PRODUCTION OF CITRIC ACID FROM SUGARCANE MOLASSES BY *Aspergillus niger* USING SUBMERGED FERMENTATION

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ABSTRACT: Citric acid (CA) has a high demand due to its various uses in the food and pharmaceutical industries. However, the natural supply of CA is minimal compared to its growing industrial demand. The increasing demand for CA can be fulfilled by using biotechnological processes. This study utilized liquid state bioconversion by *Aspergillus niger* for CA production using sugarcane molasses as the primary substrate. Sugarcane molasses which is agricultural waste consists of significant proportion of organic matters such as lipids and carbohydrates. This makes sugarcane molasses as a potential and alternative source of producing CA at a lower cost. In this study, statistical optimization was applied to improve CA production using submerged fermentation in shake flasks. *Aspergillus niger* was cultured in potato dextrose agar. Then, inoculum spores were introduced into the fermentation media for a specific duration according to the experimental design from Central Composite Design (CCD) tool under Response Surface Methodology (RSM) in Design Expert 6.0 software. Three parameters were chosen to be optimized at 32 ^{0}C *i.e.* agitation rate (160, 80, 200 rpm), substrate concentration (47, 60, 73%) and fermentation time (24, 72, 120 h). High Performance Liquid Chromatography (HPLC) and Fourier-transform infrared spectroscopy (FTIR) analyses were conducted to measure CA yield. The optimization study showed that the media incubated for 72 hours with a substrate concentration of 60% and an agitation speed of 180 rpm produced the highest CA yield (21.2 g/L). The analysis of variance (ANOVA) also showed that CCD quadratic model was significant with P-value < 0.0104 and R^2 is 0.8964.

KEY WORDS: *Aspergillus niger, citric acid, FTIR spectroscopy, HPLC, molasses, submerged fermentation.*

1. INTRODUCTION

Citric acid (CA) ($C_6H_8O_7$, 2 - hydroxy - 1,2,3 - propane tricarboxylic acid) is a natural preservative compound [1]. It is the most extensively utilized organic acid in food and pharmaceuticals fields [2]. In the industrial field, CA was initially produced from Italian lemons. Lemon juice continued to be the commercial source of CA until 1919 when the industrial process

using fungus, *Aspergillus niger (A. niger)* for CA production was established in Europe [3]. This process is mainly about the fermentation of carbohydrate source to produce CA.

One technique of fermentation process is submerged fermentation. An essential factor to take into consideration during the development of a submerged fermentation process is the choice of microorganisms. Various types of microorganisms, including yeast, bacteria and fungi, have been utilized in producing CA. However, *A. niger* is the best type of microorganism for industrial process, owing to its high yields, simplicity to handle and its ability to use various cheap raw materials in fermentation, such as sugar-cane molasses [4].

In terms of global demand, the rate of CA growth is expected to increase significantly, due to the increasing population and improving standards of living which will lead to higher consumption of food and drinks per capita [5]. As an outcome, the worldwide market for CA is going to grow more in the coming years. Considering this and the availability of affordable substrates used for CA production, a thriving CA industry should be developed. Commonly, the molasses formed by sugar industry plant has a proportion of sugar content, non-sugar content, water and inorganic salts [6]. This condition makes the molasses as a suitable raw material to be used in the production of organic acids like CA. Several studies have selected molasses as the substrate for CA fermentation [7, 8]. However, the present study was undertaken to develop methods using FTIR and HPLC to ensure the identification of the final product and measure CA quantity in the medium. This objective is different compared to other published articles that focus on the alternative methods to identify and measure CA.

Moreover, it is a great idea to convert molasses which is waste to an added-value product, which is CA. It is an application of the initiative waste-to-wealth. Therefore, sustainable development and economic system for the treatment and reusing of agro-industrial residues like molasses is important to minimize environmental problems that can occur from other fermentation process and chemical production of CA [9, 10].

There are different chromatographic methods available used to analyze CA concentration. In general, chromatographic methods allow simultaneous analysis of most organic acids, including CA. High-performance liquid chromatography (HPLC) is one of the promising and the most common used methods. Most of the procedures developed until now for food and beverage analysis utilize HPLC with a refractive index (RI), UV spectrophotometric, or electrochemical detection [11]. HPLC is able to provide a proper determination of CA amount in each sample. Chromatographic techniques are beneficial but time-consuming. Before chromatography analysis is performed, several steps are necessary including saponification or hydrolysis, filtration and clean up [12].

