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Optimization for Bioethanol Production from Luffah Sponge (*Luffa aegyptica*) by *Saccharomyces cerevisiae* and *Zymomonas mobilis*

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ABSTRACT

Energy demand and the problem of fossil fuel depletion are increasing worldwide and these challenges have led search for sources that can be renewable, sustainable, efficient, cost effective, convenient and safe. Sponge gourd is a promising energy crop providing lignocellulosic biomass that is rich in fiber. Bio-ethanol is the most important energy product derived from biomass in terms of volume, market values also environmental friendly. The research was aimed to determine the optimum condition for bioethanol production from luffah sponge by Saccaromyces cereviciae and Zymomonas mobilis. Luffah sponge was pretreated with dilute acid, enzymatically hydrolysed using Aspergillus Niger then fermented to produce ethanol. The fermentation experiment was carried out using response surface (User-Defined) method on design expert (version 11.1.0) software package. The response values for bioethanol concentration was best fitted into quadratic model considering the value of predicted R2 and adjusted R2.The optimum bioethanol concentration was 36.15g/L using the optimum conditions of 9g mass of substrate, 72hrs time of fermentation, 33.4°C temperature, 3.7 pH, inoculum size of 1 mcfarland ($3x10^8$ spores/ml) and ZymomonasMoblis as the fermenting microorganism.

Keywords: *Optimization, Bioethanol, Luffah sponge, Saccaromyces cereviciae, Zymomonas mobilis*

INTRODUCTION

The world's energy demand is increasing every day and the problem of fossil fuel depletion is also looming (Abdulfatah and Freddie, 2019). These challenges have led to new approaches focusing on energy consumption management and alternative fuel sources that can be renewable, sustainable, efficient, cost effective, convenient and safe (Yusuf and Inambao, 2018).

In a bid to maintain sustainability, production of chemicals from renewable sources is required (Zhang *et al.,* 2018). Biopolymers, which include plant biomass, forestry residues and agricultural residues, are potential feedstock for production of chemicals. Biopolymeric fibres such as lignocelluloses, offer several environmental benefits owing to their renewable nature, abundance, low cost, low energy consumption in production, etc (Zhang *et al.,* 2018). Although, bioconversion of lignocellulosic residues has received much attention because of their important application such as efficient conversion of biomass to cellulose, most plant biomasses have not been fully exploited.

Bio-ethanol is the most important product derived from biomass in terms of volume and market values (Sujan *et al.*, 2018). However, the development of bioethanol still seems under the target, so it needs efforts to increase its production. Bioethanol is a liquid biofuel derived from biomass by fermentation technique where certain species of yeast or bacteria metabolize sugars in oxygen-lean conditions to produce ethanol and carbon dioxide (Qian *et al.*, 2014; Nashwa *et al.*, 2021) Bioethanol is a fuel that is more environmentally friendly and renewable.

Sponge gourd is a promising energy crop providing lignocellulosic biomass that is rich in fiber (Adetoyese *et al.*, 2020; Nyong *et al.*, 2021) In Nigeria, *Luffa aegyptiaca* plant grows in the wild and abandoned building structures and fences walls in towns and villages and its highly underutilized.

Optimization process is defined as a way to enhance the performance of any system or process by obtaining the maximum benefit and investigating suitable parameters which give best response (Kamaldeen et al., 2019). This method of optimization is recommended due to its desirability function which gives the optimum performance levels for one or more responses.

Materials and Methods

Source of Substrate (luffah sponge)

Luffah sponge used in this work was from an uncompleted building at Yankaba, Nasarawa local government area of Kano state where it has grown in abundant. It was transported to Nigerian stored products research institute laboratory in a Ziploc bag with minimum delay.

Proximate Composition of Substrates

Proximate composition (moisture, ash, crude protein, fatty acid, crude fiber content) of the substrate were determined using AOAC method 2012.

Mechanical and Acid Pretreatment of Substrate

Luffah sponge substrate was washed thoroughly with water was allowed to air dry at room temperature before milling using ball mill, and sieved through 2mm mesh size (Jaisamut et al., 2013). The milled sample was pretreated with 1.6M sulphuric acid in beaker thus ensuring the solid-liquid ratio 1:10. (Charlie et al., 2017).

