

## Optimization of D-Amino Acid Oxidase Production by *Trigonopsis variabilis* Using Glucose Syrup from Cassava as Carbon Source

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### Abstract

Glucose syrup from cassava (GSC) contains glucose as the main sugar which suitable for D-amino acid oxidase (DAAO) production by *Trigonopsis variabilis* economically and efficiently. The aim of this study is to optimize the DAAO production by *Trigonopsis variabilis* using GSC as carbon source via response surface methodology (RSM) with Face-centered Central Composite Design (FCCD). In the first step, the effects of six factors (concentration of GSC and DL-alanine, pH, temperature, incubation time, and inoculum concentration) were screened to obtain the significant factors on DAAO production by Plackett-Burman design. As the result, incubation time, concentration of GSC and DL-alanine showed to be significant factors for DAAO production ( $P < 0.05$ ). These factors were then optimized using RSM with FCCD. The optimized RSM results demonstrated that a quadratic polynomial model was found suitable to define the relation between the incubation times, concentration of GSC and DL-alanine parameters. Moreover, the observed high  $R^2$  value (0.9782) confirms a strong evaluation of experimental data. The optimum condition for DAAO production as follows: GSC and DL-alanine concentration was 12.3% and 0.3%, respectively, and the culture was incubated for 56.1 hours. Using this condition gave DAAO activity 195.38 U/g dry weight of yeast.

**Keywords:** Glucose syrup from cassava; D-amino acid oxidase; *Trigonopsis variabilis*; Optimization; Face-centered response surface methodology

### Introduction

The production of pure chiral compounds via cost-effective method has become an important approach to the fine chemical, agrochemical and pharmaceutical industries. The used of biocatalysts for the production of pure chiral compounds has found existing all over a large area application and was becoming a cost-effective approach. The recent development of biocatalysts processes was to employ on a large-scale an inexpensive enzyme with good properties (i.e., high activity, stability and selectivity) [1]. Over the last few years, the researchers has focused on several enzyme (e.g., proteases, oxidases and hydrolases) by investigating whether on protein scaffold can be changed and got better to fulfill different applications and be taken advantage for use in various fields.

Chiral compounds containing enantiomeric amine or amino acid groups can be produced using a variety of enzymes, such as amino acid dehydrogenases (EC 1.4.1.X), amine oxidases (EC 1.4.3.22), amino acid oxidases (EC 1.4.3.X), aminotransferases (EC 2.6.4.X), ammonia lyase (EC 4.3.1.X), and lipases (EC 3.1.1.X) [2]. Deracemise a DL-mixture to the L-isomer can be done by employing a D-amino acid oxidase (DAAO, EC 1.4.3.3) and a non-selective chemical reduction step to afford enantiopure L-amino acid after numerous cycles [3]. DAAO catalyzes the oxidative deamination of D-amino acids to produce  $\alpha$ -keto and ammonia.

DAAO are used in the first step in bioconversion of cephalosporin C (CPC) to 7-ACA, whereby the 7-ACA is used as a precursor for the synthesis of the active pharmaceutical ingredient (API) [4]. This process proposed two-step cleavage with D-amino acid oxidase (DAAO) and glutaryl acylase (GAC) [5]. The recent innovation has been used a one-step mono-enzymatic process using cephalosporin C acylase [6]. DAAO was also used in biosensors, in the resolution of natural (such as methionine and phenylalanine) and synthetic (such as naphthylalanine) amino acid racemic solutions [7], and in keto acids production [8]. DAAO can be found in mammalian organs, mainly in the kidneys. DAAO can also be produced from the following types of microorganism by fermentation, such as the yeasts *Trigonopsis variabilis*, *Rhodotorula gracilis*, *Candida tropicalis*, and fungi *Neurospora crassa* [9]. *Trigonopsis variabilis* DAAO has the high catalytic activity for CPC oxidation to produce intermediate compound glutaryl-7 amino cephalosporanic acid.

