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STATISTICALLY DESIGNED SEPARATE HYDROLYSIS AND FERMENTATION PROCESS FOR OPTIMISED ETHANOL PRODUCTION FROM PUMPKIN POD

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ABSTRACT
Pumpkin pod biomass was used for bioethanol production using optimised separate hydrolysis and fermentation (SHF). Central composite design (CCD) coupled with response surface methodology (RSM) was used to model and optimise the effect of broth pH, solid substrate loading and concentration of acid used for hydrolysis. Analysis of variance (ANOVA) showed that the model was statistically significant (p value=0.0003) with a low standard deviation (0.27) and a high coefficient of determination (R^2=0.91). Ethanol production was significant at high levels of solid loading and pH while the reverse was the case for acid concentration. The optimum values of pH, solid substrate loading and acid concentration obtained from RSM were 6.0, 10 %w/w and 0.051 M respectively. Under these conditions, the ethanol concentration was obtained as 7.1 %v/v. Validation of the statistical model indicated no significant difference between experimental observations and model prediction. This study has demonstrated the feasibility of producing bioethanol from pumpkin pod an otherwise waste material. Optimisation of the process helps improve the commercial potential of the process.

Keywords: Bioethanol, Fermentation, *Saccharomyces cerevisiae*, Response Surface Methodology, Hydrolysis

INTRODUCTION
With increasing world population and industrialisation, the global demand for energy has experienced a significant increase over the past couple of decades (Betiku and Taiwo, 2015). This has consequently resulted in an increase in the deleterious effects of the use of conventional petroleum-based fuels on the environment. Hence there is need to focus on alternative fuel sources which are sustainable, renewable and environmentally friendly (Betiku and Ajala, 2014). Bioethanol has emerged as a potentially sustainable liquid fuel that can reduce the problems associated with the use of conventional petroleum based fuels (Amenaghawon *et al*., 2015). Bioethanol can either be used as a blend with gasoline to enhance octane rating or as a primary fuel (Mohan and Reddy, 2012).

Studies on bioethanol have revealed that it is produced from three main types of raw materials which include sugar-based feedstocks such as cane juice, apple juice, sorghum juice; starch based-feedstocks such as cassava, cocoyam, maize, potato and lignocellulosic feedstocks such as bagasse, straw, agro and wood residues (Gnansounou *et al*., 2005; Ballesteros *et al*., 2008; Wang *et al*., 2011; Amenaghawon *et al*., 2013). However, there are ethical concerns relating to the use of potential food resources for biofuel production thus discouraging the use of

the first two types of feedstocks (Rass-Hansen *et al*., 2007). Bioethanol produced from lignocellulosic feedstocks is potentially sustainable as lignocellulosic materials which are mainly agricultural and forest residues have the potential to be an economical source of feedstock as a result of their widespread availability, sustainable production and low cost (Sakar *et al*., 2012; Amenaghawon *et al*., 2014).

An example of an important lignocellulosic material that can be fractionated into bioethanol fuel is pumpkin pod. Pumpkin (*Telfairia occidentalis*) is a perennial crop grown predominantly in West Africa particularly Nigeria. The leaf is used primarily in preparing soups and herbal medicines (Modupeola *et al*., 2014). The seeds produced by the gourd are high in protein and fat and can therefore contribute to a well-balanced diet. However, the pumpkin pod is inedible and currently has no economic value apart from being used as livestock feed and blood tunic by indigenous tribes (Akoroda, 1990). The carbohydrate, crude fibre and cellulose content of the pod could make it a potentially viable feedstock from which sugars can be recovered for bioethanol production (Akwukwaegbu *et al*., 2016).

The objective of this study was to optimize important parameters for the production of bioethanol from pumpkin pod via separate hydrolysis and fermentation using *Saccharomyces cerevisiae.* Central composite design coupled with response surface methodology was used to study and optimise the effects of hydrolyzing acid concentration, solid substrate loading and fermentation pH on the yield of ethanol produced.