Samples Collection for Isolation of Isolates

Two grams of sand sample 2 inches away from surface was collected using a clean spatula into a 100ml zip lock bag for isolation of *Aspergillus niger*. Two mililiters of samples of pineapple juice and fermented sorghum were collected in a sterilized screw capped bottle for isolation of *Saccharomyces cereviciae* and *Zymomonas mobilis*. All samples were taken to the Nigerian Stored Products Research Institute (NSPRI) Kano central laboratory with minimal delay for analysis.

Growth Medium for Microorganism

Aspergillus niger was grown on PDA media while *Saccharomyces cerevisiae* was grown on yeast extract peptone dextrose medium (YPD) containing yeast extract (10g), peptone (20g) and glucose (20g) per 1000ml water adjusting pH 5.4. The bacteria *Zymomonas mobilis* was grown on beef extract peptone dextrose medium (BPD) containing beef extract (10g), peptone (10g) and glucose (20g) per 1000ml water adjusted pH 7. Morphological appearances of the inoculated plates were observed. Microscopic observations were made using wet mount preparation (Dubey and Maheshwari, 2004).

Biochemical Characterization of *Zymomonas mobilis* and *Saccharomyces cerevisiae*

Pure isolates suspected to be *Zymomonas mobilis* and *Saccharomyces cerevisiae* were subjected to biochemical characterization according to criteria described in Bergeys manual for determinative bacteriology (Holt *et al.*, 1994; Tambuwal *et al.*, 2018)

Figure 1: Luffah sponge (*Luffah aegyptiaca*)

Inoculum Preparation

Aspergillus niger cultures were inoculated onto PDA medium in the Petri dish, after 72 hrs, the spores were harvested using sterilized water with 0.1% Tween 80 (Ajeet *et al.,* 2014). Spore count was measured with haemocytometer and adjusted to $2x10^6$spores/ml by adjustment of optical density. Yeast Peptone Dextrose Broth (100 ml) in 250 ml Erlenmeyer flask was inoculated with 48 hours old pure colonies of *Saccharomyces cereviciae* and *Zymomonas mobilis* from agar slant with the aid of an inoculating loop. This was incubated at 28°C in an incubator for 48 hours and cells were harvested by filtration and suspended in 10ml of sterilized water and the turbidity was adjusted to 1 Mcfarland standard (3.0×10^8) cfu/ml (Zakpaa *et al.,* 2009; Wanderley *et al.*, 2014).

Enzymatic Hydrolysis

As described by Hamed et al., (2015) 1g Pepton, 2g Yeast extract, 2g KH_2PO_4 and 1g $MgSO_4.7H_2O$,10 $(NH_4)_2SO_4$per 100ml was added to a flask containing 5g substrate. The flasks were shaken to dissolve the content, plugged with cotton wool and sterilized for 30 minutes, the flask were inoculated with 2mililiters of inoculums of 4.0×10^6 cfu *Aspergillus niger* and incubated at 350C for 6 days in an orbital shaker at 100 rpm. Uninoculated flasks were used as control. All experiments were done in triplicates (Ajeet *et al.*, 2014).

Determination of Reducing Sugar Content

Reducing sugar was measured using DNS method of Miller (1959). The color tests were made with 3mls of reagent added to 3-ml of glucose solution in test tubes. The mixtures were heated for 5minutes in a boiling water bath and then cooled under running tap water to adjust to ambient temperature. Cooling to ambient temperature was made necessary by the effect of temperature on the absorbance of the color. The color intensities were measured using a spectrophotometer (T80 UV/VIS) at 540nm .The reducing sugar content was subsequently determined by making reference to a standard curve of known glucose concentrations.

Fermentation of Hydrolysate

Fermentation was carried out *using Saccharomyces cerevisiae* and *Zymomonas mobilis* to ferment the hydrolyzed sample to ethanol and carbon-dioxide. Batch fermentations were done in Erlenmeyer flasks fitted with rubber stoppers containing 100mL of hydrolysate, the samples are sterilized 121 ^{O}C for 15min. After sterilizaton, the hydrolysate was cooled to room temperature and aseptically inoculated with inoculums culture of *Saccaromyces cerevisiae* and *Zymomonas mobilis* (Zakpaa *et al.,* 2009).

