

Application of Plackett-Burman Design for Screening The Media Components for Citric Acid Production From Paddy Straw Using Solid-State Fermentation

Hayder Kh. Q. Ali^{1,*} and M.M.D. Zulkuli¹

¹*School of Bioprocess Engineering, University Malaysia Perlis, Kompleks Pusat Pengajian Jejawi 3, Arua, 02600, Perlis, Malaysia*

*Corres.author: enghayder_alsafar@yahoo.com, Phone no. 0060126141598

Abstract: The study of the screening of the media constitutions to improve the citric acid fermentation from paddy straw by *Aspergillus niger* was reported. Plackett-Burman Design (P-BD) was employed to evaluate the significant parameters that have large effect on the fermentation and with this experimental design a successful results were obtained. The screening of the 7 mineral salts (NH₄NO₃, KH₂PO₄, MgSO₄, CuSO₄, ZnSO₄, FeSO₄ and MnSO₄) to gauge the most significant nutrients on citric acid concentration, pH and final molasses concentration were concluded. It was found that NH₄NO₃ was the most significant nutrient. Moreover, KH₂PO₄ and MgSO₄ affect were editing the citric acid production indirectly through the reduction of molasses concentration and pH of the fermentation culture, respectively.

Keywords: Citric Acid, Solid-State Fermentation, *Aspergillus niger*, Media components, paddy Straw, Plackett-Burman Design.

Introduction

Citric acid is one of the microbial commercially valuable products, which consider one of the world's largest tonnages of fermentation products. It has been commercially produced by submerged fermentation (SmF) of sucrose and molasses based media using *Aspergillus niger* [1]. Citric acid has many applications such as in food and beverage industries as acidifying and flavor-enhancing agent, also play a significant role in pharmaceutical industry. The last three decades, shows more interest in solid-state fermentation (SSF) as an alternative technique to SmF due to many advantages such as low energy requirement, less risk of bacterial contamination, low waste water generation, less environmental concerns, and higher yield produced, so it consider as environmental friendly technique [2].

Many factors (physical and chemical) which were observed to have an effect on the *A. niger* ability to produce the citric acid such as temperature, pH, inoculums size, rate of mixing, aeration, moisture level, carbon source, nitrogen source and mineral salts in the media have been recorded. Considering the chemical factors such as nitrogen source and mineral salts which they have strong effects on the rate of accumulation of citric acid through the SSF [3]. Many researchers are encouraged to study the effect of these additives on the citric acid production. The work of Dawson *et al.* has shown that the nitrogen and potassium concentrations should be limited. The excess of these two components would enhance the cell to consume the carbon source towards energy and biomass instead of other metabolic products [4]. Tran *et al.* found through their study the effects of metal

ions (Fe, Zn, Mn, Cu, and Mg) on the production of citric acid [5].

This paper reported the screening of the media components to improve the citric acid production. Statistical analysis through Plackett-Burman Design was used for screening the media components.

Material and methods:

Microorganism:

The fungal strain of *Aspergillus niger* was obtained from the culture collection laboratory in the School of Bioprocess, University Malaysia Perlis. *A. niger* was grown and cultured in a selective agar containing (g/L): molasses: 140, NH₄NO₃: 2.5, KH₂PO₄: 1.0, MgSO₄: 0.25, CuSO₄: 0.0048, ZnSO₄: 0.0038, FeSO₄: 0.0022, MnSO₄: 0.001, [6]. The fungal culture was incubated for 7 days at 32 °C. The spore suspensions were suspended by using glass beads to collect the spores in distilled water. The spores were counted using haemocytometer to maintain the density of 1-2*10⁷ spores/ml. The fungal strain was maintained on the same agar slants, stored at 4 °C sub-cultured once in a twenty days.

Experimental procedure for solid-state fermentation

The paddy straw was pretreated with 0.5M NaOH (12% solid-liquid ratio) for 2 hour at room temperature. Then, it washed with water sufficiently to remove the base, and then it was dried in an oven at 70 °C overnight [7].

Molasses were treated with sulfuric acid in order to theoretically convert all the sucrose in the molasses to glucose. Subsequently the pH of the molasses was adjusted to 4.00 by adding 1N H₂SO₄. Left for 1.5hr at room temperature then followed by centrifugation at 10000 rpm for 10min. Then it was neutralized with sodium carbonate and was left over night. The supernatant was diluted to the desired concentration [8].

Subsequent experiments were conducted in 500 ml Erlenmeyer flasks. 2g of pretreated paddy straw was added into the flask and the moisture adjusted to level of 80%, an initial pH of 5.5 was adjusted and molasses concentration was 140 g/L. The amount of spore suspension inoculated was 10% of the solid substrate quantity. The flasks were incubated at 32 °C. All experiments were performed in triplicate.

