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Sewage Sludge as a Renewable Resource for Citric Acid Production: Optimization of Supplementary Nutrients

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Abstract: The optimization of supplementary nutrients in the sewage sludge as a basal medium was carried out to determine the media components on the citric acid production. Two statistical designs were employed in the citric acid productions which are Plackett-Burman (PB) and Central Composite Design (CCD). For the media screening, the two-level PB design was applied. Under the experimental conditions, it was observed that total suspended solids of sewage sludge (TSS), sugar, cassava, methanol, potassium di-hydrogen phosphate and urea to be the major factors in maximizing citric acid production. A study of single factor was conducted to find the optimum ranges of parameters for citric acid production followed by the Central Composite Design (CCD) under the responses surface methodology. A polynomial model was created to correlate the relationship between two factors (sewage sludge TSS and sugar) and citric acid production. The optimum yield of citric acid, 29.12 g L^{-1} was produced with 0.8% TSS sewage sludge and 7% sugar in four days of fermentation. The determination of coefficient (R^2) from the analysis was 0.9527, indicating that the model of this experiment is significant. This study shows an effective solution to the sludge management for further development through the production of citric acid.

Key words: Plackett-burman, bioconversion, media screening, *Aspergillus niger*, central composite design

INTRODUCTION

Sludge can be a very good source of carbon, nitrogen, phosphorus and other nutrients for many microbial processes. Sewage consists of some 99.9% by weight of water containing dissolved organic material, suspended solids (>100 μm-colloidal), microorganisms and other components. Typically sewage sludge contains 75% of suspended solids and 40% of the dissolved solids are organic (Table 1). In Malaysia, its generation is top listed (64.4%), followed by animal husbandry wastes (32.6%), agro-based (1.7%) and industrial effluent (1.3%) in terms of the BOD load (Alam *et al*., 2008). The management of the ever-increasing volume of domestic and industrial organic wastes has been one of the prime environmental issues in Malaysia. Approximately 4.2 million cubic meters of sewage sludge (pure organic waste without mixing with the industrial waste) is produced annually by Indah Water Konsortium (IWK), a national sewerage company in Malaysia and the total cost of managing is estimated at Ringgit Malaysia (RM) 1 billion (US$ 0.35 billion) (Fakhru'l-Razi *et al*., 2002). The country has to adopt a practical, economic and acceptable approach in managing and disposing sewage sludge since the treatment and disposal of sewage sludge is an important phase in sewage treatment. As an alternative to treat Sewage Treatment Plant (STP) sludge, it can be used to produce another valuable product such as citric acid, since it can be a very good source of carbon, nitrogen, phosphorus and other nutrients for many microbial processes (Jamal *et al*., 2005; Alam *et al*., 2008).

Citric acid is one of the valuable metabolic products from fermentation of microbes. Citric acid is a nearly universal intermediate product of metabolism and its traces are found in virtually all plants and animals. It is used in food and beverages industries as an acidifying and flavour-enhancing agent and also in other industries such as pharmaceuticals (Yaykash *et al*., 2005). *Aspergillus niger* is the most commonly used fungus for citric acid production because of its relatively high yield. *Aspergillus niger* is the most commonly used fungus for citric acid production because of its relatively high yield (Kim *et al*., 2006). In citric acid production, there are few main factors that affect the production: type and concentration of carbon source, nitrogen and phosphate limitation, pH, aeration, trace elements concentration and

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Table 1: Characterization of sewage sludge

Components	Concentration (mg L^{-1})
Nitrogen	4-5
Phosphorus	2-8
Potassium	7-16
Calcium	1-4
Magnesium	1-4
Copper	10-15
Iron	10000-12000
Manganese	100-200
Zinc	300-600

the morphology of the producer organism. Certain nutrients needed to be in excess (sugar, protons, oxygen) while others had to be limiting (nitrogen, phosphate) and some other components had to remain below defined limits (trace metals especially manganese) (Ali *et al*., 2002; El-Holi and Al-Delaimy, 2003; Mattey, 1992). Generally, only sugars that are rapidly taken up by the fungus allow a high yield of citric acid and sucrose is preferable to glucose as *A. niger* has potent extracellular mycelium-bound invertase that is active at low pH and rapidly hydrolyzes sucrose (Lotfy *et al*., 2007).

