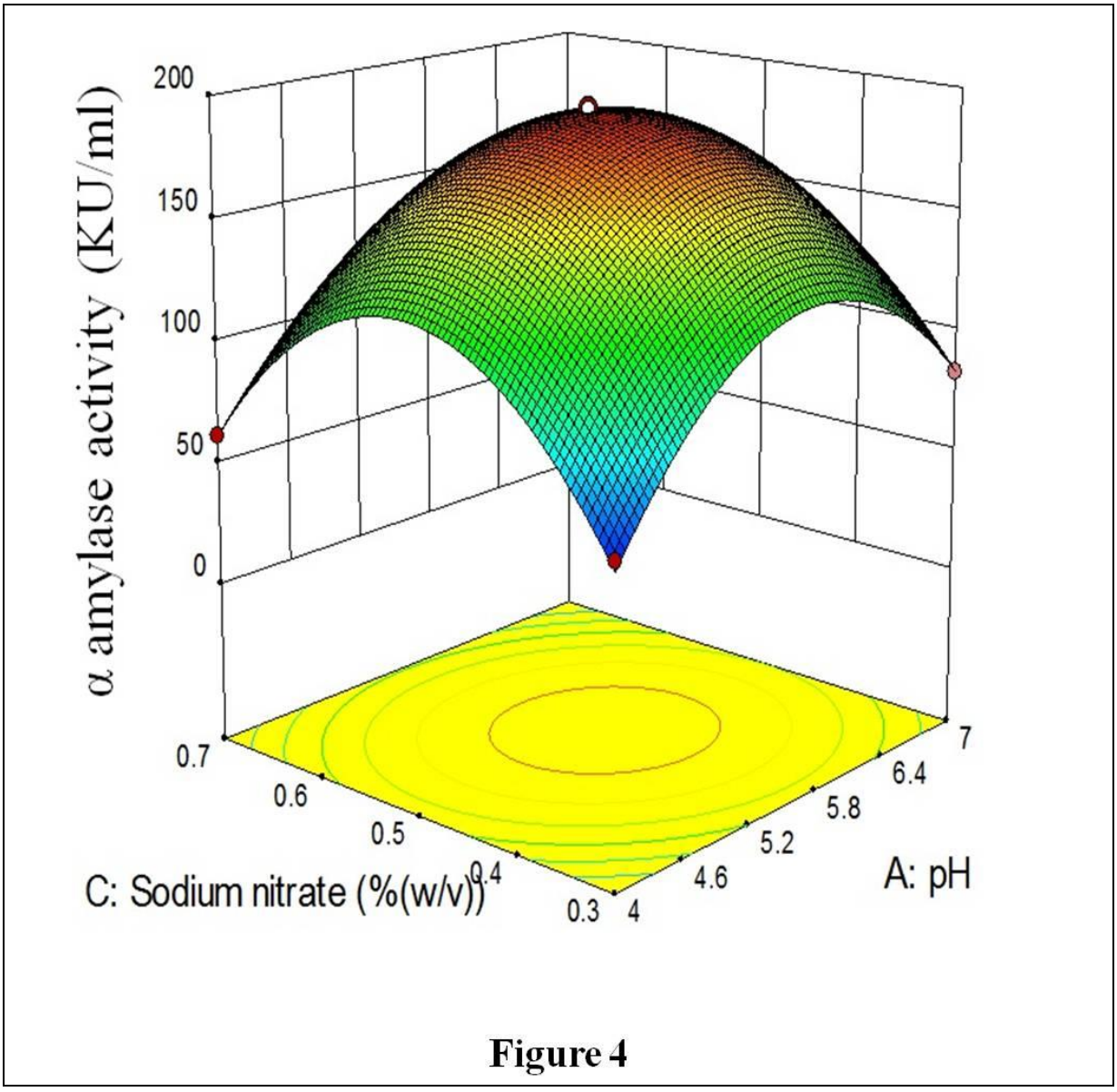

Figure 4: α-amylase production (KU/mL) observed as a response to the interaction of variables (% w/v) concentration of sodium nitrate and medium pH and concentration of starch at central point.

