Optimization of bioethanol production from simultaneous saccharification and fermentation of pineapple peels using *Saccharomyces cerevisiae*

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ABSTRACT: In this study, bioethanol production from the simultaneous saccharification and fermentation (SSF) of pineapple peels using cellulase and *Saccharomyces cerevisiae* was investigated. A three-factor Box-behnken design (BBD) and response surface methodology (RSM) were employed to study the effect of broth pH (2-6), yeast loading (2-10 g/l) and ammonium sulphate concentration (1-5 g/l) on the bioethanol production process. Optimum values of pH, yeast loading and ammonium sulphate concentration of 6.0, 8ml and 5g/l, respectively were obtained for maximum bioethanol concentration of 5.82%v/v. The results obtained show the possibility of using pineapple peels as feedstock for bioethanol production via SSF method. Moreover, the use of BBD and RSM as robust technique for determining the effect of parameters and optimum conditions for bioethanol production has been ascertained.

The excessive consumption of non-renewable energy has greatly resulted in environmental deterioration and public health problems (Kahia et al., 2016). This in turn has resulted in the need to find a source of renewable energy. Bioethanol produced by fermentation of plant biomass is considered to be an environmentally friendly alternative to fossil fuels and has the potential to suitably replace gasoline as a transportation fuel (Itelima et al., 2012). The economics of bioethanol production is significantly influenced by the cost of the raw materials and in order to reduce this cost, cheap materials are sourced as feedstock for ethanol production (Franko et al., 2016). As a result, the search for renewable biomass sources has focused primarily on plant biomasses that are usually regarded as waste and possess lignocellulosic materials (Fish, Bruton and Russo, 2009). The use of lignocellulosic residues in the production of bioethanol would ensure continuous energy supply because they are less expensive than starchy and sucrose producing crops (commonly used in bioethanol production) and are available in large quantities.

An example of an important lignocellulosic residue that can be used in bioethanol production is pineapple peels. Pineapple is the third most important tropical fruit in the world after Banana and Citrus and Nigeria, ranks 7th on the list of world producers of pineapple as well as is the leading producer of pineapple in Africa. (Adegbite et al.2014). However, there are a lot of unused excess parts of the pineapple, notably the peels, which are considered as waste and contribute to the country's garbage problem. These peels are a major component of domestic and industrial waste worldwide, rich in sugar and lignocellulosic components and account for 29-40% (w/w) of the total pineapple weight. Their high sugar and lignocellulosic components could make them a potentially viable feedstock for bioethanol production. Bio-ethanol fermentation process is usually done by species of the yeast *Saccharomyces* because it ferments glucose to ethanol and is known for its high insensitivity to temperature and substrate concentration, rapid fermentation rates as well as high ethanol tolerance. (Avril Rodiel Bries, 2008). In bioethanol fermentation from lignocellulosic materials, pretreatment and hydrolysis are usually needed to convert these materials to monomeric sugars before fermentation can take place. Enzymes are usually employed for the hydrolysis of these materials and this is considered a very viable strategy since it offers advantages over other chemical conversion routes of higher yields, minimal byproduct formation, low energy requirements, mild operating conditions, and environmentally friendly processing (Zheng, Pan and Zhang, 2009). In using the enzymatic route, studies have shown that it is advantageous to use the simultaneous saccharification and fermentation (SSF)

route in the production of bioethanol. (Avril Rodiel Bries, 2008). In this process, glucose released by the enzyme, cellulase is simultaneously converted to ethanol by the fermenting microorganism. One of the advantages of this process is that ethanol fermentation is carried out in a single bioreactor which provides a reduction in the overall fermentation time and a reduction in the investment and operational costs (Białas et al., 2010).

Optimization of significant process conditions is a very important stage in order to develop an efficient and cost-effective bioprocess. (Gade, 2009). Usually in optimization processes, the traditional one-factor-at-a-time method is employed but this method is often cumbersome and time consuming. (Nadya et al, 2012). As a result, response surface methodology (RSM) which is a useful tool that helps to identify the effects of several process variables influencing a particular response by varying them simultaneously and carrying out a limited number of experimental runs is now more commonly employed. (Cazetta et al., 2007)

The objective of this study was to optimize important parameters for the bioethanol production from pineapple peels via simultaneous hydrolysis and fermentation using *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Sample Collection and Preparation: Pineapple peels obtained from a fruit vendor were washed, cut in small pieces and then blended until a pulpy mass was obtained. It was then stored in the refrigerator prior to use.