Fourier-transform infrared spectroscopy (FTIR) has stood out as a robust instrument for studying and monitoring of biological processes. FTIR spectroscopy is fast, easy to handle and a useful biophysical technique that aims to detect molecular bond vibrations. The usage of FTIR spectroscopy is increasing in many fields [13], including food studies [14].

The objective of this study was to develop a rapid FTIR and HPLC methods for identifying and quantifying CA, respectively, which is produced from sugarcane molasses using submerged fermentation of *A. niger*. In addition, three chosen parameters at 3 different ranges namely agitation rate, substrate concentration and fermentation time were optimized using RSM in Design Expert 6.0 software.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Raw Material

Sugarcane molasses was collected from Central Sugar Refinery Sdn Bhd in Shah Alam, Selangor, Malaysia.

2.1.2 Microorganism

A. niger strain was obtained from Microbiology Laboratory, Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia.

2.2 Method

2.2.1 Pre-treatment of Sugarcane Molasses

Sugarcane molasses was pretreated according to the methodology described by [15]. This raw material was heated in an autoclave for 1 hour to inhibit any occurring of fermentation due to microbial contamination. Then, the solution was filtered using a filter paper. To breakdown complex sugars in sugar molasses to the simpler sugars, 1N sulphuric acid (H_2SO_4) was added and then neutralized with calcium hydroxide ($Ca(OH)_2$). The solution was then filtered again to remove any impurities. The pH of the solution was adjusted to 5 using potassium hydroxide (KOH) to be used in submerged fermentation.

2.2.2 Inoculum Preparation

A. niger suspension was prepared by harvesting a loopful of its spores from the purified colonies grown on potato dextrose agar plate into 10 mL of sterilized water in a test tube. The test tube was shaken vigorously to obtain a homogenous mixture of spore suspension. Spores were counted using haemocytometer and about 10^6 spores/ml was used as the inoculum for the fermentation.

2.2.3 Batch Fermentation

Fermentation medium was sterilized and dispensed into 250 mL Erlenmeyer flasks with 125 mL of fermentation media (pre-treated molasses). About 5 mL of *A. niger* suspension was inoculated into the Erlenmeyer flasks which were then kept in an incubator shaker at 32 °C under different agitation speeds and fermentation times based on the experimental design (Table 1). Sampling for each run was done after the set fermentation time has completed.

Table 1: Lowest and the highest value of each chosen parameter

Factor	Parameter	Low (-1)	Center (0)	High (1)
A	Substrate Concentration (%)	47	60	73
B	Time (h)	24	72	120
C	Agitation Speed (rpm)	160	180	200

2.3 Citric Acid Identification Test

Samples collected were diluted and then placed into a freeze dryer for three days to prevent moisture content loss in the samples. Then each sample was analyzed using FTIR with the

following operating conditions; spectral range of 4000 – 500 cm^{-1}; spectral resolution of 1 cm^{-1}. The result was obtained in strong absorption. The instrument used in this study was Nicolet iS50 FT-IR for © 2012 U.S. Thermo Fisher Scientific Inc. using OMNIC software.

2.4 Determination of Citric Acid Concentration

To determine CA amount in the samples, HPLC analysis was performed. Before conducting this test, samples were filtered using syringe filter into small vials that are specifically designed for the use of HPLC instrument equipped with a refractive index detector (RID). The column used was Aminex HPX-87H (300 x 7.8 mm, Bio-Rad, USA). The flowrate of 5 mM H_2SO_4 as the mobile phase was 0.6 mL/min at 50 °C (column temperature).

