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Production and Characterization of Bioethanol from De-oiled Seeds Cake of *Rothmannialongiflora* Using *Saccharomyces cerevisiae*

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ABSTRACT

The motive of this research is production of bioethanol from deffated seeds cake of Rothmannia longiflora and analysis of its physical/chemical properties. The fermentation process was optimized for maximum ethanol yield base onthree parameters; temperature, pH and yeast concentration. The statistical analysis revealed that pH and temperature are the most influential parameters affecting the ethanol yield. The maximum ethanol yield of 11.14 g/cm^3 was observed at the following optimum conditions; pH of 6, temperature of 30^{o}C and yeast concentration of 2 %. The produced ethanol was subjected to fuel properties analysis. The structural investigation of the derived bioethanol was conducted using FT-IR analysis and it confirmed the characteristics bands of ethanol at 3369.52 cm^{-1}, 2918.5 and 2844.0 cm^{-1}. Somefuel properties of the bioethanol were found to be consistent with ASTM standard and to those of conventional ethanol. The study revealed the viability of Rothmannia longiflora seeds cake as promising feedstock for bioethanol production.

Keywords: Bioethanol, Fermentation, Rothmannia longiflora, Seed cake.

1. INTRODUCTION

Currently, about 85% of the world's energy needs come from fossil fuels (Edenhofer *et al.*, 2010), which are much responsible for global warming and natural destruction (Nigam and Singh, 2011). The current approach is to use renewable alternative energy sources such as biomass, geothermal, solar and wind to reduce green gas emissions. The potential to produce biofuels from biomass is high (Naik *et al.*, 2010). Biodiesel is an environmentally friendly biomass fuel that has received attention as an alternative to conventional fuels. However, some problems that may arise are the handling of the seed cake. Due to their toxic properties, some seed cakes can neither be used as animal feed nor in agricultural farming. Producing biofuels from these cakes would be the best option for it'sefficient utilization (Deshmukh and Marathe, 2016).

Bioethanol is considered as important renewable fuel that can partially replace fossil-derived fuels. Due to its favorable properties, it has shown great potential as an alternative fuel in spark-ignition and compression-ignition engines. Bioethanol is an oxygenated fuel and therefore reduces particulate emissions from engines. Bioethanol also has higher octane numbers, higher heat of vaporization, and wider flammability limits compared to gasoline and diesel, resulting in enhanced fuel combustion, higher compression ratios, and shorter ignition times (Khuong *et al.*, 2016). The world production of bioethanol increased from 50 million m^3 in 2007 to over 100 million m^3 in 2012 (Kang *et al.*, 2014). Brazil and the United States are the world's leading suppliers, mainly using corn or sugar cane.

In Nigeria, bioethanol production is still in its infancy and requires the attention of policymakers and financial institutions to develop and build the industry. Bioethanol production in Nigeria uses cassava (mainly), sweet sorghum and sugarcane as feedstocks. A major uncertainty in the large-scale use of bioethanol is the risk of food crisis while achieving energy security (Phillips and Mary, 2011). Therefore, food-related raw materials need to be replaced by non-food materials.The use of common biomass and lignocellulosic wastes can significantly increase bioethanol production.

Bioethanol production from de-oiled seed cakes has not been well studied. Therefore, this study aimed to produce and characterize ethanol from de-oiled seed cakes of *Rothmannia longiflora* seeds by fermentation using *Saccharomyces cerevisiae*.

2. MATERIALS AND METHODS

2.1 Materials

The substrate used for the production of the bioethanol was seeds cake obtained after oil extraction from seeds of *Rothmannialongiflora*.

2.2 Sample Pretreatment

Sixty grams (60g) of the seed cake was soaked in water (500 cm^3) and left overnight to remove excess oil and dirt. The oil and dirt substances floating on the surface were gently decant. The sample was further subjected to chemical treatment by soaking it in a mixture of (1% H_2SO_4 and 1% NaOH) in the ratio of 1:1 and left to stand overnight and then filtered. The residue was washed with distilled water to ensure neutrality in the sample.