DAAO can be produced through fermentation using medium containing carbon source, nitrogen, mineral, etc. For industrial scale, the utilization of raw materials based on local resources for extractive and fermentative process has to be considered to achieve economic aspect [10]. Indonesia has an abundant agro-industrial product that can be used as raw material for specific purpose of product. Agro industrial products (molasses, cassava, etc.) have potentially been used as substrate in fermentation industry. Based on preliminary studies, it is known that glucose syrup from cassava is better when compared with molasses or fructose syrup from sorghum, so it can be used as an alternative carbon source. Glucose syrup from cassava (GSC) contains glucose as the main sugar [11], where glucose is the best carbon source for DAAO production [12]. GSC can act as a source of carbon and it

also contain a little amount of nitrogen which is needed during culture cultivation and also enzyme biosynthesis, that makes DAAO production economical and efficient. As agricultural country, in 2015, Indonesia is among the 3 largest cassava producing countries in the world, producing more than 24 million tons and increasing every year [13], so the availability of raw materials can be guaranteed for mass scale production.

Statistical technique, including combination of Plackett-Burman design (PBD) and central composite design (CCD) are suitable and the most widely method used for optimization of biological processes [14,15]. Plackett-Burman design (PBD), developed by Plackett and Burman. It was designed to improve the quality control process that could be used to study the effects of design parameters on the system state so that intelligent decisions can be made. Plackett and Burman (PB) devised orthogonal arrays are useful for screening, which yield unbiased estimates of all main effects in the smallest design possible [16]. CCD is the most popular of the many class of RSM. It is widely used for estimating second order response surfaces. The choice of axial distance  $\alpha$  is based on the region of interest. Choosing the appropriate values of  $\alpha$  specifies the type of CCD. Suppose it is of interest to build a second-order response surface model and the biologist is interested in predicting growth of the organism inside and on the perimeter of the cuboidal region produced by the cube. In addition, for biological reasons, one cannot experiment outside the cube, though experimentation at the extremes in the region is permissible and, in fact, desirable. This scenario, which occurs frequently in many scientific areas, suggests a central composite design in which the eight corners of the cube are centered and  $\alpha=1$ , often called the face-centered central composite design (FCCD) [17]. FCCD has been widely used in enzyme production [18,19].

The aim of this study is to optimize the production of DAAO by *Trigonopsis variabilis* using GSC as carbon source. The optimization was done by response surface methodology (RSM) to enhance the production. Important production parameters screened by Plackett-Burman design and the optimization of significant factors were done using Face-centered Central Composite Design (FCCD).

## Experimental Sections

### Materials

*Trigonopsis variabilis* was used as source of the enzyme, obtained from Biotech Center- BPPT (Serpong, Tangerang, Indonesia). Glucose syrup from cassava (GSC) obtained from PT. Rejo Madusari (Pati, Central Java, Indonesia). DL-alanine were purchased from HiMedia.  $K_2HPO_4$ ,  $KH_2PO_4$ ,  $MgSO_4 \cdot 7H_2O$ , NaCl,  $CaCl_2$ ,  $MnCl_2 \cdot 4H_2O$ ,  $ZnSO_4 \cdot 7H_2O$ ,  $CuCl_2 \cdot 3H_2O$ ,  $H_3BO_3$ ,  $FeCl_3 \cdot 6H_2O$  were purchased from Merck (Darmstadt, Germany). The other chemicals, such as thiamin, biotin, o-phenylenediamine, horseradish peroxidase were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All experiments were carried out in duplicate.

### Inoculum preparation

The strain was maintained in yeast malt medium. The cultures were kept at 4°C and sub cultured at regular intervals of 30 days. Pre culture medium (pH 6) contained: 22 g/L CGS, 4 g/L DL-alanine, 2 g/L  $K_2HPO_4$ , 0.21 g/L  $KH_2PO_4$ , 0.5 g/L  $MgSO_4 \cdot 7H_2O$ , 0.1 g/L NaCl, 0.1 g/L  $CaCl_2$ , 0.105 g/L  $MnCl_2 \cdot 4H_2O$ , 0.0231 g/L  $ZnSO_4 \cdot 7H_2O$ , 0.042 g/L  $CuCl_2 \cdot 3H_2O$ , 0.105 g/L  $H_3BO_3$ , 0.0714 g/L  $FeCl_3 \cdot 6H_2O$ , 0.24 mg/L

thiamin and 0.02 mg/L biotin. GSC and DL-alanine was sterilized using 0.2  $\mu$ m filter. The other components were sterilized in the autoclave at 121°C for 15 min. Inoculum was prepared by taking a loop full of fresh culture (72 h) and suspended in 5 mL of sterile saline water to get an appropriate suspension ( $OD_{600}=0.1-0.2$ ), then 1 mL of culture suspension was inoculated to 50 ml pre culture medium in 250 ml Erlenmeyer flask. The culture was then incubated in an orbital shaker at 30°C and 200 rpm for 24 h for inoculum development.