Analytical assay:

100 ml distilled water was added at the end of fermentation and it shaken at 150 rpm for 1 hour at room temperature for citric acid extraction. The pH of the extracted solution was determined. The citric acid was determined spectrophotometrically at 420 nm by the acetic anhydride-pyridine method that developed by Miller [9]. Total reducing sugars in molasses were measured by 3-5 dinitrosalicylic acid (DNS) method of Marier and Boulton [10]. The cellulose content in paddy straw was determined by using Updegraff method [11].

Experimental Design:

The Plackett-Burman design was used for screening of the factors (media components) that significantly influenced citric acid production. The design considers the main effect of these variables but not their interaction effects [12]. It can represent by first-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^n \beta_i x_i \quad \dots\dots (1)$$

Where Y represents the response, β_0 is the model coefficient, β_i is the liner coefficient, x_i is the variables, and n is the number of parameters (variables). Each variable was represented in two levels, i.e. high (+) and low (-).

The effect of each variable was determined by the following equation:

$$E(x_i) = \frac{\sum M_{i+} - \sum M_{i-}}{N} \quad \dots(2)$$

Where $E(x_i)$ is the response value effect of the tested variable; $\sum M_{i+}$ is the summation of the response value at high level, $\sum M_{i-}$ is the summation of the response value at low level, and N is the number of experiments.

Table 1 represented the selected variables to be evaluated, whereas, Table 2 showed the design matrix; seven assigned variables were screened in the 12 experimental runs. The citric acid, pH and consumed sugar were carried out in triplicate. The factors significant at 95% level (p -value < 0.05) were considered reliable [13]. The statistical analyses were performed by use of multiple regression and ANOVA with the Minitab V.15 software.

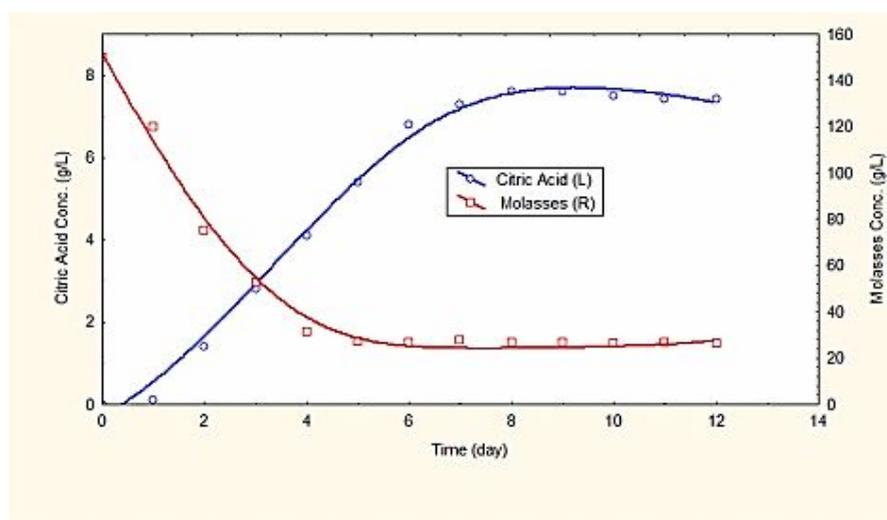


Fig 1. Concentration of citric acid and molasses during fermentation time

Table 1: The table showed the levels, actual values of the factors tested in Plackett-Burman design.

Variables	NH ₄ N O ₃	KH ₂ PO ₄	MgSO ₄	CuSO ₄	ZnSO ₄	FeSO ₄	MnSO ₄
<i>Coded</i>	A	B	C	D	E	F	G
<i>Low level (-)</i>	0	0	0	0	0	0	0
<i>High level* (+)</i>	2.5	2.5	0.25	0.00006	0.001	0.00025	0.0013

*The concentration of the components is in g/L

Table 2: Twelve trial Plackett-Burman Design with the responses

Run	A	B	C	D	E	F	G	Citric Acid ^a	Reduce Sugar ^b	pH ^c
1	+	-	+	-	-	-	+	4.44	83.86	33.89
2	+	+	-	+	-	-	-	4.36	85.46	37.13
3	-	+	+	-	+	-	-	7.47	62.93	46.53
4	+	-	+	+	-	+	-	4.63	82.14	37.59
5	+	+	-	+	+	-	+	5.20	85.46	37.59
6	+	+	+	-	+	+	-	4.32	85.46	34.82
7	-	+	+	+	-	+	+	6.70	63.05	46.53
8	-	-	+	+	+	-	+	7.28	44.82	47.30
9	-	-	-	+	+	+	-	8.47	52.58	47.61
10	+	-	-	-	+	+	+	6.74	82.01	43.29
11	-	+	-	-	-	+	+	8.39	64.77	46.68
12	-	-	-	-	-	-	-	7.12	64.16	46.53

