Optimization of medium components for amylase production using the Plackett-Burman design-A statistical approach

Chayut Intaya[1], Siriluk Teeradakorn,[2,]*

[1]*Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*
[2]*Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10330, Thailand*
**e-mail: siriluk.T@chula.ac.th*

Abstract

Amylases are one of the most important enzymes in present-day biotechnology. This work was concerned with the production from bacteria (*Basillus amyloliquefaciens* TISTR 1045), yeast (*Saccharomycopsis fibuligera* TISTR 5033) and fungus (*Aspergillus oryzae* TISTR 3086). The effect of carbon-nitrogen components were optimized based on statistical approach-the Randomized Block Design (RBD). The optimal for *Bacillus amyloliquefaciens* TISTR 1045 and *Aspergillus oryzae* TISTR 3086 were 15% grain sorghum and 0.5% $(NH_4)_2SO_4$. While *Saccharomycopsis fibuligera* TISTR 5033 gave a low amylase production. Moreover, the Plackett-Burman Design was used to screen mineral salts that influence on amylase production. It revealed that $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$ and NaCl enhanced amylase yield by *B. amyloliquefaciens* TISTR 1045 and $(NH_4)_2SO_4$. KH_2PO_4, $CaCl_2$, $FeSO_4$ enhanced amylase yield by *Aspergillus oryzae* TISTR 3086.

Keywords: amylase, Plackett-Burman design, statistical approach, carbon-nitrogen, mineral salts

Introduction

Amylases (E.C 3.2.1.1 α-amylase, β-amylase and glucoamylase) can be divided into two categories, endoamylases and exoamylases. Endoamylases such as α-amylase cleaves α-1, 4 glycosidic bonds in a random manner present in the inner part (endo) of amylose or amylopectin chain of the starch molecule. Exoamylases either cleave α-1, 4 glycosidic bonds such as β-amylase or cleave both α-1, 4 and α-1, 6 glycosidic bonds like amyloglucosidase or glucoamylase and α-glucosidase from the non-reducing end of the starch molecule (Agrawal et al. 2005). Amylases can be found in every living organism such as animals, plants, bacteria and fungi. Microorganisms with amylolytic capability include α-amylase, 1)groups of bacteria, such as *Bacillus amyloliquefaciens, B. subtilis* and *B. licheniformiss* (Zar et al.2013) 2) group of yeast, such as *Saccharomycopsis fibuligera* and *Schwanniomyces occidentalis* (Liu et al. 2009)and 3) group of fungi such as *Aspergillus oryzae*, *A. kawachii, A. flavus* and *A. niger* (Xu et al. 2008). Amylase was used in a wide range of applications in fermentation processes, food industry, pharmaceuticals and textile or paper industries. Recently this enzyme has growing interests to utilize agro-industrial residues, including cassava in order to be as the substrate for the ethanol production (Aquino et al. 2003). Since these residues are inexpensive and also eliminates large-scale accumulation of the biomass. Sorghum (Sorghum bicolor L.) is one of alternative crops used as carbon source for the production of ethanol because this plant is resistant to drought, short period (3–5 months) cultivation and high-yield. It is an attractive crop in semiarid regions of the world with high starch, nitrogen content than other grains (Zhan et al. 2003).

Optimal conditions for the production of amylase are variations of carbon and nitrogen source by statistical approach such as SPSS. It is an extensive system for analysis data that

can take data from almost any type of file and use them to generate tabulated reports, plots of distributions, descriptive statistics and complex statistical analysis. Another variables are minerals such as K^{+}, P^{+}, Mg^{2+}, Ca^{2+}, Na^{+}, Fe^{2+}(Deb et al. 2013). The Media optimization is a sequential procedure starting with screening of variables, followed by estimation of optimum levels of the screened variables. The Plackett-Burman design is a well establish statistical design for the screening of variables. The traditional one factor at a time technique is time consuming due to the number of experiments and can easily miss the interaction effect (Plackett and Burman 1946).

Therefore, in this present study, an attempt has been made to optimize condition for amylase production using the grain sorghum as carbon source. We also evaluated the effect of carbon -nitrogen sources and mineral salts for amylase production using a statistical approach.