Characterization of partially purified α- amylase

The partially purified α-amylase from *A. luchuensis*was used characterization studies. The effect of pH, temperature, contact time and substrate specificity on α amylase were evaluated. The maximum amylase activity (192 KU) was obtained at pH 5.6 when incubated at 40 °C for 15 min (Figure 5A). Enzyme activity increases with an increase in pH up to 5.5 and decreased further. The results of optimum pH are in good agreement with the reports in the literature (Laderman *et al.*, 1993; Cangenella *et al.*, 1994 and Aguilar *et al.*, 2000)

The effect of the temperature on α amylase is shown in Figure 5B. It is clear from that α- amylase activity (228 KU) was at temperature 60 °C. Results obtained in the present investigation are on par with reports of Aguilar *et al.* (2000) Savchenko *et al.* (2002) and Bhanja and Banerjee (2015). The result implies the thermo tolerant nature of α-amylase, which promises potential role instead for conventional industrial chemical process.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

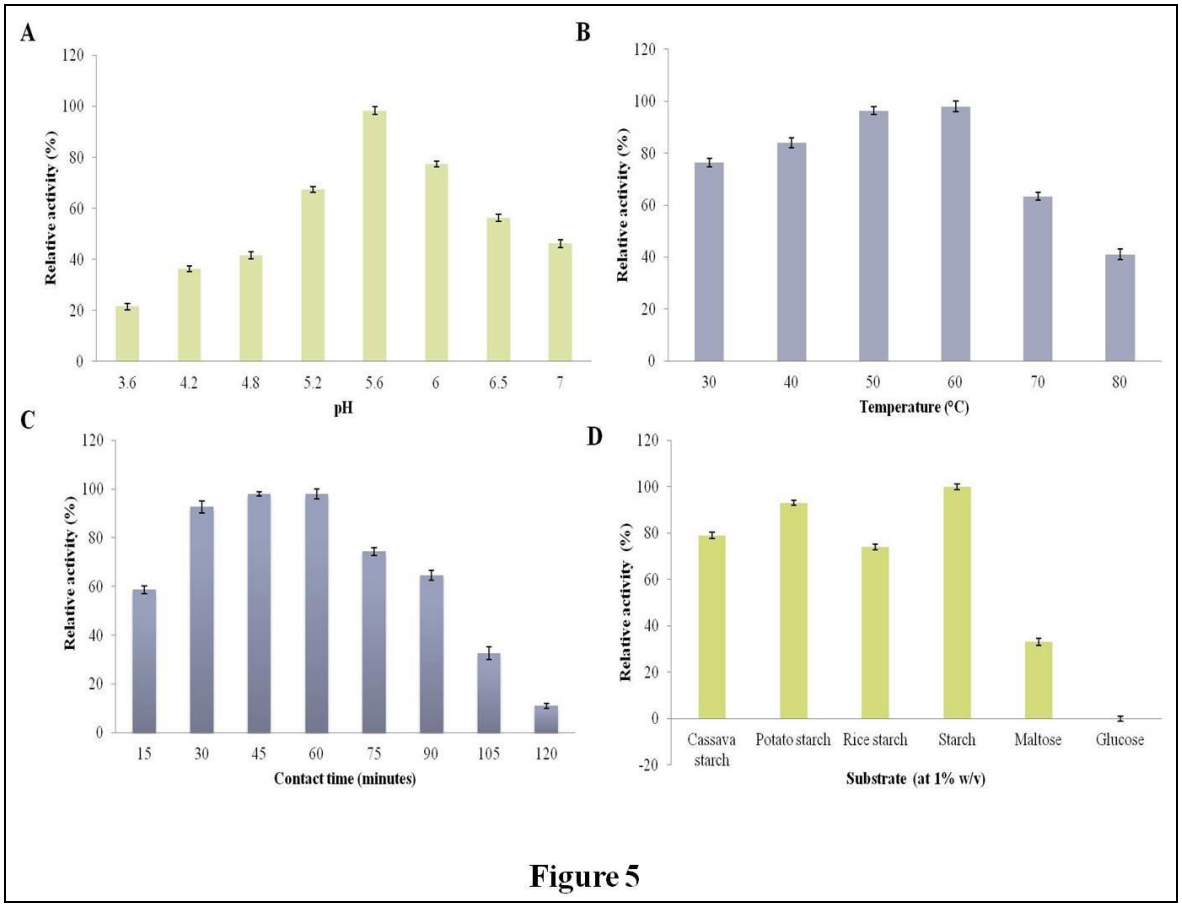

Figure 5: Effect of pH (A), temperature (B), contact time (C) on partially purified α-amylase. Substrate specificity of partially purified α-amylase (D).

Under the evaluation of contact time of amylase to the substrate results the maximum (325KU) activity attained for 60 min when incubated at optimum pH and temperature (Figure 5C). The results for the substrate specificity of α- amylase from *Aspergillus luchuensis* strain bs1 are summarized in Figure 5D. The partially purified α-amylase showed highest specificity to soluble starch (100%), followed by potato starch (93%), rice starch (73%) and maltose (33%). These results support the report that enzyme was more specific to a 1,4-glycosidic linkage cleaving (Sahnoun *et al.*, 2012; Karam *et al.*, 2017). Since the starch from different sources varies from their granular size, shape and amylose and amylopectin ratio and their structure (Bhanja and Banerjee, 2015; Karam *et al.*, 2017), the specific activities of enzyme varies.

Testing the efficiency of desizing by α-amylase

The process of desizing was experimented with triplicates and the treated fabric was analyzed for its desizing efficiency. The Figure 6A. represents the weight loss percentage of the cotton fabric upon desizing. The maximum weight loss of 9.5% was achieved at 1.8% (v/v) using purified amylase concentration respectively under process condition. Absorbency test data of treated fabric is depicted in Figure 6B. The wetting time required for the 1.8% (v/v) amylase treated fabric showed a minimum time of 5 sec, which was promising result considered as an adequate absorbency for the fabric (Chehna *et al.*, 2007). The grading of the treated fabric by Tegewa test is shown Figure 6B. The Tegewa rating of 9 for the 1.8% amylase treated fabric indicates complete removal of size from the fabric (Figure 6D) was higher than the report of Chand *et al.*, (2014). The commercially acceptable rating is 6-7 (Wurster, 1987) but present investigation shows a potential concentration of partially purified α-amylase (1.8%) for the desizing applications in textile industry.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

