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Optimization of Citric Acid Production by *Aspergillus niger* and *Candida tropicalis* for Solid State Fermentation Using Banana Peel Substrate

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Abstract

Increasing cost of production and global demand for citric acid is driving research towards optimizing process conditions to yield very high quantity of the organic acid using abundant cheap substrates and selected microorganisms. Consequently, this study was designed to optimize the production of citric acid by making use of banana (*Musa acuminata*) peels (agro-waste) through means of solid-state fermentation involving *Aspergillus niger* (Model A) and *Candida tropicalis* (Model B). In this study, a two-level, five-variable full factorial design of response surface methodology (RSM) comprising 32 experimental runs for each model were used to develop a statistical model for the optimization of fermentation conditions which include: pH, glucose, zinc, ammonium chloride and methanol. The results obtained indicate that a second order polynomial model fitted adequately and statistically significant ($p<0.0001$) and ($p<0.0410$) for Model A and B, respectively. The optimum values of the variables were: pH 4; glucose 5% w/v; zinc 2% w/v; ammonium chloride 0.5% w/v; and methanol 3% v/v. Under these conditions, the concentration of citric acid produced were 97.6 g/L with a pH of 3.85 using *Aspergillus niger* and 113.6 g/L with a pH of 3.45 using *Candida tropicalis* at 10days fermentation period. Experimental validation of the model indicated that no difference exists between the predicted and the actual yield results. Therefore, utilization of low-cost agro-waste banana peel which serve as suitable substrate for optimization of citric acid production is advocated because of their advantages such as income generation, reduction in environmental problems posed by food-waste disposal and public health hazards associated with it.

Keywords: *Citric Acid, Banana Peel, Solid State Fermentation, Candida tropicalis, Optimization, Aspergillus niger*

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I. INTRODUCTION

Citric acid is scientifically known as 2-hydroxy-1, 2, 3-propanetricarboxylic acid). Joint FAO/WHO Expert Committee on Food Additives have given approval for global recognition of citric acid as a generally regarded as safe (GRAS) organic acid considering its wide application in the pharmaceutical and food industries (Varshney, 2016; Dutta *et al*., 2019). It is a known weak acid which is also a biodegradable organic acid. Citric acid can also be described as a biochemical product usually obtained by fermentation (Oladele *et al*., 2015). Since global demand for citric acid has exceeded natural citric acid supply, the use of biotechnological fermentation processes has become imperative (Kishore and Reddy, 2011) Different substrates rich in carbon which is not limited to cassava bagasse, sucrose, brewery waste, carrot waste, cane molasses, beet molasses and glycerol have been utilized by researchers for the purpose of producing reasonable quantity of citric acid (Soccol *et al*., 2006). For

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commercial purposes, inexpensive substrates are selected for production of citric acid. Utilizing agro-industrial waste materials to be the source of carbon during the production process of citric acid is beneficial because it helps in managing waste as well as reduces the cost of production (Nadeem *et al.,* 2010). Different types of fruits contain citric acid which gives fruit a sour taste (Khairan *et al.,* 2019). In recent times, the use of fruit waste to produce citric acid is increasingly becoming more attractive to researchers because it is capable of reducing the cost of producing the organic acid (Varshney, 2016; Dutta *et al*., 2019) The use of banana and plantain peels fermented by *Aspergillus niger* to produce citric acid was successfully carried out by Khairan *et al*., 2019; Chukwuemeka *et al.* (2019).

Banana is a fruit daily consumed globally by every family which generates huge banana peels posing disposal problem in the environment (Kareem and Rahman, 2013). The increase in quantity of citric acid demanded globally makes it imperative to test alternative substrates for citric acid production. This calls for testing of banana peels rich in nutrients to ascertain its suitability as good substrate for the purpose of producing substantial quantity of citric acid.

For several decades, production of citric acid is commonly practiced by fermentation of different substrates by selected microorganisms such as bacteria, fungi and yeasts. For commercial purposes, *Aspergillus niger* as well as some yeasts like *Saccharomyces* species remains the microorganism of choice for production of citric acid (Oladele *et al*., 2015). In a recent study, Urak *et al.* (2014) reported the use of *Yarrowia lipolytica* for citric acid production. Also, Hesham *et al.* (2020) suggested the use of *Candida tropicalis* for commercial production of citric acid since it is more advantageous than *A. niger* which is a widely used fungus for that purpose. The advantages of using *Candida tropicalis* for citric acid production is due to its ability to utilize numerous substrates, reduced sensitivity to low concentrations of dissolved oxygen, associated with fewer health hazards, has ability to maintain stability against heavy metals, genetic variations and mechanical stress. Production of citric acid through fermentation is widely practiced because the operation is simple, control system involved is less sophisticated, energy requirement is low and importantly, frequent power failures do not have critical effect on the process (Kishore and Reddy, 2011). High production rate of citric acid leading to accumulation of this product is dependent on maintaining optimal level of sugar, acidity, dissolved oxygen, trace metals, nitrogen, phosphate, alcohol etc. (Jianlong *et al.,* 1998). Several factors such as aeration (Darouneh *et al*., 2009; Angumeenal *et al*., 2013), carbon substrate source and concentration (Soccol *et al*., 2006), pH (del Campo *et al.,* 2006), temperature, inoculum density (Auta *et al*., 2014), agitation (Benghazi *et al.,* 2014), moisture content (Lingappa *et al.,* 2001) etc. could affect the production of citric acid during fermentation. Therefore, optimizing these variables is aimed at achieving best performance which will cause an upsurge of citric acid yield in large quantity.