Simultaneous Saccharification and Fermentation (SSF): The batch SSF was performed at a solid loading of 15% (w/v) at room temperature for 72 hours with a final working volume of 100 ml. The unsterilized pulp was supplemented with mineral media without glucose and the pH was adjusted by adding 0.5M Sodium Hydroxide. After the enzymes were added the mixture was left for 1hour for pre-saccharification at room temperature. Thereafter the inoculum was added at a concentration of 5 g /l of wet cells. The parameters considered were: yeast loading (%v/v), pH, and concentration of ammonium sulphate (g/L).

Determination of Bioethanol produced from Pineapple broth: At the end of the fermentation, liquid samples were taken from the fermentation broth. The samples were filtered and the filtrate was used to determine ethanol concentration. Ethanol concentration was determined using High Performance Liquid Chromatography (HPLC) equipped with an Ultra violet (UV) detector and a

C18 column. The column was used to separate ethanol from samples using pure Acetonitrile as mobile phase at a flow rate of 1ml/min and injection volume of 10µl.

Process Optimization by Response Surface Methodology: A three-factor Box-Behnken Design (BBD) was employed for the experimental design. The responses obtained from the BBD were optimized using response surface methodology. Each of the factors to be optimized was coded at three levels which gave range for yeast loading (2-10% v/v), pH (2-6), concentration of ammonium sulphate $((NH_4)_2SO_4)$ (1-5 g/L). The bioethanol concentration was chosen as the response for process optimization using RSM. The experimental design carried out using Statistica version 22 (Dell inc. USA) was made up of 17 runs. Experimental observations from the fermentation process were analyzed and fitted according to Equation (1) as a second-order polynomial equation including main effects and interaction effects of each variable. Analysis of variance (ANOVA) and response surface plots were generated using Design Expert software. The optimized value of the independent variables for optimum response was determined using numerical optimization.

$$Y_i = b_o + \sum b_i X_j + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2 + e_i$$
(1)

where Y_i is the dependent variable or predicted response, X_i and X_j are the independent variables, b_o is offset term, b_i and b_{ij} are the single and interaction effect coefficients and e_i is the error term.

RESULTS AND DISCUSSION

Optimization of bioethanol production using RSM: The optimization of bioethanol production from SSF of pineapple peals was performed using BBD. The responses of the experimental runs obtained from the BBD are depicted in Table 1. The BBD is a distinctive experimental design due to occurrence of treatment combinations at the midpoints of the experimental space edge. As a result of this, it is easier to estimate the first and second order coefficients using BBD. Besides, due to fewer numbers of runs in BBD compare to CCD, the cost of running the experiment is less. The use of BBD in this study resulted in a non-linear second order model between the input variables (pH, yeast loading and $(NH_4)_2SO_4$ concentration) and the output variable (bioethanol concentration). The significance and adequacy of the RSM model shown in Equation (1) was evaluated using ANOVA (Table 2) and coefficient of determination (R^2). Optimum

conditions obtained using the regression model were pH 6, yeast loading 8 ml and concentration of ammonium sulphate, 5g/l which gave an optimum ethanol concentration value of 5.82% (v/v).

Table 1: Experimental and predicted results of the ethanol production process

Experimental runs	pH (A)	Yeast loading (g/l) (B)	Concentration of $(NH_4)_2SO_4$ (g/l) (C)	Bioethanol concentration (%v/v) (Y)
1	2	2	3	2.67
2	4	10	5	2.61
3	6	10	3	2.76
4	4	6	3	1.38
5	6	2	3	1.70
6	4	6	3	1.38
7	4	2	5	0.48
8	4	10	1	2.03
9	4	2	1	2.08
10	6	6	5	5.49
11	2	10	3	3.87
12	2	6	5	4.32
13	4	6	3	1.38
14	4	6	3	1.92
15	6	6	1	3.67
16	4	6	3	1.29
17	2	6	1	7.30

Table 2: Analysis of variance (ANOVA) for the optimization of bioethanol concentration using Box-Behnken Design