Ethanol Content

Bioethanol content from the hydrolysate was obtained by distillation using a rotary evaporator at 700 ^{O}C and condensed bioethanol was collected under ice to avoid re-evaporation (Khalil *et al.*, 2015). Analysis of ethanol content was performed using potassium dicromate spectrophotometeric method at 578nm after every 24hrs and concentration of bioethanol present was estimated from ethanol standard curve (Sumbhate *et al.*, 2012).

Modelling and Optimation of Bioethanol Production of Substrates

Modelling and optimization of bioethanol production parameters were carried out using response surface method on design expert (version 11.1.0) software package. A three-variable Box-Behnken Design (BBD) was used to develop a statistical model for the fermentation process. This is because there was a need to determine the optimum variable combination that could give the desired output with a minimum number of the experiment, without the need for studying all possible combination experimentally. The concentration of bioethanol was evaluated as dependent variables (responses). The composition variables (factors) and their corresponding responses values (quality parameters) are reported in Table. The mean values of the investigated response measured using box bennken design were fitted to get model equations. Best-fitting mathematical models were determined based on the comparison of statistical parameters such as adjusted multiple correlation coefficients (adjusted-R2), and predicted multiple correlation coefficients (predicted-R2) (Osemwengie *et al.,* 2020)

Data Analysis

Descriptive and inferential statistics was used to analyze the data obtained. Experimental analysis was carried in triplicate and data was expressed as means± standard deviation. Data was analysed using analysis of variance (SPSS 16.0 version 2007). Were significant difference was observed, the means were compared according to Tukey's test at significance level of 5% (0.05). A statistical model was developed and response surface (User-Define) method on design expert (version 11.1.0) software package was used for optimization (Kamaldeen *et al.*, 2019; Osemwengie *et al.*, 2020).

Results and Discussion

The crude fiber content of loofah sponge (35.43%).Due to the high fibre content of the biomass, it can be considered as potential feedstock for generation of monomeric sugars and bioethanol production.

The protein content (0.73%) of the loofah sponge was quite low. This makes the loofah sponge to be a very poor source of proteins; this explains why the use of these wastes in animal feed has not been reported. Amount of protein agrees with 1.17% reported by Valerie *et al.* (2022). These findings have resemblance with the results of Nyong *et al.* (2021).

Dietary fats function to increase food palatability by absorbing and retaining flavors. Fat content in luffah sponge was 0.23% and this value agrees with 0.19% and 0.33% reported by Bangash *et al.* (2011) and Hussain *et al.* (2009) respectively. Nyong *et al.,* 2021 reported a fat content of 0.30% when determining the proximate composition of sponge gourd.

Table 1. Proximate Composition of Luffah Sponge Substrate

Component	%composition
Ash	1.15±0.17
Moisture	1.34±0.04
Protein	0.70±0.20
Crude Fibre	35.43±0.2
Crude Fat	0.23±0.05

Each value represents mean of three independent tests ± standard deviation

Enzymatic hydrolysis is the process of hydrolysis of cellulose by cellulase complex enzymes produced from fungi. Enzymatic hydrolysis after pretreatment yielded the highest hydrolysis rates after 72hrs with values of 10.28mg/mL (Table 2) lufffah sponge substrate and the lowest value of reducing sugar of 2.91mg/ml at the initial stage. Reducing sugar concentration increase with the time of hydrolysis until it reaches 72hrs then it decline. This is because all the cellulose has been converted to monomeric sugars and further fermentation results in production of inhibitory compounds. The efficiency of enzymatic hydrolysis was influenced by many factors originating from the substrate and enzymes. This agrees with the findings of Ogunbayo *et al.,* (2016) that shows increase in sugar production with increase in time of hydrolysis of sawdust and corncob substrates after acid pretreatment. These results suggest that the reduced sugars are the intermediate in ethanol production.