The steps involved in using one-factor-at-a-time technique in order to optimize variables is time consuming. A strategy to get over it is by optimizing the effecting parameters using response surface methodology (RSM) which is a technique involved in empirical statistics which makes use of multiple regression analysis of quantitative data derived from experiments that are statistically designed by solving multivariate equations obtained simultaneously (Kumari *et al*., 2008; Adeoye *et al.,* 2015).

Although banana peel has proven to be a substrate which can be used to produce citric acid, limited optimization studies have so far been carried out using different microbial species capable of synthesizing the organic acid. Therefore, this work was aimed at using RSM to optimize the conditions suitable for fermentation of banana peel by *Candida tropicalis* and *Aspergillus niger* in order to ascertain whether substantial quantity of citric acid could be produced or not.

II. Materials And Methods

A. The processing of substrate

The substrate used in this work was banana (*Musa acuminata*) peels which was thoroughly washed using water obtained from tap water and rinsed using distilled water. The peels were chopped into smaller pieces and dried inside hot air oven at 40 ^{O}C for 4 h. The peels were further milled with a hand grinder to obtain fine particles and stored under aseptic conditions until they were used.

B. The proximate composition of the substrate (banana peel)

The proximate composition of the banana peel reported in wet weight basis (wb) was determined using the AOAC (1995) methods. The carbohydrate content was calculated using the difference method.

C. Isolation, characterization and screening of fungal isolates

Serial dilution was carried out using soil and fresh samples of agricultural products (cucumber and banana peels) by adopting the method used by Jalal *et al*. (2009). For the purpose of isolating *Aspergillus niger* and *Candida tropicalis*, 0.1 ml solution was transferred from dilutions 10-4 and 10-5 and spread plated in triplicates on Potato Dextrose Agar (PDA) which was added with 10% lactic acid aimed at suppressing growth of bacteria. The inoculated culture plates were kept at 28 ± 2 °C which lasted for 7 days. Selection of discreet colonies from the culture plates which were already incubated was dependent on their colonial morphologies. The selected colonies were purified by sub-culturing on freshly prepared PDA culture plates containing lactic acid using spread plate technique followed by incubation of the culture plates at 28 ± 2 °C for a period of 7 days. Pure cultures obtained were subjected to characterization, identification and screening. Determination of various capabilities of citric acid positive fungal isolates was carried out using the method described by Patil and Patil (2014).

D. Molecular characterization of screened isolates

The procedure described by Uzah *et al*. (2020) was used in carrying out molecular characterization of the fungal isolates already screened for citric acid production. Molecular accession number (KT356204.1) and (EU440768.1) were assigned to *Candida tropicalis* and *Aspergillus niger,* respectively which were the fungal isolates successfully screened for citric acid production.

E. Inoculum preparation

Inoculum was prepared using the method adopted by Pandey (1992). The inoculum consists of spores from a 4-6 day old slant cultures. Preparation of suspension for the two isolates used in the study involved adding 10 ml of sterile water which already contain 2 drops of 0.1 % tween 80 added to the surface of the slant having copious spore growth. With a sterile inoculating needle, the spores in clumps was carefully scraped under aseptic conditions and the tubes were vigorously shaken to obtain a homogenous mixture of the suspension.

F. Determination of inoculum size

The spore density was measured using Nuebauer counting chamber following the procedure described by Blessing *et al*. (2018). Using the formula stated in equation 1 below, the number of spores was calculated.

$$\text{Cell (spore ml}^{-1}) = \frac{N\ X\ DF\ X\ 10^6}{A\ X\ D} \text{ --------- (Equation 1)}$$

Represented with letter A is the area counted = 5 x 1 m^2; Represented with letter D is depth of the counting chamber = 0.1 mm; Represented with letter N is the number of cells counted; DF represents the dilution factor.

G. Experimental design for optimization of citric acid production

Optimization of citric acid production was achieved using response surface methodology (RSM) and full factorial design as adopted by Adeoye *et al.* (2015). A total of thirty-two (32) experimental runs for each fungal isolate (*Aspergillus niger* and *Candida tropicalis*) with varied input values (two levels and five factors) were generated and carried out for optimization model development. The 2^5 full factorial design was deployed while investigating the interaction effect of input parameters which the experimental model indicated had optimum citric acid yield are dependent on the following factors - pH (A), Carbon (B), Trace element (C), Nitrogen (D) and Methanol (E). The five factors or parameters used for the optimization were pH, carbon source (glucose), nitrogen source (ammonium chloride), trace element (Zinc), and methanol. Although, addition of glucose as a carbon source has a minimal cost implication, the benefit which was aimed at enhancing microbial growth and citric acid synthesis is important because the substrate (banana peel) contains polysaccharide instead of simple sugar. The benefits of adding glucose to the medium is expected to outweigh the cost implication. Fermentation was carried out in a sucrose medium (g/L) which comprise sucrose (150 g), KH_2PO_4 (2.5 g), $MgSO_4.7H_2O$ (0.23 g) and NH_4NO_3 (3.0 g). Based on the experimental design, pH of the media was adjusted by adding HCL / NaOH. Fifty gram (50 g) of milled *Musa accuminata* peel was poured inside each of the 250 ml capacity Erlenmeyer flask and moistened with 25 ml of the fermentation medium, autoclaved at 121 ^{o}C at 15 psi for 15 min. Thereafter, the content of the flask was removed from the autoclave for cooling to take place until room temperature (28±2 ºC) was achieved before 10 ml of the inoculum suspension was inoculated into the flasks and incubated at 28 ±2 ^{O}C for 12 days.