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	48.24545	9	5.360605	132.8884	< 0.0001
A-pH	2.572769	1	2.572769	63.77848	< 0.0001
B-yeast loading	2.353969	1	2.353969	58.35447	0.0001
C-conc of ammonium sulphate	0.59308	1	0.59308	14.70235	0.0064
AB	0.004225	1	0.004225	0.104737	0.7557
AC	5.791328	1	5.791328	143.566	< 0.0001
BC	1.190244	1	1.190244	29.50595	0.0010
A^2	23.05349	1	23.05349	571.492	< 0.0001
B^2	4.714474	1	4.714474	116.871	< 0.0001
C^2	8.09001	1	8.09001	200.5499	< 0.0001
Residual	0.282374	7	0.040339		
Lack of Fit	0.025075	3	0.008358	0.12994	0.9374
Pure Error	0.257299	4	0.064325		
Cor Total	48.52782	16			
R- squared	0.9942				
Adjusted R-squared	0.9867				
Predicted R-squared	0.9834				
Adequate Precision	44.14976				

The ANOVA results for the BBD of the bioethanol production from SSF is depicted in Table 2. The quadratic model obtained in coded form for the optimization of the bioethanol production is shown in Equation (1).

$$Y = 1.47 - 0.57A + 0.54B - 0.27C - 0.032AB + 1.20AC + 0.55BC + 2.34A^2 - 1.06B^2 + 1.39C^2 \quad (1)$$

Based on the ANOVA results in Table 2, it can be seen that the p-value which determine the statistical significance of the model obtained from the RSM is greater < 0.0001, an indication that the model has over 95% confidence level in terms of predictability. In addition, the robustness of the model can also be ascertained from the values of the R^2 (0.9942). This implies that the experimental data was well fitted into

the RSM model. The predicted R^2 (0.9834) value is a measure of how good a prediction of the model gives to the response value while the adjusted R^2 (0.9867) value represents the amount of variation in the design model. Both the predicted R^2 and the adjusted R^2 values should be within approximately 0.2 of each other to be in reasonable agreement.

The significance of the statistical model shown in Table 2 was also evaluated by the F-test. The F-value of 132.89 indicate that the f-distribution under the null hypothesis is statistically significant. Further proof of the model adequacy in explaining the data is the R-squared value. This shows that the regression model equation gives an accurate description of the experimental data. Another factor which measures

the adequacy of the model is the value of adequate precision obtained. A ratio greater than 4 is usually desirable. In this study, a ratio of 44.150 was obtained which indicates an adequate signal and as a result, this model can be used to navigate the design space.

effects between variables and the response and also to give the optimal levels of each variable needed to obtain maximum ethanol concentration.
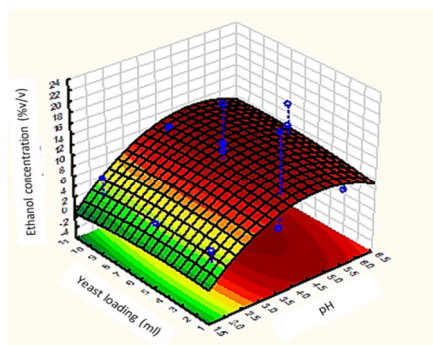

Fig 1: 3D response plots showing the effect of pH and yeast loading on ethanol production

Figure 1 shows the effect of pH and yeast loading on the bioethanol concentration. It can be seen that both yeast loading and pH significantly influence the bioethanol concentration. This is evident from the ANOVA analysis that gave p-values < 0.0001 for both yeast loading and pH. However, a close observation shows that pH has more significant effect on the bioethanol concentration compare to yeast loading. The analysis of the response plots show that an optimum pH value of 6 was obtained for maximum ethanol production. pH is one of the important factors that affect the performance of Simultaneous Saccharification and Fermentation and various studies have shown that the optimum pH value for ethanol production is between 5 and 6 (Afifi et al, 2011) which is in line with what was obtained in this study. It can also be observed that ethanol concentration increases with increase in yeast loading up to maximum of 8 ml and drops thereafter. This may be due to the fact that beyond this value, yeast cells present are increased and so competition for available substrate sets in which brings about reduction in efficiency of yeast cells.

Figure 2 depicts the effect of pH and ammonium sulphate on the bioethanol concentration. Interestingly, both pH and the ammonium sulphate influence the bioethanol concentration as clearly seen in the p-value which is less than 0.0001. Besides, an increase in the concentration of ammonium sulphate favoured the increased production of ethanol. This is as a result of the fact that addition of ammonium sulfate in sufficient quantities supports high

production of bioethanol. With the introduction of sufficient nutrients to the fermentation process, the yeast can multiply quickly and consume glucose to produce ethanol more effectively.
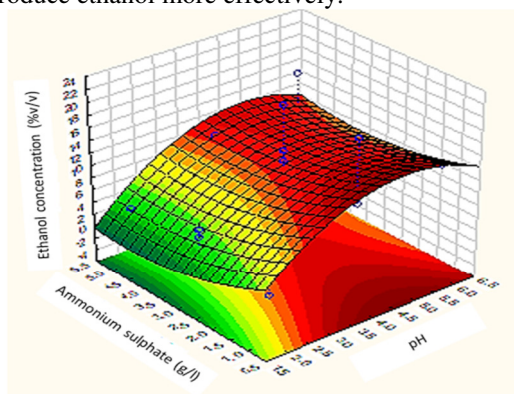