2.3 Determination of Proximate Parameters of the Seeds Cake

Crude Protein was determined using Kjeldahl method as described by Chang (2003) while crude fibre by method given by James (1995). Carbohydrate was estimated by difference using (equat. 1) as given by Raghuramulu *et al.* (2003).

$$\text{Carbohydrate} = 100 - (\text{Moisture} + \text{Fat protein} + \text{Ash} + \text{Oil/Fats}) \quad (1)$$

2.4 Enzymatic Hydrolysis

Thirty grams (30g) of pre-treated sample was put into conical flask (500 cm^3), to which distilled water (300 cm^3) was added, andthen allowed to form homogeneous mixture. *Aspergilusniger*was used for enzymatic pre-treatment. The volumetric flask was fitted with cotton and aluminium foil which was then placed in an incubator for five days at a temperature of 36 ^oC. It was allowed to stay for hydrolysis to take place (in order to breaks the hemicellulose) in the sample. The glucose obtained was subjected to reducing sugar test (Gupta *et al.*, 2012; Sebayang *et al.*, 2016).

2.5 Determination of Reducing Sugar

The reducing sugar content of the substrate was determined by Bertrand method (Demirbas, 2005) and spectrophotometry. This was done by adding 2 cm^3 of dinitrosalicylic acid (DNS) reagents to the sample in a test tube. The mixture was placed in water bath for some minutes; a reddish-brown colour was observed which was allowed to cool. The absorbance of the sample was measured at 575 nm using spectrophotometer.

$$C.R.S = \frac{Absorbance\ of\ sample}{Absorbance\ of\ glucose\ standard} \times 100 \quad (2)$$

2.6 Culture Media Preparation

An agar solution containing 3g of peptone (bacteria peptone), 0.2g of potassium chloride, 0.1g of magnesium sulphate, 0.25g of sodium chloride, 0.25g sodium trioxonitrate ($NaNO_3$) and 20g of glucose was prepared in 100cm^3 of distilled water. 0.5g of yeast (*Sacharomycescerevisae*) was added to the mixture and then placed in a shaking incubator.

2.7 Fermentation Process

The fermentation was carried out on the hydrolysate using *Sachhromyces cerevisae*. The biochemical reaction was allowed to proceed for seven (7) days at 30 ^oC, the volume of the distillate obtained was measure and the quantity of ethanol produced (in g/cm^3) was determined by multiplying the volume of the distillate by the density of ethanol (0.8033g/cm^3) (Onyeleke and Jibrin, 2009).

2.8 Optimization Study and Statistical Analysis

Bioethanol production from the pretreated seeds cake was optimized by varying temperature (30 - 40 ^oC), pH (4 – 8) and yeast concentration (2 – 6 %) using Response Surface Methodology in Design-Expert Version 6. Analysis of variance was used to generate model equation that fits the result of bioethanol yields obtained. Interactive effects of the parameters studied were visualized using3-D surface plots.

2.9 Determination of Fuel Properties and FTIR Spectroscopy of the Ethanol

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Carry630 spectrometer at the range of 4000-650 cm^{-1} was used to evaluate the chemical structure of the ethanol. Fuel properties of the produced ethanol such as flash point, cloud point, sulphur content, density and viscosity were evaluated using ASTM D93, ASTM D25100-8, ASTM D2622, ASTMD1298-99 and ASTM D445 respectively while the moisture content using a method adopted by Suleiman *et al*, (2016).

3. RESULT AND DISCUSSION

3.1 Proximate Analysis of the Seed Cake

Feedstocks with high levels of proteins, carbohydrates or lipids are good sources for many applications such as biofuel/food production, food additives and nutraceuticals (Arif *et al.*, 2020). Hence proximate investigation of the deffated seed cake of *Rothmannia longiflora* was carried out and shown in (Table 1). Proximate analysis shows the % fibre of 15.83 in the seed cake. Crude fibre measures the cellulose, hemicellulose and lignin content of a material. Cellulose and hemicellulose are polysaccharides that can be hydrolyzed into monosaccharides that can be used in ethanol fermentation. Ethanol production is directly related to the concentration of cellulose and hemicellulose and other sugars present in the feedstock.The lignin concentration in biomass is an obstacle to microbial fermentation. The seed cake was found to have carbohydrates content of 61.97 %. The high carbohydrate content indicates that the cake may produce good amount of reducing sugar and bioethanol. The protein content was measured to be 4.25 %. Proteins and carbohydrates are the main components of feedstocks for ethanol and higher alcohol production (Arif *et al.*, 2020). Considerable protein and carbohydrate content in the seed cake suggests its potential for production of higher alcohols.