H. Extraction of citric acid

At 48 h interval of incubation, the citric acid content was extracted. After fermentation, 100 ml of sterile distilled water was poured inside each of the flask in order to obtain slurry. Using a glass rod the content of each flask was stirred for 1 h and the slurry filtered using Whatman ^{TM} Qualitative filter paper No. 1 to separate the sediment. Further centrifugation of the filtrate was carried out at 10000 rpm for 10 minutes. The clear filtrate from each flask was then used to estimate citric acid and pH (Pandey, 1992; Usami *et al.,* 1998; Soccol *et al*., 2006).

I. Estimation of citric acid

Determination of the concentration of citric acid present in the culture filtrate was carried out by means of titration. Ten millilitre (10 ml) culture broth was withdrawn and 3 drops of phenolphthalein was added as an indicator (Imandi *et al*., 2007; Khosravi and Zoghi, 2008). Exactly 0.1M NaOH was titrated against 10 ml (equivalent to 10g) culture broth until the end-point was reached when the change in colour was noted. The quantity of NaOH used was read off as titre and the value was recorded. The formula which was adopted by AOAC (1995) was used to calculate the citric acid (%) released.

$$\% \text{ Citric acid} = \frac{\text{Normality} \times \text{Volume of NaOH} \times \text{Equiv. wt. of CA} \times \text{Dilution factor}}{\text{Weight of Sample (g) X 10 ml}}$$

---- (Equation 2)

J. Statistical analysis

DESIGN EXPERT version 11 enabled the use of Response Surface Methodology (RSM) as well as Full Factorial Design for the purpose of developing optimization model for citric acid production. The parameters were done in triplicates and results obtained were analyzed using one-way analysis of variance (one-way ANOVA) as well as multiple range tests in order to determine the differences in the means at 5 % (0.05) significant level.

III. RESULTS

The details of morphological characterization of the fungal isolates used in this study is presented in a recently published report by Uzah *et al*. (2020) which forms part of this publication. Figure 1 A and B are the colonial morphology of *Aspergillus niger* and *Candida tropicalis* which were screened for their ability to produce citric acid. Figure 1, C and D shows *Aspergillus niger* and *Candida tropicalis*, respectively with a yellow zone around their colonies which is an indication that the isolates produced organic acid.

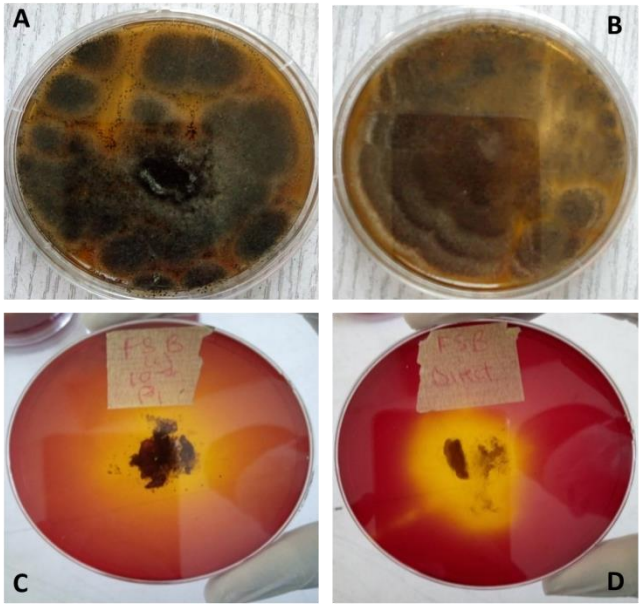


Figure 1. Colonial morphology of *Aspergillus niger* and *Candida tropicalis*. A: Colonial morphology of *Aspergillus niger* on Potato dextrose agar. B: Colonial morphology of *Candida tropicalis* on Potato dextrose agar. C: Culture plate showing organic acid production with yellow zones around colonies of *Aspergillus niger*. D: Culture plate showing organic acid production with yellow zone around colonies colonies of *Candida tropicalis*.

The proximate composition of the banana peel is presented in Figure 2. The moisture, ash, protein, fat, crude fiber and carbohydrate content is 86.28 %, 1.76 %, 0.60 %, 1.15 %, 4.67 % and 5.55 %, respectively. The nitrogen content of the banana peel is 0.10±0.007 %.

