Original research article

Optimization of some fermentation conditions for bioethanol production from yam peels (*Dioscorea rotundata*) using Box-Behnken Design (BBD)

Abstract

Aim: The aim of this research was to optimize the production of bioethanol from a select second generation feedstock (yam peels).

Methodology: Bioethanol was produced from yam peels using Box Behnken Design (BBD). The independent variables selected for optimization of bioethanol yield were; fermentation temperature (˚C), fermentation time (hours) and yeast concentration (%w/v). A set of 17 experiments were considered by design expert software. All experiments were run in triplicates. Test for bioethanol was carried out using acidified potassium dichromate method and absorbance was taken using a spectrophotometer at 600nm.

Results: From the experiments carried out above, the optimum conditions under which the highest bioethanol (yield%) was produced were a fermentation temperature of 30℃, yeast concentration of 5.50%w/v and fermentation time of 96hours under which a bioethanol yield of 45.79% was produced.

Conclusion: Yam peels may therefore have the potentials to serve as substrates for bioethanol production rather than using food crops such as sugarcane juice and cassava flesh which may lead to food shortages and crisis especially is developing countries such as Nigeria.

Key: Bioethanol, *Saccharomyces cerevisiae*, Lignocellulose, Renewable energy, yam peels, Separate hydrolysis and fermentation (SHF), Simultaneous sacchrification and fermentation (SSF)

1 Introduction

Bio-ethanol is a form of renewable energy that can be produced from agricultural feed stocks. It can be made from very common crops such as sugarcane, potato, cassava and corn. There has been considerable debate about how useful bio-ethanol is in replacing petroleum. Concerns about its production and use relate to increased food prices due to the large amount of arable land required for crops, as well as the energy and pollution balance of the whole cycle of ethanol production, especially from corn. Recent developments with cellulosic ethanol production and commercialization may relieve some of these concerns. Cellulosic ethanol offers promise because cellulose fibers, a major and universal component in plant cell walls can be used to produce ethanol. According to the International Energy Agency, cellulosic ethanol could allow ethanol fuels to play a much bigger role in the future [1].

Even though the production of ethanol from first generation feedstock (starch biomass) represents the most convenient and technically advanced option for bioenergy, such practices would result in severe competition between energy and food supplies. It may therefore be unjustifiable to use such crops for bioethanol production [2]. The raw materials account for 40-

70% of the total ethanol production costs based on current sugar- or starch-containing feed stocks. Reduction of the production cost would increase the competitiveness of ethanol with fossil fuels. Lignocellulosic biomass is believed to be less expensive and more plentiful than either starch- or sucrose-containing feed stocks [2].

Second generation bioethanol is bioethanol produced from sustainable feedstock that cannot be used directly for food production. Wastes that could potentially be converted into bioethanol include agricultural wastes, domestic wastes, forest residues and dedicated energy crops such as perennial and elephant grasses which require a series of pretreatment and hydrolysis steps to recover fermentable sugars. The conversion of these potential sources of feedstock for bioethanol offers the double benefit of a reduction in global waste and the generation of valuable transportation fuels. Third generation bioethanol is produced using algae, sewage sludge and municipal solid wastes [1].

Bio-ethanol is a liquid biofuel which can be produced from several different biomass feed stocks and conversion technologies. However, agro wastes are made up of lignocelluloses and cannot be effectively exploited without adequate pretreatment. Lignocellulosic feedstocks are composed of cellulose, hemicelluloses and lignin in an intricate structure, which is recalcitrant to decomposition. However, the major problem is low accessibility of cellulose because of the rigid association of cellulose with lignin. This leads to difficulties within the conversion process; therefore, breaking down lignin seal in order to make cellulose more accessible to enzymatic hydrolysis for conversion is one main aim of pretreatment. In other words, pretreatment is the crucial and costly unit process in converting lignocellulosic materials into fuels [1]. Various methods are therefore used for pretreatment which may be physical (milling/grinding, microwave, irradiation ultrasonic), chemical (acid or alkaline) or biological (bacteria, fungi or enzymatic) [3].

Bioethanol fermentation process is usually carried out using *Saccharomyces cerevisiae* because of its ability to ferment glucose to ethanol. It also has the ability to withstand relatively high temperatures, high substrate concentrations, rapid fermentation rates and high ethanol concentrations [4]. For efficient bioethanol production from feed stocks, optimum yeast concentrations must be used [5].SHF has an advantage over SSF of being possible to carry out cellulose hydrolysis and fermentation reactions at their optimum temperatures of between 45 and 50°C and between 30 and 37°C respectively since this is the optimum growth temperatures for most fermenting microorganisms [6], unlike SSF where a compromise between the optimum temperature for hydrolysis and fermentation is usually made thereby setting the temperature between 35-40°C which may not be the optimum for either reactions, depending on the select microorganism [7]. [6] Reported a slightly higher yield of ethanol using SHF than SSF when corn cob was fermented with *Trichoderma reesei*. Notwithstanding, SSF has the advantage of occurring over a short period of time and also a minimized risk of microbial contamination due to the presence of ethanol in the flask [7]. Fermentation temperature influences the growth rate of microorganisms [5]. Shorter fermentation periods cause wasteful fermentation because of insufficient growth of microorganisms. However, when fermentation continues for a long period of time, it negatively affects yeast growth especially in batch mode, due to high concentration of ethanol in the fermented broth. A decrease in water availability because of the production of bioethanol may cause the inhibition of key glycolytic enzymes and these proteins may be denatured. Bioethanol inhibits yeast growth even at moderately low concentrations, thereby preventing cell division. At high concentrations, ethanol reduces cell vitality and increases cell

death. Exposing yeast cells to high concentrations of ethanol by increasing the fermentation time causes an increase in membrane fluidity thereby decreasing membrane integrity [8]. According to [5], the optimum yeast concentrations for bioethanol production are between 3-10% (v/v).

