Indian Journal of Biotechnology
Vol 10, January 2011, pp 65-69

Production of ganoderic acid by *Ganoderma lucidium* MTCC 1039 from cottonseed oil cake: Statistical screening of process variables

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Production of ganoderic acid (GA) using submerged fermentation has received great attention in recent years for its increased demand in the treatment of hepatitis-B and to reduce blood pressure, blood cholesterol and platelet aggregation. Present study aims to produce GA by *Ganoderma lucidium* MTCC 1039 from cottonseed oil cake and to identify the important media components and operating conditions. The effect of variables, such as cottonseed oil cake, glucose, ammonium chloride, di-sodium hydrogen phosphate, sodium di-hydrogen phosphate, magnesium sulfate, *p*H and inoculums size was studied using two level Plackett-Burman design. The experimental GA production was subjected to statistical analysis using MINITAB 15 and statistical significance of the variables was analyzed. The first order linear model showing the effect of variables on GA production was validated with regression coefficient (R^2) 0.92 and normal probability plot. The statistical significance of the variables was studied using student's t-test and pareto chart. It was found out that the cottonseed oil cake and ammonium chloride are the highly significant and glucose and magnesium sulfate are the most important factors on the production of GA by *G. lucidium* MTCC 1039.

Keywords: Ganoderic acid, Pareto chart, Plackett-Burman design, process variables, submerged fermentation.

Introduction

Ganoderma lucidum is one of the most famous traditional Chinese medicinal mushrooms. The first mention of this mushroom dates back to the period of the first emperor of China, Shih-huang of the Chin Dynasty (221-227 BC). *G. lucidum* mushroom is used in herbal remedies for the last thousands of years to promote immune function. Its fruiting body is called 'Reishi or Mannentake' in Japanese and 'Lingzhi' in Chinese language. In regions of China, Japan, Korea and Taiwan, Lingzhi has been a popular folk-oriental medicine used to treat various human diseases. Evidences concerning medicinal application of *G. lucidum* in the treatment of various diseases, such as cancers and immunological disorders, as well as its biotechnological utilization in recent years have made this mushroom very popular[1]. In a symposium at 230th National Meeting of American Chemical Society, held at Washington, DC during 28th August to 1st September 2005, research on traditional medicines, including edible-medicinal mushroom Reishi, were highlighted[2].

G. lucidum has been reported to have immunity regulation properties as well as anti-tumour, anti-viral, gastric cancer and hepato-protective activities[3-6]. Polysaccharide produced by *G. lucidum* is a type of carcinostatic agent, which has anti-tumour and hypoglycemic activities[7,8]. The fungus can also produce many species of oxygenated triterpenes, especially ganoderic acid (GA), with various biological functions, such as cytotoxicity to hepatoma cells, inhibition of cholesterol synthesis and absorption, as well as stimulation of platelet aggregation. It is also reported that water extracts of *reishi* reduce blood pressure and platelet aggregation, cholesterol in blood, affect histamin-releasing inhibition and promote anti-tumour activity[9]. GA produced by this mushroom possesses very interesting anti-tumour and anti-HIV-1 activities[10].

It usually takes several months to cultivate the mushroom and the product yield has been low in soil cultivation. As a result, it has been proving difficult to supply the great demand of market with high-quality *G. lucidum* products. Many attempts have been made to increase its intracellular as well as extracellular polysaccharides and other useful components[11].

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Note: This paper was presented in the International Conference on 'Challenges in Biotechnology and Food Technology' organized at Annamalai University, Annamalai Nagar during 8-10 October, 2009.

The present work is aimed to evaluate the effect of medium components and operating conditions on the production of extracellular GA by *G. lucidum* MTCC 1039 in shake culture fermentation by two level Placket-Burman (PB) statistical design using cottonseed oil cake as substrate.

Materials and Methods

Microorganism

G. lucidum MTCC was obtained from Microbial Type Culture Collection, IMTECH, Chandigarh, India.

Cottonseed Oil Cake Powder

The dried cottonseed oil cake (COC), purchased from local market (Chennai, India), was dried overnight at 65ºC to remove moisture, if any, and powdered and sieved. The COC powder passed through 80-mesh and retained by 120-mesh sieve was used as natural substrate for production of GA.