MATERIALS AND METHODS

Feedstock Collection and Pretreatment

Pumpkin pods were graciously provided by the University of Benin model farm, Faculty of Agriculture, University of Benin, Benin City, Nigeria. The pods were chopped, washed to remove adhering dirt and dried to a constant weight at 60 ^{o}C in a hot air oven. The dried biomass was milled to obtain 2 mm particles using an electronically operated laboratory mill. Delignification was carried out by soaking the biomass in 2 M NaOH solution and autoclaving at 121 ^{o}C for 2 hours. The resulting material was neutralized by continuous washing with warm distilled water until neutral pH was obtained. The delignified sample was oven dried at 60 ^{o}C until constant weight was attained (Agbodike *et al*., 2013).

Hydrolysis of Elephant Grass

The delignified biomass was subjected to hydrolysis using dilute sulphuric acid at 121^{o}C for 60 minutes. The concentration of the acid was varied within the range 0.051-2.04 M to determine the effect of acid concentration on hydrolysis. At the end of the hydrolysis process, the solid residue was separated by centrifugation and the hydrolysate was detoxified according to the method of Silva *et al*. (1998). The detoxified hydrolysate was stored in a glass vessel in a refrigerator for further use.

Culture Media and Fermentation

The method reported by Agbodike *et al*. (2013) was adopted for the fermentation process. A complex medium containing the pumpkin pod hydrolysate was prepared. The composition of the medium (per 100 mL) was as follows: $FeNH_4(SO_4)_2$, 0.1 g; $(NH_4)_2HPO_4$, 0.25 g; Urea, 0.3 g; and Peptone, 0.5 g. In order to investigate the effect of solid substrate loading, the amount of biomass was varied in the range 2 to 10 w/w%. The medium was dispensed into 250 mL Erlenmeyer flasks containing 100 mL of the medium. The flasks containing the fermentation medium were sterilized in an autoclave at 121 ^{o}C, 15 psi for 15 min. The flasks were removed from the autoclave and cooled to room temperature with cold water. The pH of the fermentation medium was adjusted to 5.5 with sterilised 5 M NaOH solution. The flasks were inoculated with 10 mL of *Saccharomyces cerevisiae* with a cell concentration of 3 x 10^8 cells/mL and then incubated at ambient temperature on an orbital shaker set at 200 rpm for 3 days.

Analytical Methods

Liquid samples were taken from the fermentation broth at the end of fermentation. The samples were filtered using a Whatman's No 4 filter paper and the amount of ethanol produced was determined according to the method reported by Hadeel *et al*. (2013).

Experimental Design

A three variable and five level CCD consisting of 20 experimental runs with six replications at the centre point was used for optimisation of the reaction variables (fermentation pH, solid substrate loading and H_2SO_4 concentration). The ranges of these variables are shown in Table 1. Ethanol concentration was chosen as the response for process optimisation using RSM. Experimental observations from the fermentation process were analysed using Design Expert® 7.0.0 (Stat-ease, Inc. Minneapolis, USA) 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 and the optimised value of the independent variables for optimum response was determined using a numerical optimisation.

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

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.

Table 1: Experimental ranges and levels of independent variables

Independent Variable	Symbols	Coded and Actual Levels				
		-1.68	-1	0	+1	+1.68
Fermentation pH	X_1	4	4.41	5	5.59	6
Solids loading (% w/w)	X_2	6	6.81	8	9.19	10
H_2SO_4 concentration (M)	X_3	0.50	0.80	1.25	1.70	2

RESULTS AND DISCUSSION

Statistical Modelling and Analysis

Analysis of the experimental data using the Design Expert software revealed that the quadratic model was significant for ethanol production. This was further confirmed by ANOVA results which showed that the p value of the model was less than 0.05 indicating statistical significance (Table 3). The final response function for predicting ethanol production after estimating the regression coefficients is given in Equation 2.

$$Y = 19.35 - 3.38X_1 - 2.69X_2 + 3.61X_3 + 0.074X_1X_2 - 0.99X_1X_3 - 0.26X_2X_3 + 0.37X_1^2 + 0.19X_2^2 + 1.28X_3^2 \quad (2)$$

where X_1, X_2 and X_3, represent the pH of fermentation, solid substrate loading, and concentration of H_2SO_4, respectively. The values of ethanol concentration as predicted by Equation (2) are presented in Table 2 alongside the experimental data for comparison. The results of analysis of variance (ANOVA) carried out to determine the fit of the statistical model are presented in Tables 3 and 4.