[9] Reported the optimum conditions for bioethanol production from calabash using RSM as a temperature of 28^{o}C, pH of 6.08 and an inoculum size of 10% (v/v). [10] also reported the optimum conditions for bioethanol produced from lignocellulosic biodegradable municipal solid wastes (BMSW) using RSM as; an initial substrate concentration of 75g/L, pH of 6.0, fermentation time 39hours and a yeast concentration of 2ml/L. Similarly, [11] reported the highest ethanol concentration of 5.82%v/v produced under the optimum conditions of pH 6.0, ammonium sulphate concentration of 5g/L and a yeast oncentration of 8% (v/v). Bioethanol production from cheese whey using *sacccharomyces cerevisiae* DIV13ZZ087COVS strain was also optimized using CCD and the optimum conditions for fermentation temperature, pH and yeast extract concentration were found to be 28.38^{o}C, 4.31 and 3.969 g/L respectively under which an ethanol concentration of 18.53g/L after 24hours incubation time was produced [12].

The feedstock used for first generation fuel ethanol production is mainly sugarcane in tropical areas such as India, Brazil and Colombia, while it is dominantly corn in other areas such as the United States, European Union, and China. Ethanol production from sugar crops such as sugarcane and sugar beet account for about 40% of the total bio-ethanol produced and nearly 60% corresponding to starch crops. Due to its increase in demand as a food source and its rising price, the availability and feasibility of using corn as a feedstock is at stake. The further expansion of ethanol production from many of these feed stocks therefore causes debate on food/feed versus fuel thereby limiting the use of first generation feed stock for ethanol production. Consequently, for sustainable fuel grade ethanol production, non-food feedstock should be used. There is an urgent need for development of second generation bioethanol [13].

2 Methods

2.1 Preparation of raw biomass

Yam peels were collected from domestic wastes and were washed and sun dried. The samples were subjected to physical pretreatment (grinding) to reduce the size and increase the surface area of contact of the biomass. The ground samples were then used for the study.

2.2 Fermenting microorganism

Instant dry yeast *(Saccharomyces cerevisiea)* was used as the fermenting microorganism.

2.3 Mineral solution

Before fermentation was initiated, yeast extract (5% (w/v) and mineral solution (5%v/v) consisting of $(NH_4)2SO_4$ (3.75g/L), K_2HPO_4 (2.1g/L), $MgSO_4.7H_2O$ (0.375g/L), $CaCl_2.2H_2O$ (0.5g/L) were added into flasks. The mineral stocks were prepared and autoclaved separately and kept at room temperature [14].

2.4 Enzymatic Hydrolysis of yam peels

Yam peels were hydrolyzed using cellulase enzyme to liberate reducing sugars. Cellulase enzyme (10mg/ml/g of dry substrate) was used for hydrolysis with citrate-Na_2HPO_4 buffer (Mc-Ilvaine buffer) (0.05M, pH5.0) [15]. Yam peels were hydrolyzed in 250ml flasks with a solid loading of 10% (w/v) after autoclaving for 15minutes at 121^{o}C. Enzymatic hydrolysis reactions were performed on an orbital shaker at 150 rpm at a temperature of 50^{o}C, pH of 5.0 and hydrolysis time of four (4) days. Fermentation process was initiated by decreasing the temperature to 30^{o}C and adding yeast (*Saccharomyces cerevisea*) [14].

2.5 Test for ethanol (Acidified Potassium Dichromate Method)

Potassium dichromate (34g) was dissolved in 300ml of distilled water. Concentrated H_2SO_4 (325mL) was added. The volume was completed to one (1) liter using distilled water.

Samples (fermented broth) were poured in a round bottom flask and distilled at 78^{o}C to ensure complete condensation and recovery of alcohol distillate. Distillate (2mL) was oxidized at 60^{o}C with dichromate solution (10ml) for 15minutes. Alcohol concentration was determined spectrophotometrically at 600nm [16].

3 Results

Optimization of some fermentation conditions for bioethanol production from yam peels using Box-Behnken Design (BBD)

Table 1 shows results of actual and predicted bioethanol yields (%) produced from yam peels for different conditions of temperature (^{o}C), yeast concentration % (w/v) and incubation time (hours). The responses obtained for each experimental run and the predicted responses were closer to each other. It was observed that; increase in temperature and incubation time above 35(^{o}C) and 96hours respectively led to decrease in bioethanol yield (%). However, the ethanol yield %) increased positively with increase in yeast concentration of up to 5.50 % (w/v). Maximum ethanol yield of 45.79% was obtained with a yeast concentration of 5.50% (w/v) at 96hours of incubation and a temperature of 30^{o}C (Run 8, Table 1). The least bioethanol yield %) of 0.88% was obtained with an inoculum size of 1% (w/v) at 96hours of incubation and a temperature of 25^{o}C (Run 16, Table 1).