Table 2. Reducing Sugar Content of Luffah Sponge Substrate after Hydrolysis

Time	Luffah sponge	Control
0hr	2.91±0.14^{a}	0.68±0.00^{a}
24hrs	9.59±0.10^{b}	0.75±0.00^{c}
48hrs	9.69±0.17^{c}	0.74±0.00^{c}
72hrs	10.28±0.73^{d}	0.70±0.00^{b}
96hrs	9.73±0.16^{c}	0.70±0.00^{b}

Each value represents mean of three independent tests ± standard deviation. Means displayed with same homogenous superscript within the same column are not significantly different p> 0.05

Table 3.Model Summary Statistics for Optimization of Bioethanol Production using Corncob

Model	Standard Deviation	R^2	AdjustedR^2	PredictedR^2
Linear	6.52	0.7576	0.7405	0.7167
2FI	6.44	0.8051	0.7467	0.6650
Quadratic	4.63	0.9065	0.8691	0.8126

Key: 2FI= Two Factors Interaction

The investigation was carried out on linear, two factor interaction, and quadratic models to determine the best model that is statistically significant and best describes the relationship between the response and independent variables i.e. substrate mass, time of fermentation, pH and temperature. From the model summary statistics shown in table 3, it is seen that the quadratic model has the maximum predicted and adjusted R2 value.

Thus, it can be concluded that the quadratic model bests describe the relationship between the response and the independent variables; the coefficient of determination (R2) was obtained as 0.852. This indicated that the model satisfactorily represents the relationship between the independent and the response (ethanol yield). The R2 value indicates that 98.9% of the variability in the response could be explained by the statistical model, while 1.1% could not be accounted for by the independent variables (Amenaghawon *et al.*, 2013). The R2 value indicates the degree to which the model was able to predict the response. The closer the R2 value is unity, the better the model can predict the response (Qi *et al.*, 2009). The "Predicated R-Squared" of 0.696 is in reasonable agreement with the "Adjusted R-Squared" of 0.793, that is, the difference is less than 0.2.

Table 4. Statistical Analysis from the Modeling Bioethanol Production Using Luffah sponge

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	4766.69	41	116.26	14.47	< 0.0001	Significant
A-Substrate mass	1938.93	1	1938.93	241.39	< 0.0001	
B-Time	838.55	1	838.55	104.40	< 0.0001	
C-Temperature	198.05	1	198.05	24.66	< 0.0001	
D-pH	0.6992	1	0.6992	0.0870	0.7692	
E-Innoculum size	6.77	1	6.77	0.8430	0.3630	
F-Organism	111.63	1	111.63	13.90	0.0005	
AB	120.20	1	120.20	14.96	0.0003	
AC	26.72	1	26.72	3.33	0.0742	
AD	2.26	1	2.26	0.2811	0.5983	
AE	1.72	1	1.72	0.2142	0.6455	
AF	81.76	1	81.76	10.18	0.0025	
BC	2.09	1	2.09	0.2603	0.6121	
BD	27.53	1	27.53	3.43	0.0700	
BE	0.5565	1	0.5565	0.0693	0.7935	
BF	86.03	1	86.03	10.71	0.0019	
CD	0.0351	1	0.0351	0.0044	0.9475	
CE	6.07	1	6.07	0.7560	0.3887	
CF	55.36	1	55.36	6.89	0.0115	
DE	1.21	1	1.21	0.1505	0.6997	
DF	5.81	1	5.81	0.7228	0.3993	
EF	3.80	1	3.80	0.4725	0.4950	
A^2	0.0540	1	0.0540	0.0067	0.9350	
B^2	72.92	1	72.92	9.08	0.0041	
C^2	669.47	1	669.47	83.34	< 0.0001	
D^2	300.46	1	300.46	37.41	< 0.0001	
E^2	23.90	1	23.90	2.98	0.0907	
ABF	15.54	1	15.54	1.93	0.1704	
ACF	5.92	1	5.92	0.7366	0.3948	
ADF	0.1128	1	0.1128	0.0140	0.9061	
AEF	0.8256	1	0.8256	0.1028	0.7498	
BCF	0.1485	1	0.1485	0.0185	0.8924	
BDF	6.62	1	6.62	0.8248	0.3682	
BEF	0.5356	1	0.5356	0.0667	0.7973	
CDF	13.55	1	13.55	1.69	0.2000	
CEF	4.79	1	4.79	0.5963	0.4436	
DEF	3.52	1	3.52	0.4388	0.5108	
A^2F	8.80	1	8.80	1.10	0.3002	
B^2F	35.72	1	35.72	4.45	0.0400	
C^2F	229.76	1	229.76	28.60	< 0.0001	
D^2F	0.2470	1	0.2470	0.0308	0.8615	
E^2F	20.71	1	20.71	2.58	0.1146	
Residual	401.62	50	8.03			
Lack of Fit	347.60	40	8.69	1.61	0.2138	not significant
Pure Error	54.03	10	5.40			
Cor Total	5168.31	91				

