Pak. J. Bot., 48(6): 2557-2561, 2016.

PRODUCTION OPTIMIZATION BY USING PLACKETT-BURMAN DESIGN AND PARTIAL CHARACTERIZATION OF AMYLASE FROM *ASPERGILLUS TUBINGENSIS* SY 1

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Abstract

Amylases are omnipresent and exceedingly demanded industrial enzymes. In this study, the conditions of extracellular amylase production from a fungal strain *A. tubingensis* SY1 were statistically optimized by applying Plackett-Burman equation, under submerged fermentation conditions. Maximum enzyme activity was noted in a medium containing (g/L); Starch (5.0), Peptone (10.0), KH_2PO_4 (2.0), NH_4NO_3 (3.0), KCl (1.0), $MgSO_4.7H_2O$ (1.0), $FeSO_4.7H_2O$ (0.01), Agitation (200 rpm) with an inoculum size 1%. As witnessed from Pareto chart; variables that were most important for amylase production were Peptone, agitation; $MgSO_4.7H_2O$ followed by inoculum size. Partial characterization of the crude enzyme revealed that the optimum temperature and pH for enzyme activity was 60°C and 5.6, respectively. The residual activity of the enzyme was reduced to 50% after storage for ~3h at 44°- 64°C. The enzyme was, however, stable at a pH range of 3.6 - 5.6 for up to 3h.

Key words: Plackett-Burman design, Amylase, *A. tubingensis* SY 1.

Introduction

Starch is a polysaccharide that is abundantly distributed in nature as a reserve of energy in plants. It can be degraded to oligosaccharides, disaccharides, which are ultimately converted into mono saccharides such as glucose through the action of group of enzymes known as amylases. There are three major classes of amylases namely α-amylases, β-amylases and glucoamylases, based on the bonds they break and the product of degradation. Amylases have received a great deal of attention because of their perceived biotechnological applications ranging from food, fermentation, textile, paper and pharmaceutical to sugar industries (Aboud-Zeid, 1997; Gupta *et al*., 2003; Kandra, 2003; Pandey *et al*., 2000). Amylases contribute to nearly 25% of the international enzyme market and have almost entirely substituted chemical hydrolysis of starch in the starch utilizing industry (Rajagopalan & Krishnan, 2008; Reddy *et al*., 2003).

Amylases can be obtained from several microorganisms, plants and animals. Among the microorganisms, amylases from filamentous fungi typically *Aspergillus niger*, *Rhizopus oryzae* and *Aspergillus awamori* have been considered significant for industrial applications (Bakri *et al*., 2009). According to the Association of Manufacturers of Fermentation Enzyme Products (AMFEP), amylases for commercial needs are mostly produced by filamentous fungi. Fungal amylases are mostly preferred for commercial production because fungal enzymes are more efficient compared to those obtained from bacteria (Abdullah *et al*., 2014; Djekrif-Dakhmouche *et al*., 2006; Hernández *et al*., 2006; Saxena *et al*., 2004).

Designing an effective fermentation medium for maximum enzyme production is a critical and vital process as the medium composition can considerably affect the product-yield (Ahmed *et al*., 2015; Djekrif-Dakhmouche *et al*., 2006; Ooijkass *et al*., 1998). Conventional method for process optimization is time consuming and arduous task of altering one factor at a time while keeping others at constant levels. Alternative and more effective approach today is the use of statistical approach such as Plackett-Burman (PB) design. This design has been successfully established for its efficacy in screening the important factors in few experimental runs. So far, few studies have been reported on statistical optimization of amylase production from *Aspergillus* strains (Kammoun *et al*., 2008; Qunhui *et al*., 2008).

Keeping in view the broad industrial importance of amylase, nine medium components were screened for their impact on amylase production by *A. tubingensis* SY1 under submerged fermentation using PB design. Moreover, partial characterization of the crude amylase preparation was also investigated.