Methodology

Microorganisms

Three kinds of microorganisms were investigated in this study; bacteria, yeast and fungi. *Bacillus amyloliquefaciens* TISTR 1045, *Saccharomycopsis fibuligera* TISTR 5033 and *Aspergillus oryzae* TISTR 3086 were obtained from The Thailand Institute of Scientific and Technological Research (TISTR).

Inoculum preparation of *B. amyloliquefaciens* TISTR 1045

The strain was maintained on nutrient agar (NA) slant consisted of 3 g/l beef extract and 5 g/l peptone. Then the strain was incubated at 37 °C for 18 h and stored at 4°C. The slant culture was transferred to nutrient broth (NB). Cultivation conditions were 37°C with 150 rpm shaking for 12 h. Next, 10% v/v submerged culture was used as the inoculum for amylase production.

Inoculum preparation of *S. fibuligera* TISTR 5033

The strain was maintained on Yeast Extract Peptone Dextrose Agar (YPDA) slant consisted of 10 g/l yeast extract, 20 g/l peptone, and 20 g/l dextrose (20 g). Then incubated at 30°C for 36 h and stored at 4°C. The slant culture was transferred to Yeast Extract Peptone Dextrose Broth (YPDB). Cultivation conditions were 30°C with 150 rpm shaking for 18 h. Next, 10% v/v submerged culture was used as the inoculum for amylase production.

Inoculum preparation of *A. oryzae* TISTR 3086

The strain was maintained on Potato Dextrose Agar (PDA) slant consisted of 200 g potato-broth, 20 g/l dextrose and made up volume with distilled water (1000mL). Then incubated at 30°C for 72 h and stored at 4°C. The slant culture was transferred to Potato Dextrose agar (PDA) plate. Incubation conditions were 30°C for 72 h. A spore suspension was made by adding 10 ml of sterile water and scraped aseptically with inoculating loop. The spore suspensions were filtered through a sterile muslin cloth into sterile flask. Next, 10% v/v of this spore suspension (10^{6}spore/ml) was used as the inoculum for amylase production.

Substrate preparation

Grain sorghum was selected as substrate in the present study for the production of amylase. It was obtained form Suphanburi Field Crop Research Center, Thailand. Grain sorghum from the field was dried by keeping in oven at 80°C for 12 h, then grinded pass through a sieve 500 µm. 10% (w/v) of this powder is used as the carbon source.

Amylase production

The experiments were carried out in shaken flasks using 125 ml Erlenmeyer flasks consisted of 10% (w/v) grain sorghum, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.1% $MgSO_4$, 0.01%, $CaCl_2$ and 0.01% NaCl. Cultivations condition were 30°C (for *S. fibuligera* and *A. oryzae*) or 37 °C (for *B. amyloliquefaciens*) with 150 rpm for 72 h. The culture was sampling every 6 hrs. The culture broth was centrifuged at 10,000 rpm for 5 minutes to remove the fine particles. The supernatant was used for determination of amylase activity.

Amylase determination

Amylase activity was measured following the method described by Bernfeld (1955). A reaction mixture consisted of 400 µl of 1% soluble starch solution in 0.1 M sodium acetate buffer (pH 5.8) and 100 µl of diluted enzyme solution was incubated at 50°C for 10 min. The reaction was terminated by adding 500 µl of DNS solution (1 g of 3,5-dinitrosalicylic acid dissolved 20 ml of 2M NaOH, 30 g of sodium potassium tartarate and water were added to make it 100 ml). Reaction mixtures were boiled for 5 min and after cooling, 1 ml of distilled water was added. Absorbance was measured at 540 nm using glucose as the standard. One unit (1 U) of enzyme activity was defined as the amount of enzyme required to produce 1 micromole of glucose as reducing sugar in one minute from soluble starch under the assay conditions.

Optimal conditions of carbon–nitrogen sources for amylase production

Grain sorghum used as carbon source was varied in the range 5-15% (w/v).The effect of the following 0.5% (w/v) nitrogen sources were investigated; urea, $(NH_4)_2SO_4$, yeast extract and peptone. Cultivations condition were 30°C (for *S. fibuligera* and *A. oryzae*) or 37 °C (for *B. amyloliquefaciens*) with 150 rpm for 48 h. The culture was sampling every 12 h. The culture broth was centrifuged at 10,000 rpm for 5 minutes to remove the fine particles. The supernatant was used for determination of amylase activity.