It can be observed from results in table 4 that the model shows p value 0.0001 for ethanol concentration, implying that the chance of the model error is less than 5%. This indicates that the model is suitable for determining ethanol concentration. The model can be employed as a significant model for predicting response over the independent input variables. The reduced equation of the ethanol concentration response is as follow:

Ethanol concentration=19.95+7.78A+5.12B-2.49C-2.38D-3.05E+3.88AB+1.60AF+1.64BF+1.32CF-6.19C2-4.15D2+1.43B2F+3.63C2F

Description: Y= ethanol concentration (g/L)

A= mass of substrate (g)

B= time of fermentation (hours)

C= temperature (^{O}C)

D= pH

E= size of inoculum (McFarland standard)

F= fermenting organism

From the statistical analysis in table 4, it can be observed that the factors which significantly ($p < 0.05$) affect the ethanol concentration were the substrate mass (A), time of fermentation (B), and temperature (C), inoculum size (E), fermentation organisms (F),interaction between mass of substrate and fermentation time(AB),interaction between time and fermenting organism (AF),interaction between temperature and fermenting organism (BF),square of time (B2), the square of temperature2 (C2), square of pH2 (D2) and interaction between the square of temperature and fermenting organism (C2F) while the rest did not significantly ($p > 0.05$) affect the ethanol concentration. Negative values of coefficients C, D, E, F, C2 and D2 in the equation showed that high temperature, pH, size of inoculum and type of fermenting organism significantly affect the ethanol concentration.

The concentration of ethanol increases in the quadratic curve with increase mass of substrate and temperature from 30 to 35^{O}C then it reduces.

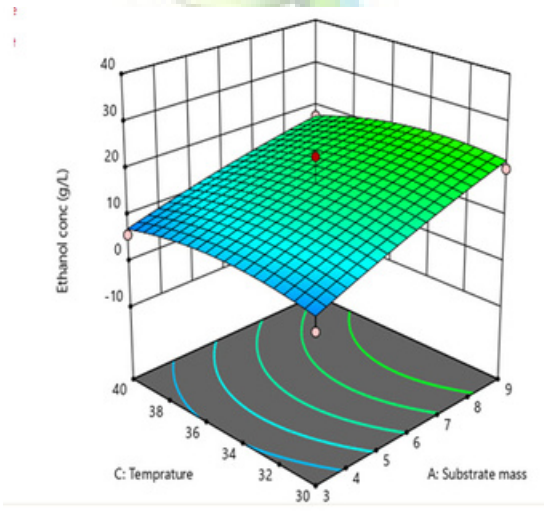

Fig 1. The interactive effect of temperature, substrate mass and ethanol concentration using *Saccharomyces cerevisiae*

This reduction can be due to the fact that higher temperatures render the fermenting organisms inactive or even kill them. This effect can be seen in both figure 1 and 2, there is a sharp decrease in ethanol production after 34 to 35^{O}C in figure 2 but figure a has a steady decrease, it may be due to the fact that *Zymomonas mobilis* is easily affected by high temperature than Saccaromycese but even at that Zymomonas produces higher amount of ethanol than Saccaromyces from the figures.