Table 1: Experimental and predicted bioethanol yields (%) produced under different conditions of fermentation temperature (^{o}C) yeast concentration % (w/v) and time (hours) from yam peels

Exp. Runs	Temperature (^{o}C)	Yeast concentration % (w/v)	Incubation Time (hrs)	Experimental yield %)	Predicted yield %)
1	35.00	5.50	120.00	20.22±0.71	20.20
2	25.00	5.50	72.00	8.73±0.88	8.75
3	35.00	10.00	96.00	38.15±0.99	38.25

4	30.00	10.00	72.00	38.77±1.04	38.76
5	30.00	5.50	96.00	45.77±0.20	45.58
6	30.00	1.00	120.00	12.73±0.25	12.74
7	35.00	5.50	72.00	19.13±0.97	19.04
8	30.00	5.50	96.00	45.79±0.91	45.58
9	30.00	5.50	96.00	45.70±1.97	45.58
10	35.00	1.00	96.00	3.32±0.99	3.34
11	25.00	5.50	120.00	9.23±1.01	9.32
12	30.00	1.00	72.00	15.60±0.36	15.67
13	30.00	5.00	96.00	45.57±1.18	45.58
14	25.00	10.00	96.00	19.66±1.07	19.64
15	30.00	10.00	120.00	43.50±0.79	43.42
16	25.00	1.00	96.00	0.88±0.01	0.78
17	30.00	5.00	96.00	45.06±0.86	45.58

Table 2: shows the experimental and fitted model results of bioethanol yield produced from yam peels. From the result, temperature (^{o}C), yeast concentration % (w/v) and fermentation time (hours) had significant *(P> 0.05)* effects on bioethanol yield %) with *P values* of $P < 0.0001$, $P < 0.0001$, and $P< 0.0015$ respectively. As illustrated in table 4.15, the analysis of variance for a *P-value < 0.05* indicates a significant effect on the response. The model F- value of 15357.58 implies that the model is significant. There is only a 0.01% chance that a "Model F- Value" this large could occur due to noise. *P values* less than 0.05 indicate model terms are significant. In this case, A, B, C, A^2, B^2, C^2, AB and BC are significant model terms. The "Lack of Fit F-value" of 0.18 implies there is a 29.63% chance that a "Lack of Fit F-value" this large could occur due to noise. Regression analysis produced the following second- order polynomial fit with a satisfactory coefficient of determination ($R^2 = 0.9973$).

Ethanol yield (%) = -908.56540 +52.03677*A+0.49881*B+3.06817*C-0.86796 A^2-0.41365B^2-0.016582*C^2+0.17833*A*B+1.22917E-003*A*C+0.017593*B*C……Equation…1

Where A, B and C represent Temperature, inoculum size and incubation time respectively. AB, AC and BC are the interactions and A^2, B^2, and C^2 are the quadratic terms.

Table 2: Analysis of variance result for bioethanol yield produced from yam peels

Source	Sum of Squares	Mean Square	F-Value	P- Value

Model	4648.62	516.51	8727.94	<0.0001 Sig
A	223.87	223.87	3782.96	< 0.0001
B	1445.88	1445.88	24432.12	< 0.0001
C	1.49	1.49	25.14	0.0015
A^2	1982.51	1982.51	33500.10	< 0.0001
B^2	295.43	295.43	4992.20	< 0.0001
C^2	384.13	384.13	6490.97	< 0.0001
AB	64.40	64.40	1088.23	< 0.0001
AC	0.087	0.087	1.47	0.2646
BC	14.44	14.44	244.00	< 0.0001
Lack of Fit	0.049	0.016	0.18	0.9050 not Sig

Adeq Precision = 240.097 C.V = 0.90

KEY: A= Temperature, B= Inoculum size and C=Fermentation time

Figure 1 depicts a correlation between the bioethanol yields produced from yam peels and the predicted values. The cluster distribution between the observed and predicted bioethanol yields signify satisfactory correlation between the observed values and the predicted values in the parity plot.

Also, figure 2 shows the parity graph showing the distribution of residual and predicted values of bioethanol yields produced from yam peels. The clustered points around the diagonal line indicate goodness of fit of the model since there is less deviation between the observed and predicted values