Table 2: Central composite design matrix for the optimisation of variables and the response values

Run No	Factors						Response	
	Coded values			Actual values			Ethanol conc. (%v/v)	
	X_1	X_2	X_3	X_1	X_2	X_3	Observed	Predicted
1	0	0	0	5.0	8.0	1.25	3.09	3.24
2	0	1.68	0	5.0	10.0	1.25	4.74	4.94
3	1	1	-1	5.6	9.2	0.80	4.93	4.79
4	-1	1	-1	4.4	9.2	0.80	4.72	4.57
5	0	0	1.68	5.0	8.0	2.00	3.64	3.80
6	1	1	1	5.6	9.2	1.70	3.93	3.80
7	1	-1	-1	5.6	6.8	0.80	3.44	3.30
8	0	0	0	5.0	8.0	1.25	3.08	3.24
9	0	0	0	5.0	8.0	1.25	3.07	3.24
10	1	-1	1	5.6	6.8	1.70	2.98	2.87
11	0	0	-1.68	5.0	8.0	0.50	3.90	4.12
12	1.68	0	0	6.0	8.0	1.25	3.07	3.25
13	0	0	0	5.0	8.0	1.25	3.15	3.24
14	-1	-1	1	4.4	6.8	1.70	4.04	3.91
15	-1.68	0	0	4.0	8.0	1.25	3.77	3.96
16	0	-1.68	0	5.0	6.0	1.25	2.91	3.09
17	0	0	0	5.0	8.0	1.25	3.38	3.24
18	0	0	0	5.0	8.0	1.25	3.74	3.24
19	-1	-1	-1	4.4	6.8	0.80	3.44	3.30
20	-1	1	1	4.4	9.2	1.70	4.77	4.64

The model was statistically significant as shown in the very small p value of 0.0003 (Table 3). The model also showed non-significant lack of fit as shown in the p value of 0.4951. Model terms with p values < 0.05 were significant while those with p values > 0.1 were not significant. The pH and solid substrate loading were significant indicating that these variables should be maintained at their optimal levels to maintain optimum metabolic activity during fermentation. The quadratic model had an R^2 value of 0.91 and adjusted R^2 value of 0.84 (Table 4). This indicates that 91% of the total variation around the mean could be explained by the model. The low value of the coefficient of variation (CV) indicates that the runs were carried out with high precision and reliability (Hou and Chen, 2008). The adequate precision was greater than the recommended minimum value of 4, showing that the model can be used to navigate the design space (Cao *et al*., 2009).

Table 3: Analysis of variance (ANOVA) for quadratic model

Sources	Sum of Squares	df	Mean Squares	F value	p value
Model	7.53	9	0.84	11.65	0.0003
X_1	0.60	1	0.60	8.36	0.0161
X_2	4.15	1	4.15	57.83	< 0.0001
X_3	0.12	1	0.12	1.66	0.2268
X_1X_2	0.022	1	0.022	0.31	0.5922
X_1X_3	0.55	1	0.55	7.69	0.0197
X_2X_3	0.15	1	0.15	2.08	0.1801
X_1^2	0.24	1	0.24	3.39	0.0955
X_2^2	1.08	1	1.08	15.08	0.0030
X_3^2	0.93	1	0.93	12.98	0.0048
Residual	0.72	10	0.072		
Lack of Fit	0.36	5	0.072	1.01	0.4951
Pure Error	0.36	5	0.071		
Cor Total	8.25	19			

Table 4: Statistical information for ANOVA

Parameter	Value
R^2	0.91
Adjusted R^2	0.84
Mean	3.69
Standard dev.	0.27
CV %	7.26
Adeq. Precision	10.96

Effect of Independent Variables on Ethanol Production

The response surface plots shown in Figs. 1 and 2 were based on the final model in which one variable was kept constant at the middle value and the other two were varied within the experimental range. It is clear from Fig. 1 that increasing the substrate concentration enhanced ethanol production. Maximum ethanol concentration was recorded at a solids loading of 10 w/w%. Using more of the solid substrate during fermentation creates provision for the production of more fermentable sugars during hydrolysis. Since ethanol production is enhanced at increased sugar concentration, it will be expected that increasing the solids loading will also have a similar effect. The increase in ethanol concentration in the course of fermentation has been attributed to the consumption of sugar substrate by the