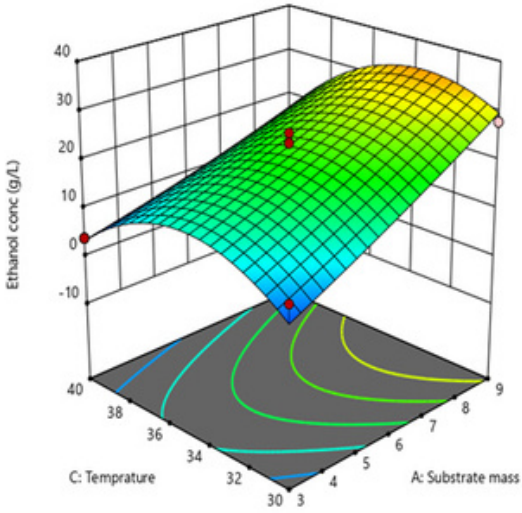

Fig 2. The interactive effect of temperature, substrate mass and ethanol concentration using *Zymomonas mobilis*

It can be observed from both figure 3 and 4 that ethanol concentration increases with time of fermentation from 34hrs to 72hrs, likewise with increase in mass of substrate form 3g to 9g. The highest ethanol concentration was seen at 72hrs of fermentation using 9g of substrate. Although from figure 1 there was reduction in ethanol concentration after 72hrs of fermentation with 3g of substrate, this was due to total conversion of glucose in the fermenting medium by bacteria faster than yeast (figure 4).

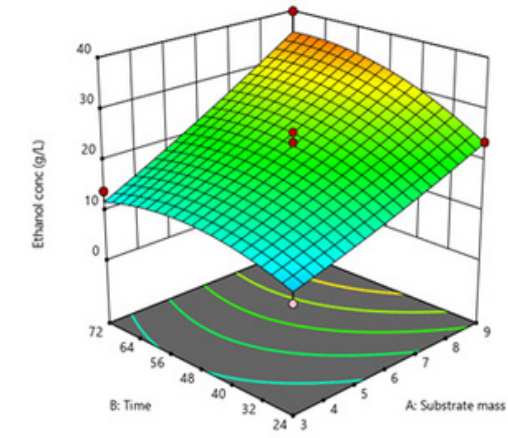

Fig 3. Effect of time and substrate mass on ethanol concentration using *Zymomonas mobilis*

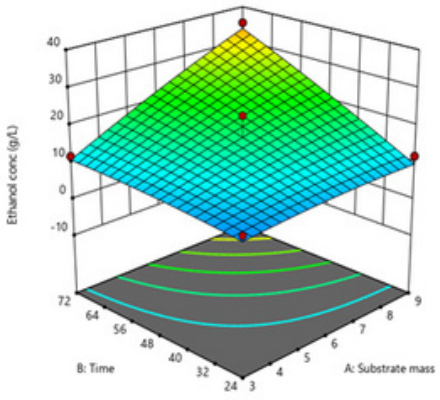

Fig 4. Effect of time and substrate mass on ethanol concentration using *Saccharomyces cerevisiae*

From the figure 5, there is no effect in innoculum size on ethanol concentration as the only effect seen is with increase in mass of substrate from 3 to 9g. But in fig 6, there is a slight effect observed as the ethanol concentration increased at inoculum size of 2 both from mass of 3g up to 9g.

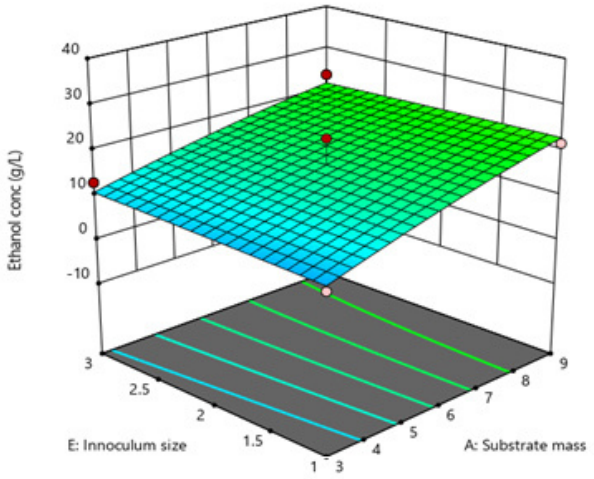

Fig 5: Effect of innoculum size and substrate mass on ethanol concentration using *Saccharomyces cerevisiae*