Materials and Methods

Microorganism and cultivation: An environmental isolate, *A. tubingensis* SY 1 was used in this study. Fungal spores grown on SDA plate were transferred to tubes containing sterile saline after 4-5 days of incubation. An aliquot (0.1 ml; ~2 X 10^4 spores/ml) from this spore suspension was transferred to 10 ml fermentation medium in 100 ml Erlenmeyer flasks, incubated in an orbital shaker at 30°C with agitation at 150 rpm for 4 days. To determine the enzyme activity, mycelia were removed by centrifugation at x 6000*g* for 20 min at 4°C and the clear cell-free culture supernatant (CFCS) was used as crude enzyme preparation.

Optimization of culture conditions using Plackett-Burman (PB) design: The important fermentation parameters that affect amylase production by *A. tubingensis* SY 1 were screened by employing Plackett-Burman design (Placket & Burman, 1946) under shake-flask conditions. Nine independent factors (variables)

consisting of medium components and operating conditions were selected for this study. Each factor was studied at two levels, high (+) and low (−), as shown in Table 1. Twelve trials were conducted to evaluate the effects of these factors. Table 2 shows the Plackett-Burman experimental design matrix in which each column represents variables and each row represents an experiment. The fungal spore suspension was inoculated into the medium and incubated for 96 h at 30°C on a rotary shaker according to the experimental design.

Table 1. Variables studied at different levels in Plackett-Burman design for amylase production under shake-flask condition.

Variables	Variable code	Units	Low level (-)	High level (+)
Starch	X1	g/L	2.5	5.0
Peptone	X2	g/L	5.0	10.0
KH_2PO_4	X3	g/L	1.0	2.0
NH_4NO_3	X4	g/L	3.0	5.0
KCl	X5	g/L	0.5	1.0
$MgSO_4.7H_2O$	X6	g/L	0.5	1.0
$FeSO_4.7H_2O$	X7	g/L	0.01	0.02
Agitation	X8	rpm	150	200
Inoculum size	X9	ml	10	20

Statistical package, Minitab version 14 was used for the design of experiment and analyzing the experimental data. Experiments were performed, in triplicate, and the estimated mean of amylase production were used as the experimental response (dependent variable). PB experimental design is based on the first order model as given in equation 1.

$$Y = \beta_0 + \Sigma\beta_i x_i \ldots\ldots\ldots\ldots\ldots\ldots \text{Equation 1}$$

where, Y is the dependent variable (response in terms of enzyme activity), β_0 is the model intercept, i is the variable number, β_i is variable estimated coefficient and x_i are independent variables. F-test was for analysis of variance (ANOVA), to determine the significance of each term in equation and statistical significance of the model Student's t-test was used to evaluate the statistical significance of regression coefficients. The adequacy of the model can be articulated by the coefficient of multiple determinations (R^2) and lack-of fit value. The variables whose p-value was less than 0.1 were considered to have significant influence on the amylase productivity.

Table 2.Plackett-Burman experimental design applied on trial runs: (+) high level variables, (-) low level variables.

Run No.	X1	X2	X3	X4	X5	X6	X7	X8	X9	Amylase activity (IU/ml)
1	-	+	+	+	-	+	+	-	+	2.695
2	+	-	-	-	+	+	+	-	+	1.26
3	-	-	-	+	+	+	-	+	+	1.87
4	-	+	-	-	-	+	+	+	-	2.67
5	+	+	-	+	-	-	-	+	+	2.28
6	+	+	-	+	+	-	+	-	-	2.125
7	-	-	+	+	+	-	+	+	-	2.096
8	-	-	-	-	-	-	-	-	-	1.095
9	-	+	+	-	+	-	-	-	+	1.29
10	+	-	+	+	-	+	-	-	-	0.97
11	+	+	+	-	+	+	-	+	-	3.75
12	+	-	+	-	-	-	+	+	+	1.77

Amylase assay: Soluble starch (25µl; 0.5 % w/v) was mixed with 25µl of the crude enzyme preparation and incubated at 60°C. The reaction was stopped by adding 150 µl of 1% DNS and boiling for 5 min. The reaction mixture was chilled on ice for 5 min, added 720 µl of distilled water and A_{500} recorded on a spectrophotometer (Beckman Coulter, UV / Vis, DU 730). One unit of amylase activity is defined as micromoles of reducing sugar produced by one ml of enzyme in one minute. The amount of reducing sugar was calculated using a standard curve of glucose (Miller, 1959).