3. RESULTS AND DISCUSSION

3.1 FTIR Spectroscopy Analysis

Qualitative estimation of CA in the fermentation media was determined from FTIR analysis. The standard/pure CA was evaluated together with the experimental samples. Then, the calibration series of CA and the samples were compared in order to identify and assure the existence of CA in the collected samples. CA spectrum is shown in Fig. 1a and it can be seen that many peaks appeared as reported by previous research [16, 17]. According to [18, 19] and [20], the chemical structure of CA has three carboxylic groups. Therefore, one of the significant proofs for the existence of CA in the samples is the appearance of the carbonyl stretch (C=O) as a full band from 1760-1690 cm^{-1} [21]. The exact position of this broadband depends on whether the carboxylic acid is saturated or unsaturated, dimerized, or has internal hydrogen bonding. It can be seen from Fig. 1b that the intensity of CA absorbance has its peak between the wavelength of 1699 and 1745 cm^{-1} that falls into the range of the carboxylic acid band (1723 cm^{-1}) [22]. In order to determine the identity of CA in the samples, the comparison was made with pure CA as in Fig. 1a. Some differences are obviously seen in the calibration between the pure CA and samples due to the samples are mixtures of various components like moisture content, metals and nutrients, etc. that will have different peaks and absorbance ranges. For example; the towering peak of CA at 1760-1690 cm^{-1} remained constant in all calibration standards and was unaffected by varying concentrations of samples as shown in Fig. 1b.

3.2 Optimization of Submerged Fermentation Parameters using Statistical Analysis

The optimization study showed that the media incubated for 72 hours with a substrate concentration of 60% and an agitation speed of 180 rpm produced the highest CA yield (21.2 g/L). Further analysis was carried out to understand the interactions of selected parameters with CA yield using analysis of variance (ANOVA) and RSM. ANOVA analysis demonstrates that the F-value and P-value > F were 9.62 and <0.0007, respectively which indicates that the model obtained is significant as shown in Table 2. The regression coefficient (R^2) was recorded to be 0.8964 which indicates that a high degree correlation between the experimental and predicted values. The predicted R^2 (0.8964) is acceptable since the difference with the adjusted R^2 (0.8032) was less than 0.2, in which it indicates a high degree of significance of the model.

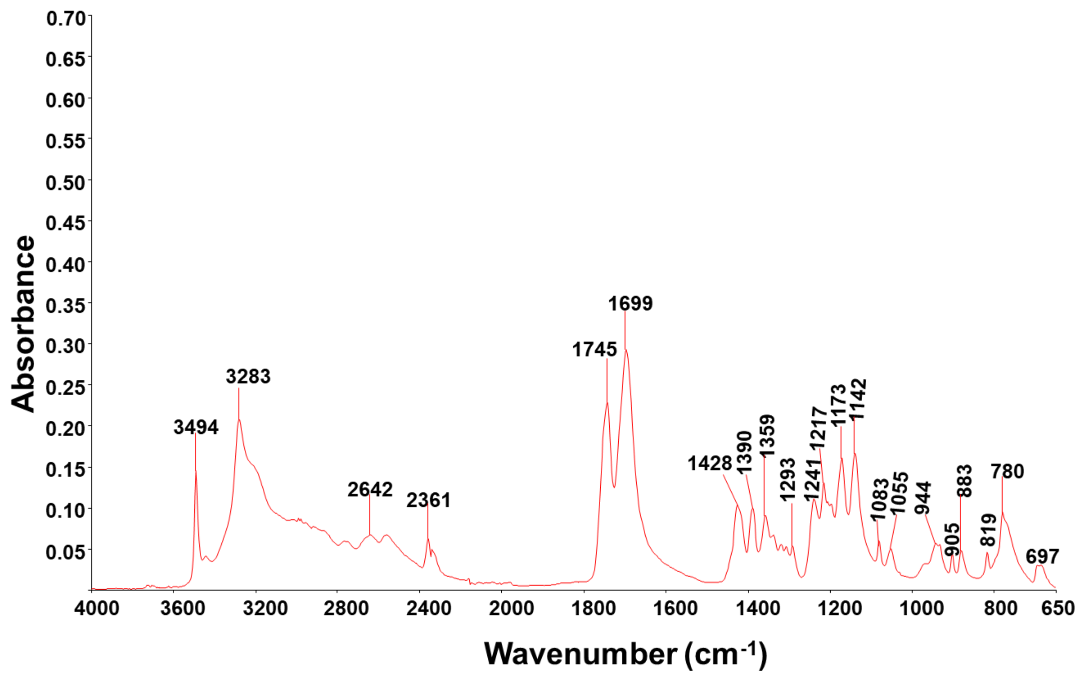

Fig. 1a. FTIR spectra of pure CA.

