



## Response Surface Optimization of Bioethanol Production from Sugar Bagasse by *Aspergillus flavus*

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### ABSTRACT

Sugarcane bagasse presents an appealing opportunity as a bioethanol source due to its abundance, affordability, and renewable characteristics. Employing response surface methodology to optimize operational parameters affecting bioethanol production from sugarcane bagasse offers a cost-effective and efficient approach to enhance bioethanol yields. In this investigation, sugarcane bagasse served as the substrate for bioethanol production, with the microbial strain *Aspergillus flavus* being utilized. The study aimed to explore the impacts of temperature, pH, and inoculum concentration on bioethanol yield through a central composite design. The findings revealed that the optimal conditions for bioethanol production were observed at a temperature of 26°C, pH of 6, and substrate concentration of 50 g/L. Consequently, the projected bioethanol yield under these conditions reached 36.4 g/L. These results contribute valuable insights into the potential of sugarcane bagasse as a bioethanol feedstock, emphasizing the significance of optimizing process parameters to maximize yields. It is evident from this study that the optimization of process parameters plays a critical role in enhancing bioethanol yield from sugarcane bagasse. Furthermore, the study showcases the effectiveness of utilizing *Aspergillus flavus* as a microbial strain for bioethanol production.

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### INTRODUCTION

The global energy consumption has increased tremendously in the last four decades due to massive urbanization and industrialization of emerging economies. Since 2000, the energy consumed globally has tripled, and this growing trend of energy consumption is projected to continue in the foreseeable future [1]. According to the projection of the International Energy Agency (IEA), if current trends of energy consumption is maintained, the global energy consumption will reach 740 million terajoules by 2040 [2]. It was established that the rate of energy consumption is directly related to industrial development, there the unprecedented rise in energy consumption should be a good thing. But unfortunately, it is not, because almost 83% of the

energy used in the world are derived non-renewable, and environmentally harmful carbon sources. The main source of energy used for electricity generation, home heating, industrial heating, and transportation are fossil fuels such as petroleum, coal, and natural gas. Burning carbon-based fuels releases CO<sub>2</sub> into the atmosphere, significantly contributing to the increase in the amount of greenhouse gases in the atmosphere and ultimately to global warming [3].

Over-dependence on fossil fuels as the chief source of energy is an existential threat in the world, because a significant increase in the global temperature will trigger a series of catastrophic event that might bring the world to an end. This necessitates finding alternative and renewable source of energy to reduce the level of

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CO<sub>2</sub> emission and global warming. There are numerous sources of renewable energy that can provide clean energy to satisfy the increasing global demand [4]. However, one of the most interesting renewable sources of energy are biofuels, fuels derived immediately from living things. A major advantage of biofuels is in their utility to serve as a potential alternative to transportation fuels. According to the United States Energy Information Administration (EIA), although biofuels are predominantly used as for transportation, they can also be used for electricity generation. Biofuels provide alternative source of clean energy that can mitigate the effect of climate change using readily available renewable resources. In recent years, biofuel has attracted attention of researchers, governments, and industries, due to their potential as a renewable, biodegradable and non-toxic alternative to fossil fuel [5].

Although at a slow phase, there is an upward trend in the use of renewable fuels such as biodiesel, biogas and bioethanol in place of conventional fossil fuels. Bioethanol is massively produced from different biomass sources such as beets, sugarcane, whole grains and cereals [6]. However, there is an increasing concern over the use these edible sources of biomass for energy production, they are good sources of carbohydrate for human consumption. Therefore, non-edible biomass alternatives that can be used for biofuel production were actively explored. The upgrading of non-edible biomass into biofuels is one of the routes being considered. Sugarcane is used worldwide as a feedstock for ethanol and sugar production. In Brazil, for instance, *circa* 570 million tons of sugarcane were produced in 2009 (UNICA, 2010). After sugarcane is milled for juice extraction, bagasse is obtained as a residue, which corresponds to about 25% of the total weight and contains 60% to 80% of carbohydrates [7]. The fermentation of these carbohydrates could significantly improve bioethanol productivity and sustainability but, instead, bagasse is discarded as agricultural waste or burned for energy supply in sugar and ethanol mills. Both alternatives are, however, pollutant and inefficient in making use of the chemical energy available in the biomass [8].