Fig 2: 3D response plot showing the effect of pH and ammonium sulphate on ethanol production

It can also be observed that in the presence of relatively high concentrations of ammonium sulphate, the rate of fermentation increased up to a pH optimum of 6 which is line with studies by Nadya et al. (2012) who reported that an optimum pH value of 6 was obtained for the production of ethanol from pineapple peel extract.
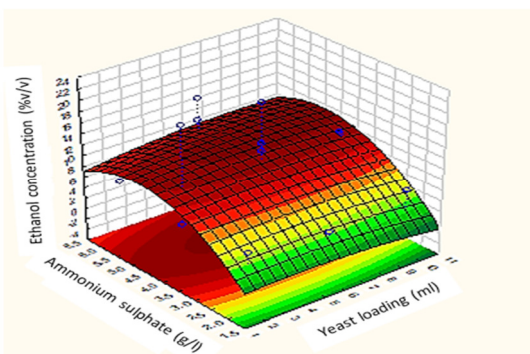

Fig 3: 3D response plot showing the effect of yeast loading and ammonium sulphate on ethanol production

Figure 3 above shows the effect of yeast loading and ammonium sulphate on ethanol production. It is seen that there is the interaction between the amounts of ammonium sulphate introduced into the SSF broth and the yeast loading has significant influence on the bioethanol concentration. However, the amounts of ammonium sulphate have more effect on the bioethanol concentration compare to yeast loading. The reason for this can be explained by various studies that have been carried out. Irhan et al. (2010) reported that an increase in innoculum size brings about an increase in biomass concentration and a corresponding increase in bioethanol concentration

although an optimum size is required beyond which the ethanol concentration reduces as a result of the fact that competition for food by yeast cells increases. Also, studies by Mendes-Ferreira et al, (2004) have shown that supplementation with ammonium sulphate during fermentation increases fermentation rate. In line with these studies, it is seen from Figure 3 that the bioethanol concentration increased as the concentration of ammonium sulphate increased up to an optimum value of 5g/l and yeast loading increased up to an optimum value of 8 ml beyond which it is noticed that ethanol concentration begins to reduce which can be accounted for by the fact that at this point competition by yeast cells start to occur.

Comparison between the Observed bioethanol concentration and the predicted values: The parity plots showing the comparison between the observed bioethanol concentration and the RSM predicted values are depicted in Figure 4. It can be seen that the observed values of the bioethanol concentration from the experimental runs is in good correlation with the RSM predicted values. This shows the robustness of the RSM as a good predictive tool besides being used for optimization. Moreover, it also indicate that the regression model equation gives an accurate representation of the experimental data.
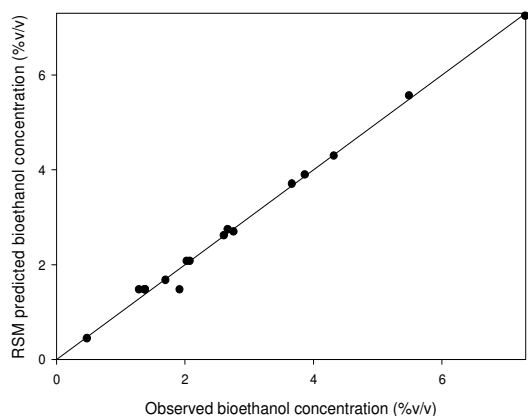

Fig 4: Parity plot of the observed and predicted values of the bioethanol concentration

Conclusion: Response Surface Methodology has been employed for the optimization of bioethanol production from simultaneous saccharification and fermentation of pineapple peels using *Saccharomyces cerevisiae*. The interaction effects from the RSM shows that pH, yeast loading and ammonium sulphate significantly influence the bioethanol concentration as indicated by their respective p-values (<0.0001). The RSM optimization resulted in maximum bioethanol concentrations of 5.82% (v/v)

at pH of 6, yeast loading of 8 ml and ammonium sulphate,concentration of 5g/l.

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