Statistical analysis using the Randomized Block Design (RBD)

The optimal carbon-nitrogen sources were performed using Randomized Block Design. The amount of the nitrogen source was treatments and the amount of carbon source was into blocks. All of the experiments were performed in triplicate and the related data were expressed as average values. Amylase activity experimental data were analyzed using SPSS software with one-way analysis of variance (ANOVA) followed by Tukey's multiple range method test to compare means. Differences in means were judged to be significant when *p* values for the null hypothesis were 0.05 or less.

Optimal conditions of mineral salts for amylase production

The medium consisted of 15% (w/v) grain sorghum, nitrogen source and mineral salts were as follows $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $CaCl_2$, NaCl, $FeSO_4$, Agitation rate at 150 rpm for 24 h. Then collected the culture broth was centrifuged at 10,000 rpm for 5 minutes. The supernatant was used for determination of amylase activity.

Statistical analysis using the Plackett-Burman design

To determine which mineral salts significantly affect amylase production, the Plackett-Burman design was used. Six variables were considered, with each variable represented at two levels, high and low denoted by (+) and (-) respectively. The variables were as follows: $(NH_4)_2SO_4$ (A), KH_2PO_4 (B), $MgSO_4$ (C), $CaCl_2$ (D), NaCl (E) and $FeSO_4$ (F). The variables with its actual levels were shown in (Table 1) for *A. oryzae* and (Table 2) *for B. amyloliquefaciens*. The statistical software package "Minitab 17", was used for analyze the

experimental data. The effect of each variable was determined by the following first-order polynomial model equation:

$$Y = \beta o + \Sigma \beta i x i \ (i = 1,\ldots.k) \quad (1)$$

Where, Y is the response function (amylase production), βo is the intercept term, βi is regression coefficient and xi is the coded independent variables.

Table 1 Experimental design using the Plackett–Burman method for amylase production by *A. oryzae* TISTR 3086, the cultivation condition was at 30°C, 150 rpm for 24 h

Variables	Components	Low level (-) values (g/l)	High level (+) values (g/l)
A	$(NH_4)_2SO_4$	1	10
B	KH_2PO_4	0.5	5
C	$MgSO_4$	0.1	2
D	$CaCl_2$	0.5	5
E	NaCl	0.5	5
F	$FeSO_4$	0.1	1

Table 2 Experimental designs using the Plackett–Burman method for amylase production by *B. amyloliquefaciens* TISTR 1045, the cultivation condition was at 30°C, 150 rpm for 24 h

Variables	Components	Low level (-) values (g/l)	High level (+) values (g/l)
A	$(NH_4)_2SO_4$	1	10
B	KH_2PO_4	0.5	5
C	$MgSO_4$	0.1	2
D	$CaCl_2$	0.5	5
E	NaCl	0.5	5

Results and Discussion

Amylase production

In the present study, three strains of *A. oryzae* TISTR 3086, *B. amyloliquefaciens* TISTR 1045 and *S. fibuligera* TISTR 5033 were investigated for amylase production. Figure 1 showed that high amount of amylase activity at 24 h, 14.363 U/ml and 7.475 U/ml were obtained by *A. oryzae* TISTR 3086 and *B. amyloliquefaciens* TISTR 1045, respectively, whereas *S. fibuligera* TISTR 5033 exhibited a tiny amylase activity (1.668 U/ml).