Fractionation of bagasse components and their conversion to fermentable sugars is essential in enabling this renewable feedstock to be used for biofuel production. Similarly, to other plant cell walls, sugarcane bagasse is mainly formed by two carbohydrate fractions (cellulose and hemicellulose) embedded in a lignin matrix. Lignin is a phenolic macromolecule, resistant to enzyme attack and degradation, and thus its content and distribution are recognized as the most important factors determining cell wall recalcitrance to hydrolysis [9].

Pretreatment technologies applied to lignocellulosic substrates are necessary to decrease this recalcitrance and to improve the yields of monomeric fermentable sugars that are liberated by enzymatic hydrolysis. Different pretreatment methods have singular action mechanisms. They may decrease cellulose crystallinity and/or the degree of polymerization, increase accessible surface areas or selectively remove hemicellulose and lignin from the lignocellulosic matrix. An effective pretreatment strategy should also minimize carbohydrate degradation and the production of enzyme inhibitors and toxic products for fermenting microorganisms [10]. A variety of pretreatments have been applied to different lignocellulosic matrices. These include physical processes such as milling and irradiation; physical-chemical treatments, using hot water and/or steam explosion ammonia explosion, organic and ionic solvents, supercritical fluids, diluted acids and/or bases, and finally, biological pretreatments using bacteria and fungi [11].

Experimental design and data analyses are important process that are used to optimize process parameter that affect the production of bioethanol. Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for modeling problem [12]. RSM is also a statistical approach for designing experiments, evaluating the effect of various factors, and finding the optimal condition for desirable response and reducing the number of required experiments. A Central Composite Design (CCD) is employed to fit an empirical, second-order polynomial model. Coupled with

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second-order polynomial model, it gives a very powerful combination that usually provides an adequate representation of most continuous response surfaces over a relatively broad factor domain. CDD was employed in this study to reduce the total number of experiments needed to determine the best combination of parameters for optimization of the process. In this work, the process parameters influencing bioethanol production from sugarcane bagasse were investigated to obtain optimal parameters. The influence of four experimental conditions on bioethanol yield produced from sugarcane bagasse were to the objective is to find the optimum condition of four parameters used that have the most significant impact on the concentration of bioethanol produced.

## MATERIALS AND METHODS

### Substrate

The substrate used in this study was sugarcane bagasse, which was sourced from local area (Gusau, Nigeria). The substrate was first dehydrated to remove water molecules and then milled to obtain a fine powder and fibers, which can be easily used to perform further experiments. Specifically, the biomass was chopped and washed to remove extraneous matters. It was then oven-dried at 70°C for 3 days, grounded with a hammer mill and sieved to a mesh size of 1 mm. dried samples were analyzed for moisture content and ash content. Analyses of main compositions of raw material; cellulose, hemicellulose and lignin, were carried out according to AOAC Official Method 973.18. all analyses to determine the chemical composition of the biomass were conducted both before and after pretreatment to determine the impact of pretreatment on the substrate's composition.

### Source and Preparation of Microorganism

The microorganism used in this study (*A. flavus*) was isolated, identified and prepared as follows; firstly, the cultures of the microorganism was grown and maintained on Potato dextrose agar (PDA) slants. Sub-culturing was performed at 15 days interval. The cultures were stored in a refrigerator at 4°C until needed for fermentation.

Preparation of Inoculum using a 24days old culture *A. flavus*. Briefly, a loopful of the cultures were inoculated in the medium and then incubated for 48 hours at 30°C on a rotary shaker. Centrifugation was performed for 48 hours at 200 rpm to collect the cells. The inoculum concentration of 0.1% (dry weight/volume) was used for the fermentation process

### Substrate Pretreatment

The dried substrate was pretreated using NaOH solution as follows; Fourty grams of powdered substrate was material was suspended in 400 ml of 5% w/v NaOH solution to form a substrate-NaOH suspension. 10ml aliquot of the suspension was transferred to a screw-capped Erlenmeyer flask and then heated for 2hour in water bath maintained at 100°C. After heating for 2-hours, the solid residue was recovered by vacuum filtration and washed repeatedly with water until it reached a neutral pH. Thereafter, its moisture content was determined before storing in a sealed plastic bag at 4°C to be used as substrate for the cellulose saccharification experiment. The pretreated substrate was not dried prior to enzyme digestion to avoid pore collapses that can occur in the micro-structures of the biomass, which will lead to decrease in the release of enzymatic glucose during the cellulose saccharification process.