In maximizing the production of citric acid, standardization of media and fermentation process is vital. A conventional way of media optimization, where a single dimensional search involving changing in one variable while fixing the others at a certain level is laborious and time-consuming, especially when it involve large numbers of variables to be measured (Ambati and Ayyanna, 2001). Thus, an alternative approach is the statistical methods where it is more efficient in optimizing the parameters measured in an experiment. Response Surface Method (RSM) has been widely applied in optimization of media and process conditions of an experiment and it is preferred because it can simultaneously consider several factors at many different levels and corresponding interactions among others. The aim of RSM is to find out the optimum operating conditions for a give system, or the way which a particular response is affected by a set of variables over some specific regions of interest. The first step in RSM was to find a suitable approximation of the true functional relationship between dependent variable (response) and the set of independent variables (factors) (Kang *et al*., 2004).

This study put forward a potential less cost in the citric acid production, since STP sludge is used as substrate which is ever-available material source. The study of citric acid production from sewage sludge is still rare thus it will add some information as it proposed an approach to fully utilize the waste water as well as to produce citric acid in less expensive way. The effects of lignocellulosic materials (empty fruit bunch and rice straw), carbon sources (sugar and cassava), trace metals/minerals (Mg, Mn, Zn, Cu), nitrogen sources (urea, yeast extract, ammonium nitrate), alcohol (methanol) and KH_2PO_4 as well as total suspended solids of sewage sludge on citric acid production were studied. Media optimization was determined through Plackett-Burman, a two-level factorial design, where the concentration of potential contributing factors in the maximal citric acid production was optimized. Central Composite Design (CCD) was applied to determine the statistical factor in maximizing the production of citric acid through sewage sludge by *A. niger*.

MATERIALS AND METHODS

Sample collection and inoculum preparation: Sewage Treatment Plant (STP) sludge used in this study was collected from Indah Water Konsortium (IWK) Taman Shamelin Cheras, Kuala Lumpur and stored in a cold room at 4°C to conserve the microorganism's activity.

Culture of *Aspergillus niger* is obtained from lab stock, Bioenvironmental Engineering Lab, IIUM which are isolated from orange, lemon and IWK wastewater. The strains were subcultured on Potato Dextrose Agar (PDA) plates for 4-6 days for incubator at 32°C. Approximately 100 mL of distilled water was poured evenly on four PDA plates which contained the 4-6 days old culture. The fungal suspension (1×10^7 spores mL^{-1}) was filtered using Whatman filter paper No. 1 into a shake flask. The filtrate was used as inoculum. All flask, funnel, filter paper, distilled water was sterilized prior to use. The study was carried out in the year 2010.

Fermentation process

Fermentation medium and culture conditions: In this experiment, eleven parameters which are low and high concentrations are tested. Twelve runs are performed according to Plackett-Burman design (Plackett and Burman, 1946). Fermentation medium was 50 mL and the initial pH was adjusted to 5.0±0.01 with 1 M and 5 M HCl. Fermentation was carried out with 150 rpm at room temperature for four days. 3% (v/v) of inoculum *A. niger* O103A was introduced into the media.

Experimental design-plackett-burman design: Media optimization is very important in defining the maximum production of a product. In developing standard media and process of fermentation conditions, the standardization of both elements is crucial. Media optimization is easier to be done by applying statistical methods which are response surface methods (Ambati and Ayyanna, 2001). Plackett-Burman design