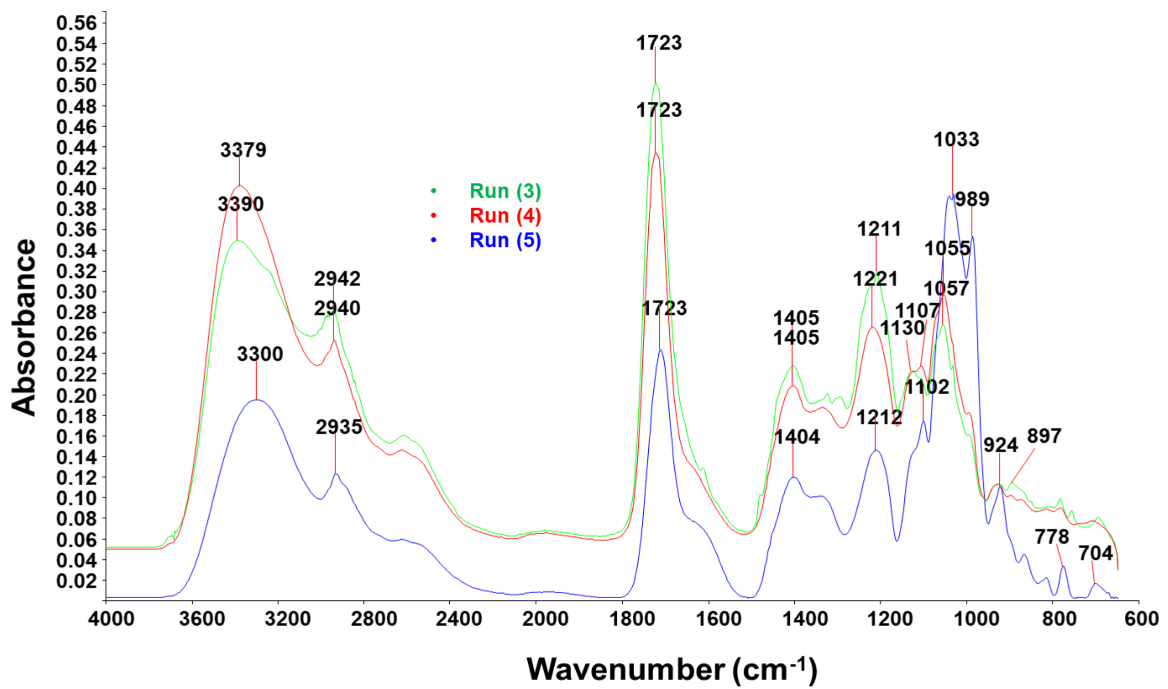

Fig. 1b. FTIR spectra of samples from Run 3, 4 & 5 resulted from submerged fermentation.

Table 2: Summary of the model analysis for HPLC

Standard Deviation	2.64	**R^2**	0.8964
Mean	12.54	**Adj R^2**	0.8032
C.V	21.07	**Prediction R^2**	0.3298
PRESS	451.81	**Adequate Precision**	7.354
P-value > F	0.0007	**Lack of Fit**	11.31

As mentioned earlier, based on ANOVA, F-value and P-value > F are 9.62 and <0.0007, respectively which indicate that the model obtained is significant. In statistical analyses, a model can be considered as significant if it satisfied the condition of having less than 0.05 for P-value > F. Additionally, if the model has P-value > F less than 0.001, it is considered as highly significant. In addition, from Table 3, the term C^2 (agitation speed) is significant, whereas the rest of the terms were not significant. If there are many insignificant model terms (not counting those required to support hierarchy), the model reduction may improve the model.