### Production of DAAO

Production medium (pH 6) contained: 32 g/L GSC, 6.2 g/L DL-alanine, 2 g/L  $K_2HPO_4$ , 0.21 g/L  $KH_2PO_4$ , 0.5 g/L  $MgSO_4 \cdot 7H_2O$ , 0.1 g/L NaCl, 0.1 g/L  $CaCl_2$ , 0.105 g/L  $MnCl_2 \cdot 4H_2O$ , 0.0231 g/L  $ZnSO_4 \cdot 7H_2O$ , 0.042 g/L  $CuCl_2 \cdot 3H_2O$ , 0.105 g/L  $H_3BO_3$ , 0.0714 g/L  $FeCl_3 \cdot 6H_2O$ , 0.24 mg/L thiamin and 0.02 mg/L biotin. GSC and DL-alanine was sterilized using 0.2  $\mu$ m filter. The other components were sterilized in the autoclave at 121°C for 15 min.

Production of DAAO were carried out by transferring 10% inoculum ( $OD_{600}=0.7-0.8$ ) to the production medium and then incubated in an orbital shaker at 30°C and 140 rpm for 72 h.

### Permeabilization of cell

The cells were harvested and neutralized to pH 6.0 using potassium hydroxide, then centrifuged at 10,000 g at 4°C for 10 min to obtain the cell, and then washing with potassium phosphate buffer (pH 8.0) twice. Certain volume of cells were taken and centrifuged, washed with distilled water and dried at 105°C to reach a constant mass to determined dry cells weight [20]. The rest of washed cells were suspended using the same buffer. The suspension cells were permeabilized using 5% toluene-ethanol (1:1) and held for 1 h at 37°C. The permeabilized cells were used for enzyme assay.

### Enzyme assay

The DAAO activity were measured by o-phenylenediamine/horseradish peroxidase coupling assay [21]. The reaction mixture contained 30 mM D-alanine, 0.03% o-phenylenediamine, 1700 U horseradish peroxidase and DAAO of interest in 100 mM potassium phosphate buffer (pH 8.0). The reaction was monitored by an increase in absorbance at 450 nm for 3 min at 25°C.

One unit of enzyme activity was defined as the enzyme needed to produce 1 micromole of  $H_2O_2$  per min at 25°C and pH 8.0.

### Experimental design for optimization

Optimization were applied in two stages, first to identify the most significant factors for DAAO production using Plackett-Burman design and later the significant factors were optimized by using RSM with face-centered central composite design. The experimental design and statistical analysis were done using Design Expert software (version 7.1)

### Plackett-Burman design

The Plackett-Burman design is very useful for screening the most important factors with respect to their main effects. This model does not describe interaction among factors, it is used to screen and evaluate the important factors that influence the response. Six different independent variables including carbon (glucose syrup from cassava)

concentration, (DL-alanine) concentration, pH, incubation time (period), temperature and inoculum quantity were selected for screening. Each variable was tested at two levels, namely a high level denoted by (+1) and a low level denoted by (-1) as listed in Table 1. Six variables were screened by twelve experiments conducted by Plackett-Burman design (Table 2). All experiments were carried out in duplicate and the average value of DAAO activity was used for statistical analysis.

Code	Variables	Low level (-1)	High Level (+1)
A	GSC (%)	10	15
B	DL-Alanine (%)	0.3	0.7
C	pH	6	8
D	Temperature (°C)	28	32
E	Incubation time (Hours)	48	72
F	Inoculum (%)	5	10

**Table 1:** Six factors and their levels used in Plackett-Burman design.

Run No.	A	B	C
1	1	1	-1
2	-1	0	0
3	-1	1	-1
4	0	0	-1
5	-1	-1	1
6	1	1	1
7	-1	1	1
8	0	0	0
9	1	0	0
10	0	0	0
11	0	0	1
12	0	0	0
13	0	-1	0
14	1	-1	-1
15	0	0	0
16	1	-1	1
17	0	0	0
18	-1	-1	-1
19	0	0	0
20	0	1	0

**Table 2:** Matrix of Face-centered central composite design.

### Face-centered central composite design (FCCD)

This step involved optimization of the levels and the interaction effects between various significant variables. In this study, the experimental plan consisted of 20 trials and the independent variables were studied at three different levels: low (-1), middle (0), and high (+1) [22]. The center points were repeated six times in order to evaluate the curvature and the experiment replication facilitated the pure error estimation, so that the significant lack of fit of the models could be predicted. All the experiments were done by duplicate and the average of DAAO activity (U/g yeast cell dry weight) obtained were taken as the dependent variable or response (Y).