Table 2: Media optimization from plackett-burman design

Experimental run	*S (TSS%)	EFB (% w/v)	RS (% w/v)	Su (% w/v)	C (% w/v)	Me (% v/v)	Mi (% v/v)	K (% w/v)	U (% w/v)	N (% w/v)	YE (% w/v)	Citric acid (g L^{-1})
1	0.25 (-1)	1.5 (+1)	1.5 (+1)	2 (-1)	3 (+1)	0.5 (-1)	0.0 (-1)	0.0 (-1)	0.4 (+1)	0.4 (+1)	0.4 (+1)	0.80
2	0.25 (-1)	1.5 (+1)	0.25 (-1)	2 (-1)	1 (-1)	2 (+1)	4 (+1)	0.2 (+1)	0.0 (-1)	0.4 (+1)	0.4 (+1)	0.60
3	0.25 (-1)	0.25 (-1)	1.5 (+1)	6 (+1)	3 (+1)	0.5 (-1)	4 (+1)	0.2 (+1)	0.0 (-1)	0.4 (+1)	0.0 (-1)	0.87
4	1 (+1)	0.25 (-1)	1.5 (+1)	6 (+1)	1 (-1)	2 (+1)	0.0 (-1)	0.0 (-1)	0.0 (-1)	0.4 (+1)	0.4 (+1)	1.58
5	1 (+1)	0.25 (-1)	0.25 (-1)	2 (-1)	3 (+1)	2 (+1)	4 (+1)	0.0 (-1)	0.4 (+1)	0.4 (+1)	0.0 (-1)	3.31
6	1 (+1)	1.5 (+1)	0.25 (-1)	6 (+1)	3 (+1)	0.5 (-1)	4 (+1)	0.0 (-1)	0.0 (-1)	0.0 (-1)	0.4 (+1)	1.85
7	1 (+1)	0.25 (-1)	1.5 (+1)	2 (-1)	1 (-1)	0.5 (-1)	4 (+1)	0.2 (+1)	0.4 (+1)	0.0 (-1)	0.4 (+1)	1.01
8	1 (+1)	1.5 (+1)	1.5 (+1)	2 (-1)	3 (+1)	2 (+1)	0.0 (-1)	0.2 (+1)	0.0 (-1)	0.0 (-1)	0.0 (-1)	3.59
9	0.25 (-1)	1.5 (+1)	1.5 (+1)	6 (+1)	1 (-1)	2 (+1)	4 (+1)	0.0 (-1)	0.4 (+1)	0.0 (-1)	0.0 (-1)	1.29
10	1 (+1)	1.5 (+1)	0.25 (-1)	6 (+1)	1 (-1)	0.5 (-1)	0.0 (-1)	0.2 (+1)	0.4 (+1)	0.4 (+1)	0.0 (-1)	2.68
11	0.25 (-1)	0.25 (-1)	0.25 (-1)	6 (+1)	3 (+1)	2 (+1)	0.0 (-1)	0.2 (+1)	0.4 (+1)	0.0 (-1)	0.4 (+1)	4.69
12	0.25 (-1)	0.25 (-1)	0.25 (-1)	2 (-1)	1 (-1)	0.5 (-1)	0.0 (-1)	0.0 (-1)	0.0 (-1)	0.0 (-1)	0.0 (-1)	20.98

S = Sludge; EFB = Empty fruit bunch; RS = Rice straw; Su = Sugar; C = Cassava; Me = Methanol; Mi = Minerals (Mn, Cu, Mg, Zn); K = KH_2PO_4; U = Urea; N = NH_4NO_3; YE = Yeast extract, Within parenthesis are levels, *Three levels: -: Low; +: High; 0: Centre

was used to define the best media composition in citric acid production (Plackett and Burman, 1946). Eleven factors were measured in this experiment (Table 2). All trials were performed in triplicate and citric acid yield is treated as response. The main effect of each variable was simply calculated as the difference between the average of measurements made at the high setting (+) and the average of measurements observed at low setting (-) of that factor. Plackett-Burman experimental design is based on the first order model:

$$Z = b_0 + \sum b_i x_i$$

where, Z is the response (citric acid yield), b_0 is the model intercept and b_i is the linear coefficient and x_i is the level of the independent variable (Lotfy *et al*., 2007).

The effects of these factors were examined and it was identified that there were contributing and least contributing factors which led to the single factor optimization. The identified factors for the single factors determination are carbon sources (sugar and cassava) while the others shown that although they contributed to the production of citric acid, their effects is insignificant thus they are neglected in this study.