^a (g/L) final Conc. of citric acid

^b (%) the amount of consume sugar

^c (%) reduce of acidity

Table 3: ANOVA for the three responses

	<i>Citric Acid</i>				<i>Reduce Sugar</i>				<i>pH</i>			
	<i>Effect</i>	<i>Coef.</i>	<i>p-value</i>	<i>S.</i>	<i>Effect</i>	<i>Coef.</i>	<i>p-value</i>	<i>S.</i>	<i>Effect</i>	<i>Coef.</i>	<i>p-value</i>	<i>S.</i>
<i>NH₄NO₃</i>	-2.623	-1.312	0.001	*	25.347	12.673	0.001	*	-9.478	-4.739	0.003	*
<i>KH₂PO₄</i>	-0.373	-0.187	0.283		6.260	3.130	0.050	*	-1.155	-0.577	0.468	
<i>MgSO₄</i>	-0.907	-0.453	0.040	*	-2.030	-1.015	0.418		-2.028	-1.014	0.232	
<i>CuSO₄</i>	-0.307	-0.153	0.367		-4.947	-2.473	0.093		0.335	0.168	0.828	
<i>ZnSO₄</i>	0.640	0.320	0.101		-5.030	-2.515	0.089		1.465	0.732	0.367	
<i>FeSO₄</i>	0.563	0.282	0.135		0.553	0.277	0.818		1.258	0.629	0.432	
<i>MnSO₄</i>	0.397	0.198	0.259		-1.460	-0.730	0.552		0.845	0.422	0.589	
<i>R²</i>	96.04%				97.33%				92.32%			
<i>p-value</i>	0.012				0.005				0.041			

S. the significant sign

Results and discussion:

The fermentation of paddy straw to citric acid was shown in Fig. 1, which shown the concentration of reduces sugar in molasses and citric acid with time. The fungus consuming almost all the molasses in the first four days (79%) for germinate and built up the hyphae after that the fungus will start to consume the cellulose and convert it to citric acid by producing enzymes that able to convert the cellulose to glucose and then to citric acid. The benefit of use low concentration of molasses at the beginning in order to enhance the cell to grow and built the hyphae. Citric acid considered as a secondary metabolic product, so it is important to enhance the cell to produce the citric acid rather than to grow and produce biomass. The concentration of citric acid appear to rise after the second day (0.1 g/L) and keep rising till it reach to a constant value in the 8th day (7.6 g/L), this value kept constant for 3 days then the concentration start to decrease because of oxidize of citric acid, that mean the maximum concentration for citric acid was reached in the 8th day and no need to make the fermentation time more than eight days. As it shown in Fig. 1, *A. niger* was able to consume 82% of the molasses that used during the fermentation time.

Screening of Media Components:

The nutrients concentrations have shown an important role in the fermentation of citric acid from paddy straw. The significant parameter (nutrients) was chosen due to their effect on the final concentration of citric acid, pH, and rate of consuming sugar. The Plakett-Burman Design was used for screening the parameters (nutrients) to find the significant one. The *p*-value was considered as a tool for evaluating the significance of each of the coefficients. The parameters with confidence levels greater than 95% were considered as influencing the response significantly. Table 3 showed the ANOVA for the

three responses. It was obviously seen that NH_4NO_3 was the most significant nutrient due to the low *p*-value in all the responses where it noted (0.001, 0.001 and 0.003) in (citric acid, reduce sugar, and pH) response, respectively. KH_2PO_4 was observed to affect the citric acid fermentation indirectly through the effect on the final concentration of molasses response where its *p*-value was 0.050, MgSO_4 also a significant nutrient where it effect on the final concentration of citric acid response which its *p*-value was 0.040

The main effect of each response was evaluated according to Eq. 2, as the difference between both averages of measurement made at the high level (+1) and the low level (-1) of that factor. A large contrast coefficient either positive or negative indicates that a factor has a large impact on fermentation; while a coefficient close to zero means that a factor has little or no effect. It is another indicator to confirm the chosen of the significant nutrients which was selected according to the *p*-value. As it seen in table 3, all the insignificant parameters are close to zero.