The levels of factors used for experimental design are given in Table 3. For three factors, a fractional factorial design with six replications of center points to have a total number of 20 runs.

Run no.	A	B	C	D	E	F	DAAO activity (U/g YCDW)
1	10	0.3	8	28	72	10	87.6509
2	10	0.3	6	28	48	5	85.8477
3	15	0.3	8	32	72	5	43.8850
4	15	0.7	8	28	48	5	66.3733
5	10	0.7	8	32	48	5	68.5606
6	15	0.7	6	28	48	10	66.6269
7	15	0.7	6	32	72	10	32.6922
8	10	0.7	6	32	72	5	41.7822
9	10	0.7	8	28	72	10	50.2795
10	15	0.3	6	28	72	5	52.4000
11	15	0.3	8	32	48	10	79.5999
12	10	0.3	6	32	48	10	87.0552

**Table 3:** Plackett-Burman screening, representing the response of DAAO production as influenced by GSC (A), DL-alanine (B), pH (C), temperature (D), incubation time (E), and inoculum (F).

## Results and Discussion

### Screening by Plackett-Burman design

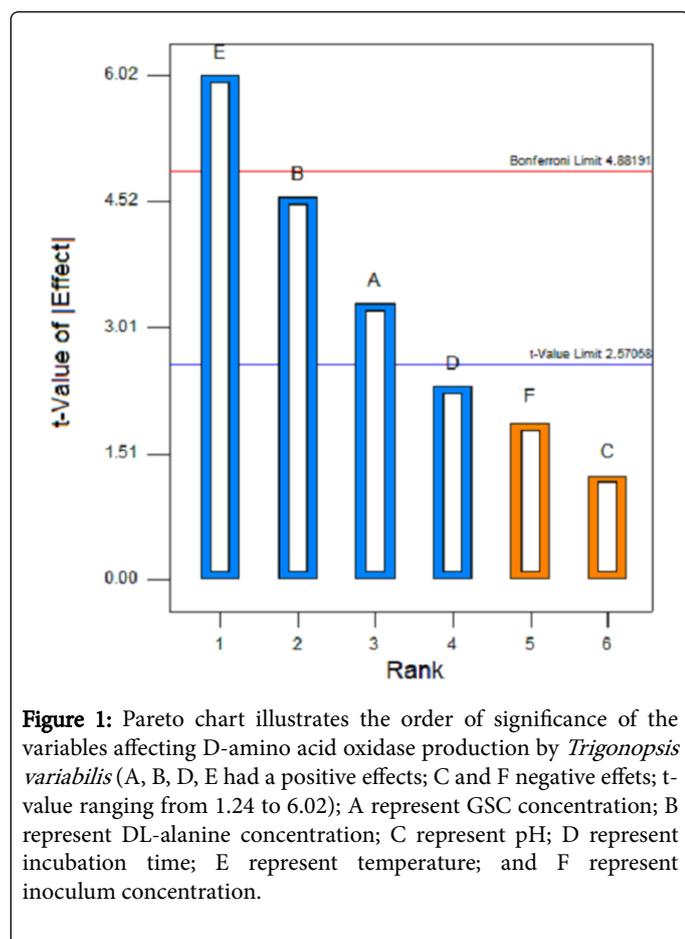
Plackett-Burman design was used to identify the important parameters for DAAO production. Table 3 represents the results of the screening of significant variable for D-amino acid oxidase production and the corresponding response (Y) using Plackett-Burman design. Statistical analysis of the DAAO activity were performed and represented in Table 3. With respect to the main effect of each variable, it shows that two variables named pH and inoculum concentration positively effect on DAAO production, where the other four variables named GSC and DL-alanine concentration, temperature and incubation time negatively effect on DAAO production. The T and P values were used to identify the effect of each factor on DAAO production as shown in Table 4. Incubation time, GSC and DL-alanine concentration had a significant effect on DAAO activity (P<0.05). The R<sup>2</sup> values provide a measure of how much variability in the observed response values can be explained by the experimental factors. In this

study, the value of the  $R^2$  were 0.9400, indicates that 94% of the variability in the response were attributed to the given independent variables and only 6% of the total variations are not explained by the independent variables. The Pareto chart (Figure 1) illustrate the order

of significance variables affecting DAAO production in Plackett-Burman experimental design, showed the incubation period, concentration of GSC and DL-alanine were the significant parameters.