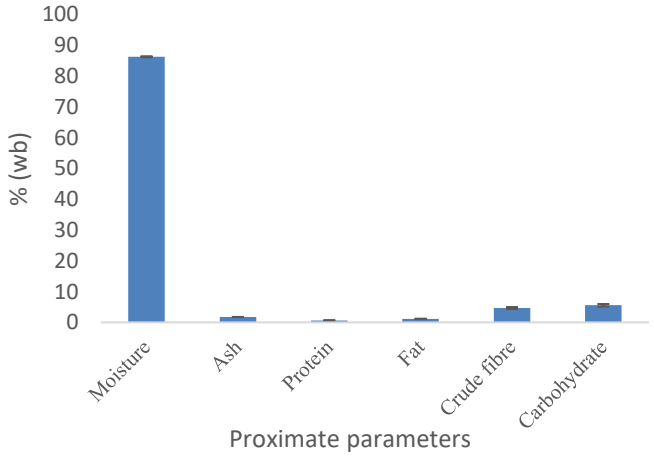


Figure 2. Proximate composition of banana peel; Key: Wb – Weight wet basis

The result of the response from optimized production of citric acid depicted in Table 1 indicates that optimization A setup (*Aspergillus niger*) had the highest mean value of 47.2 g/L on runoder 16 and least mean value of 12.96 g/L on runorder 11 while optimization B setup (*Candida tropicalis*) had the highest mean value of 37.6 g/L on runorder 22 and least mean value of 9.76 g/L on runorder 32.

The quadratic model analysis of variance (ANOVA) using *Aspergillus niger* and *Candida tropicalis* are shown in Table 2 and 4, respectively. This is an indicator that model F-value of 6.86 and $p < 0.05$ suggests that the model terms were significant. With respect to this study, 'B', 'AE', 'ADE' and 'ABCD' are model terms which are significant. Also, Lack of Fit F-value 0.59 suggests that Lack of Fit is not significantly relative to the pure error. This indicates that model F-value 4.36 and $p<0.05$ suggests that the model terms are significant. In this situation, 'CE' is a significant model term. Also, the Lack of Fit F-value 1.14 suggests that Lack of Fit is not significant relative to the pure error.

The coefficient estimate of the fitted model denotes the predictable change which occurred in response per unit change in factor value in a situation all the factors remaining are kept constant are shown in Table 3 and 5. Using *Aspergillus niger* for the process optimization model, Table 2 shows an intercept coefficient of 27.30 with VIF of 1.0 while *Candida tropicalis* (Table 4) shows an intercept coefficient of 25.58 and VIF of 1.0.

Surface graph using contour plots (Figures 3-5) shows the variations and interaction effects between two input parameters on response. In process optimization model (*Aspergillus niger*), Fig. 3 shows the interaction between pH and methanol which indicates that the citric acid yield was at its peak of 28 g/L when pH is at 5.5 and methanol 2.6 %. Figure 4 shows no interaction between pH and nitrogen. In process optimization model (*Candida tropicalis*), Figure 5 shows the interaction between methanol and trace element which indicates that the citric acid yield was at its peak of 26 g/L when methanol is at 2.4 % and trace element 1.4 %.

In Figures 3 and 4, the response surface plots can be seen as being nearly plane whereas the contour displayed a nearly parallel straight lines which can be attributed to statistically low interaction coefficients.

Also considering the contrary twists in all response, the surface graphs could easily be observed especially with interaction terms having higher coefficient in mathematical model.

Table 1. Full factorial design procedure and response towards the yield of citric acid

Runorder	Different coded factors					The actual factors					Response (g/L)	
	A	B	C	D	E	pH	Carbon (Glucose) % w/v	Trace element (Zinc) % w/v	Nitrogen (Ammonium chloride) % w/v	Methanol %v/v	Optimization A (*Aspergillus niger*)	Optimization B (*Candida tropicalis*)
1.	1	1	1	1	1	6	5	1	0.25	2	30.72	33.6
2.	1	1	1	1	2	6	5	1	0.25	3	28	36.8
3.	1	1	1	2	1	6	5	1	0.5	2	20.16	31.36
4.	1	1	1	2	2	6	5	1	0.5	3	27.2	24.825
5.	1	1	2	1	1	6	5	2	0.25	2	20	26.88
6.	1	1	2	1	2	6	5	2	0.25	3	23.2	27.04
7.	1	1	2	2	1	6	5	2	0.5	2	14.4	13.76
8.	1	1	2	2	2	6	5	2	0.5	3	20	21.92
9.	1	2	1	1	1	6	10	1	0.25	2	13.6	30.08
10.	1	2	1	1	2	6	10	1	0.25	3	23.84	33.6
11.	1	2	1	2	1	6	10	1	0.5	2	12.96	31.68
12.	1	2	1	2	2	6	10	1	0.5	3	31.2	30.88
13.	1	2	2	1	1	6	10	2	0.25	2	26.4	24.96
14.	1	2	2	1	2	6	10	2	0.25	3	33.92	29.12
15.	1	2	2	2	1	6	10	2	0.5	2	22.72	29.92
16.	1	2	2	2	2	6	10	2	0.5	3	47.2	28.8
17.	2	1	1	1	1	4	5	1	0.25	2	40.8	28.8
18.	2	1	1	1	2	4	5	1	0.25	3	19.04	18.72
19.	2	1	1	2	1	4	5	1	0.5	2	25.92	28.64
20.	2	1	1	2	2	4	5	1	0.5	3	46.08	31.2
21.	2	1	2	1	1	4	5	2	0.25	2	27.2	28.16
22.	2	1	2	1	2	4	5	2	0.25	3	27.2	37.6
23.	2	1	2	2	1	4	5	2	0.5	2	29.12	27.36
24.	2	1	2	2	2	4	5	2	0.5	3	20	30.4
25.	2	2	1	1	1	4	10	1	0.25	2	30.88	19.04
26.	2	2	1	1	2	4	10	1	0.25	3	22.4	12.32
27.	2	2	1	2	1	4	10	1	0.5	2	38.88	28.64
28.	2	2	1	2	2	4	10	1	0.5	3	24.8	14.88
29.	2	2	2	1	1	4	10	2	0.25	2	44.32	12.8
30.	2	2	2	1	2	4	10	2	0.25	3	39.84	21.92
31.	2	2	2	2	1	4	10	2	0.5	2	28	13.12
32.	2	2	2	2	2	4	10	2	0.5	3	13.6	9.76