Single factor optimization: The optimization was performed due to the selection of potential sources that can maximize the production of citric acid. As for carbon sources which are sugar and cassava, the optimization was done to identify the most potential source for the maximum citric acid production. As for this study, few factors that were believed to have positive effects for the increment of citric acid production, as identified in the Plackett-Burman design was also introduced. They are methanol (2%) (v/v), KH_2PO_4 (0.2%) (w/v) and urea (4%) (w/v). The study of Total Suspended Solids (TSS) in sludge also conducted where the concentration of TSS is varied (0.25, 0.5, 0.75. 1.0 and 1.5%). Lastly the effect of methanol was studied and the concentrations are 1, 2, 3 and 4%.

A 50 mL of 0.5% TSS of sludge were added with 1, 3 and 5 (%) (w/v) of sugar and cassava flour and it was done in triplicates. The media also were added with addition of 2% (v/v) methanol, 0.2% (w/v) KH_2PO_4 and 0.4% (w/v) urea. Three percent (v/v) of inoculum of *A. niger* O103A is added into the media and the pH of the media was adjusted to 5.0±0.01 and fermentation was carried out with 150 rpm at room temperature for four days.

Effect of TSS sludge and various sugar concentrations in citric acid production: Fermentation was done with the effects of 0.25, 0.5, 0.75, 1.0 and 1.5% TSS (w/w) of sludge and 2, 4, 6 and 8 (%) (w/v) sugar for the production of citric acid. Three percent (v/v) of inoculum of *A. niger* O103A is added into the media and the pH of the media was adjusted to 5.0±0.01 and fermentation was carried out with 150 rpm at room temperature for four days.

Effect of methanol in citric acid production: The addition of lower alcohols, ethanol, methanol, n-propanol, in fermentation substrate can increase the production of citric acid. Yigitoglu (1992) stated that the optimal concentration of methanol from 1 to 4% by volume was said to be more effective in the citric acid production from ethanol. Thus this experiment is designed to determine the effect of the addition of methanol in the fermentation media in order to increase the yield of citric acid.

Four different concentrations of methanol (1, 2, 3 and 4%) of methanols (v/v) were added into 50 mL of 0.25 and 0.5% TSS sludge with the addition of sugar as co-substrate in the fermentation media. Three percent (v/v) of inoculum *A. niger* O103A is added into the media and initial pH is adjusted to 5.0± 0.01. Fermentation is carried out with 150 rpm at room temperature for four days.

Design of experiment and statistical analysis: response surface methodology (RSM): Design of Experiment (DOE) and statistical analysis in this study were done using statistical software Stat-Ease 6.0.8. DOE was designed using Central Composite Design (CCD) with two factors, sludge and sugar with three levels for parameters measured. The three levels are-1, 0 and +1 which indicate that the low, middle and the high level of the parameters studied. Thirteen experimental runs were conducted with five runs at centre point and the reaction is observed through this second order polynomial model equation:

$$Y = \beta_0+\beta_1 A+\beta_2 B+\beta_{11} A^2+\beta_{22} B^2+\beta_{12} AB \qquad (1)$$

where, Y is the dependent variable or predicted response, in this study is citric acid; A and B are the independent variables (sludge and sugar). In addition, β_0 is the constant coefficient; β_1 and β_2 are the linear effects; β_{11} and β_{22} are the squared effects and β_{12} is the interaction term (Ambati and Ayyanna, 2001).

Determination of citric acid and sugar concentrations: Citric acid was determined according to method of (Marrier and Boulet, 1958). Pyridine and acetic anhydride was added to the samples with 1:1.3:5.7 ratios in fume hood. Then the solution was incubated in water bath at 32°C for 1 h and the absorbance is read at 420 nm using spectrophotometer. Total sugar was determined according to method of DuBois *et al.* (1956). The addition of 5% phenol and 98% sulfuric acid to samples with 1:5:1 ratio and the solution were allowed to cool down at room temperature (25-32°C) before read the absorbance at 490 nm using spectrophotometer. pH of the samples was measured at room temperature before and after fermentation.