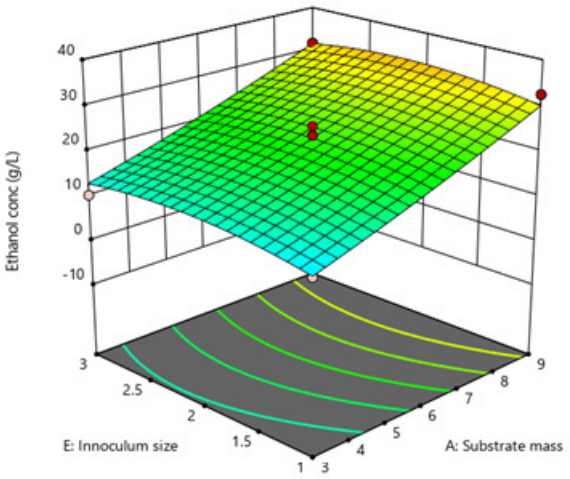

Fig 6: Effect of innoculum size and substrate mass on ethanol concentration using *Zymomonas mobilis*

The effect of pH on ethanol concentration as seen in both fig 7 and 8 shows an increase in concentration from pH from 3 to pH of 3.5 increases bioethanol concentration and reduction in concentration after pH 3.5 to 4. But still the pH of 4 produces higher ethanol concentration than pH 3 from both figures.

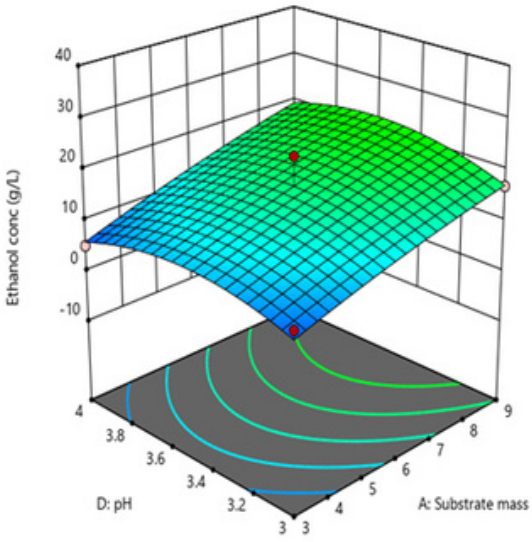

Fig 7: Effect of pH and substrate mass on ethanol concentration using *Saccharomyces cerevisiae*

Zymomonas mobilis has higher ability to ferment glucose to ethanol than *Saccharomyces cereviciae* .Fermenting organism consumes sugar for growth and production of other metabolic products. Fermentation of the product of waste biomass hydrolysis by the yeast, *Saccharomyces cerevisiae* and bacteria *Zymomonas mobilis* yielded ethanol in accordance with the sugar concentration obtained. Hydrolysate of luffah sponge with high sugar concentration yielded the highest concentration of ethanol. This is an indication that the yield of ethanol is directly proportional to concentration of sugar in the fermenting fluid. Moreover since growth commences during the aerobic phase, some amount of sugar get used up before the anaerobic stage which is characterized by ethanol production (Saliu and Sani, 2012).

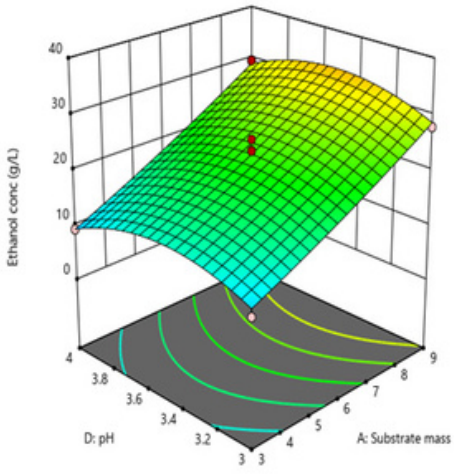

Fig 8: Effect of pH and substrate mass on ethanol concentration using *Zymomonas mobilis*

Sivasakthivelan *et al.,* (2014) recorded highest ethanol yieled in *Zymomonas mobilis* than *Saccharomyces cerevisiae* when studying ethanol production using sun flower head waste.

Szambelan (2018) stated that *Zymomonas mobilis* is more efficient in ethanol production than *Saccharomyces cerevisiae* with values 61.48g/L and 58.38g/L respectively when comparing the organisms in fermentation with stillage reusing.