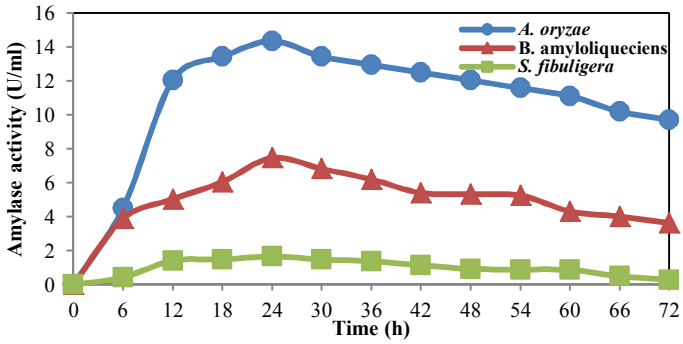

Figure 1 Amylase production by *A. oryzae* TISTR 3086, *B. amyloliquefaciens* TISTR 1045 and *S. fibuligera* TISTR 5033 in 125 ml Erlemeyer flask

Optimal conditions of carbon–nitrogen sources for amylase production using the Random Block Designs

Initial screening of the most significant carbon and nitrogen to maximize the amylase production were performed by two variables at a time approach. The concentration of carbon source (grain sorghum) was varied in the range 5-15% w/v. The nitrogen sources such as urea, $(NH_4)_2SO_4$, yeast extract and peptone were used to individually test. Optimal conditions of amylase production was presented statistic significant with confidence interval 95% follow by separated the mean by Tukey's and Duncan at α =0.05 (RBD). The results revealed maximal amylase activity of 18.003 U/ml for *A. oryzae* TISTR 3086 (Table 3) and of 12.548 U/ml for *B. amyloliquefaciens* TISTR 1045 (Table 4) were obtained with 15% (w/v) grain sorghum and 0.5% $(NH_4)_2SO_4$.

Table 3 Amylase production by *A. oryzae* TISTR 3086 in 125 ml Erlemeyer flask

Nitrogen source 0.5% (w/v)	Carbon source (%w/v)		
	5%	10%	15%
Urea	12.477	14.081	16.220
$(NH_4)_2SO_4$	13.903	13.725	18.003
Yeast extract	12.299	14.081	17.646
Peptone	12.655	14.081	17.468

Table 4 Amylase production by *B. amyloliquefaciens* TISTR 1045 in 125 ml Erlemeyer flask

Nitrogen source	Carbon source (%w/v)		
0.5 % (w/v)	5%	10%	15%
Urea	6.441	8.270	12.216
$(NH_4)_2SO_4$	6.132	7.866	12.548
Yeast extract	6.797	7.272	12.263
Peptone	6.417	7.320	11.835

Optimal conditions of mineral salts for amylase production using the Plackett–Burman design

A statistical approach-the Plackett–Burman design was used for screening significant mineral salts influencing amylase production by *A. oryzae* TISTR 3086 and *B. amyloliquefaciens* TISTR 1045. Total six variables of *A. oryzae* TISTR 3086 and five variables of *B. amyloliquefaciens* TISTR 1045 were analysed at two concentration levels. The average of maximum amylase activity was taken as response Y (Prediction equation). To examine the fitting quality of the model, the proximate correlation coefficient (R^2) to 0.999 indicated better fitting of the predicted values from the equation to the experimental values. The magnitude and direction of the factor coefficient in the equation explained the influence of the medium compositions on the amylase production from grain sorghum. The greater magnitude of the coefficient indicated a large effect on the response. Table 5 and Table 7 represented the experimental designs and the effect of each variable was determined by the first order polynomial equation (Equation 2 and 3).

Table 5 The Plackett–Burman experimental designs for the screening of significant mineral salts for amylase production by *A. oryzae* TISTR 3086

Run No.	Variables						Amylase activity (U/ml)	
	A	B	C	D	E	F	Exp.	Pred.
1	1	-1	-1	-1	1	1	13.153	13.960
2	-1	-1	-1	1	1	1	19.477	19.509
3	-1	1	1	1	-1	1	18.030	19.121
4	1	1	-1	1	1	-1	17.149	16.908
5	-1	-1	1	1	1	-1	21.774	22.267
6	1	1	1	-1	1	1	12.932	12.618
7	-1	1	1	-1	1	-1	18.628	17.852
8	1	-1	1	-1	-1	-1	16.551	17.673
9	-1	1	-1	-1	-1	1	16.048	16.048
10	1	-1	1	1	-1	1	19.603	17.988
11	1	1	-1	1	-1	-1	17.621	17.862
12	-1	-1	-1	-1	-1	-1	20.988	20.149

Prediction equation for amylase activity by *A. oryzae* TISTR 3086

$$Y = 17.663 - 1.495\,A - 0.928\,B + 0.257\,C + 1.280\,D - 0.477\,E - 1.122\,F \quad (2)$$