Table 3: ANOVA for CCD quadratic model

Source	**Sum of Squares**	**DF**	**Mean Square**	**F-Value**	**P-value > F**	
Model	604.30	9	67.14	9.62	0.0007	**Significant**
A-substrate concentration (%)	4.80	1	4.80	0.69	0.4263	
B-time (h)	1.16	1	1.16	0.17	0.6927	
C-agitation speed (rpm)	0.12	1	0.12	0.018	0.8970	
A^2	0.94	1	0.94	0.13	0.7220	
B^2	11.88	1	11.88	1.70	0.2214	
C^2	229.89	1	229.89	32.92	0.0002	
AB	0.18	1	0.18	0.026	0.8746	
AC	0.082	1	0.082	0.012	0.9158	
BC	3.39	1	3.39	0.49	0.5016	
Residual	69.83	10	6.98			
Lack of Fit	64.16	5	12.83	*11.31*	0.0093	**not significant**
Pure Error	5.67	5	1.13			
Cor Total	674.12	19				

The 2D contour and 3D are plotted to test the interactions of the variables between each other and to set for each variable, the optimum value required to produce CA by *A. niger* strain. To specify the optimal level for each variable in order to maximize the production of CA, 3D response surface graphs were plotted by plotting the response (CA production) on the Z-axis versus any two independent variables, while maintaining other variables at their optimal levels. These plots hold a single constant variable at its center, while the other two were varied within their experimental ranges generated by the software. It is stated in [23] that to identify the highest predicted value, it is the value that is confined by the surface in the smallest ellipse in the contour diagram. Elliptical contours observed when there is a perfect interaction between the independent variables.

Fig. 2 represents that an increase in sugarcane molasses concentration to 60% w/v and time of 72 hours, can lead to maximum CA production to the optimum value. Referring to the 3D graph; it can be observed that further increasing of CA production can be achieved when the substrate concentration is increased. The same concept applied in Fig. 3, which shows the relationship

between substrate concentration and agitation speed. From the 3D graph, it could be deduced that increasing substrate concentration to 60% w/v at an agitation speed of 180 rpm can raise CA yield.

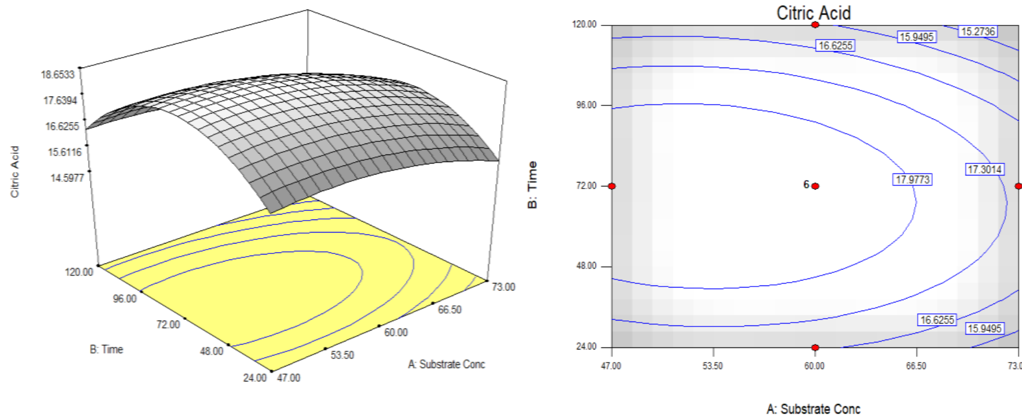

Fig. 2. 3D (left) and 2D (right) plot for CA production using HPLC as a function of time and substrate concentration.