Table 2. Analysis of variance (partial sum of squares) for optimization setup A (*Aspergillus niger*) using Response surface quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	Level of Significance
Model	1732.97	4	433.24	6.86	0.0001	Significant
B-Carbon	459.67	1	459.67	7.28	0.0091	
AE	416.16	1	416.16	6.59	0.0128	
ADE	494.62	1	494.62	7.83	0.0069	
ABCD	362.52	1	362.52	5.74	0.0198	
Residual	3725.54	59	63.14			
Lack of Fit	1241.00	27	45.96	0.5920	0.9159	Is not significant
Pure Error	2484.53	32	77.64			
Cor Total	5458.51	63				

With respect to the 3D graph of combination effect, our results revealed that pH played the most significant effect in achieving citric acid yield compared with other input parameters.

Table 3. Estimated coefficients which is for the fitted model considering the coded factors for optimization A (*Aspergillus niger*)

Factor	Coefficient	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	27.30	1	0.9933	25.31	29.29	
B-Carbon	-2.68	1	0.9933	-4.67	-0.6924	1.0000
AE	-2.55	1	0.9933	-4.54	-0.5624	1.0000
ADE	-2.78	1	0.9933	-4.77	-0.7924	1.0000
ABCD	-2.38	1	0.9933	-4.37	-0.3924	1.0000

Table 4. Analysis of variance (partial sum of squares) for optimization setup B (*Candida tropicalis*) using Response surface quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	Level of Significance
Model	236.04	1	236.04	4.36	0.0410	significant
CE	236.04	1	236.04	4.36	0.0410	
Residual	3358.31	62	54.17			
Lack of Fit	1736.19	30	57.87	1.14	0.3558	Is not significant
Pure Error	1622.12	32	50.69			
Cor Total	3594.35	63				

Table 5. Estimated coefficients which is for the fitted model considering the coded factors for optimization B (*Candida tropicalis)*

Factor	Coefficient	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	25.58	1	0.9200	23.74	27.42	
CE	1.92	1	0.9200	0.0815	3.76	1.0000

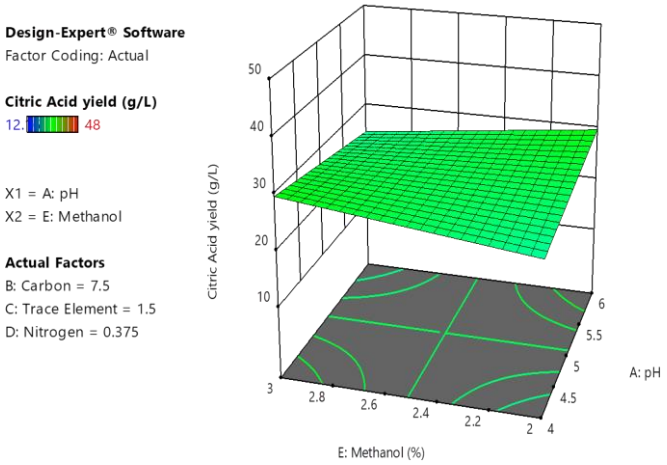


Figure 3. Response surface graph for the model optimization (*Aspergillus niger*) showing the interaction between methanol and pH for the production of citric acid.

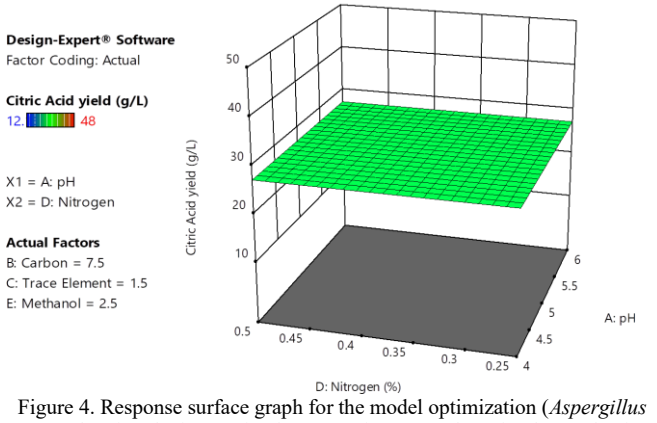


Figure 4. Response surface graph for the model optimization (*Aspergillus niger*) showing the interaction between nitrogen and pH for the production of citric acid