Table 6 Estimated effects and coefficients of the Plackett–Burman design for *A.oryzae* TISTR 3086

Term	Effect	Coef.	SE Coef.	T-Value	P-Value
Constant		13.566	0.353	50.04	0.000
A	-2.989	-1.495	0.353	-4.23	0.008
B	-1.856	-0.928	0.353	-2.63	0.047
C	0.514	0.257	0.353	0.73	0.499
D	2.559	1.28	0.353	3.62	0.015
E	-0.954	-0.477	0.353	-1.35	0.234
F	-2.245	-1.122	0.353	-3.18	0.025

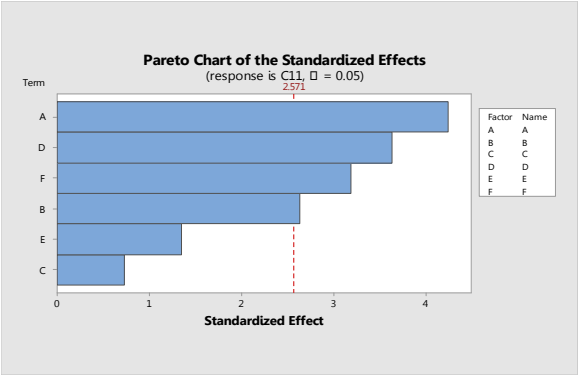

Figure 2 Pareto chart showing the effect of mineral salts on amylase production by *A. oryzae* TISTR 3086

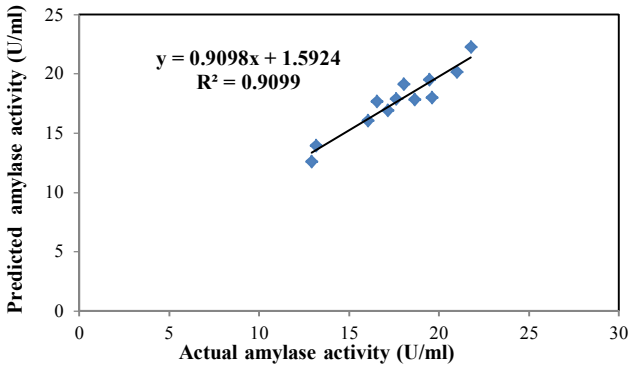

Figure 3 Parity plot between the actual and predicted values of important variables for *A.oryzae* TISTR 3086

The six components of the mineral salt medium were screened using twelve experimental runs as per the design. The effect of the variables and the associated T and *P* values was given in Table 6. *P* value less than 0.05 indicates that the effect is significant. From the Pareto chart shown in Figure 2, the four variables including $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2$ and $FeSO_4$ were found to be significant from standardized effect for amylase production. Parity plot represented in Figure 3 showed a satisfactory correlation between the experimental and predicted responses and the deviation between the values was minimal. The R^2value of 0.9099 indicates that the model could predict 90.99% variability in the response.

Table 7 The Plackett–Burman experimental designs for the screening of significant mineral salts for amylase production by *B. amyloliquefaciens* TISTR 1045

Run No.	Variables					Amylase activity (U/ml)	
	A	B	C	D	E	Exp.	Pred.
1	1	1	1	-1	1	12.272	11.839
2	1	-1	1	-1	-1	12.885	12.862
3	1	-1	1	1	-1	12.696	12.303
4	-1	1	-1	-1	-1	14.348	13.829
5	1	1	-1	1	-1	12.319	11.941
6	-1	-1	-1	1	1	15.717	15.819
7	-1	-1	-1	-1	-1	15.198	15.615
8	1	-1	-1	-1	1	14.584	15.049
9	-1	1	1	1	-1	11.516	11.847
10	-1	-1	1	1	1	14.962	14.396
11	-1	1	1	-1	1	13.924	13.168
12	1	1	-1	1	1	12.366	12.704

Prediction equation for amylase activity by *B. amyloliquefaciens* TISTR 1045

$$Y = 13.556 - 0.712\,A - 0.775\,B - 0.523\,C - 0.303D + 0.405\,E \quad (3)$$

Table 8 Estimated effects and coefficients of the Plackett–Burman design for *B. amyloliquefaciens* TISTR 1045