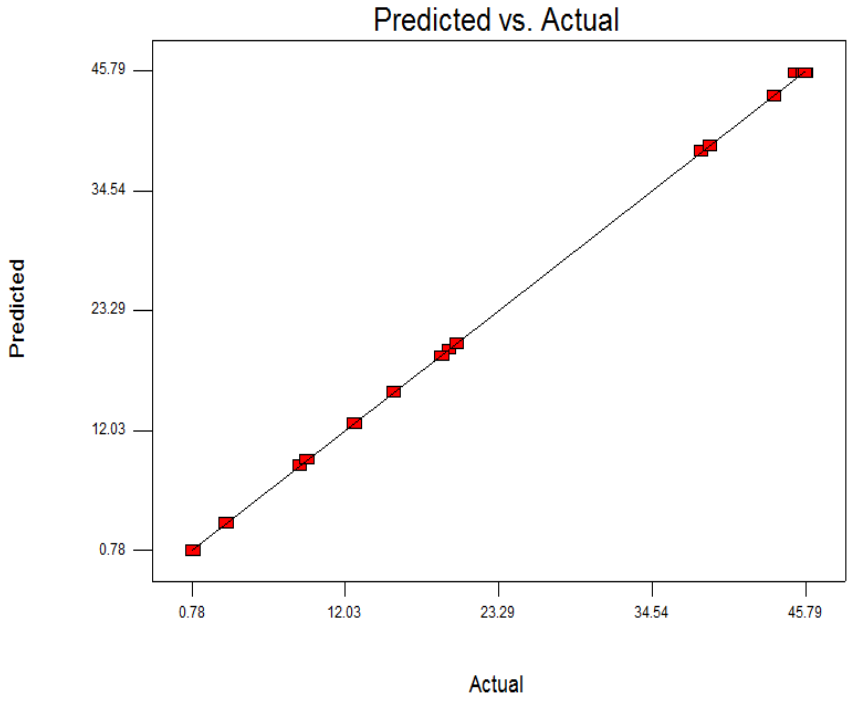

Figure 1: Parity graph showing the distribution of actual vs. predicted yields of bioethanol produced from yam peels

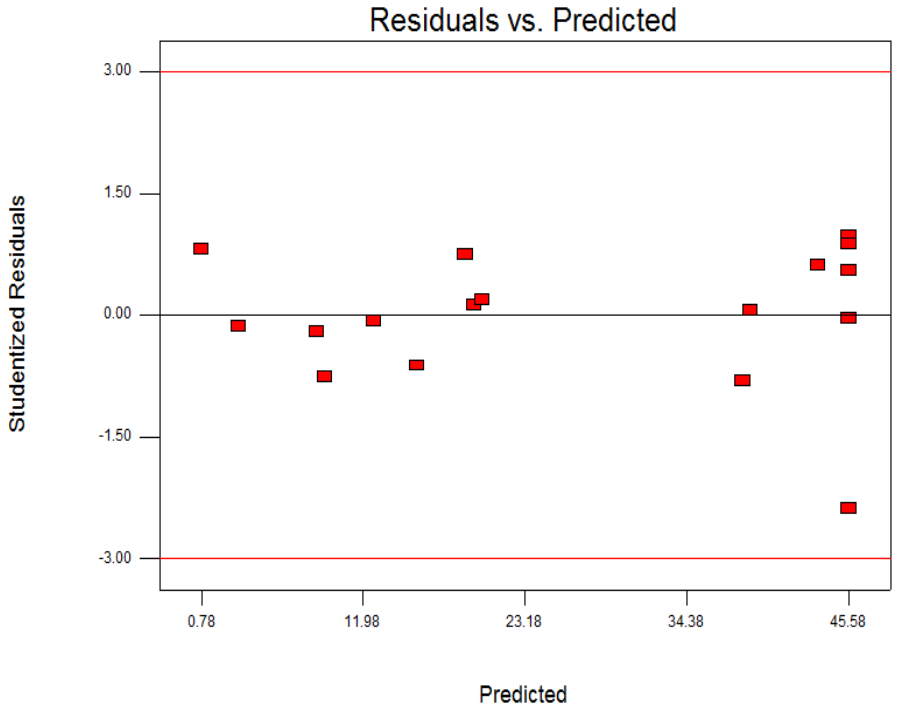

Figure 2: Parity graph showing the distribution of residual vs. predicted yields of bioethanol produced from yam peels

Interactions between yeast concentration (%w/v), fermentation temperature (°C) and fermentation time (hours) for optimization of bioethanol production from yam peels

Response surface plots were generated by plotting the response (bioethanol yield) on the y-axis against any two independent variables on the x-axis, while keeping the other independent variables at its center part. Therefore, two responses were obtained by considering the possible combinations.

Figures 3 and 4 represent the three-dimensional and contour surface plots for the optimized conditions. The plot illustrates the main and the interactive effects of the independent variables on the dependent ones. The response surface plots were generated by plotting the response on the y-axis. Figure 3 shows the effects and interaction between fermentation temperature (°C) and yeast concentration % (w/v) on bioethanol production while fermentation time was kept constant at 96.00 hours. Bioethanol yield was observed to increase with increase in fermentation temperature and yeast concentration of up to 30°C and 5.50% (w/v) respectively, after which further increases in the parameters caused a decrease in bioethanol yield produced from yam peels. However, bioethanol yield was higher at a fermentation temperature of 50°C and a yeast concentration of 5.50% (w/v). The elliptical 3-dimensional surface shows the interactive effect between fermentation temperature and yeast concentration on bioethanol yield.