### Enzymatic Hydrolysis

Enzymatic hydrolysis was performed according to the method reported in earlier studies [28] with slight modifications. Hydrolysis was performed using citrate buffer 50 mM, pH 4.8, with 0.02% sodium azide. The conventional hydrolysis kinetics at solid load 10% was performed at enzyme loads of 10, 20, and 40 FPU/g bagasse at a reaction volume of 30 mL. Samples from hydrolysate was centrifuged at 400 rpm, 4°C, for 10 min. the supernatants liquid was collected and used for determining the quantity of glucose. Conversion of biomass (X) was calculated according to the following formula.

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$$X = \left( \frac{S_g \times V_H}{W_{dry\ bagasse} \times F_g} \right) 0.9 \times 100\% \dots \dots \dots [1]$$

Taking into account the amount of soluble sugars in the liquid phase after hydrolysis ( $S_g$ , is glucose equivalent concentration;  $V_H$ , the volume of the solution where the hydrolysis was performed; and the correction factor for hydration 0.9, in order to correct for the water molecule added upon hydrolysis [29], and the initial amount of glucose equivalent in the solid cellulose sample before hydrolysis ( $W$ , the weight of dry lignocellulosic sample, and  $F_g$ , the fraction of cellulose in the lignocellulosic sample).

**Determination of Reducing Sugar**

The reducing sugar content of the hydrolysates was determined according to the method [30]. Specifically, equal volumes (3ml) of 3, 5- dinitrosalicylic acid (DNS) reagent and hydrolysate was added into different test tubes. The test-tubes were shaken to mix the contents thoroughly, and heated in boiling water (100°C) for 10 minutes until reddish brown color was developed. Moreover, 40% potassium sodium tartrate solution was added to stabilize the reactions and then allowed to cool to a room temperature under running tap water. Absorbance measure measurement was then carried out spectrophotometrically at 490nm. The concentrations of the reducing sugar were estimated by extrapolation with a prepared standard curve of known glucose concentration.

**Sugar Fermentation**

Fermentation of the hydrolyzed samples was performed according to the method described [10]. For each fermentation run, 50ml of the hydrolysates was autoclaved in 100 ml conical flasks at 120°C for 15 minutes and then allowed to cool prior to inoculation. Different concentration of inoculum was then aseptically added in the autoclaved hydrolysates. The concentration of the bioethanol produced will be quantified by treating 4ml fermented samples with 2mls of chromium reagent. The mixture was then incubated for 1 hour before taking the absorbance spectrophotometrically at 588nm. For the determination of the percentage yield of bioethanol, the initial and final reducing sugar content of the medium was recorded and equation 2 was used to compute the bioethanol yield.

$$\text{Ethanol yield (\%)} = \frac{\text{Ethanol produced} \times 100}{\text{Reducing sugar Utilized}} \dots \dots \dots [2]$$

**RESULTS AND DISCUSSION**

**Response Surface Optimization**

Four parameters or independent variables, including inoculum concentration, pH, temperature and fermentation time which were reported to be the most significant factors that influence the bioethanol production [13]. These factors at different levels (Table 1) were used to build a randomized response surface design matrix based on faced centered central composite design. The influence of these parameters on fermentation for bioethanol production were optimized was further investigated using Analysis of variance to indicate factors that significantly influence the response.