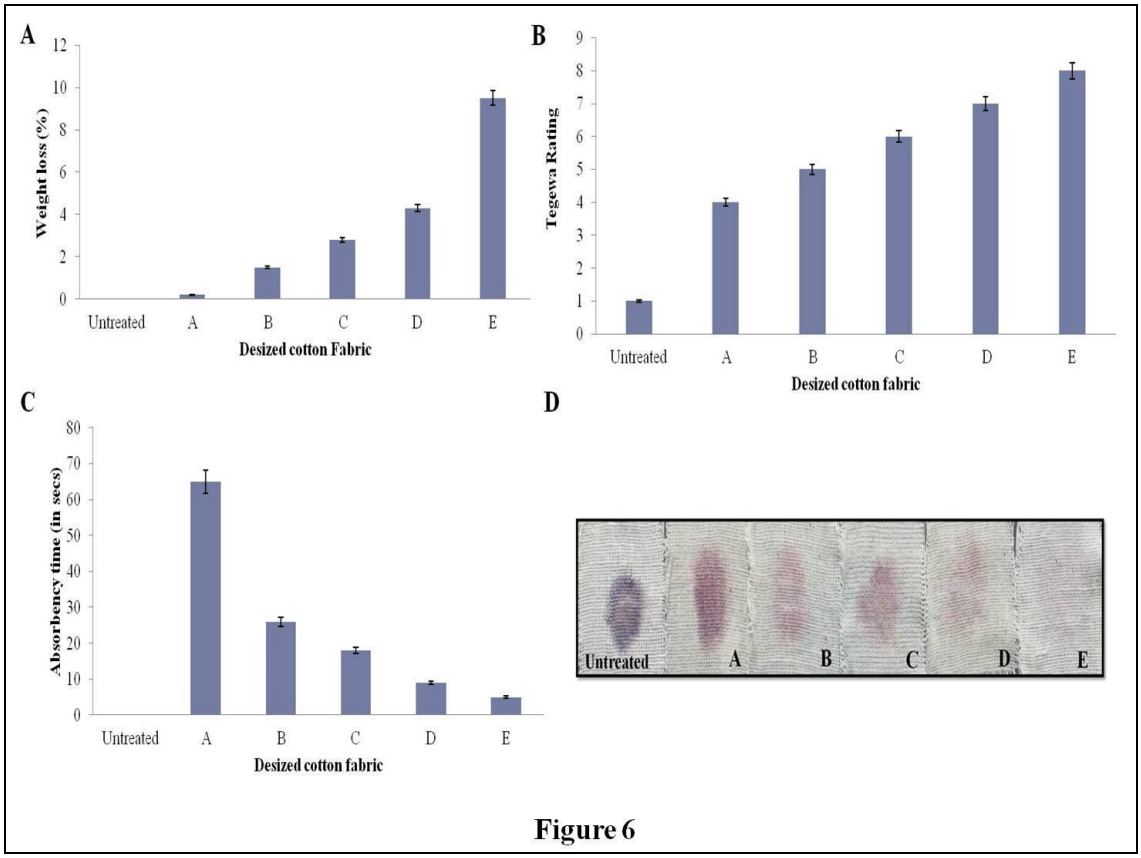

Figure 6

Figure 6: Weight loss percentage of desized cotton fabric (Figure 6A). Tegewa rating of desized cotton fabric (Figure 6B). Absorbency time in sec for desized cotton fabric (Figure 6C). Potassium iodine drop test of desized cotton fabric (Figure 6D). A represents control treatment without amylase and B, C, D and E denotes the treatment of fabric with α-amylase at different concentration 0.3, 0.8, 1.3 and 1.8% respectively.

Table 6: Comparison between the original and optimized media.

Media	Nutrient Name	Concentration %(w/v)	α-amylase activity KU/mL
Original	Starch	3	127.81±0.02
	Sodium nitrate	0.25	
	Yeast extract	0.15	
	Peptone	0.15	
	K_2HPO_4	0.2	
	KH_2PO_4	0.2	
Optimized	Starch	3	192.89±0.03
	Sodium nitrate	0.5	
	Yeast extract	0.25	
	K_2HPO_4	0.3	
	KH_2PO_4	0.2	

CONCLUSION

A fungal strain isolated from the soil sample was selected for its potential to produce α-amylase. The strain was identified as *Aspergillus luchuensis* for the first time ever in India. The process parameters were optimized sequentially by one factor at atime, Plackett-Burmann Design and Box-Behnken Design. The effect of three variables namely starch, sodium nitrate and medium pH were positive driving factors that increased the α-amylase yield (192KU). The partially purifiedα-amylase was used in desizing the sized cotton fabric. This study proposes a thermo tolerant α-amylase which can be used for textile and food industrial applications.