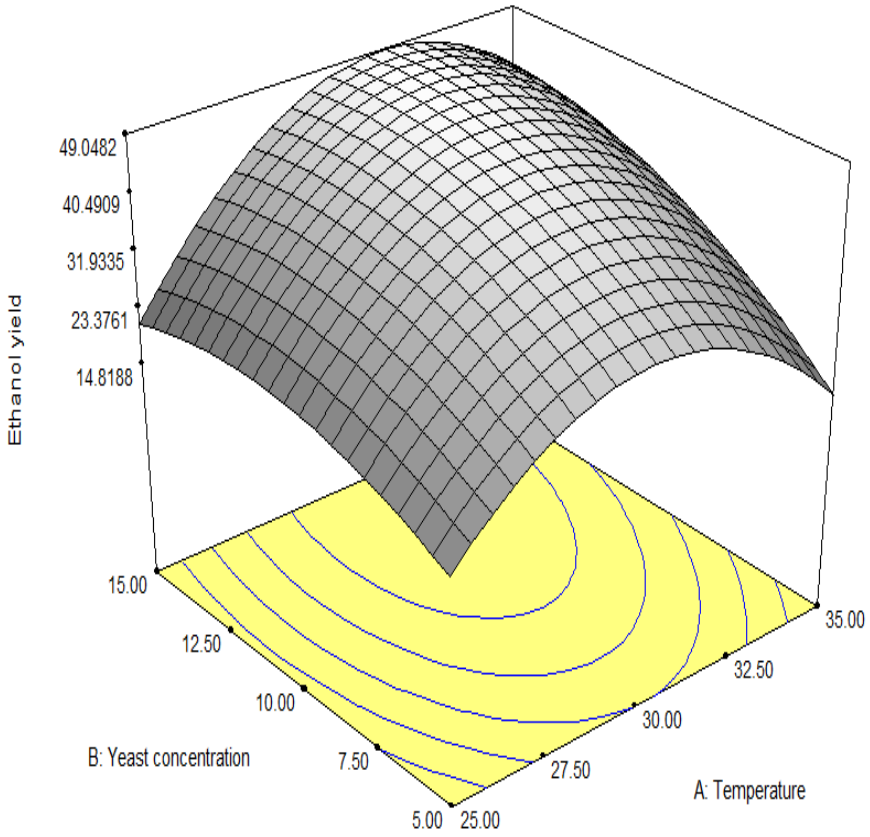

Figure 3: 3-Dimensional presentation showing interaction between fermentation temperature (°C) and yeast concentration % (w/v) for bioethanol production from yam peels. Fermentation time was kept constant at 96hours.

Figure 4 shows the effects of yeast concentration %w/v) and fermentation time on bioethanol production from yam peels. Fermentation temperature was maintained at 30^{o}C. Similarly, bioethanol yield was observed to increase with increase in yeast concentration and fermentation time until the optimum values were reached after which increases in the parameters caused a decrease in bioethanol yields produced from yam peels. However, bioethanol yield was higher at a yeast concentration of 5.50% (w/v) and fermentation time of 96.00 hours. The elliptical 3-dimensional surface shows the interactive effects between yeast concentration and fermentation time for bioethanol produced from yam peels. There was no significant interaction between fermentation temperature and fermentation time. Subsequently, the 3-D and contour plots were not generated.

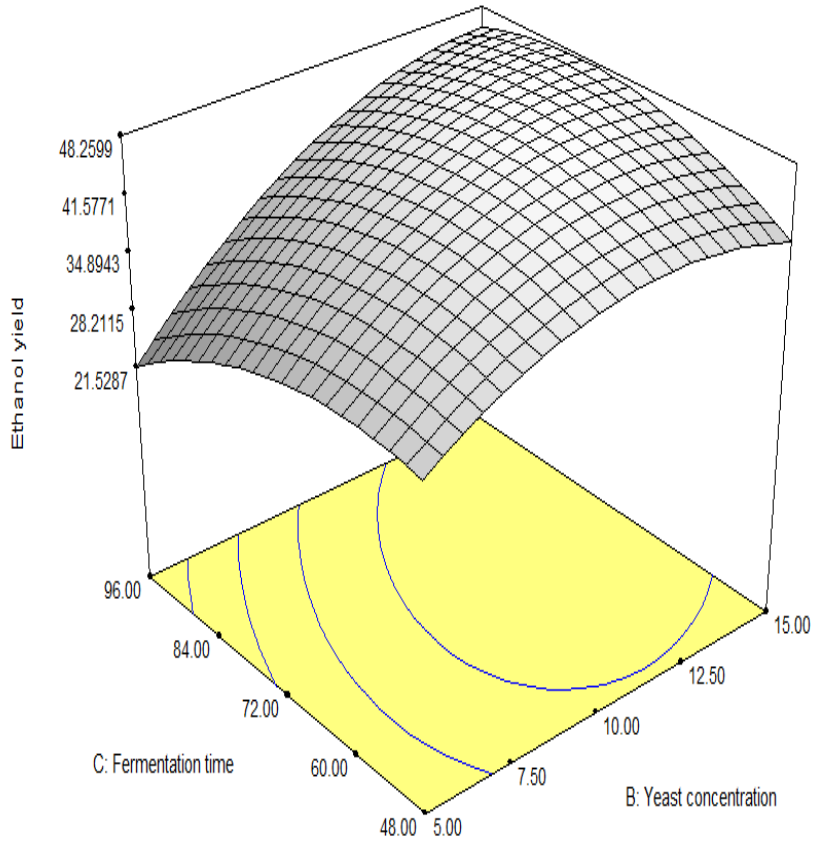

Figure 4: 3-Dimensional presentation showing interaction between yeast concentration % (w/v) and fermentation time (hours) for bioethanol production from yam peels . Fermentation temperature was kept constant at 30°C.