Table 1: Some Proximate Composition of the Seed Cake

Parameters	Values
Carbohydrates (%)	61.97 ± 0.650
Crude Fibre (%)	15.83 ± 0.577
Protein (%)	4.25 ± 0.120

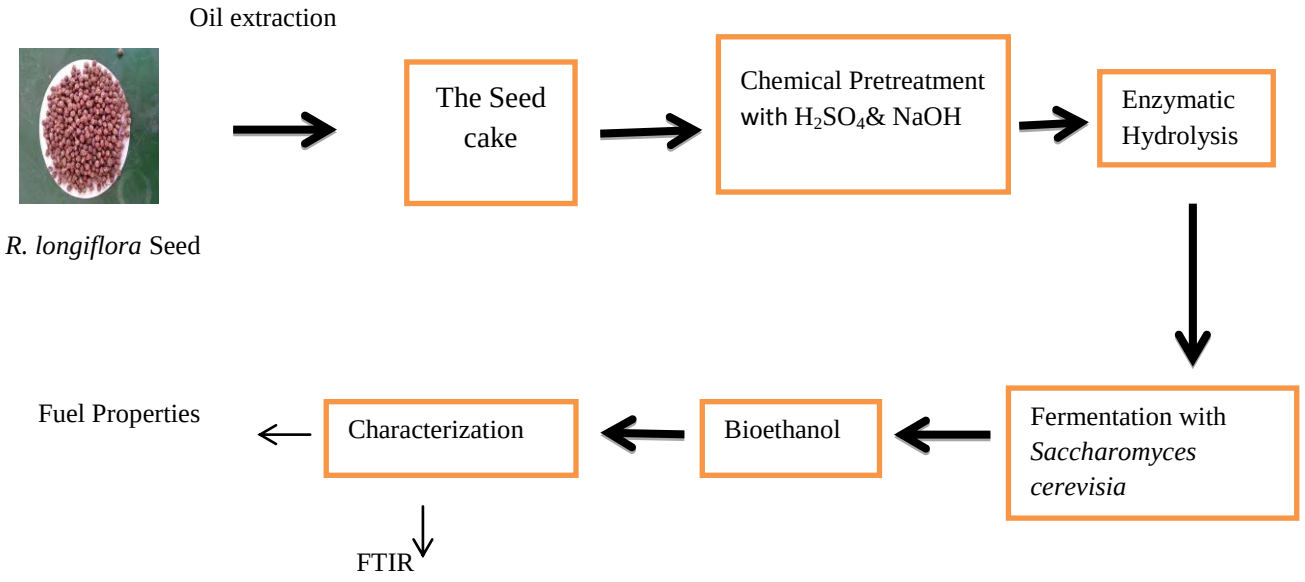

Figure 1: Process Flow Chart

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Table 2: Design Matrix with Experimental Bioethanol Yield

Std	Run	A:pH	B:Catalyst conc	C:Temperature (^oC)	Bioethanol Yield (g/cm^3)
1	1	8	6	35	7.43
6	2	6	2	40	8.35
14	3	6	4	35	9.70
5	4	6	4	35	9.56
16	5	6	4	35	9.94
8	6	6	2	30	11.14
10	7	6	4	35	9.90
7	8	4	4	40	10.62
11	9	6	6	40	10.86
3	10	6	4	35	9.34
12	11	4	2	35	8.55
2	12	6	6	30	9.59
9	13	8	2	35	5.85
17	14	8	4	40	6.92
13	15	4	4	30	8.65
4	16	8	4	30	9.50
15	17	4	6	35	7.80