RESULTS AND DISCUSSION

Identification of contributing factors for the production of citric acid by plackett-burman design: Eleven components were studied in this design for the increment of citric acid production (Table 2). Plackett-Burman is often used for the media screening, it is effective in screening the media components and also less time-consuming as well as it can be done in shorter time. As it can be observed from Table 2, the production of citric acid varies from 0.606 to 20.979 g L^{-1} depending on the media components. Main effects are shown in Fig. 1.

From Fig. 1, it is observed that sludge, sugar, cassava, methanol, potassium di-hydrogen phosphate and urea give the positive effect in contributing to the higher citric acid yield. The highest effect would be from cassava flour and methanol most likely. It is observed that higher concentration of methanol would result in higher yield of citric acid (Rohr, 1998). The presence of methanol in the media may increase the citric acid production since

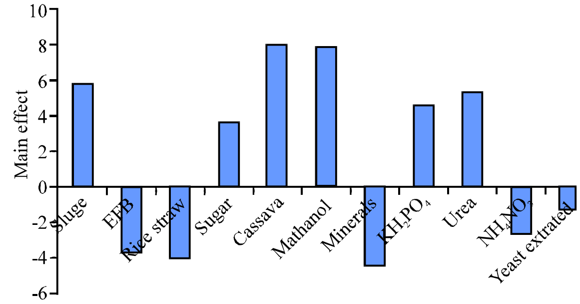

Fig. 1: Main effects of media constituents on citric acid production according to the experimental results by Plackett-Burman design

it has profound effect on the metabolism on the metabolism of sugar by *A. niger*. However, the mechanism on how methanol stimulates the citric acid production is not clear (Yigitoglu, 1992).

It is followed by sludge, urea, di-hydrogen phosphate and finally sugar. Sugar or sucrose is the traditional substrate for citric acid production (El-Holi and Al-Delaimy, 2003). It indicates that more positive value obtained, citric acid production is higher while EFB, rice straw, minerals (Mg, Zn, Cu and Mn), ammonium nitrate (NH_4NO_3) and yeast extracts is favorable if they exist in lesser quantity for higher production of citric acid. The minerals (Mg, Mn, Cu and Zn) is most favorably not to be introduced to the media since it showed the most negative effect for the maximum production of citric acid. Although in some studies it is found that they contributed to the increment of the citric acid yields but in this study using sludge as main substrate the effect of these minerals cannot be determined. Mn^{2+} presented a strong negative effect on citric acid production and the absence of this component is important for the microbial growth thus affecting the citric acid production. Zn^{2+} deficiencies are also favorable in maximizing citric acid production. Although copper and magnesium play important roles in producing large amount of citric acid (Yigitoglu, 1992) their effect on this study is neglected.

The introduction of lignocellulosic materials which are empty fruit bunch and rice straw, was not increasing the production of citric acid yield. Although it is well known that *Aspergillus niger* are capable of synthesizing lignocellulosic materials (Ikram-Ul-Haq *et al.*, 2004), however, with the abundance of the substrate it seem that synthesis of lignocellulosic materials is ignored. Ammonium nitrate and yeast extract also show negative effects to the citric acid production. As mentioned before nitrogen limitation is very important in producing high yield of citric acid. Thus for single factor of carbon source optimization, the validation of sugar and cassava were determined while maintaining 0.5% methanol (v/v), 0.2% KH_2PO_4 (w/v) and 0.4% urea (w/v) in the fermentation media.

Single factor optimization: Single factor optimization is to select the most potential carbon source as co-substrate in the fermentation media. Sugar and cassava flour were used as the main carbon sources in the media composition. From media optimization, the sum of responses was analyzed according to the factors that contribute to the maximum production of citric acid. In this experiment, also the effect of methanol, potassium di-hydrogen phosphate and urea were taken into consideration in contributing to the increase of citric acid production. From Fig. 2, it is observed that the highest production of citric acid was contributed by 5% sugar (w/v) with 26.416 (g L^{-1}) while the lowest also by 1% (w/v) sugar with 0.492 (g L^{-1}). Although co-substrate cassava also produced high yield of citric acid, however, the highest amount was produced with the sugar as co-substrate in the media. Thus from the analysis sugar was identified as a potential co-substrate in increasing the citric acid production compared to cassava flour. Many researches has been done using sucrose as their substrate/co-substrate in the citric acid fermentation since it gave higher yield of citric acid namely sugarcane molasses, sugarcane bagasse, beet molasses (Ikram-Ul-Haq *et al*., 2004; Kumar and Jain, 2008) as compared to polysaccharides (cassava). This is due to the slow hydrolysis of polysaccharides caused by low activity of hydrolytic enzyme's activity at low pH which is very important in citric acid production.