Stock and Preculture of *G. lucidum*

G. lucidum MTCC 1039 strain was cultivated in malt extract agar slants at 25ºC for 10 d and stored at 4ºC. 100 mL of preculture media consisted of the following components: COC 2% (w/v), glucose 2%, ammonium chloride 0.05%, di-potassium hydrogen phosphate 0.05%, potassium di-hydrogen phosphate 0.05% and magnesium sulfate 0.05%, initial *p*H 5.5, was prepared in a 250 mL Erlenmeyer flask. The glucose was autoclaved separately. 5 mL mycelium suspension from slant culture was inoculated and incubated at 30°C, 150 rpm for 7 d.

Screening of Medium Components and Operating Conditions by Plackett-Burman Design

Plackett-Burman (PB) design is an efficient screening design when main effects of the medium components are to be considered. PB design offers a good and fast screening procedure and mathematically computes the significance of a large number of factors in one experiment, which is time saving and gives the effect of change in more than one factors in single experiment[12-15].

PB experimental design is based on the first order model and it is given by equation 1.

$$Y = \beta_0 + \sum \beta_i X_i \quad \ldots (1)$$

Where, "Y" is the response (GA production in mg/L), "β_0" is the model intercept, "β_I" is co-efficient of the variable "i" estimates, "X_i" is independent variable and "i" is the variable number. This model describes no interaction among the factors that influences GA.

In this work, PB design in 12 experimental run was carried out to evaluate the effect of 8 factors of medium components and operating conditions on GA production. Eight assigned and three unassigned variables (commonly referred as dummy variables) were screened in PB design of 12 experiments. Dummy variable (D_1, D_2 and D_3) are used to estimate experimental errors in data analysis[13,14]. All the factors are prepared at two levels "–1" for low level and "+1" for high level. The factors (%, w/v), such as COC (X_1), glucose (X_2), ammonium chloride (X_3), di-potassium hydrogen phosphate (X_4), potassium di-hydrogen phosphate (X_5), magnesium sulfate (X_6), *p*H (X_7) and inoculum size in mL (X_8) were studied. The actual values of the variables at low level (-1) and high level (+1) are given in Table 1. Table 2 shows the factors considered for investigation, the PB design in 12 experimental run and the experimental GA production.

Production of GA

A 100 mL of the production medium was prepared in 250 mL flasks based on two-level Plackett-Burman design (Table 2), inoculated with 4 mL/6 mL of mycelium suspension from preculture and incubated for 5 d at 30°C on a rotary shaker (160 rpm). The samples were taken for each flask and filtered through filter paper under suction.

Extraction of GA

In order to determine the extracellular GA, the fermentation broth filtrate was added with 4 volume of 95% ethanol and left overnight at 4°C to precipitate the crude GA. The precipitated GA was collected by centrifugation at 10000 rpm for 10 min, dried at 60°C to remove the residual ethanol[11].

Phenol-Sulphuric Acid Assay of GA

The dried GA precipitate was suspended in 2 mL of distilled water. 50 µL of suspended sample solution was mixed with 50 µL of 80% phenol solution. The mixture was vortexed with addition of 2 mL concentrated sulfuric acid in a stream. Then the mixture was incubated for 10 min at room temperature and absorbance was read at 490 nm[16].

Result and Discussion

Evaluation of Medium Components and Operating Conditions

The data on GA production using PB experiments showed a wide variation from 211.74 to 964.36 mg/L

for COC used as substrate. The data on GA production level given in Table 2 was subjected to statistical evaluation using MINITAB 15.0 version through multiple linear regression analysis, student's t-test for *P*-value and confidence level. Estimated t-value, *P*-value and confidence level giving the effect of variables on GA production are shown in Table 3.

The t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. In this work, variables with confidence levels greater than 90% were considered as significant. On analysis of student's t-test for calculated t-values, *P*-values and confidence level of the variables, KH_2PO_4 (X_5), $MgSO_4.7H_2O$ (X_6) and *p*H (X_7) have shown positive effects and COC (X_1), glucose (X_2), NH_4Cl (X_3), K_2HPO_4 (X_4) and inoculum size (X_8) have shown negative effects on GA production. On the basis of the calculated *p*-values at 90% confidence level (α=0.10), cottonseed oil cake (confidence level=95.9%) and ammonium chloride (confidence level=94.5%) were identified as the significant media components on GA production.