Term	Effect	Coef.	SE Coef.	T-Value	P-Value
Constant		13.566	0.143	95.04	0.000
A	-1.424	-0.712	0.143	-4.99	0.002
B	-1.550	-0.775	0.143	-5.43	0.002
C	-1.046	-0.523	0.143	-3.66	0.011
D	-0.606	-0.303	0.143	-2.12	0.078
E	0.810	0.405	0.143	2.84	0.030

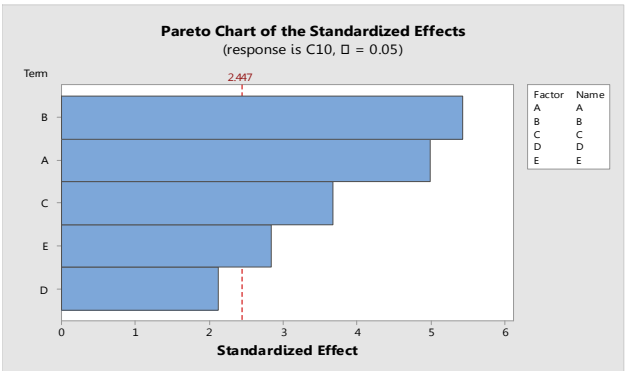

Figure 4 Pareto chart showing the effect of mineral salts on amylase production by *B. amyloliquefaciens* TISTR 1045

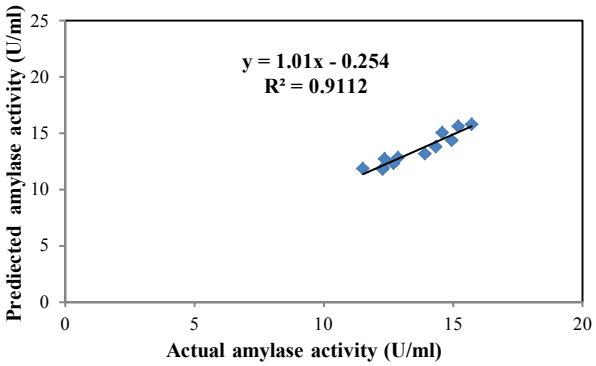

Figure 5 Parity plot between the experimental and predicted values of important variables for *B. amyloliquefaciens* TISTR 1045

The five composition of the mineral salt medium were screened using twelve experimental runs as per the design. The effect of the variables and the associated T and *P* values was given in Table 8. *P* value of less than 0.05 indicates that the effect is significant. From the Pareto chart shown in Figure 4, the four variables including $(NH_4)_2SO_4$, KH_2PO_4 $MgSO_4$ and NaCl were found to be significant from standardized effect for amylase production. Parity plot represented in (Figure 5) shows a satisfactory correlation between the experimental and predicted responses and the deviation between the values was minimal. The R^2value of 0.9112 indicates that the model could predict 91.12% variability in the response.

Conclusion

In this work, three strains were comparison for amylase ability. *A. oryzae* TISTR 3086 and *B. amyloliquefaciens* TISTR 1045 showed potential amylase abilities, whereas *S. fibuligera* TISTR 5033 exhibited a tiny amylase activity. An attempt to optimize the carbon-nitrogen components required for the optimal amylase production. A statistical approach, Randomized Block Design (RBD) by SPSS was used. *A. oryzae* TISTR 3086 yield a high amylase production with 15% grain sorghum, 0.5% $(NH_4)_2SO_4$ at 30°C, 150 rpm for 24 h and at 37°C, 150 rpm for 24 h for *B. amyloliquefaciens* TISTR 1045. Further study, the Plackett-Burman design was used to screen the mineral salts in the medium. Among the variables, four factors as follows: $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2$ and $FeSO_4$ were found significant optimal values for *A. oryzae* TISTR 3086. Four factors as follows: $(NH_4)_2SO_4$, KH_2PO_4 $MgSO_4$ and NaCl were found significant optimal values for *B. amyloliquefaciens* TISTR 1045. These statistical approaches showed a satisfactory correlation between the experimental and predicted response.

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