ACKNOWLEDGEMENT

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

REFERENCES

Aguilar, G., Morlon-Guyot, J., Trejo-Aguilar, B. and Guyot, J. P. (2000). Purification and characterization of an extracellular α--amylase produced by *Lactobacillus manihotivorans* an amylolytic lactic acid bacterium. *Enzyme and Microbial Technology* **27, 406-413.**

Ahlawat, S., Dhiman, S. S., Battan, B., Mandhan, R. P. and Sharma, J. (2009). Pectinase production by *Bacillus subtilis* and its potential application in biopreparation of cotton and micropoly fabric. *Process Biochemistry* **44, 521-526.**

Ali, I., Akbar, A., Yanwisetpakdee, B., Prasongsuk, S., Lotrakul, P., and Punnapayak, H. (2014). Purification, characterization, and potential of saline waste water remediation of a polyextremophilic α-amylase from an obligate halophilic *Aspergillus gracilis*. *BioMed Research International* **2014, 1-7.**

Bhanja, T. and Banerjee, R. (2015). Biocatalysis and agricultural biotechnology purification, biochemical characterization and application of α-amylase produced by *Aspergillus oryzae* IFO-30103. *Biocatalysis and Agricultural Biotechnology* **4, 83-90.**

Bruns, T. D., Vilgalys, R., Barns, S. M., Gonzalez, D., Hibbett, D. S., Lane, D. J., Simon, L., Stickel, S., Szaro, T. M., Weisburg, W. G. and Sogin, M. L. (1992). Evolutionary relationships within the fungi: Analyses of nuclear small subunit rRNA sequences. *Molecular Phylogenetics and Evolution* **1, 231-241.**

Canganella, F., Andrade, C. and Antranikian, G. (1994). Characterization of amylolytic and pullulytic enzymes from thermophilic archaea and from a new *Ferividobacterium* species. *Applied Microbiology and Biotechnology* **42, 239-245.**

Chehna, A. B., Agrawal, N., Ricard, L. B., Smith, GM. and Varley, A. R. (2007). AATCC Technical Manual, American Association of Textile Chemists and Colorists, North Carolina. **pp. 83.**

Chi, Z., Chi, Z., Liu, G., Wang, F., Ju, L. and Zhang, T. (2009). *Saccharomycopsis fibuligera* and its applications in biotechnology. *Biotechnology Advances* **27, 423-431.**

Cripwell, R., Favaro, L., Rose, S. H., Basaglia, M., Cagnin, L., Casella, S. and van Zyl, W. (2015). Utilisation of wheat bran as a substrate for bioethanol production using recombinant cellulases and amylolytic yeast. *Applied Energy* **160, 610-617.**

Couto, S. R. and Sanromán, M. A. (2006). Application of solid-state fermentation to food industry- A review. *Journal of Food Engineering* **76, 291-302.**

Dey, G., Mitra, A., Banerjee, R. and Maiti, B. R. (2001). Enhanced production of amylase by optimization of nutritional constituents using response surface methodology. *Biochemical Engineering Journal* **7, 227-231.**

Djekrif-Dakhmouche, S., Gheribi-Aoulmi, Z., Meraihi, Z. and Bennamoun, L. (2006). Application of a statistical design to the optimization of culture medium for a-amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder. *Journal of Food Engineering* **73, 190-197.**

Francis, F., Sabu, A., Nampoothiri, K.M., Ramachandran, S., Ghosh, S., Szakacs, G. and Pandey, A. (2003). Use of response surface methodology for optimizing process parameters for the production of α-amylase by *Aspergillus oryzae*. *Biochemical Engineering Journal* **15, 107-115.**

Feitkenhauer, H. (2003). Anaerobic digestion of desizing wastewater: Influence of pretreatment and anionic surfactant on degradation and intermediate accumulation. *Enzyme and Microbial Technology* **33, 250-258.**

Gangadharan, D., Sivaramakrishnan, S., Nampoothiri, K.M. and Pandey, A. (2006). Solid culturing of Bacillus amyloliquefaciens for α-amylase production. *Food Technology and Biotechnology* **44, 269-274.**

Gargas, A., and Taylor, J. W. (1992). Polymerase chain reaction (PCR) primers for amplifying and sequencing 18S rDNA from lichenized fungi. *Mycologia* **84, 589-592.**

Gavrilescu, M. and Chisti, Y. 2005. Biotechnology-a sustainable alternative for chemical industry. *Biotechnology Advances* **23, 471-499.**