Table 3: Summary of ANOVA Result

Source	SS	DF	MS	F-Value	Prob > F
Model	33.7	9	3.74	61.12	0.0001
A	4.97	1	4.97	81.14	0.0001
B	0.25	1	0.25	4.02	0.085
C	0.57	1	0.57	9.26	0.0188
A^2	12.45	1	12.45	203.27	0.0001
B^2	1.82	1	1.82	29.69	0.001
C^2	3.83	1	3.83	62.59	0.0001
AB	0.94	1	0.94	15.42	0.0057
AC	5.18	1	5.18	84.48	0.0001
BC	4.12	1	4.12	67.26	0.0001
Residual	0.43	7	0.061		
Lack of Fit	0.18	3	0.061	0.99	0.4823
Pure Error	0.25	4	0.062		
Cor Total	34.13	16			

Table 4: Model Summary

R-Squared	0.9874
Adj R-Squared	0.9713
Pred R-Squared	0.9031
Adeq Precision	27.547
Std. Dev.	0.25
Mean	9.02
C.V.	2.74
PRESS	3.31

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The analysis of variance presented in (Table 3) revealed that the model have low probability value (p = 0.0001) demonstrating significant of the generated model. Similarly, higher correlation coefficients (R^2 = 0.9874), prediction coefficients (Pred.R^2 = 0.9031), and adjustment coefficients (Adj.R^2 = 0.9713) indicate the ability of the model to predict bioethanol production under given process conditions. pH (A) and temperature (C) are important model terms (p-values < 0.05), indicating that changes in these factors significantly influenced the ethanol production. Another factor which measures the adequacy of the model is the value of adequate precision. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this study, adequate precision of 27.547 was obtained which implies adequate signal to noise ratio, so the model can be used to navigate the design space. Among the parameters studied, catalyst concentration (B) had the lowest coefficient (0.18) in the linear model term and was found to be insignificant within the range studied.

$$\text{Bioethanol} = +9.69 - 0.79A + 0.18B - 0.27C - 1.72A^2 - 0.66B^2 + 0.95C^2 + 0.49AB - 1.14AC + 1.02BC \quad (3)$$

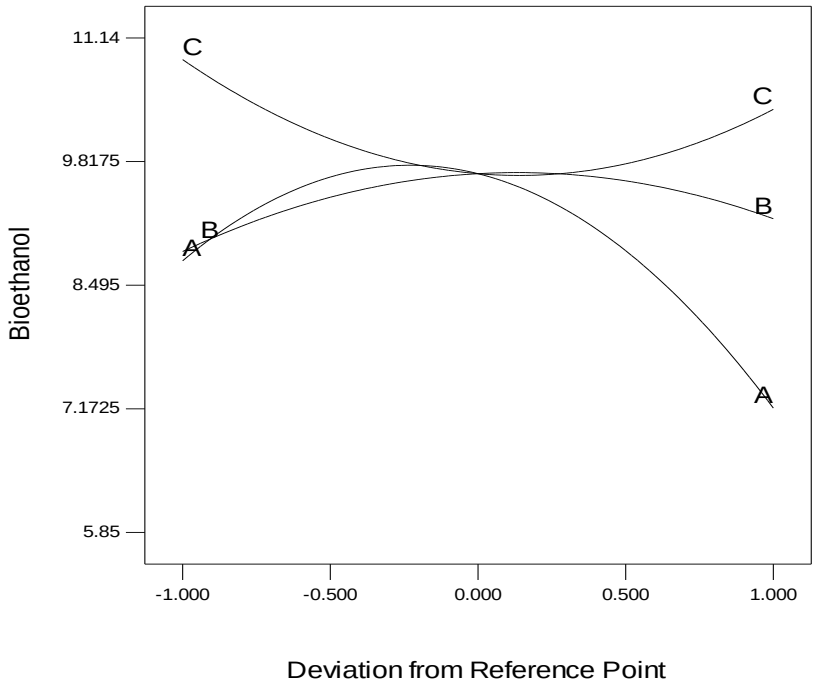

Figure 2: Perturbation Plot of the Effect of Independent Parameters on Bioethanol Yield

The perturbation plot shows a comparative effect of all the factors at a particular reference point (Figure 2). The graph helps identify the factors that have greatest impact on the response. The good steepness of the graph indicates that bioethanol production is sensitive to the factors studied. The higher curvature of pH (A) and temperature (C) demonstrates their high sensitivity. pH changes the solubility of ions and affects the dissociation of molecules, which determines their availability to microorganisms. At the proper pH, the nutrients needed by the microorganisms are fully utilized, which increases the growth rate and thus the ethanol production. Similarly, in a given medium, ethanol production can be affected by temperature, as microorganisms grow faster at certain temperatures. Thus, the pH and temperature were observed to be the most influential factors on the ethanol production. Therefore, it is very crucial to monitor pH and temperature appropriately for better growth of organisms and ethanol yield.