Effect of Temperature on amylase activity and stability: Optimum temperature for amylase activity was determined by conducting the assay at a temperature range of 50°C–74°C. To determine the thermal stability, the crude enzyme preparation was incubated at different temperatures (44°C–70°C) for 3h and aliquots were withdrawn after every 30 min to measure residual enzyme activity and $t_{1/2}$ determined.

Effect of pH on activity and stability of amylase: Effect of pH on amylase activity was determined by measuring the amylase activity at 60°C using 0.05M Na-acetate (pH 3.0-5.6), Citrate-phosphate (pH 6.0-6.6), Tris-HCl (pH 7.0-8.5), Glycine-NaOH (pH 9.0-10.5) buffers. To determine the pH stability, the crude enzyme was incubated at room temperature (28°C ± 2) for 3 h in respective buffers, aliquots were withdrawn after 30 min interval and amylase activity determined under standard conditions.

Results and Discussion

Amongst fungi capable of producing beneficial enzymes, *Aspergilli* are predominantly attractive due to easy cultivation and elevated production of extracellular enzymes for industrial utilization. In fact, the first microbial enzyme used in food industry was a starch-hydrolyzing enzyme (Bennett, 1998; Deker, 2003; Lin *et al.,* 1997).

Keeping in view the worldwide importance of amylases, the present study was conducted to statistically optimize the production and to partially characterize amylase from a fungal strain *A. tubingensis* SY 1. Nine factors were investigated to determine the optimum medium components suitable for amylase production. The amylase activities from the twelve runs are shown in Table 2. Fractional factorial Plackett-Burman design was used to screen and evaluate the significant variables that can influence enzyme yield because this model does not explain the interaction among various variables (Motol & Agharkar, 1992). The results (Table 2) indicate a variation in amylase production in the range from 0.97 to 3.75 IU/ml by *A. tubingensis* SY1. These variations revealed the importance of medium optimization to obtain better amylase yield (Kumar & Satyanarayan, 2007; Soni *et al.*, 2006). Maximum Amylase activity was obtained in run number 11 containing (g/L); Starch (5.0), Peptone (10.0), KH_2PO_4 (2.0), NH_4NO_3 (3.0), KCl (1.0), $MgSO_4.7H_2O$ (1.0), $FeSO_4.7H_2O$ (0.01), Agitation (200 rpm), and an inoculum size of 1%.

The data on enzyme activity in Table 2 was subjected to multiple linear regression analysis to estimate t- and p-values of each component. On analysis of t-values, all factors have shown positive effect on amylase production except inoculum size (X9) which showed negative effect. The statistical model itself is significant with a p-value of 0.012. However, there was no factor whose p-value is smaller than 0.1 as indicated in Table 3, which means that all factors analyzed were not statistically significant; though they played varying role in amylase production. These results are in agreement with those of other reports (Qunhui *et al*., 2008). The goodness of fit model was checked by the coefficient of determination (R^2) which indicated that the model could explain up to 84.0% variation of the data (Table 3).

Based on the PB design, the effect of independent variables on amylase production is set by the first-order linear model and is given by equation 2. Table 4 shows the Analysis of Variance (ANOVA) for linear model on effect of independent variables on amylase production from *A. tubingensis* SY1.

Y = 1.99 + 0.037 X1*+ 0.479 X2* + 0.106 X3* + 0.017 X4* + 0.076 X5* + 0.213 X6*+ 0.113 X7* + 0.417 X8*- 0.128 X9* …….. Equation 2

* Refer to Table 1 for variable codes

Table 3. Statistical analysis applied on Plackett-Burman design for amylase production from *A. tubingensis* SY 1.