Variables	Main effect	Coef.	T	P value	Source	Sum Squares	of	df	Mean Square	F Value	p-value Prob > F
A-GSC	-13.27	-6.63	-3.3	0.0215	Model	344.27		6	57.38	12.69	0.0068*
B-DL-alanine	-18.35	-9.18	-4.56	0.006	GSC	53.87		1	53.87	11.91	0.0182*
C-pH	4.99	2.5	1.24	0.2696	Inducer	87.61		1	87.61	19.37	0.0070*
D-Temperature	-55.6	-4.63	-2.3	0.0694	pH	2.75		1	2.75	0.61	0.4709
E-Incubation time	-4.04	-12.11	-6.02	0.0018	Temperature	24.71		1	24.71	5.46	0.0666
F-Inoculum	7.51	3.75	1.87	0.1208	Incubation time	161.62		1	161.62	35.73	0.0019*
					Inoculum	13.7		1	13.7	3.03	0.1422
					Residual	22.62		5	4.52		
					Cor Total	366.89		11			

**Table 4:** Statistical analysis results from Plackett-Burman design showing major effects, coefficient values, t-test (T), P-values for each variable affecting on DAAO production and analysis of variance. T: Student's test; P: corresponding level of significance; R square 0.9400 and adjusted R square 0.8680.



### Optimization by face-centered central composite design

Face-centered central composite design was employed to study the optimal levels and the interactions among the selected significant factors. The others factors were maintained at a constant level which gave maximal yield in the Plackett-Burman experiments. In this study, a total of 20 experiments with different combination of GSC concentration (A), DL-alanine concentration (B), and incubation time (C) were performed and the results of experiments for studying the effect of three independent variables on DAAO activity are presented along with predicted response and residuals (Table 5). The minimum DAAO activity (46,799 U/g YCDW) were observed in run number 6, while the maximum DAAO activity (193.880 U/g YCDW) were achieved in run number 8.

Run no.	A	B	C	DAAO activity (U/g yeast cell dry weight)	
				Experimental	Predicted
1	15	0.7	48	115.280	113.984
2	10	0.5	60	155.235	158.368
3	10	0.7	48	132.521	129.619
4	12.5	0.5	48	141.311	147.529
5	10	0.3	72	94.320	93.662
6	15	0.7	72	46.799	44.664
7	10	0.7	72	77.155	77.401
8	12.5	0.5	60	193.880	173.939
9	15	0.5	60	129.079	133.762
10	12.5	0.5	60	176.962	173.939

11	12.5	0.5	72	85.828	87.425
12	12.5	0.5	60	168.451	173.939
13	12.5	0.3	60	189.201	190.930
14	15	0.3	48	130.277	128.077
15	12.5	0.5	60	163.189	173.939
16	15	0.3	72	59.137	60.085
17	12.5	0.5	60	188.640	173.939
18	10	0.3	48	144.371	144.552
19	12.5	0.5	60	168.142	173.939
20	12.5	0.7	60	169.668	175.754

**Table 5:** Face-centered central composite design represents the response of DAAO production as influenced by GSC (A), DL-alanine