Saccharomyces cerevisiae cells to produce ethanol (Ocloo and Ayernor, 2010). The highest concentration of ethanol was produced at an acid concentration of 0.051 M and the lowest at a concentration of 2.04 M. The trend observed could be attributed to the fact that at high acid concentrations, monomeric sugars could be decomposed under the very low pH conditions created to form inhibitory products such as furfural, 5-hydroxymethylfurfural (HMF), levulinic acid, acetic acid, formic acid, uronic acid, 4-hydroxybenzoic acid, vanilic acid, vanillin, phenol, cinnamaldehyde, formaldehyde. These degradation products serve as fermentation inhibitors and they have been reported to affect cell growth and product formation during fermentation (Bakker *et al.*, 2004).

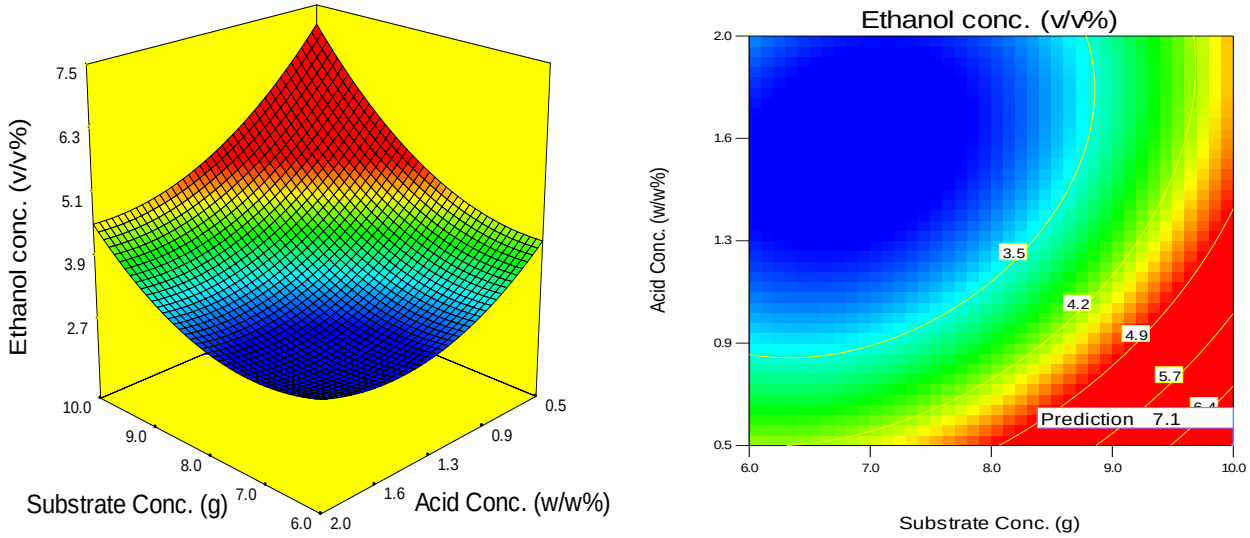

Fig. 1: Response surface and contour plot showing the effect of solids loading and acid concentration on ethanol production

Fig. 2 shows the effect of pH on the fermentation process. The pH of the fermentation medium is an important variable during fermentation. This is because the structure and activity of the enzymes responsible for fermentation are dependent on the pH of the solution. Maximum ethanol concentration was recorded at a pH of 6.0. There is usually an optimum pH value for the yeast cells to function. The growth of the cells is adversely affected if the pH of the medium deviates from the optimum value. This has the consequence of also adversely affecting the production of ethanol during fermentation (Graves *et al.*, 2006). According to Oberoi *et al.* (2011), yeast cells perform best in a medium in the vicinity of pH 5.0 and too low a pH value can cause stress to the yeast cells thereby reducing the fermentative ability of the cells. Similar values have been reported by other researchers. Ado *et al.* (2009) reported that the optimal pH for growth and ethanol production by *Saccharomyces cerevisiae* was between pH 5 and 6 while Wang *et al.* (2008) reported that during the production of ethanol from various waste materials such as bread residue, citrus peel, kitchen garbage and pineapple cannery waste, *Saccharomyces cerevisiae* performed well within the pH range of 4.18 – 6.