Effect of TSS sludge and various sugar concentrations in citric acid production: From Fig. 3, it is observed that the highest production of citric acid is obtained from 0.25% TSS sludge with 8% sugar (25.1 g L^{-1}), followed by 0.25% TSS sludge with 6% sugar (24.1 g L^{-1}) and 0.5% TSS sludge with 4% sugar (23.1 g L^{-1}). The lowest yield of citric acid obtained is from 1.5% of TSS sludge with 2% sugar (0.6 g L^{-1}). It is also observed that the higher % of TSS sludge, the lower yield of citric acid (g L^{-1}) is produced. The higher amount of residual sugar in higher % TSS sludge indicates that the conversions of sugar into citric acid is low and thus give the low amount of citric acid. Although in Plackett-Burman design it is noticed the higher concentration of total suspended solids in sewage sludge, higher yield of citric acid is produced. However, by this study, it is determined that the higher concentration of the total suspended solids of sludge, lower amount of citric acid is produced. Several studies

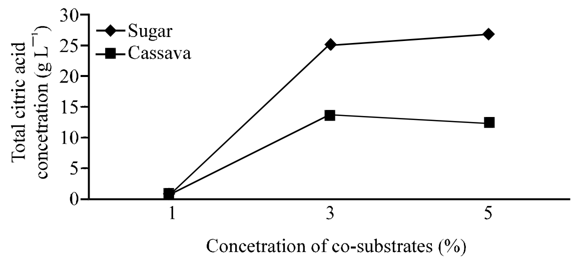

Fig. 2: Production of citric acid by different concentrations of co-substrates (sugar and cassava in 0.5%TSS of sludge)

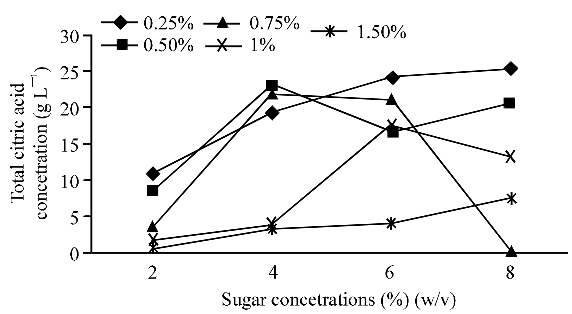

Fig. 3: Effect of sugar and TSS of sewage sludge concentrations on the citric acid production

have been conducted in production of citric acid using solid substrate (empty fruit bunches), palm oil mill effluent etc. which are not direct comparable as the substrate for the present is new (Bari *et al*., 2009; Alam *et al*., 2008).

Effect of methanol in citric acid production: Figure 4a and b show the production of citric acid and the residual sugar in four days of fermentation. It is observed that with the increasing of methanol concentrations (v/v) (%) the production of citric acid also increased. The highest amount of citric acid obtained is 22.93 g L^{-1} (0.5% TSS sludge with 4% sugar). Even though methanol increases the amount of citric acid (g L^{-1}) the addition of methanol is considered insignificant because if compared to previous study where without methanol addition in the fermentation media, the total yields of citric acid were high. The researches showed that optimum level of methanol stimulates the production of citric acid by using it as a carbon source and by increasing the permeability of cell membrane for the secretion of citric acid (Navaratnam *et al*., 1998; Jianlong, 2000).