Final production of citric acid from the developed process optimization models are depicted in Fig. 6-7. The line plot which describes production of citric acid in relation to pH and time shown in Fig. 6 indicates that the highest yield of citric acid of 97.6 g/L at day 10 with final pH of 3.85 using *Aspergillus niger* while Fig. 7 shows the line plot of citric acid production using *Candida tropicalis* which indicates that maximum possible yield of citric acid was 113.6 g/L at day 10 with final pH of 3.45. To estimate the quantity of citric acid released by *Aspergillus niger*, 10 ml of citric acid which is equivalent to 10 g was neutralized by 19.64 ml of NaOH. Similarly, estimation of citric acid released by *Candida tropicalis* involved 22.87 ml of NaOH which neutralized 10 ml of citric acid being equivalent to 10 g.

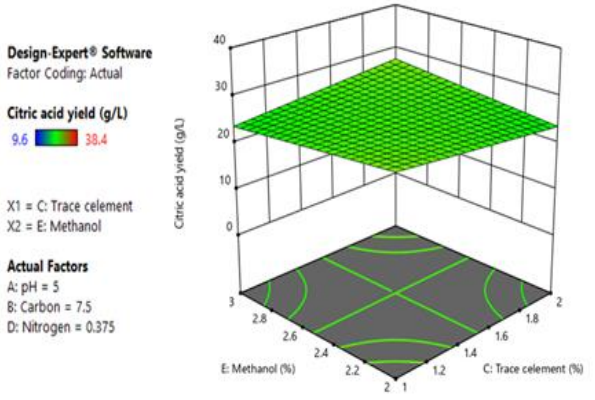


Figure 4. Response surface graph for the model optimization (*Candida tropicalis*) showing the interaction between methanol and trace element for the production of citric acid

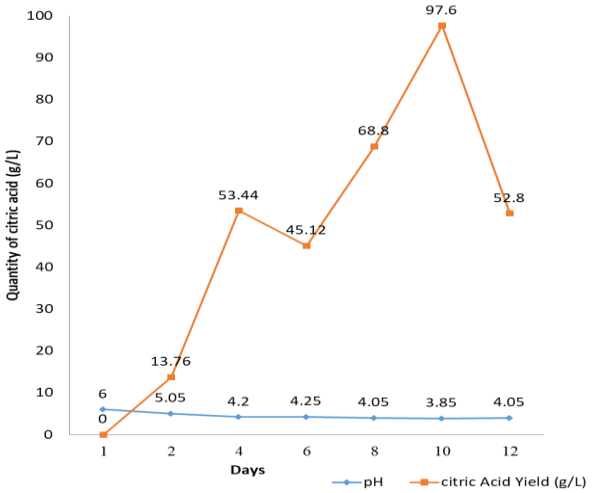


Figure 6. Line plot of citric acid production from process optimization model (*Aspergillus niger*)

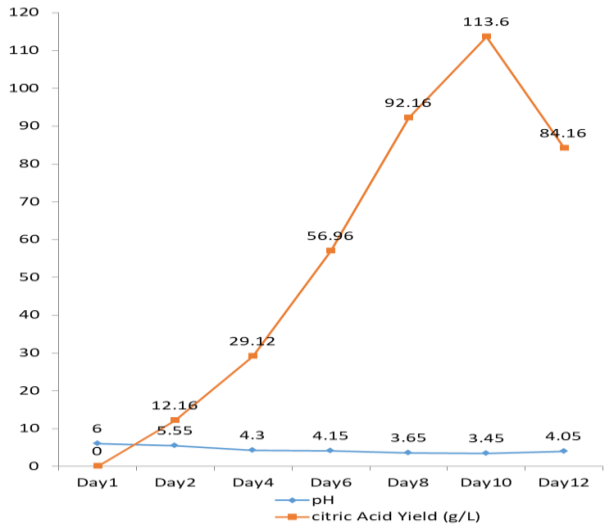


Figure 7. Line plot of citric acid production from process optimization model (*Candida tropicalis*)

IV. Discussion

A. Proximate composition of banana peels

According to Anhwange *et al*. (2009), moisture, ash, protein, crude lipid, carbohydrate, and crude fibre content of banana peel is 6.70, 8.50, 0.90, 1.70, 59, and 31.70 %, respectively. This result partially agrees with the values obtained from this study. The time of harvesting, varieties of banana and cultural practices could be responsible for the variations in the proximate composition of the banana peel. Nitrogen constitute the basic part of cell proteins, capable of inducing the formation of pellets in filamentous fungi. Therefore, it plays a significant role in the production of citric acid. Furthermore, studies have demonstrated that the specific type of nitrogen source employed in citric acid production usually influences the yield of citric acid Kudzai *et al.* (2016). The carbohydrate content (5.55 %) of the banana peels which serves as the carbon source was low. This forms part of the reason glucose was added to the fermentation medium to enhance yield of the citric acid. Similarly, ammonium chloride was also added as a nitrogen source since the nitrogen content of the banana peel was 0.10 %.