Term	Main Effect	Coefficient	StDev Coefficient	T-value	P-value
Constant		1.9892	0.2178	9.13	0.012*
X1	0.0732	0.0366	0.2178	0.17	0.882
X2	0.9582	0.4791	0.2178	2.20	0.159
X3	0.2118	0.1059	0.2178	0.49	0.675
X4	0.0335	0.0168	0.2178	0.08	0.946
X5	0.1518	0.0759	0.2178	0.35	0.761
X6	0.4265	0.2133	0.2178	0.98	0.431
X7	0.2268	0.1134	0.2178	0.52	0.654
X8	0.8335	0.4167	0.2178	1.91	0.196
X9	-0.2568	-0.1284	0.2178	-0.59	0.615

S = 0.7544 R-Sq = 84.0%

T –value= student t- test; *p – value= corresponding level of significance

Table 4. Analysis of variance for the experiment on optimization of amylase production by *A. tubingensis* SY 1.

Source	DF	SS	MS	F	P
Regression	9	5.9596	0.6622	1.16	0.545
Error	2	1.1381	0.5691		
Total	11	7.0977			

DF= Degree of freedom; SS= Sum of squares; ASS= Adjusted sum of squares; MSS= Mean sum of squares; F= Fishers's function; P= Corresponding level of significance

Table 5. Half-life ($t_{1/2}$) of amylase from *A. tubingensis* SY 1.

Strain	Temperature (^oC)	$T_{½}$ (min)
A. tubingensis SY1	44	180
	50	175
	54	170
	60	170
	64	155
	70	18

Pareto chart of effects was plotted for identifying the factors important for enzyme production and shows the factors main effect estimates on the horizontal axis. Ranking of the factors is done according to their importance. It is evident from the Pareto chart of process variables (Fig. 1) that the factors important for amylase production by *A. tubingensis* SY1 were peptone, agitation and $MgSO_4.7H_2O$.

Studies on the thermal and pH stability of an enzyme are important aspects for its use in industries (Haki & Rakshit, 2003.). Therefore, in this work, the physico-chemical characteristics of amylase were determined within a broad range of temperature (50°C-74°C). Results explained that the enzyme activity increased with increasing temperature and reached the maximum at 60°C (Fig. 2). Further increases in temperature of incubation, resulted in a drastic decrease in the amylase activity. Temperature optima vary depending upon the producing microorganism (Bakri *et al*., 2009, Thorsen *et al*., 2006). However, 50°C-60°C is the optimum range for amylase activity (Norouzian *et al*., 2000). The CFCS of *A. tubingensis* SY 1 was also used to determine the thermal-stability and half-life of amylase. The data showed that the enzyme remained stable at the temperature range of 44°C-64°C (Table 5). Hence, the respective enzyme is thermo-stable and is suitable for use in starch processing industries as many of the industrial processes are performed at high temperatures. The results obtained were comparatively better than obtained by others, according to their reports that the amylases they worked with had had a half-life of much less than 3 h (Kumar & Satyanarayana, 2003, Frolova *et al*., 2002, Okolo *et al*., 2000).

The stability of amylases at acidic pH is principally important for the efficient degradation of starch (Akpan *et al*., 2005). The pH profile of amylase, was assayed using buffered-starch prepared in a pH range of 3.5–10.5. The results indicate that *A. tubingensis* SY 1 amylase is active with in the pH range 3.0-6.6 and the maximum activity was observed at pH 5.6 (Fig. 3). The results are in agreement with those reported in earlier studies on fungal amylases (Kajiwara *et al*, 1997; Kandra, 2003). The enzyme activities drastically declined at alkaline pH. Amylases produced by most fungi generally have pH optima falling within the range of 4.0-6.0 (Cereia *et al*., 2006; Thorsen *et al*., 2006). The amylase from *A. tubingensis* SY 1 showed high degree of stability at acidic pH (3.6-5.6) for up to 3h; the enzyme, however, lost ~ 80% of the activity within 30 min when incubated under alkaline conditions (Fig. 4).