Statistical optimization by central composite design: For statistical optimization, two factors which are sewage sludge (A) and sugar (B) were selected in optimizing the

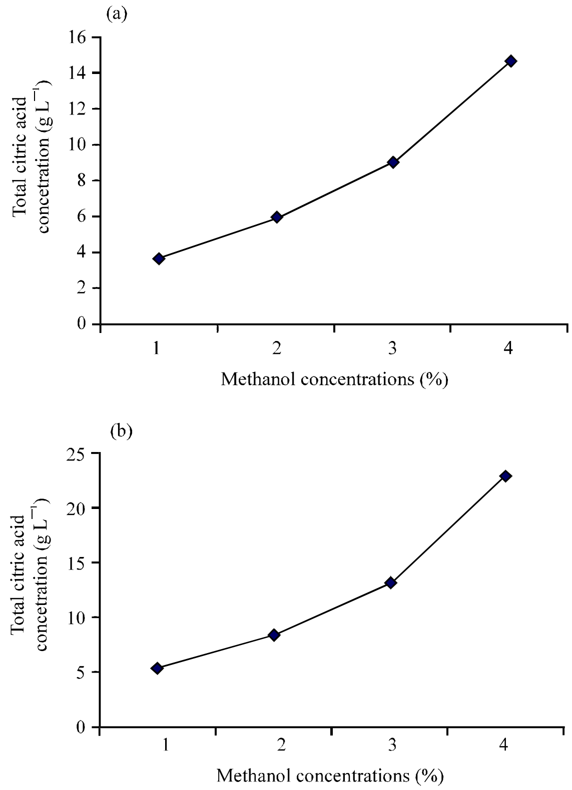

Fig. 4: (a) Effect of methanol on citric acid with 0.25% TSS sludge and 8% (w/v) sugar and (b) Effect of methanol on citric acid with 0.5% TSS sludge and 4 (w/v) sugar

production of citric acid. The experiment was designed based on Central Composite Design (CCD). From the analysis, the suggested model is quadratic and the final equation in term of coded factors can be described as:

$$Y\ (\text{citric acid, g L}^{-1}) = +23.79+7.10\ A+8.42\ B-4.42\ A^2-10.59\ B^2+6.42\ AB \quad (2)$$

The citric acid production obtained is considered as dependent variable of response Y while the effects of independent variables such as sludge (A) and sugar (B). The theoretical results calculated by the Eq. 2 and experimental results by statistical design (CCD) are presented in the Table 3. The production of citric acid (experimental) was analyzed and developed a polynomial regression model using Design Expert software. The results showed that the maximum citric acid production (29.2 g L^{-1}) was found in the experimental run 12. Analysis of variance (ANOVA) is shown in Table 4. The R^2 value (0.9527) as determined from ANOVA indicated that 95.27% of the variables were supported by the

Table 3: Production of citric acid from experimental and predicted using Central Composite Design (CCD) with the value of coded and actual factors

Experimental run	*Sludge (% w/v) (A)	Sugar (% w/v) (B)	Citric acid (g L^{-1}) Experimental	Predicted
1	0.2 (-1)	4 (0)	6.678	12.27
2	0.8 (+1)	4 (0)	28.667	26.48
3	0.2 (-1)	1 (-1)	2.916	0.32
4	0.2 (-1)	7 (+1)	6.053	3.68
5	0.5 (0)	4 (0)	24.233	23.79
6	0.5 (1)	1 (-1)	0.375	1.05
7	0.5 (0)	4 (0)	24.419	23.79
8	0.5 (0)	4 (0)	24.589	23.79
9	0.5 (0)	4 (0)	26.686	23.79
10	0.5 (0)	4 (0)	23.461	23.79
11	0.5 (0)	7 (+1)	20.757	21.62
12	0.8 (1)	7 (+1)	29.212	30.73
13	0.5 (0)	1 (-1)	2.223	4.78

*Three levels: -: Low; +: High; 0: Centre. Within parenthesis are levels

Table 4: Analysis of variance for the response surface quadratic model

Source	Sum of squares	F-value	p-value > F
Model	1433.76	28.19	0.0002*
Sludge (A)	302.73	29.76	0.0010*
Sugar (B)	425.21	41.80	0.0003*
A^2	53.87	5.30	0.0549
B^2	310.01	30.48	0.0009*
AB	164.99	16.22	0.0050*

*p-value indicates that model is significant

response. The adjusted R^2 was also high (0.9189) indicating that the model is highly significant. ANOVA quadratic regression model demonstrated also shows that the model was significant, since F value is less than 0.05 (p value >F = 0.0002). The model F value of 28.19 implies the model is significant and there is only 0.02% chance that a model F value this large could occur to noise.