B. Model development for process optimization

The traditional 'one-factor at a time' technique usually adopted to optimize a multivariable system is challenging because precious time is wasted in the process. Apart from that, the technique fails to capture the interactions that come into play between the variables (Abonama *et al*., 2014). It is evident that this method involves carrying out a large number of experiments before the optimum levels can be determined. The setback occasioned by single factor optimization process can be triumphed by optimizing all the affecting factors using full factorial or central composite design (Box and Wilson, 1951), of response surface methodology (RSM).

Response surface methodology (RSM) which involves 2 levels and 5 factors was used to develop quadratic model for process optimization which involves fermentation of citric acid by *Aspergillus niger* and *Candida tropicalis* making use of banana peels as a substrate. Findings from our study shows that increase in citric acid yield occurred while fermentation lasted. Low P-value (0.001) and high F-value (6.86) for *Aspergillus niger* as well as low P-value (0.0410) and high F-value (4.36) for *Candida tropicalis* further substantiate statistical significance and non-significant lack of fit for both microbial genera is good and makes the model to fit. This observation agrees with the findings reported by Adeoye *et al.* (2015), which stated that the greater the F-value from a unit, the more certain it is that the factors explain adequately the variation in the data about its means and effect of estimated factors are real. Based on results obtained from RSM applied in this study, adequate precision signal to noise ratio was 9.356 for *Aspergillus niger* and 2.9522 for *Candida tropicalis* gives an indication that adequate signal for the model will be used to navigate the design space.

Result obtained from this study shows an intercept coefficient of 27.30 with Variance Inflation Factor (VIF) of 1.0 using *Aspergillus niger* while *Candida tropicalis* shows an intercept coefficient of 25.58 and VIF of 1.0. Thus, the intercept in an orthogonal design depicts the overall average response which involves all the runs. The coefficients describe the adjustments around the average considering the factor settings. Situations whereby the factors are orthogonal, the VIFs are 1; VIFs >1. This suggests its multi-collinearity. Therefore, each time the VIF becomes higher, the correlation of factors becomes more severe. Based on existing rough rule, VIFs < 10 are tolerable (Hair *et al.,* 1995, Ringle *et al.,* 2015).

C. Input parameters interaction and their effects on production of citric acid

This study has shown that pH, carbon, trace element, nitrogen and methanol influenced the experimental citric acid yields whereas incubation period and temperature remain the constant variables. The high yield is attributable to the fermenting organisms, substrate and nutritional contents of fermentation medium (Kubicek, 1998; Kobomoje *et al*., 2013). Large variations in citric acid yields 12.96 - 47.2 g/L by *Aspergillus niger* and 9.76 -37.6 g/L by *Candida tropicalis* shown in Table 1 demonstrate that the bioprocess was highly susceptible to the input parameters considered in this study. Our results revealed that increase in carbon and other input parameters above the optimum point reduced citric acid yields. In the first model which involved *Aspergillus niger*, pH was identified as a factor which had the most effect on citric acid production followed by methanol. Meanwhile, trace

element was found to be most effective in the second model that involved *Candida tropicalis*.

Surface results gives an indication that input parameters becoming higher will directly lower the citric acid yields. These results showed consistency with the coefficient in the statistical model. 3D modelling result obtained as well as their contours clearly shows the direction sensitive towards citric acid yield. Thus, observation of curved contour lines could be attributed to high interaction effects (Bingol *et al*., 2010). Fig. 3-5 shows that if input parameters namely pH, carbon, trace element, nitrogen and methanol were maintained at 5.5, 5 %, 2 %, 0.25 % and 2 % respectively, what would happen will be a consequential increase in yield of citric acid. This result is in agreement with findings reported by Akdeniz *et al.* (2012). However, further increase in these input parameters will not greatly impact on production of citric acid (Adeoye *et al.,* 2015). Therefore, these observations with the various input parameters are essentially significant in the course of production of citric acid in a large scale. In addition, 2% optimum methanol concentration obtained from this study indicates the impact of low molecular weight alcohol (methanol) in citric acid production which is in agreement with the investigations of some researchers (Yu *et al.,* 2017, Assadi and Nikkhah, 2002), who reported a remarkable increase in citric acid yield with 2 -3% methanol. Furthermore, the addition of 3-4 % (v/v) methanol to the fermentation medium could retard the fungal growth and sporulation, thereby improving the citric acid yield. Also, it is suggested that the presence of methanol may increase the permeability of the cell to citrate, and the cell responds to the diminished intracellular level by increasing production via repression of 2-oxoglutarate dehydrogenase. Although the exact role of methanol in citric acid production is not clear, it is suggested that methanol serve as a source of acetyl CoA which might bring about increase in the transfer of nutrients across the cell membrane. Consequently, an upsurge in the excretion of citric acid is bound to occur (Kapoor *et al*., 1982; Jianlong, 1998; Navaratnam *et al.*, 1998).

D. Citric acid Production from the optimization model

Validation of experimental design of the optimized profile as shown in Fig. 6 and 7 made it known that maximum yield of citric acid (97.6 g/L) was achieved using *Aspergillus niger* and 113.6 g/L using *Candida tropicalis in* the laboratory with improvement of 36 -70 g/L over the optimization response. The results obtained indicate that citric acid yield was higher as to compare with research findings of Kareem and Rahman (2013) which reported that maximum yield of citric acid 82.12 g/Kg was obtained using banana peel, 1 % methanol and trace element (10 ppm) at 30 ^{o}C.