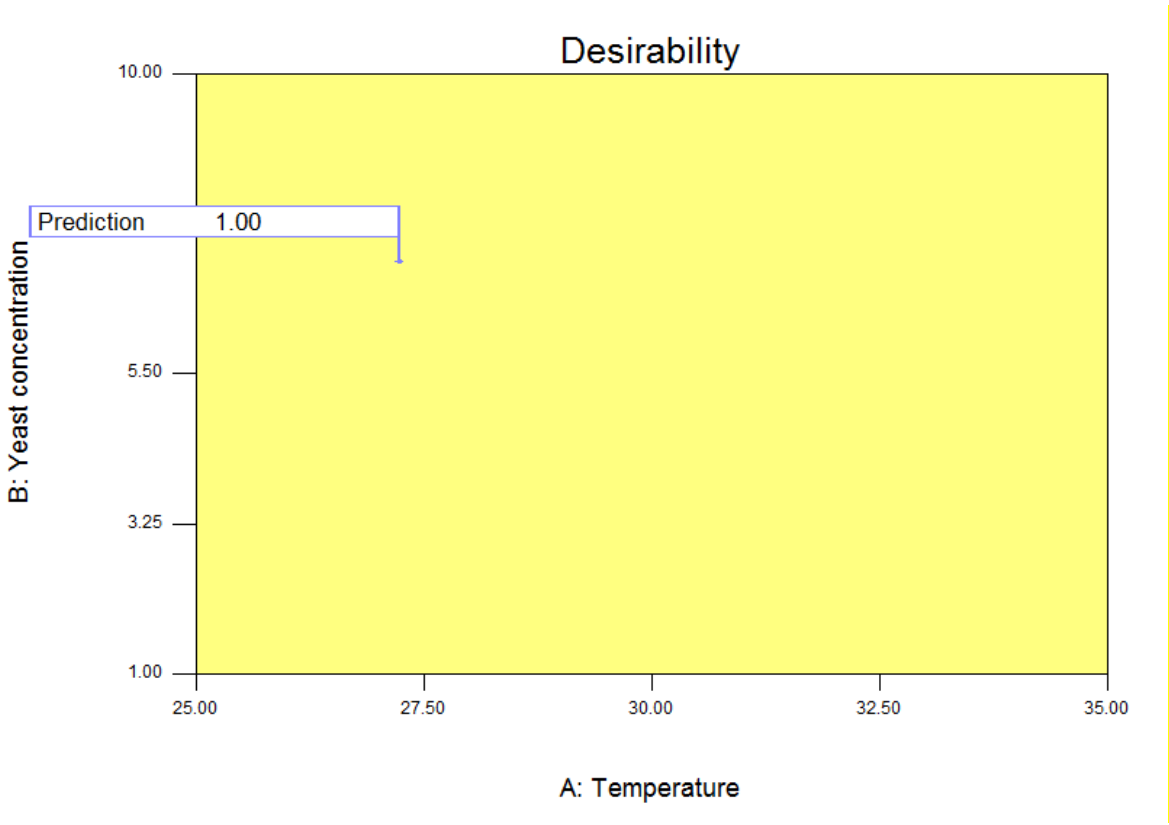

Figure 5: Desirability chart for bioethanol production from yam peels

Discussions

Three independent variables; A=Fermentation temperature (^oC), B=yeast concentration % (w/v) and C=fermentation time (hours) were selected while bioethanol yields (%) produced from yam peels was the dependent variable studied using Design expert software. A total of seventeen (17) experiments were designed using Box Behnken Design (BBD) each for yam peels and millet husks fermentation reactions. Shaking rate of 150rpm and an initial pH of 5.0 were kept constant throughout the fermentation step.

The model was significant while lack of fit for bioethanol production from yam peels was not (Table 2). This implies that the model fits well and the independent variables have significant effects on the response/dependent variable. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 240.097 indicates adequate signal for bioethanol production from yam peels. *P* values less than *0.05* imply that model terms are significant. A, B and C represented fermentation temperature (^oC), yeast concentration % (w/v) and fermentation time (hours) respectively for bioethanol production each from yam peels and millet husks. A^2, B^2 and C^2 were their quadratic terms while AB, AC and BC represented interaction effects between the variables. For bioethanol production from yam peels, A, B, C, A^2, B^2, C^2, AB and BC had significant effects on the response while AC had a *P value* of 0.2646 and was therefore not significant.

Temperature, yeast concentration and fermentation time significantly affect bioethanol production. Temperature affects the enzymatic activity and membrane fluidity of yeast cells. It affects the rate at which yeast cells multiply and metabolize sugars [17]. Temperature had both positive and negative effects on bioethanol yield (%) produced from yam peels in that, it increased bioethanol yields at the optimum temperatures but decreased the yields at higher or lower temperatures. Temperature had a significant effect on bioethanol yields (%) produced from yam peels with *P values* of $P<0.0001$ during bioethanol production.

Similarly, the quadratic terms of temperature was also significant with *P values* of $P<0.0001$. There were significant interactions between fermentation temperature (^oC) and yeast concentration % (w/v) and yeast concentration % (w/v) and fermentation time (hours) during bioethanol production from yam peels as shown in Table 2. Similarly, interactions between fermentation temperature (^oC) and yeast concentration % (w/v), fermentation temperature (^oC) with fermentation time (hours) and yeast concentration % (w/v). It was observed that, as fermentation temperatures (^oC) and yeast concentrations % (w/v) increased during fermentation, bioethanol yields increased and vice versa. Similarly, as yeast concentrations % (w/v) and fermentation times (hours) during bioethanol production from yam peels increased, yields (%) also increased until the optimum conditions were reached after which lower yields (%) were produced. There was no significant interaction between fermentation time (hours) and yeast concentration % (w/v) during yam peels fermentation (Table 2). The ellipsoidal contours seen in the plots suggest significant interactions.