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K. and Chauhan, B. (2003). Microbial α-amylases: A biotechnological perspective. *Process Biochemistry* **38, 1599-1616.**

Gupta, A., Gupta, V. K., Modi, D. R. and Yadava, L. P. (2008). Production and characterization of α-amylase from *Aspergillus niger*. *Biotechnology* **7, 551-556.**

Ghorai, S., Banik, S. P., Verma, D., Chowdhury, S., Mukherjee, S. and Khowala, S. (2009). Fungal biotechnology in food and feed processing. *Food Research International* **42, 577-587.**

Irfan, M., Nadeem, M. and Syed, Q. (2012). Media optimization for amylaseproduction in solid state fermentation of wheat bran by fungal strains. *Journal of Cell and Molecular Biology* **10, 55-64.**

Jukes, T. H., and Cantor, C. R. (1969). Evolution of protein Molecules. New York: Academic Press. **pp. 21-132.**

Karam, E. A., Abdel Wahab, W. A., Saleh, S. A. A., Hassan, M. E., Kansoh, A. L. and Esawy, M. A. (2017). Production, immobilization and thermodynamic studies of free and immobilized Aspergillus awamori amylase. *International Journal of Biological Macromolecules* **102, 694-703.**

Khan, J. A. and Yadav, S. K. (2011). Production of α-amylase by *Aspergillus niger* using cheaper substrates employing solid state fermentation. *International Journal of Plant, Animal and Environmental Science* **1, 100-108.**

Khuri, A. I. and Cornell, J. A. (1987). Response Surfaces: Design and Analysis. Marcel Dekker, Inc., New York.

Kim, H., Kim, J., Bai, D. and Ahn, B. (2011). Identification and characterization of useful fungi with α-amylase activity from the Korean traditional *nuruk*. *Mycobiology* **39, 278-282.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Koushik, C. V. and Josico, A. I. (2003). Chemical processing of textiles: Preparatory processes and dyeing. NCUTE. India.

Krishna Prasad, K., Venkata Mohana, S., Sreenivas Raob, R., Ranjan Pati, B. and Sarma, P. N. (2005). Laccase production by *Pleurotus ostreatus* 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. *Biochemical Engineering Journal* **24, 17-26.**

Laderman, K., Davis, B., Krutzsch, H., Lewis, M., Griko, Y., Privalov, P. and Anfinsen, C. (1993). The purification and characterization of an extremely thermostable α-amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. *The Journal of Biological Chemistry* **268, 24394-24401.**

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent *The Journal of Biological Chemistry* **193, 265-275.**

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determinationof reducing sugars. *Analytical Chemistry* **31, 426-428.**

Mulimani, V. H., Patil, G. N. and Ramalingam, (2000). α-Amylase production by solid state fermentation: a new practical approach to biotechnology courses. *Biochemical Education* **28, 161-163.**

Ghobadi, N., Ogino, C., Yamabe, K., Ohmura, N. (2017). Characterizations of the submerged fermentation of *Aspergillus oryzae* using a Fullzone impeller in a stirred tank bioreactor. *Journal of Bioscience and Bioengineering* **123, 101-108.**

Chand. N., Sajedi. R. H., Nateri, A. S., Khajeh, K. and Rassa, M. (2014). Fermentative desizing of cotton fabric using an α-amylase-producing *Bacillus* strain: Optimization of simultaneous enzyme production and desizing. *Process Biochemistry* **49, 1884-1888.**

Oner, E. T. (2006). Optimization of ethanol production from starch by an amylolytic nuclear petite *Saccharomyces cerevisiae* strain.*Yeast **23*, 849–856.**

Pedersen, S., Jensen, B. F., Dijkhuizen, L., Jørgensen, S.T. and Dijkstra, B. W. (1995). A better enzyme for cyclodextrins. *International Journal of Chemtech Research* **12, 19-25.**