Variables	Coefficient	SE coefficient	T	P	Source	Sum Squares	df	Mean Square	F Value	p-value Prob>F
A	114.92	24.589	4.673	0.001	Model	36220.1	9	4024.46	42.99	<0.0001*
B	-269.97	190.093	-1.42	0.186	A-GSC	1513.64	1	1513.64	16.17	0.0024*
C	46.4	5.123	9.057	0	B-DL-alanine	575.85	1	575.85	6.15	0.0325*
A × A	-4.46	0.933	-4.778	0.001	C-Incubation time	9031.39	1	9031.39	96.48	<0.0001*
B × B	235.08	145.859	1.612	0.138	AB	0.35	1	0.35	0	0.9523
C × C	-0.39	0.041	-9.678	0	AC	146.24	1	146.24	1.56	0.2398
A × B	0.42	6.841	0.061	0.952	BC	0.88	1	0.88	0.01	0.9246
A × C	-0.14	0.114	-1.25	0.24	A <sup>2</sup>	2136.71	1	2136.71	22.83	0.0007*
B × C	-0.14	1.425	-0.097	0.925	B <sup>2</sup>	243.16	1	243.16	2.6	0.1381
					C <sup>2</sup>	8766.89	1	8766.89	93.65	<0.0001*
					Residual	936.11	10	93.61		
					Lack of Fit	174.63	5	34.93	0.23	0.9341
					Pure Error	761.48	5	152.3		
					Cor Total	37156.21	19			

**Table 6:** Statistical analysis of face-centered central composite design showing coefficient values, standard error (SE) coefficient, t-test (T), P-values and analysis of variance. A: the coded value of GSC, B: the coded value of DL-alanine, and C: the coded value of incubation time; T : Student's test; P : corresponding level of significance; F : Fisher's function R square 0.9748 and adjusted R square 0.9521.

All the interactions (A × B; A × C; and B × C) are not significant, indication that there is no significant correlation between each two variables and they did not help much in increasing the production of DAAO. In order to evaluate the relationship between dependent and independent variables and to determine the maximum DAAO production were proposed to calculate the optimum levels of the variables. By applying the multiple regression analysis on experimental data, the equation that defines predicted response (Y) can be shown as follows:

(B), and incubation time (C) along with the predicted DAAO production. A: the coded value of GSC, B: the coded value of DL-alanine, and C: the coded value of incubation time.

Analysis of variance (ANOVA) was used to analyze the data (Table 6). The goodness of fit of the model was checked by the coefficient of determination (R<sup>2</sup>), which found to be 0.9782. the present R<sup>2</sup>-value reflected a very good fit between the observed and predicted responses and implied that the model is reliable for DAAO production in the present study. The P-values denote the significance of the coefficients and are also important in understanding the pattern of the mutual interactions between the variables. Interactions between two factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient).

Table 6 shown that the GSC (A), DL-alanine, incubation time (C), quadratic effect of cassava glucose syrup (A) and incubation time (C) are significant.

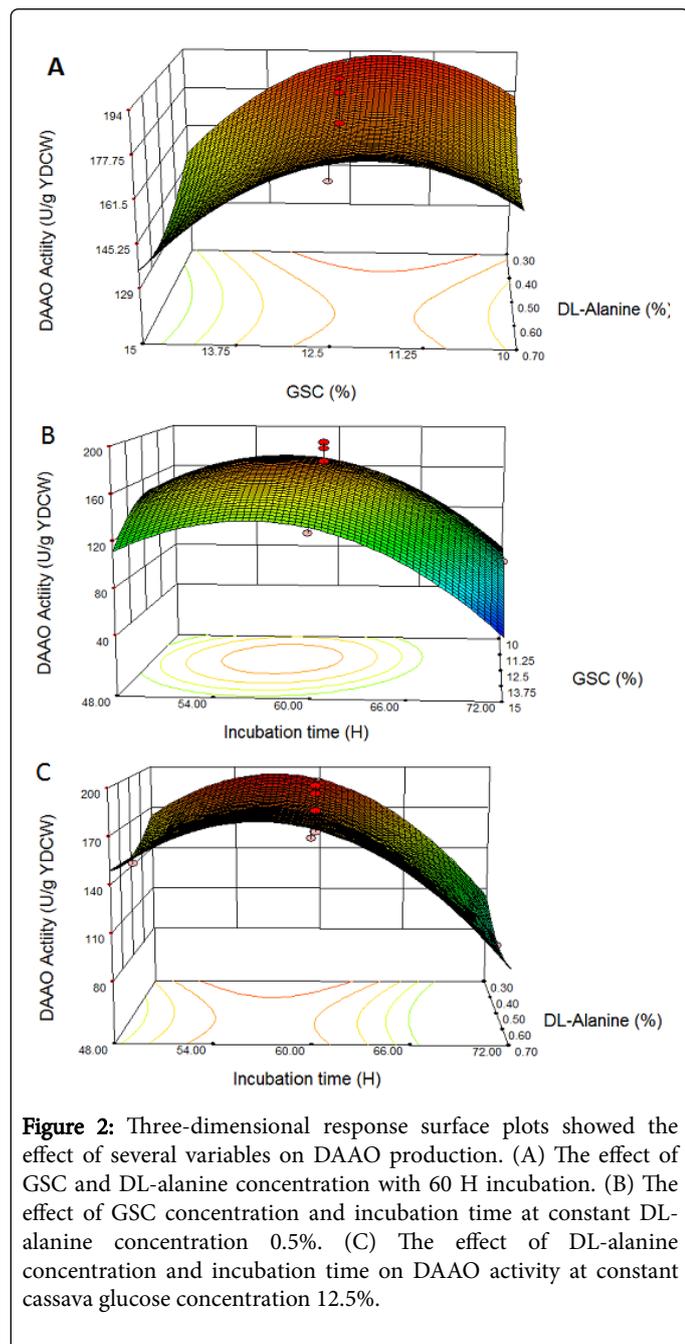
$$Y(\text{DAAO activity})=173.94-12.30 \times A-7.59 \times B-30.05 \times C+0.21 \times A \times B-4.28 \times A \times C-0.33 \times B \times C-27.87 \times A^2+9.40 \times B^2-56.46 \times C^2 \quad (1)$$