**Table 1:** Ranges of factors Used in Response Surface Optimization

Factors	Name	Units	Minimum	Maximum
A	Temperature	°C	25.00	45.00
B	Fermentation Time	hours	50.00	90.00
C	Inoculum Concentration	%v/v	5.00	20.00
D	pH		5.00	7.00

The design matrix of all the independent variables and the results of 30 runs in triplicates of

experimental and predicted bioethanol concentration are shown in Table 2. It can be

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observed that there was a good correlation between experimental and predicted bioethanol concentration from various conditions. This clearly shows the high accuracy of response surface model constructed in this study. Second order polynomial model was suggested as the model that has the best describe the relationship between the dependent and independent variables in the model, with the model p-value of 0.0003, which is significantly less than the 0.05

threshold for a significant model. The Adequate precision measure of the model (9.540), which is a measure of the model's signal to noise ratio, was greater than 4. Indicating that the model can be successfully used to navigate the design space. The model's F-value of 6.85 further indicate the significance of the model. Furthermore, the model Lack of Fit F-value of 1.90 indicate that the model's Lack of fit is not significant relative to pure error.

**Table 2:** Design Matrix for the Response Surface Optimization of Bioethanol Production

Number of Experiments	Temperature °C	Fermentation Time (hours)	Inoculum Concentration %v/v	pH	Bioethanol Concentration g/L
1	35	70	12.5	6	5.5
2	25	90	20	7	9.1
3	25	90	5	7	9.5
4	25	50	20	5	9.7
5	45	50	5	7	4.3
6	25	90	5	5	8.4
7	25	50	20	7	5.3
8	45	50	20	5	3.8
9	45	50	20	7	4.2
10	45	90	20	7	9.6
11	35	70	12.5	7	8.2
12	35	70	12.5	6	7.8
13	25	70	12.5	6	9.4
14	45	70	12.5	6	8.2
15	35	70	5	6	7.4
16	45	90	20	5	8
17	35	70	20	6	8.7
18	35	50	12.5	6	3.1
19	35	90	12.5	6	8.6
20	25	90	20	5	9.2
21	25	50	5	5	3.5
22	45	90	5	5	8.8
23	35	70	12.5	6	7.3
24	35	70	12.5	6	7.7
25	25	50	5	7	3.2
26	45	90	5	7	8.4
27	35	70	12.5	5	5.2
28	35	70	12.5	6	7.1
29	45	50	5	5	2.8
39	35	70	12.5	6	8

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The results of the model's analysis of variance (Appendix I) indicate that B, C and B<sup>2</sup> are significant model terms with p-values <0.05 were found to highly influence the fermentation rate and the concentration of bioethanol produced.

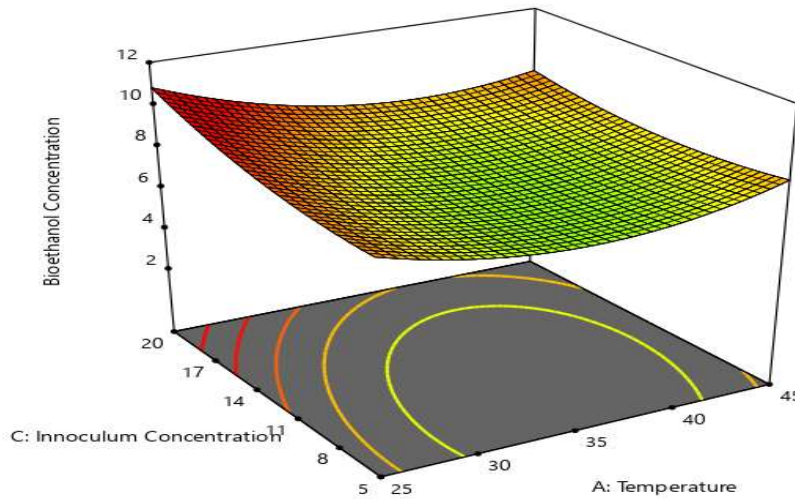
$$\text{Bioethanol Concentration} = 7.36 + 0.51A + 2.20B + 0.62C + 0.133 \times D + 0.32AB + 0.46AC + 0.42AD + 0.52BC + 0.31BD + 0.27CD + 1.29A^2 - 1.65B^2 + 0.54C^2 + 0.80D^2 \dots\dots\dots(1)$$

The quadratic model (Equation 1) can be used to predictions of bioethanol concentration that can be produced given a given level of each operational parameter. Response surface optimization of bioethanol production was performed to investigate the optimum values of operational parameters that can be used to obtain high rate of bioethanol. Response surface optimization was carried out to find the optimal operational parameters for the condition for the fermentation process. The optimization analysis of the experimental data suggested optimum levels of the operational variables determined by the quadratic model of faced centered central composite design were 20% inoculum

concentration, pH 5, Temperature 25, and fermentation time 90 hours. The predicted bioethanol concentration obtained using the optimal operational parameters was 9.96g/L as shown Figure 2 of the supplementary information.