Prajapati, V. S., Trivedi, U. B., and Patel, K. C. (2015). A statistical approach for the production of thermostable and alklophilic alpha-amylase from *Bacillus amyloliquefaciens* KCP2 under solid-state fermentation. *3 Biotech* **5(2), 211-220.**

Prakash, O. and Jaiswal, N. (2009). Alpha-amylase: An ideal representative of thermostable enzymes. *Applied Biochemistry and Biotechnology* **162,2123-2124.**

Plackett, R. L. and Burman, J. P. (1946). The design of optimum multifactorial experiments. *Biometrika* **33, 305-325.**

Ramachandran, S., Patel, A. K., Nampoothiri, K. M., Francis, F., Nagy, V., Szakacs, G. and Pandey, A. (2004). Coconut oil cake – a potential raw material for the production of α-amylase. *Bioresource Technology* **93, 169–174.**

Sahnoun, M., Bejar, S., Sayari, A., Triki, M. A., Kriaa, M. and Kammoun, R. (2012). Production, purification and characterization of two α-amylase isoforms from a newly isolated *Aspergillus oryzae* strain S2. *Process Biochemistry* **47, 18-25.**

Savchenko, A., Vieille, C., Kang, S., Zeikus, J. G., (2002). *Pyrococcus furiosus* α-amylase is stabilized by calcium and zinc. *Biochemistry* **41, 6193-6201.**

Singh, S., Singh, S., Bali, V., Sharma, L., Mangla, J., (2014). Production of fungal amylases using cheap, readily available agriresidues, for potential application in textile industry. *BioMed Research International* **2014(215748), 9.**

Shigechi, H., Koh, J., Fujita, Y., Matsumoto, T., Bito, Y., Ueda, M., Satoh, E., Fukuda, H. and Kondo, A. (2004). Direct production of ethanol from raw corn starch via fermentation by use of a novel surface-engineered yeast strain codisplaying glucoamylase and α-Amylase. *Applied and Environmental Microbiology* **70, 5037-5040.**

Shore, J. (1995). Cellulosics Dyeing. Society of Dyers and Colorists. England.

Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K. M., Soccol, C. R. and Pandey, A. (2006). α-Amylases from microbial sources-an overview on recent developments. *Food Technology and Biotechnology* **44,173-184.**

Spohr, A., Carlsen, M., Nielsen, J. and Villadsen, J. (1998). α-Amylase production in recombinant *Aspergillus oryzae* during Fed-batch and continuous cultivation. *Journal of Fermentation and Bioengineering* **86, 49–56.**

Sudo, S., Ishikawa, T., Sato, K. and Oba, T. (1994). Comparison of acid stable α-amylase production by *Aspergillus kawachii* in solid-state and submerged cultures*. Journal of Fermentation and Bioengineering* **77, 483-489.**

Thompson, D. O. (1997). Cyclodextrins-enabling excipients: Their present and future use in pharmaceuticals. *Critical Reviews in Therapeutic Drug Carrier System* **14, 1-104.**

Tonkova, A. (2006). Microbial starch converting enzymes of the α-Amylase family. *In:* Microbial Biotechnology in Horticulture, Ray R. C. and Wards O. P. (eds.). Science Publishers, New Hampshire, USA. **pp. 421-472.**

Viswanathan, P. and Surlikar, N. P. (2001). Production of α-amylase with *Aspergillus flavus* on amaranthus grains by solid-state fermentation. *Journal of Basic Microbiology* **41, 57-64.**

Vohra, A. and Satyanarayana, T. (2002). Statistical optimization of medium components by response surface methodology to enhance phytase production by *Pichia anomala*. *Process Biochemistry* **37, 999-1004.**

Wurster, P. (1987). The TEGEWA drop test, a method for the rapid determination of textile fabric absorption. Melliand Textilberichte, Germany. **68, pp. 581-583.**

Li, Z., Wang, D. and Shi, Y.-C. (2017). Effects of nitrogen source on ethanol production in very high gravity

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

fermentation of corn starch. *Journal of the Taiwan Institute of Chemical Engineers* **70, 229-235.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538