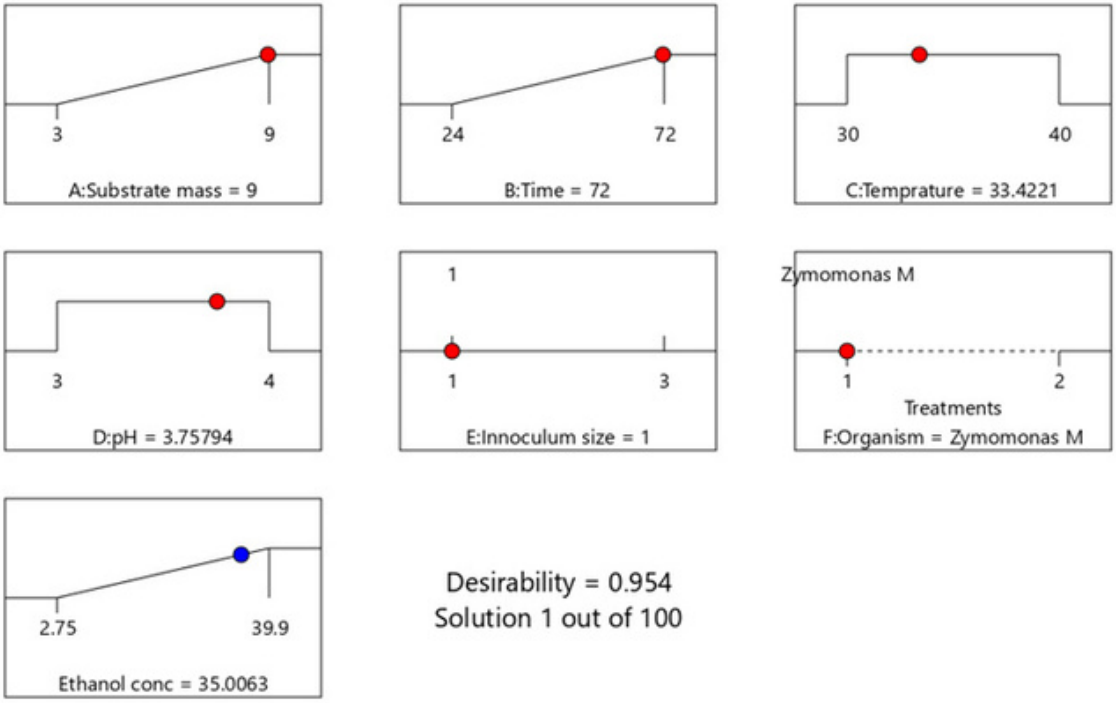

Fig 9. Optimum condition for ethanol production using luffas sponge

Validation of Statistical Model

To confirm the validity of the statistical model, three confirmation experimental runs were performed at the chosen optimum fermentation conditions representing maximum point of concentration of ethanol. The experiment was conducted at optimum conditions of (9g, 72 hrs, 33.40C, pH of 3.7 and 1 inoculum size). The result shows that there was no significant difference ($p>0.005$) between the maximum experimental ethanol concentrations of 36.15g/L to the predicted value of 35g/L.

CONCLUSION

Aspergillus niger, *Saccharomyces cereviciae* and *Zymomonas mobili*s were isolated and used in bioethanol production from luffah sponge substrate. Fiber content of the substrate (35.43%) was pretreated and hydrolysed using *Aspergillus niger* to produce of maximum reducing sugar concentration of 10.28g/L after 72hrs. This resulted in production of optimum ethanol concentration of 36.15g/L using a set of optimum conditions of 9g, 72hrs, 33.40C, 3.7 pH, innocullm size of 1with *Zymomonas mobilis* as fermenting organism which agreed well with the predicted value of 35g/L. This indicates the success of the model in optimizing the fermentation conditions.

AUTHORS CONTRIBUTIONS

Ahmad, F. U. design the study, carry out analysis and drafted the manuscript, Bukar, A. and Kawo, A. H. checked manuscript and supervised the enter research. All authors read and approved the manuscript.

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CONFLICT OF INTEREST

There was no any conflict of interest in this research.

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