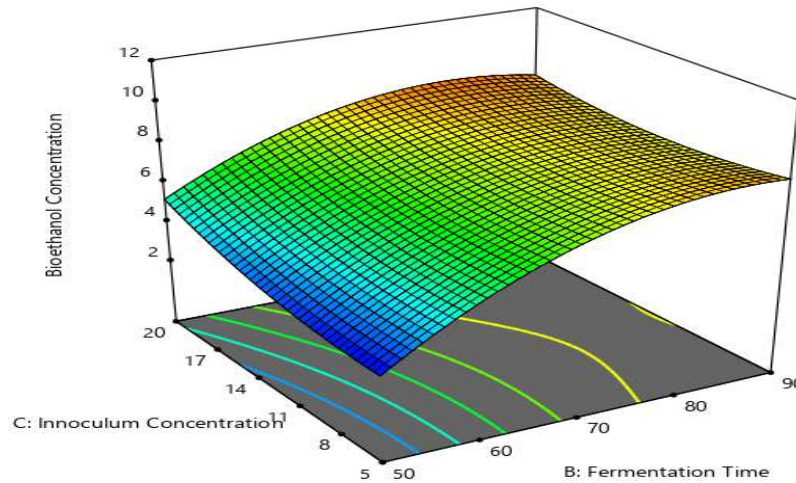
**Effect of Inoculum Concentration**

The concentration of bioethanol produced from the fermentation of sugarcane bagasse is affected by the concentration of inoculum used in the process. The result of the analysis of variance indicate that inoculum concentration is significant with p-value of 0.03, which is less than 0.05 threshold. Figures 1 and 2 shows the 3D response surface plot of inoculum concentration against temperature and fermentation time respectively. It can be observed (Figure 1) from that increase in inoculum concentrations increase the rate of the bioethanol produced. This is consistent with literature on bioethanol production from other biomass sources. Optimal production of bioethanol was obtained at inoculum concentration of 20 % (v/v), further increase does not show any appreciable increase in bioethanol concentration.



**Figure 1:** Response Surface Plot of Inoculum Concentration and Temperature

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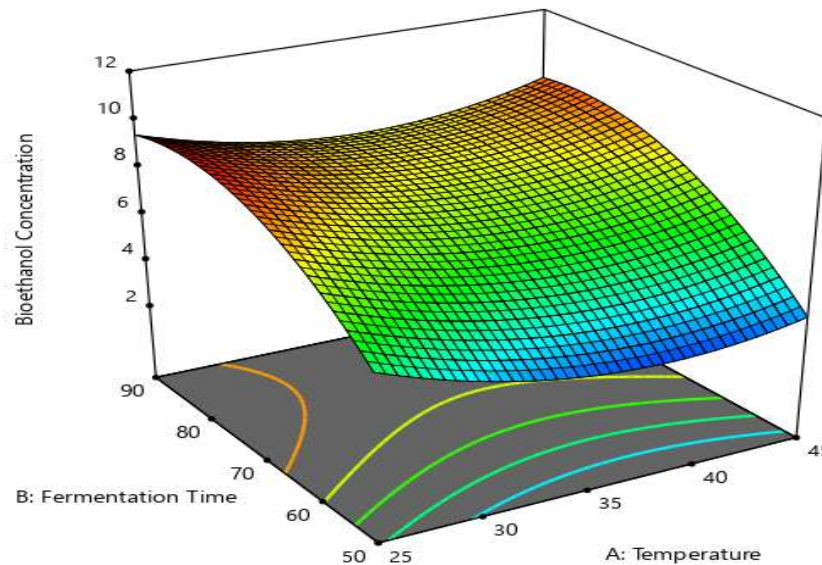