The interaction effects and optimal levels of the variables were determined by plotting the three-dimensional response surface curves (Figures 2a-2c) when one of the variables is fixed at optimum value and the other two are allowed to vary.

Figure 2a represents the effect of varying GSC and DL-alanine concentration on DAAO production at constant incubation time 60 h. The increase of DAAO yield occurred with an increase of GSC concentration at DL-alanine concentration from 0.3% to 0.4%. Further

increase of DL-alanin concentration would decrease the DAAO yield. According to this interaction effects, the maximum yield of DAAO activity were  $\approx 193.88$  U/g at GSC  $\approx 12.5\%$  and DL-alanin concentration  $\approx 0.3\%$ .

Figure 2b shows the cooperative effect of GSC concentration and incubation time at constant DL-alanin concentration 0.5%. As shown in Figure 2(b), at low and high value of GSC concentration and incubation time, the DAAO activity decreases. The maximum DAAO activity  $\approx 193.88$  U/g were obtained at GSC concentration  $\approx 12.5\%$  and incubation time  $\approx 56$  h.



**Figure 2:** Three-dimensional response surface plots showed the effect of several variables on DAAO production. (A) The effect of GSC and DL-alanine concentration with 60 H incubation. (B) The effect of GSC concentration and incubation time at constant DL-alanine concentration 0.5%. (C) The effect of DL-alanine concentration and incubation time on DAAO activity at constant cassava glucose concentration 12.5%.

Figure 2c shows the effect of both DL-alanin concentration and incubation time on DAAO activity at constant cassava glucose

concentration 12.5%. It is obvious that with the increase of DL-alanin concentration above 0.3%, the DAAO activity decreases. The maximum DAAO activity  $\approx 193.88$  U/g obtained at lowest DL-alanin concentration  $\approx 0.3\%$  and incubation time  $\approx 56$  h.

### Verification of the model

In order to determine the accuracy of the model and to verify the result, an experiment under the optimal conditions from RSM were performed and compared with the predicted data. The measured DAAO activity obtained were 195.38 U/g, close to the predicted one 196.38 U/g, indicating a high degree of accuracy. The predicted optimal levels of the process variables for DAAO production by *Trigonopsis variabilis* were 12.3% of GSC, 0.3% of DL-alanine and 56.1 hours of incubation.

### Conclusions

The experimental design is useful to enhance the DAAO production. Plackett-Burman design can reduce the factors that not significantly affect to the production. Results of Plackett-Burman design showed that the incubation time, concentration of cassava glucose syrup and DL-alanine was significant factor for DAAO production. Optimization of significant factors was done using Face-centered Central Composite Design (FCCD) and the results were evaluated using RSM. The optimum condition for DAAO production as obtained from RSM were as follows: GSC concentration 12.3%, DL-alanine (inducer) concentration 0.3% and 56.1 hours of incubation. At these optimum levels, DAAO activity reaches 195.38 U/g dry weight of yeast. The results of this study could be used to design a suitable medium using GSC as carbon source, for economically and efficiently production of DAAO.

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