For the model can be check by the determination of R^2 (regression coefficient) which it provides a measure of how much variability in the observed response values can be explained by the experimental factors, the closer R^2 to 1, the better the model predicts the response [14, 15]. The following first-order polynomial equation for citric acid concentration:

$$y = 6.260 - 1.312A - 0.187B - 0.453C - 0.153D + 0.320E + 0.282F + 0.198G \dots\dots(2)$$

Here the R^2 value was 96.04%, which indicating that 96.04% of the variability in the response could be explained by the model, and it indicates an acceptable agreement between experimental and predicted values and implies that the mathematical model is very reliable for citric acid production in the present study. The model R^2 (97.33%

for consuming reduces sugar and 92.32% for reducing in pH) suggested that the fitted linear models could explain 97.33% and 92.32% of the total variation.

Nitrogen plays a major role in the metabolism of citric acid. The cell needs nitrogen in form of ammonium to build up cell substances. On the other side too much nitrogen inhibits the production of citric acid due it will enhance the cell to grow and produce biomass [16]. Potassium and phosphate in (KH_2PO_4) considered also a growth enhanced nutrient so it must be limited to enhance the cell to produce citric acid

rather than growth and produce biomass. Magnesium affects the rate of sugar utilization by the cell that relates to rise in mycelia weight (biomass rise) [17].

Conclusion:

Three nutrients (NH_4NO_3 , KH_2PO_4 and MgSO_4) were significant for citric acid production. The maximum citric acid concentration obtained was 8.47 g/L.

References:

- [1] Heinzle E., Biver A.P. and Cooney C.L., Development of sustainable Bioprocess: Modeling and Assessment, John Wiley and Sons, England, 2007.
- [2] Singhanian R.R., Patel A.K., Soccol C.R. and Pandey A., Recent advances in solid-state fermentation. A review, Biochemical Engineering Journal, 2009, 44, 13–18.
- [3] Kun L.Y., Microbial Biotechnology, Principle and Applications, World Scientific Publishing Co. Pte. Ltd., Singapore, 2006.
- [4] Dawson M.W., Madox I.S. and Brooks J.D., Evidence for nitrogen catabolite repression during citric acid production by *Aspergillus niger* under phosphatelimited growth conditions, Biotechnol. Bioeng., 1989, 33, 1500–1504.
- [5] Tran C.T., Sly L.I. and Mitchell D.A., Selection of a strain of *Aspergillus* for the production of citric acid from pineapple waste in solid-state fermentation, World Journal of Microbiology & Biotechnology, 1998, 14, 399–404.
- [6] Krishnan, M.R.V., Citric Acid, Process, In: the Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation, Flickinger M.C. and Drew S.W., eds., John Wiley and Sons Inc., New York, 1999, 651-661.
- [7] Pradhan R., Sawada K., Tobioka H. and Tasaki I., Effect of Dip-treatment of Rice Straw with Sodium Hydroxide Solution on Chemical Composition and in vitro Dry Matter Digestibility, Proc. Sch. Agric. Kyushu Tokai Univ., 1993, 12, 33-37.
- [8] UI Haq I, Ali S., Qadeer M.A., Iqbal J., Stimulatory effect of alcohols (methanol and ethanol) on citric acid productivity by a 2-deoxy DD-glucose resistant culture of *Aspergillus niger* GCB-47, Bioresource Technology, 2003, 86, 227–233.
- [9] Miller G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugars, Anal Chem, 1958, 31, 426–429.
- [10] Marier J.R. and Boulet M., Direct determination of citric acid in milk with improved pyridine-acetic anhydride method, J Dairy Sci, 1958, 41, 1683–1692.
- [11] Updegraff D.M., Semimicro Determination of Cellulose in Biological Materials, Analytical Biochemistry, 1969, 32, 420–424.
- [12] Plackett R.L. and Burman J.P., The design of optimum multifactorial experiments, Biometrika, 1946, 33, 305–325.
- [13] Montgomery D.C., Design and Analysis of Experiment, John Wiley and Sons, England, 2005.
- [14] Aghaiea E., Pazoukib M., Hosseinia M.R., Ranjbara, M. and Ghavipanjehb F., Response surface methodology (RSM) analysis of organic acid production for Kaolin beneficiation by *Aspergillus niger*, Chemical Engineering Journal, 2009, 147, 245–251.
- [15] Alam M.Z., Jamal P. and Nadzir M.M., Bioconversion of palm oil mill effluent for citric acid production: statistical optimization of fermentation media and time by central composite design, World J Microbiol Biotechnol, 2008, 24, 1177–1185.
- [16] Wiczorek S. and Brauer, H., Continuous production of citric acid with recirculation of the fermentation broth after product recovery, Bioprocess Engineering, 1998, 18, 1–5.
- [17] SHU P. and Johnson M.J., The interdependence of medium constituents in citric acid production by submerged fermentation, J. of bacteriology, 1948, 56, 577–585.