**Figure 2:** Response Surface Plot of Inoculum Concentration and Fermentation Time

**Effect of Fermentation Time**

The economic viability of bioethanol production is a function of fermentation time, making it one of the most important operational parameters that needs to be optimized. The importance of the variable is indicated by its p-value (<0.0001), which is much less than the 0.05 p-value threshold for important parameters. The 3D response surface plot of the fermentation time with temperature and pH as shown in figures 3 and 4 indicate that the rate of bioethanol production

increases with increase in the time allowed for the fermentation process to occur. The optimal fermentation time was 80 hours, which give the maximum concentration of bioethanol produced. Different values were reported in the literature as the optimal fermentation time depending on types of reducing sugar generated and the unique metabolic regulation of each. Glucose consumption by *S. cerevisiae* was faster than other types of sugars.



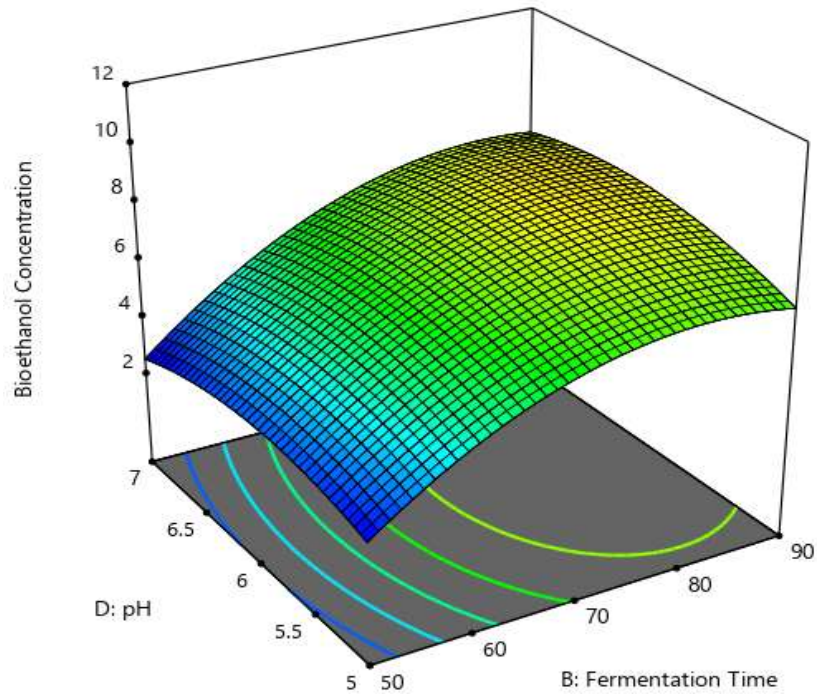
**Figure 3:** Response Surface Plot of Inoculum Concentration and Temperature

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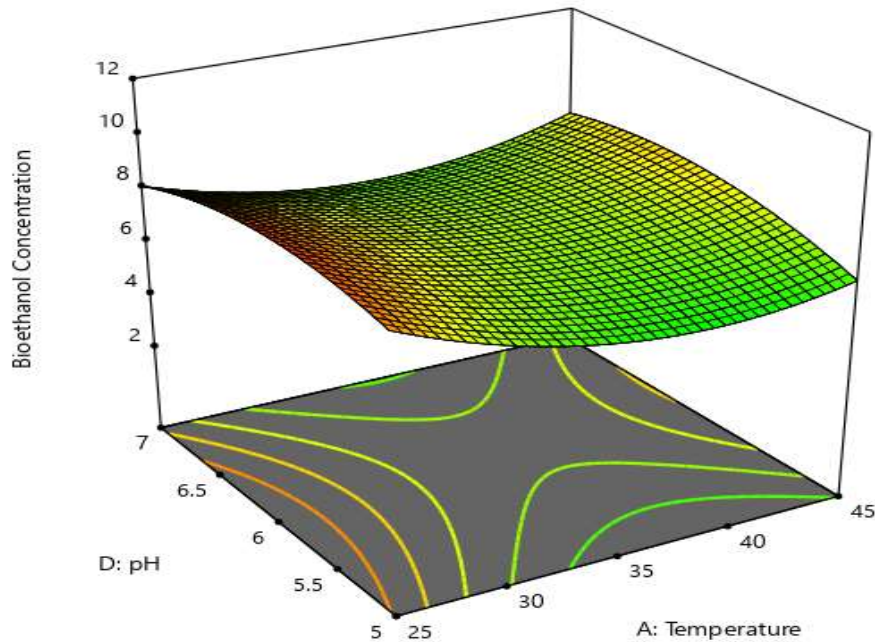
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**Figure 4:** Response Surface Plot of pH and Fermentation Time