At a low temperature of 25^oC, lowest bioethanol yields (%) were produced from yam peels (Table 1, run 16). Furthermore, at 25^oC, the yield of bioethanol produced was low even when 5.5 (w/v) yeast concentration was used and respective fermentation reactions took place at 72hours (Table 1, run 2). At low temperatures, the rates of reaction may be slow due to insufficient energy to activate the enzymes involved in fermentation [4]. However, when fermentation

temperature was raised to 30°C, bioethanol yield (%) increased (Table 1, run 8). This implies that the optimum temperature for bioethanol production from yam peels was 30°C. This temperature is nonetheless, close to that reported by [9] where the highest bioethanol yield of 6.97% was produced from calabash using CCD at an optimum temperature of 28°C. At a fermentation temperature of 35°C, decrease in ethanol yield (%) was observed (Table 1, run 10). These low yields (%) obtained under high temperatures may be due to severe intracellular changes which may have resulted in inactivation or denaturation of enzymes. High temperatures have been reported to inhibit or denature some enzymes. This may lead to yeast death and a high concentration of volatile compounds, mainly esters and higher alcohols [18].

Yeast concentration % (w/v) also had significant effects on bioethanol yields (%) produced from yam peels (Tables 1). The quadratic terms of yeast concentration % (w/v) during fermentation of both feed stocks were also significant (Tables 4.15 and 4.18). Yeast concentration % (w/v) interacted significantly with temperature (°C) and fermentation time (hours) during fermentation of yam peels as shown by the significant *P* value (Table 1). The highest bioethanol yield (Table 1, run 8) was produced when a yeast concentration of 5.5%. This may be because an increase in yeast cell concentration up to the optimum level allows cells to grow rapidly thereby metabolizing sugars into ethanol [4]. Conversely, [9] reported an optimum inoculum size of 10% (v/v) under which a yield of 6.97% was produced from calabash using CCD.

The fermentation time (hours) for bioethanol production from yam peels was significant (Table 1) when fermented with *Saccharomyces cerevisiae*. The quadratic terms of yeast concentration % (w/v) during fermentation of yam peels was also significant as reported in Table 1. However, there was no significant interaction *(P>0.2646)* between temperature (°C) and fermentation time (hours) Table 1. The optimum fermentation time for bioethanol production from yam peels in this study was 96hours (Table 1, run 8). [19] Reported that, shorter fermentation periods caused wasteful fermentations because of insufficient growth of microorganisms. However, when fermentation continued for a long period of time, it negatively affected yeast growth especially in batch mode due to high concentrations of bioethanol in the fermented broth. A decrease in water availability because of the production of bioethanol may cause the inhibition of key glycolytic enzymes and these proteins may be denatured [8]. Exposing yeast cells to high concentrations of ethanol by increasing fermentation time causes an increase in membrane fluidity thereby decreasing membrane integrity [8].

In this study, the best fermentation conditions for bioethanol production from yam peels using BBD were a fermentation temperature of 30°C, yeast concentration of 5.5%w/v and fermentation time of 96hours under which a bioethanol yield of 45.79 was produced using SHF. Nonetheless, [9] reported the optimum conditions for bioethanol production from calabash using CCD as a temperature of 28°C, pH of 6.08 and an inoculum size of 10% (v/v) under which a yield of 6.97% was produced. [10] also reported the optimum conditions for bioethanol produced from lignocellulosic biodegradable municipal solid wastes (BMSW) using RSM as; an initial substrate concentration of 75g/L, pH of 6.0, fermentation time 39hours and a yeast concentration of 2ml/L. Similarly, [11] reported the highest ethanol concentration of 5.82%v/v produced under the optimum conditions of pH 6.0, ammonium sulphate concentration of 5g/L and a yeast oncentration of 8% (v/v). Bioethanol production from cheese whey using *sacccharomyces cerevisiae* DIV13ZZ087COVS strain was also optimized using CCD and the optimum conditions for fermentation temperature, pH and yeast extract concentration were found to be

28.38°C, 4.31 and 3.969 g/L respectively under which an ethanol concentration of 18.53g/L after 24hours incubation time was produced [12].

In similar studies using BBD, [20] reported 4.11% as the highest bioethanol yield produced when *Haplophragma adenophyllum* was pretreated with 0.5% NaOH concentration, 10% substrate concentration and 5s residence time and subsequently hydrolyzed using commercially available cellulase enzyme (40FPU) and fermented using SSF for 96hr with *Saccharomyces cerevisiae*. However, [21] reportedly produced the highest ethanol yield of 54.54g/L after 96hrs fermentation of *Bombax ceiba* wastes pretreated with NaOH and steam.

Conclusion

This research was aimed at optimizing bioethanol production from yam peels using box-behnken design and it was observed that; yam peels have the potential to serve as feedstock for bioethanol production which may be used in place of food crops such as cassava flesh and sugarcane juice especially in developing countries such as Nigeria.

Conflict of interest

The authors hereby declare that there are no conflicting interests.