Based on the PB design, the effect of independent variables on GA production is given by the first order linear model and it is given by equation 2 (given below). The regression coefficient of the model (R^2=0.92) validates that the model is well fitted with the experimental results. Analysis of variance (ANOVA) given in Table 4 shows that the main effect of the independent variables studied are valid with 88.3% confidence level on GA. The effect of the independent variables on GA production is given by first order linear model in equation 2.

$$Y = 619.6 - 157.7X_1 - 96.7X_2 - 104.5X_3 - 30.7X_4 + 48.3X_5 + 1000.9X_6 + 73.5X_7 - 16.3_8 \quad \ldots (2)$$

Table 1—Coded and actual values of the variables

Process variables % (w/v)	COC (X_1)	Glucose (X_2)	NH_4Cl (X_3)	K_2HPO_4 (X_4)	KH_2PO_4 (X_5)	$MgSO_4$ (X_6)	*p*H (X_7)	Inoculum size (mL) (X_8)
Low Level (-1)	2	2	0.1	0.03	0.03	0.03	4.5	4
High Level (+1)	4	4	0.2	0.06	0.06	0.06	6.5	6

Table 2—Two-level Plackett-Burman design for GA production

Experiment No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	D_1	D_2	D_3	GA, mg/L
1	+1	-1	+1	+1	-1	-1	-1	+1	+1	-1	+1	263.103
2	+1	+1	-1	-1	-1	-1	-1	-1	+1	+1	-1	211.740
3	-1	+1	+1	+1	+1	-1	-1	-1	+1	+1	+1	335.429
4	+1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	375.262
5	+1	+1	-1	-1	+1	-1	-1	+1	-1	-1	+1	536.687
6	+1	+1	+1	+1	+1	+1	+1	-1	-1	-1	-1	440.252
7	-1	+1	+1	+1	-1	+1	+1	+1	+1	-1	-1	659.329
8	-1	-1	+1	+1	+1	-1	-1	+1	-1	+1	-1	800.838
9	-1	-1	-1	-1	+1	+1	+1	-1	+1	-1	+1	949.685
10	+1	-1	-1	-1	+1	+1	+1	+1	+1	+1	-1	944.444
11	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1	953.878
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	964.361

Table 3—Estimated effects and coefficients for analysis of Plackett-Burman design on GA production

Variable	Main effect	Coefficients	t-value	P-value	Confidence level, %
Constant		619.6	13.53	0.001	
X_1	-315.3	-157.7	-3.44	0.041	95.9
X_2	-193.4	-96.7	-2.11	0.125	87.5
X_3	-281.1	-104.5	-3.07	0.055	94.5
X_4	-61.3	-30.7	-0.67	0.551	54.9
X_5	96.6	48.3	1.05	0.369	63.1
X_6	201.8	100.9	2.20	0.115	78.5
X_7	146.9	73.5	1.60	0.207	79.3
X_8	-32.7	-16.3	-0.36	0.745	25.1

Table 4—ANOVA for linear model on effect of independent variables on GA production

Source	Degree of freedom (DF)	Sum of squares (SS)	Mean square (MS)	F-value	P-value
Main effects	8	876961	109620	4.36	0.127
Residual error	3	75481	25160		
Total sum of squares	11	952442			

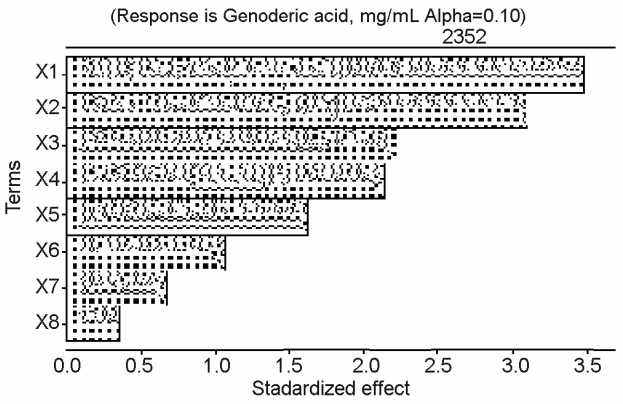

Fig. 1—Effect of medium components and operating conditions on GA production.