**Figure 5:** Response Surface Plot of pH and Temperature

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### Validation Experiment

The validation of fermentation experiment was performed under optimum conditions of 16.337 (v/v) inoculum concentration, pH 6, temperature 26.5°C and 80 hours of fermentation time. The bioethanol concentration obtained after undergoing fermentation under optimized conditions was 9.77 g/L (0.40 g/g yield, 78% conversion efficiency) which was in close agreement with model prediction value of 9.98 g/L as shown in Figure 6. The difference between the

predicted and experimental value of the concentration of bioethanol produced was negligible. Hence, it can be concluded that the response surface generated in this study could be reliably used to predict the production of bioethanol from fermentation process. The optimization of fermentation alone employing RSM software is hardly found which make this present study as one of the pioneer references for future research method.

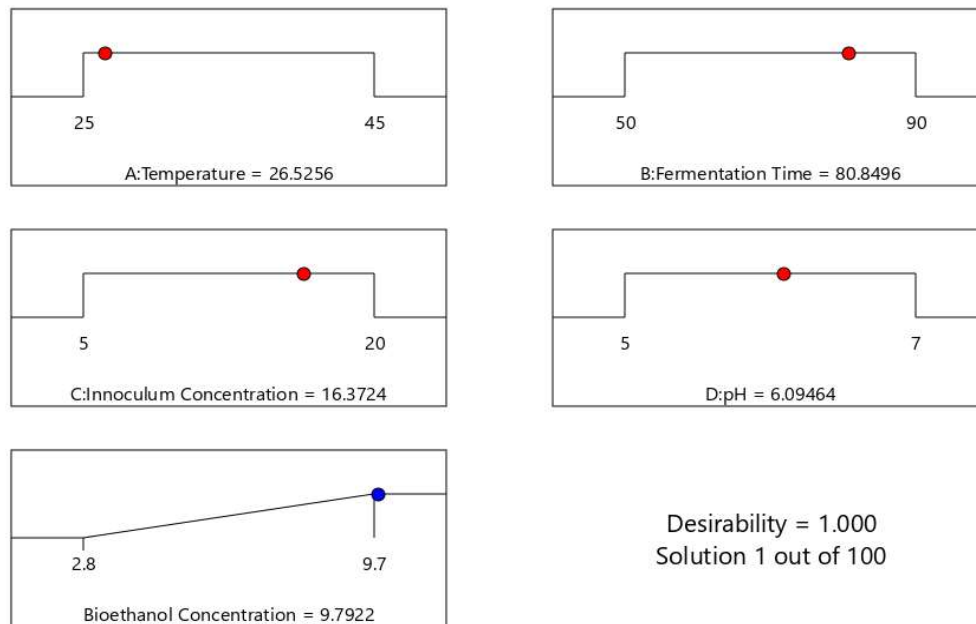


Figure 6: Optimal values of Operational Parameters Used for Validation Experiment

### CONCLUSION

Response surface optimization of bioethanol production from sugar bagasse was investigated using central composite design. The optimal value of fermentation time, temperature, pH and inoculum concentration were found to be 80hours, 26°C, 6 and 16%v/v respectively. By the use of the developed quadratic model, response surfaces, and contour plots, the investigation for the variation of the bioethanol yield depending on the changes in the process variables, pH, molasses concentration, temperature, mixing rate, and time, and the maximization of the bioethanol yield was found to be easily studied and predicted.

Further work is undertaken by the research group to investigate the effect of the difference sugarcane bagasse constituents and the secondary fermentation byproducts on the fermentation process and the bioethanol yield.

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