References

1. Awoyale, A.A. and Lokhat, D. (2021). Experimental determination of the effects of pretreatment on selected Nigerian lignocellulosic biomass in bioethanol production. *Scientific reports*, 11:557. https://doi:10.1038/s41598-020-78105-8
2. Nanda, S., Rana, R., Sarangi, P.K., Dalai, A.K. and Kozinski, J.A. (2018). A broad introduction to first, second and third generation biofuels. In: Sarangi, P., Nanda, S., Mohanty, P. (eds). *Recent advancements in biofuels and bioenergy utilization. Springer, Singapore*, pp1-25.
3. Bala, S., Garg, D., Sridhar, K., Inbaraj, B.S., Singh, R., Kamma, S., Tripathi, M. and Sharma,M. (2023). Transformation of agro wastes into value added bio-products and bioactive compounds: micro/nano formulations and applications in the agri food pharma sector. *Bioengineering* 10(152):1-17.
4. AL-Azhar M., Abdullah, R., Jambo,A., Marbawi, H., Gansau, J. A., Faik, M. A. and Rodrigues, F. K. (2017). Yeasts in sustainable bioethanol production. *Biochemistry and Biophysics Reports*,10: 52-61. https://doi:10.1016/J.bbrep.2017.03.003
5. Abubakar, M., Abolarin, M.S., Okegbile, O.J. and Eterigho, E.J. (2021). Influence of temperature and agitation speed on fermentation process during production of bioethanol fuel from cassava. *International Journal Of Engineering and Advanced Technology Studies*.9 (3):40-46. https://doi.org/10.37745/ijeats.13.
6. Santos, F., Eichler, P., Queiroz, J.H. and Gomes, F. (2020). Production of second generation ethanol from sugarcane. *Sugar bio refinery, technology and prospective*, 11;195-228.

7. Kassim, M.A., Meng, T.K., Kamaludin, R., Hussain, A.H. and Bukhari, N.A. (2022). Bioprocessing of sustainable renewable biomass for bioethanol production. *Value-chain of biofuels*, pp 9:195-234.
8. Stanley, D., Bandara, A., Fraser, S., Chambers, P.J. and Stanley G.A. (2010). The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. *Journal of applied microbiology*, 109: 13-24. https://doi:10.1111/j.1365-2672.2009.04657.x.Epub
9. Nwogwugwu, U.N., Abu, G.O. and Akaranta, O. (2021). Bioethanol production from an underutilized plant, calabash (*Cresentia cujete)* using co culture of *Saccharomyces cerevisiae* and *Cronobacter malonaticus. Journal of advances in microbiology* 21 (4): 17-33.
10. Hayder, N.H., Flayeh, H.M. and Ahmed, A.W. (2018). Optimization of biodegradeable municipal solid waste using Response Surface Methodology (RSM). *Journal of engineering and sustainable development* 22(1):47-64.
11. Oiwoh, O., Ayodele, B.V., Amenaghawon, N.A., Okieimen, C.O. (2017). Optimization of bioethanol production from simultaneous saccharification and fermentation of pineapple peels using *Saccharomyces cerevisiae*.
12. Boudjema, K., Naimi, F.F. and Hellal (2015). Optimization of bioethanol production on sweet cheese whey by saccharomyces cerevisiae DIV13-Z087C0VS using response surface methodology (RSM). *Romanian biotechnological letters* 20(5):10814-10825.
13. Amin, F.R., Khalid, H., Zhang, H., Rahman, S.U., Zhang, R., Liu, G. and Chen, C. (2017). Pretreatment methods of lignocellulosic biomass for anaerobic digestion. *AMB Express*7:72 1-12. https://doi.org/10.1186/s13568-017-0375-4.
14. Karagoz, P. and Ozkan, M. (2014). Ethanol production from wheat straw by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co culture in batch and continuous system. *Bioresource Technology*, 158, 286-293. https://doi: 10.1016/j.biortech.2014.02.002.Epub.
15. Li, J. L., Xia, W.J., Ma, G.P., Chen, Y. L. and Ma, Y.Y. (2019). A study on the enzymatic properties and reuse of cellulase immobilized with carbon nano tubes and sodium alginate, *AMB Express*, 9:122. https://doi.org/10.1186/s13568-019-0835-0
16. Crowell, E.A. and Ough, C.S. (1978). A modified procedure for alcohol determination by dichromate oxidation. *American Journal of Enology and Viticulture* 30(1):61-63. https://doi:10.5344/ajev.1979.30.1.61.
17. Tesfaw, A. and Assefa, F. (2014). Current trends in bioethanol production by *Saccharomyces cerevisiae.* Substrate, inhibitor reduction, growth variables, coculture and immobilization. *International scholarly research notices*, 532852 1-11. https://dx.doi.org/10.5897/BMBR2014.0223.
18. Kucharczyk, K. and Tuszynski, T. (2016). The effect of temperature on fermentation and beer volatiles at an industrial scale. *Journal of inst. Brew*. 124:230-235. https://doi.org/10.1155/2014/957102.
19. Zabed, H., Faruq, G. and Sahu, J.N. (2014). Bioethanol production from fermentable sugar juice, *Sci. World J.* 1–11. https://doi:10.1155/2014/957102.
20. Rubab, N., Ghazanfar, M., Adnan, S., Ahmad, I., Shakir, H.A., Khan, M., Franco, M. and Irfan, M. (2023). Microwave assisted alkali pretreatment of *Haplophragma adenophyllum* leaves for bioethanol production. *Cellulose chem. Technol.* 57 (3-4), 345-358.

21. Ghazanfar, M., Nadeem, M., Shakir, H.A., Khan, M., Franco, M., Chen, L. and Irfan, M (2022). Volarization of *Bombax ceiba* waste into bioethanol through separate hydrolysis and fermentation and simultaneous saccharification and fermentation. *Fermentation* 8, 386.