It has been established that fermentation time is very important in substrate utilization. Therefore, it influences the yield of citric acid. Fig. 6 and 7 shows that maximum citric acid yield 97.6 g/L and 113.6 g/L was obtained after fermentation lasted for 10 days at 28 ± 2 ^{o}C using *Aspergillus niger* and *Candida tropicalis,* respectively. This observation corroborates the findings of Varsha (2015) and Khairan *et al*., (2019) who reported that high yield of citric acid occurred after fermentation lasted for 10 days (240 h). However, the result is not in agreement with Nadeem *et al.* (2010) and Blessing *et al*. (2018) which reported that highest citric acid yield occurred after 192 h fermentation. In another related study, Kareem and Rahman (2013) reported optimum yield of citric acid (82.2 g/kg) after 92 h fermentation. The result obtained at Day 1 shows that production of citric acid did not occur. This could be due to prolonged lag phase which is in agreement with the findings of Blessing *et al.* (2018) and Varsha (2015).

Since wild fungal isolates were selected for citric acid production and vegetative cycle of the isolates takes 3-4 days to complete before sporulation could commence, this could have resulted in the extension of the fermentation period for optimum citric acid production up to 10 days. Hence, the need for genetic enhancement of the isolates for efficient performance. After 10 days fermentation at 28 ± 2 ^{o}C, the results obtained shows that citric acid yield increased progressively to an optimum point with reduction in pH from the initial value of 6.0 in the medium. Maximum yield of citric acid 97.6 g/L at pH of 3.85 was obtained using *Aspergillus niger* and 113.6 g/L at pH of 3.45 using *Candida tropicalis* after 10 days fermentation. Sawant *et al*. (2018) reported that solid substrate fermentation resulted in 82.12 g/L yield of citric acid using banana peels and *Aspergillus niger* UABN 210. Higher yield of citric acid reported in this study compared with the quantity reported by Sawant *et al.* (2018) could be as a result of the optimization condition adopted and strain of the *Aspergillus niger* and *Candida tropicalis* used for the production process. Result from this study shows that pH of 3.45 was most suitable for citric acid production using banana peel as substrate and *Candida tropicalis* as the fermenting yeast. Abonama *et al.* (2014) and Afolabi *et al.* (2018) reported highest citric acid yield of 30 g/L and 4.2 g/L, respectively using *Candida tropicalis* under submerged fermentation. Hence, the result obtained for this study further substantiate the fact that *Candida tropicalis* performs best under solid state fermentation than submerged fermentation. According to Kareem and Rahman (2013), during the early stages of fermentation, it is important to maintain pH for a specific amount of biomass to be formed. This condition is very important to achieve maximum yield, growth and metabolic activities of the microorganisms. In our study, consistent decrease in pH was observed as fermentation time increased.

This could be attributed to citric acid formation and accumulation. When ammonia was absorbed by the germinating spores, it is probable that protons were released. This condition might have resulted in the release

of hydrogen ions which resulted in lowering pH of the medium (Max *et al*., 2010). Notably, our findings corroborate with earlier studies by Thiruvengadam and Thangavel (2016) which observed that maximum citric acid yield occurred when the initial pH was 6.0. The result obtained from this study is close to that which was reported by Khosravi and Zoghi (2008) and Blessing *et al.* (2018) which reported that yield of citric acid was at its maximum when initial pH was 5.5. Thus, fungal strains is suggested to thrive best in acidic medium which is within pH range of 3.0-6.0 (Fawole and Odunfa, 2003) A pH that range from 2.0-6.0 is frequently maintained for solid state and submerged fermentation (Adham, 2002). Thus, these conditions have great impact on citric acid production (Ajala *et al*., 2020).

Since this study was able to demonstrate that banana peel which is a cheap and readily available agricultural waste could serve as a substrate to produce citric acid, it is expected that after carrying out detailed cost analysis based on the conditions suitable for the process which is recommended from this study, the production cost will be low and highly competitive with conventional methods.

V. CONCLUSION

The 2^5 full factorial design deployed in this study to investigate the effect resulting from interaction of input parameters as well as the experimental model indicated that optimum citric acid yield was dependent on various factors namely pH, carbon, trace element, nitrogen and methanol. The maximum optimization responses for both models were 47.2 g/L at runorder (16) using *Aspergillus niger* and 37.6 g/L at runorder (22) using *Candida tropicalis*. Results obtained showed that all the five factors impacted greatly on citric acid yield. The main effects were found to be in coded form (ADE, B, AE, and ABCD) using Aspergillus niger and (CE) using *Candida tropicalis*. The effects of experimental variability and their interactions on the response (citric acid) indicate that increase in carbon and other input parameters above it optimum point reduced citric acid yields. The most effective factor was pH followed by methanol and trace element in first and second model, respectively. Therefore, the experimental validation of the process optimization brought about maximum yield of 97.6 g/L citric acid using *Aspergillus niger* and 113.6 g/L using *Candida tropicalis* within the period of 10days fermentation. This study has successfully projected the prospects of using banana peels which ordinarily constitute an environmental challenge in terms of waste disposal to create wealth through production of citric acid.

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