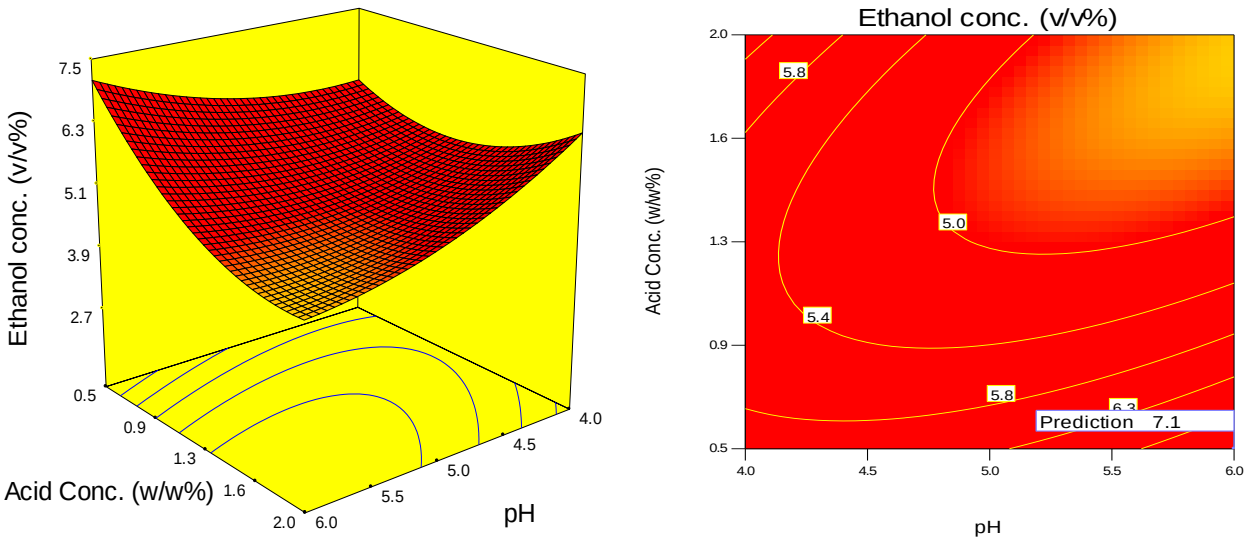

Fig. 2: Response surface and contour plot showing the effect of pH and acid concentration on ethanol production

3.3 Numerical Optimisation

Numerical optimisation of the variables was done to maximise ethanol concentration. The values of the reaction variables during numerical optimisation were fixed in the ranges shown in Table 1. The Design Expert software suggested 29 solutions out of which the one with the highest desirability (0.82) was chosen. Thus, after evaluating the model graphs and the combinations suggested by the numerical optimisation package, the chosen optimum condition was pH 6.0, solids loading 10 %w/w and acid concentration 0.051 M and this resulted in a maximum ethanol concentration of 7.1 % v/v.

3.4 Validation of Statistical Model

In order to confirm the validity of the statistical model, three confirmation experimental runs were performed at the chosen optimal fermentation conditions. The maximum experimental ethanol concentration obtained was close to the value predicted by the statistical model. The excellent correlation between the predicted and measured values of these experiments shows the validity of statistical model.

CONCLUSION

This study demonstrated that Pumpkin pod could serve as an ideal substrate for the production of bioethanol through separate hydrolysis and fermentation. Since the conditions of fermentation are important in deciding the final product yield and commercial potential of the process, CCD coupled with RSM which was executed by Design Expert software was helpful in optimising the process. High level of solid loading was favourable for ethanol production indicating a positive interaction between the variables while the reverse was the case for pH and acid concentration. The optimal values of pH, solid substrate loading and acid concentration were 6.0, 10 %w/w and 0.051 M respectively. Under these conditions, the maximum ethanol concentration was obtained as 7.1 %v/v.

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