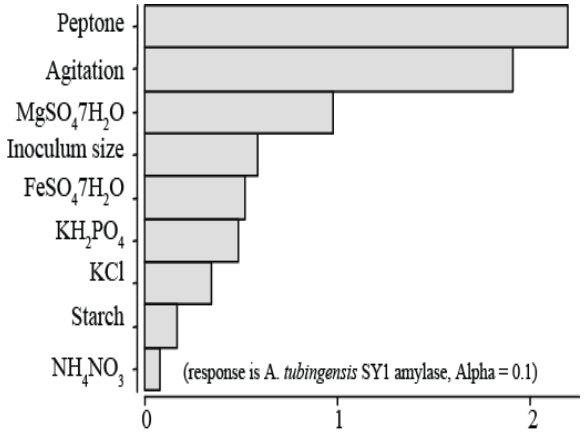

Fig. 1. Pareto chart for nutrients and conditions of significant variables for amylase produced from *A. tubingensis* SY 1.

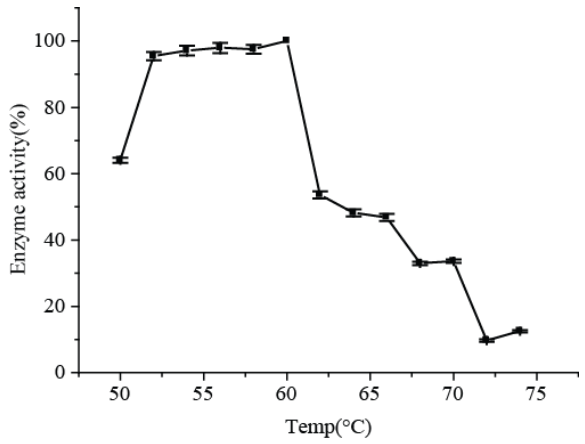

Fig. 2. Optimum temperature for amylolytic activity in CFCS of *A. tubingensis* SY 1.

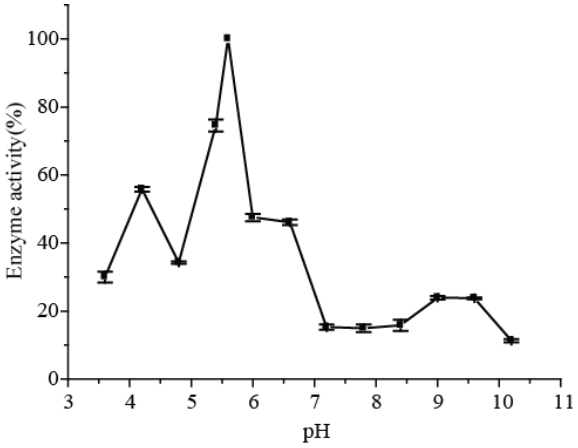

Fig. 3. Effect of pH on *A. tubingensis* SY 1 amylase activity.

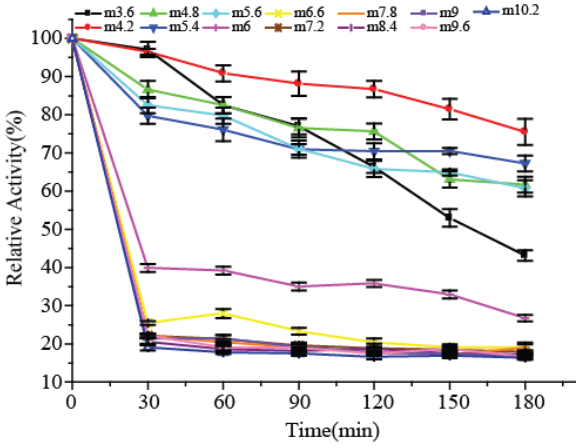

Fig. 4. pH stability profile of amylase activity from *A. tubingensis* SY 1.

Conclusions

The results of the present study indicate the possibility of Plackett-Burman trials for determining the factors that have a positive effect on enzyme production. The amylase from *A. tubingensis* SY 1 is stable at a wide range of temperature and acidic pH. Owing to the significance of these findings, further studies will focus on the development of methods for utilization of this enzyme in industrial processes.

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(Received for publication 11 October 2015)