According ANOVA also, the variable A which is sludge is significant since the p-value is 0.0010 indicating that is less than 0.1000. The same goes to variable B which is sugar where the value is 0.003 also less than 0.1000. The third and fourth variable also indicate that the interaction is significant since their p-value is less than 0.005.

The 3D response surface shows the regression equations in order to determine the optimum values of the variables within the range considered (Alam *et al*., 2008). From the Fig. 5, the maximum production of citric acid is observed at highest concentration of sludge and sugar (%, w/v). About 7% of sugar and 0.8% of TSS sludge (Run 12) gives the maximum production of citric acid (29.12 g L^{-1}). The increasing concentration of both sludge and sugar shows the highest production of citric acid. The production decreased when the higher concentration of total suspended solids in sludge was added with lower concentration of sugar in the media. Lower concentration of sugar with high TSS sludge also gave lower yield of citric acid.

Table 5: Validation of developed quadratic model optimum medium constituent

Experimental run	Sludge (% w/v) (A)	Sugar (% w/v) (B)	Citric acid (g L^{-1}) Predicted	Experimental	Deviation (%)
1	0.5	4.0	23.79	22.38	4.50
2	0.5	5.0	23.79	25.04	5.25
3	0.8	4.5	26.47	25.39	4.10
4	0.8	5.0	26.47	25.18	4.90
5	0.8	7.0	30.72	28.26	8.00

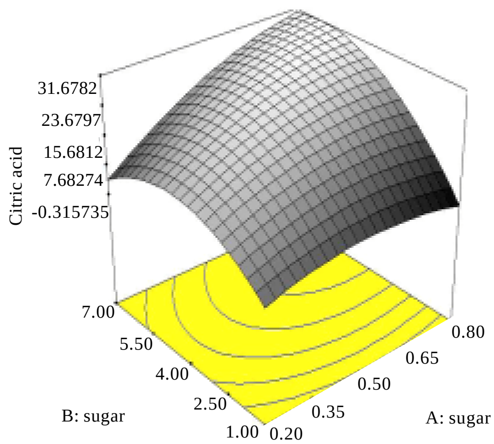

Fig. 5: A 3D response surface showing the effect of sugar (%, w/v) and sludge (%, w/v) on the citric acid production (g L^{-1})

As to verify the optimization results and validate the model developed, a set of experiment with three replications was performed according to the media constituent presented in Table 5. From the validation experiment, the production of citric acid reached around 28.26 g L^{-1} in optimum conditions which is slightly lower than the predicted value (30.72 g L^{-1}). It shows fluctuation in the production of citric acid since total amounts of components and trace elements in the sewage sludge as major substrate can be varied in every set of experiments. The production of citric acid with the variation of different nutrients such carbon, nitrogen, phosphorous, potassium and minerals sources has been observed by the several authors and found significant effects on the production (Alam *et al*., 2008; Bari *et al*., 2009; Ikram-Ul-Haq *et al*., 2004).

CONCLUSION

In screening factors affecting production of certain metabolite, it is very important to test as many factors as possible and to identify the significance each of them. Applying Central Composite Design (CCD) in the optimization process for maximal production is an efficient method that tests the effect of factors interaction. Six factors seem to be the positive contributing factors in increasing the production of citric acid. They are sludge, sugar, cassava, methanol, potassium di-hydrogen phosphate and urea. While the others show that the lesser concentration in the media, the higher citric acid can be produced thus their effect in the study is neglected. An increasing concentration of TSS sludge (%, w/v) and sugar (%, w/v) also shows that the maximum production of citric acid was obtained where 0.8% TSS sludge with 7% sugar in the fermentation media gave 29.21 (g L^{-1}). The study indicated that with higher concentration of total suspended solids of sewage sludge and sugar, as co-substrate, higher of citric acid can be obtained.

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