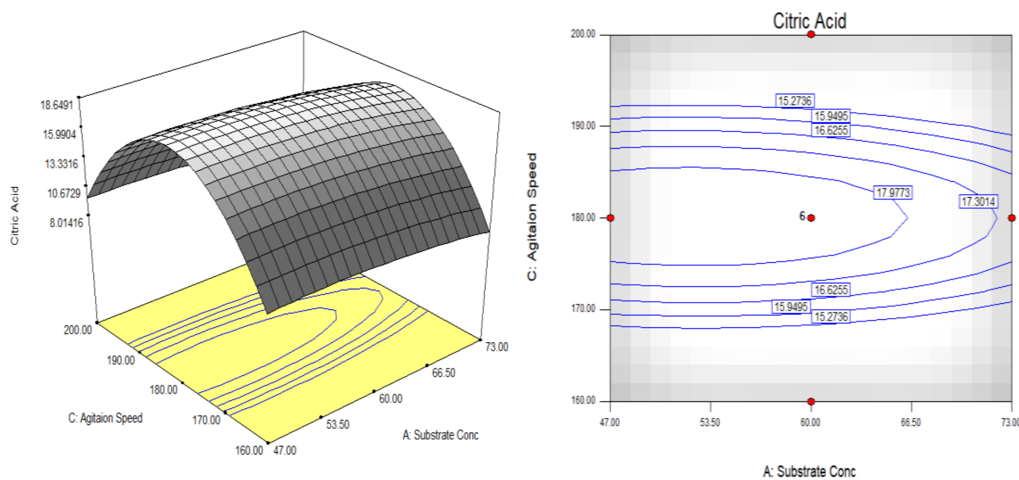

Fig. 3. 3D (left) and 2D (right) plot for CA production using HPLC as a function of substrate concentration and agitation speed.

In Fig. 4, the relationship between time and agitation speed shows that CA has not reached the maximum value. Though, agitation speed of 180 rpm and time of 72 hours approved to produce the maximum yield of CA using such parameters. It was also observed that as fermentation period was prolonged, there was a reduction in CA production. This might be due to the germination capacity of the spores. The fungus has reached its stationary phase and hence less CA was produced.

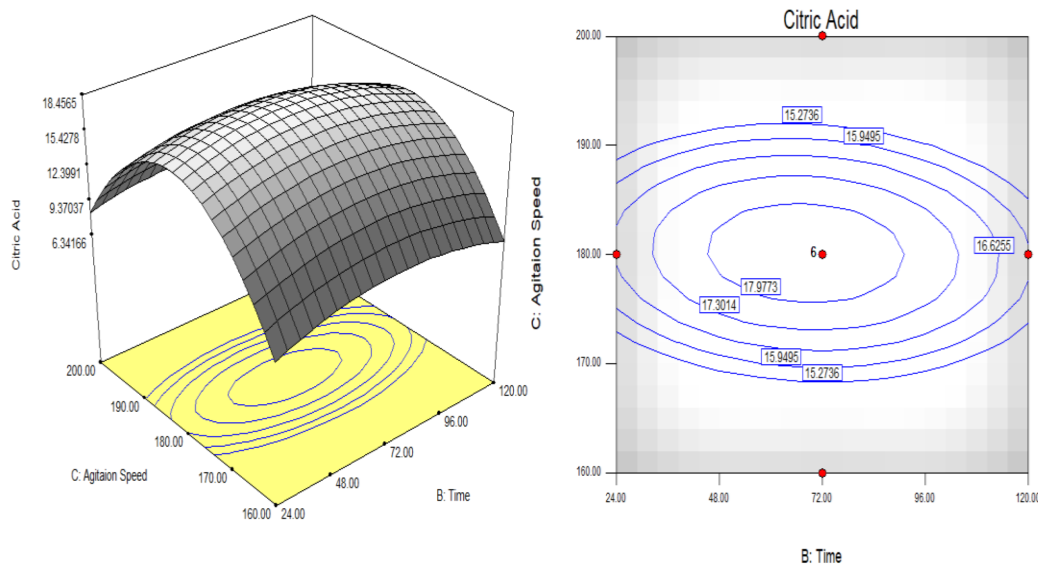

Fig. 4. 3D (left) and 2D (right) plot for CA production using HPLC as a function of agitation speed and time.

4. CONCLUSION

The objective of the study was fulfilled in which HPLC and FTIR were used to identify and quantify CA, respectively. As shown in the result and discussion, the final product was identified as CA using FTIR and the amount produced in each sample was measured by HPLC. By using the HPLC method, the maximum yield of CA was obtained at the conditions as follows: substrate concentration (60%), fermentation time (72 hours) and agitation speed (180 rpm). The range of highest production varied between 18.57 and 21.1 g/L. The highest amount of CA produced was 21.2 g/L. Optimum timing recorded at 72 hours. At this duration of time, *A. niger* shows that best adaptability to the fermentation medium. In regards to the agitation speed, the highest production observed with a speed of 180 rpm. Rather than 160 and 200 rpm, which are maybe too slow and too fast for the fungus to adapt, 180 rpm was the best for CA yield. From the three diagnostic plots, it can be concluded that the model has satisfied the assumptions of the analysis of variance.

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