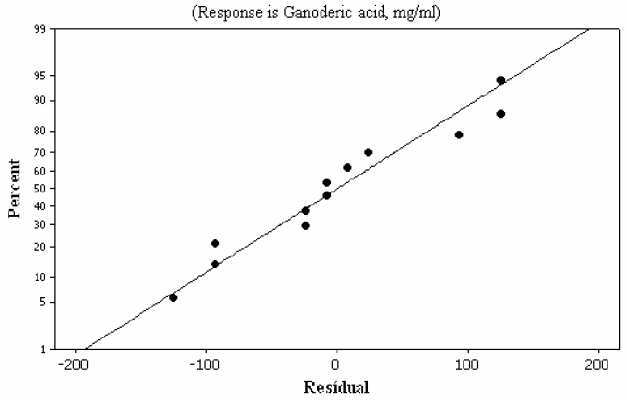

Fig. 2—The normal probability plot of GA production from cotton seed oil cake.

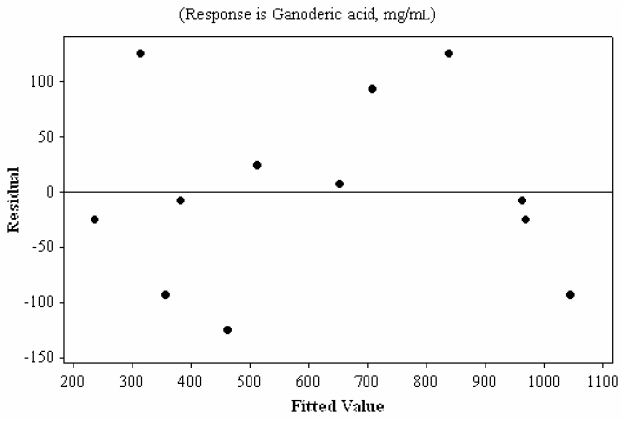

Fig. 3—Residual distribution plot of GA production using cotton seed oil cake.

Pareto chart provides facts needed for setting priorities on media components and process variables, concentrating improvement efforts on these few variables will have a greater impact and be more cost-effective on bioprocess development. Hence, the main effect of media components and process variables on GA production was also studied graphically using Pareto chart as shown in Fig. 1. It shows that the ammonium chloride (X_3) and COC (X_1) are the significant factors at 90% confidence level and the magnesium sulfate (X_6) and glucose (X_2) are as the other 2 most important factors on GA production by *G. lucidum*. The normal probability plot and residual distribution plot are the important diagnostic tools to detect and explain the systematic departure response from the assumptions. In Fig. 2, the normal probability plot of the residuals, is an important diagnostic tool to detect and explain the systematic departures from the assumptions. The residual was plotted against normal distribution of the model and it was approximate linear line for GA production, which shows that the errors are normally distributed and are independent of each other, and the error variances are homogenous. An excellent normal distribution confirmed the normality assumption and the independence of the residuals. This indicates that the model was well fitted with the experimental results. As the residuals from the fitted model are normally distributed, all the major assumptions of the model have been validated. The residual plot in Fig. 3 shows equal scatter of the residual data above and below the x-axis, indicating that the variance was independent of the GA production, thus supporting the adequacy of the model fit.

Conclusion

The effect of media components and operating conditions on the production of GA by *G. lucidum* MTCC 1039 was studied using PB design. The GA production using PB experiments showed a wide

variation from 211.74 to 964.36 mg/L. PB statistical screening results show that the media components and operating conditions influence the GA production by *G. lucidum* with in their selected level. Cottonseed oil cake and ammonium chloride were found to be the significant factors and magnesium sulfate and glucose were the other two most important factors on GA production by *G. lucidum* using a low-cost natural substrate, cotton seed oil cake, in submerged fermentation.

Acknowledgement

Authors wish to acknowledge the authorities of St. Joseph's College of Engineering, Chennai for providing laboratory facilities and constant encouragement to carry out this research work.

Nomenclature

ANOVA – Analysis of variance
GA – Ganoderic acid
F – Fishers's function
i – Variable number
P – Corresponding level of significance
PB – Plackett-Burman
t – Student's test
X - Independent variables
Y – Predicted response
β_0 – Model intercept
β_i – Variable estimate

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