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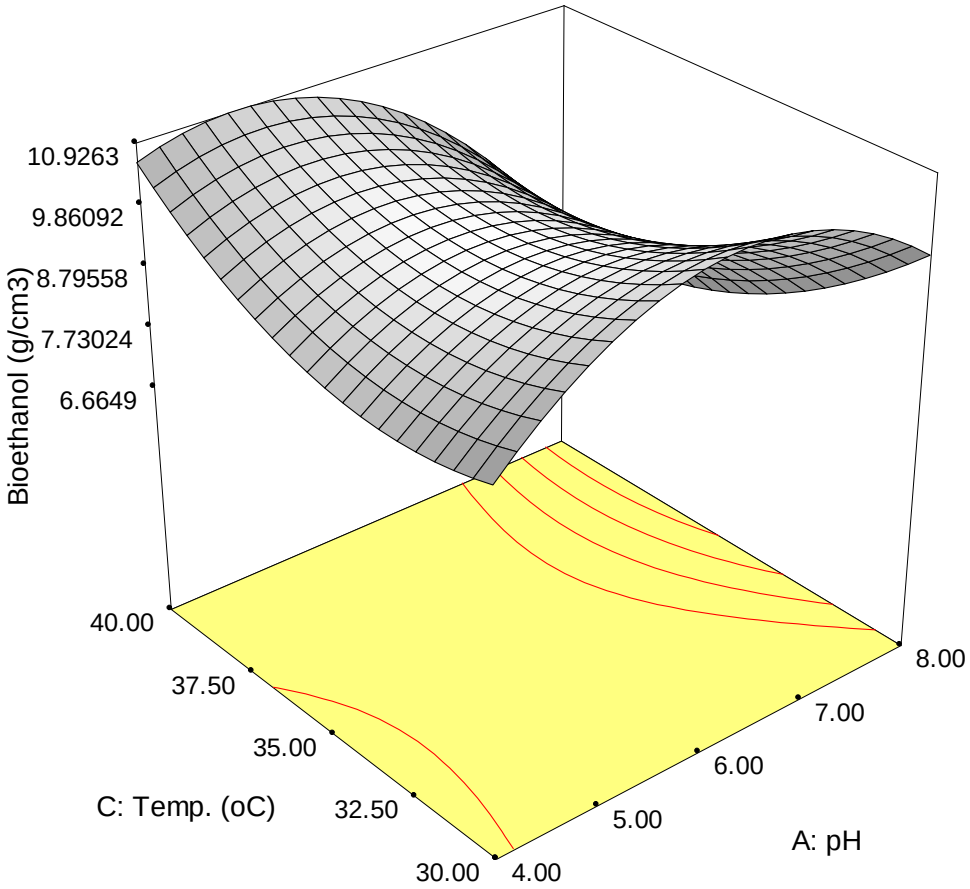

Figure 3: Response surface plots for the interaction between (A) pH and Temperature (C)

Fig.3, shows the effect of pH and temperature on the ethanol yield. It can be seen that pH and temperature have a significant interaction effect on the yield. pH has a greater effect on the yield than the temperature which was clearly proved by the p-value (< 0.0001) for pH and 0.0188 for temperature (Table 3). The 3-D surface plots shows increase in yield of the bioethanol with both increased in temperature and pH. The ethanol production was higher at pH around 7.5. Reungsang *et al*, (2013) and Varrone *et al*, (2012) reported that ethanol production was strongly influenced by pH and found an optimum pH of 8. Decreased in ethanol production was observed with further increase in pH above optimal level. The value obtained in this study is within the pH range of 6.5 - 9.0 reported by Muhaj and Sutjahjo, (2018).

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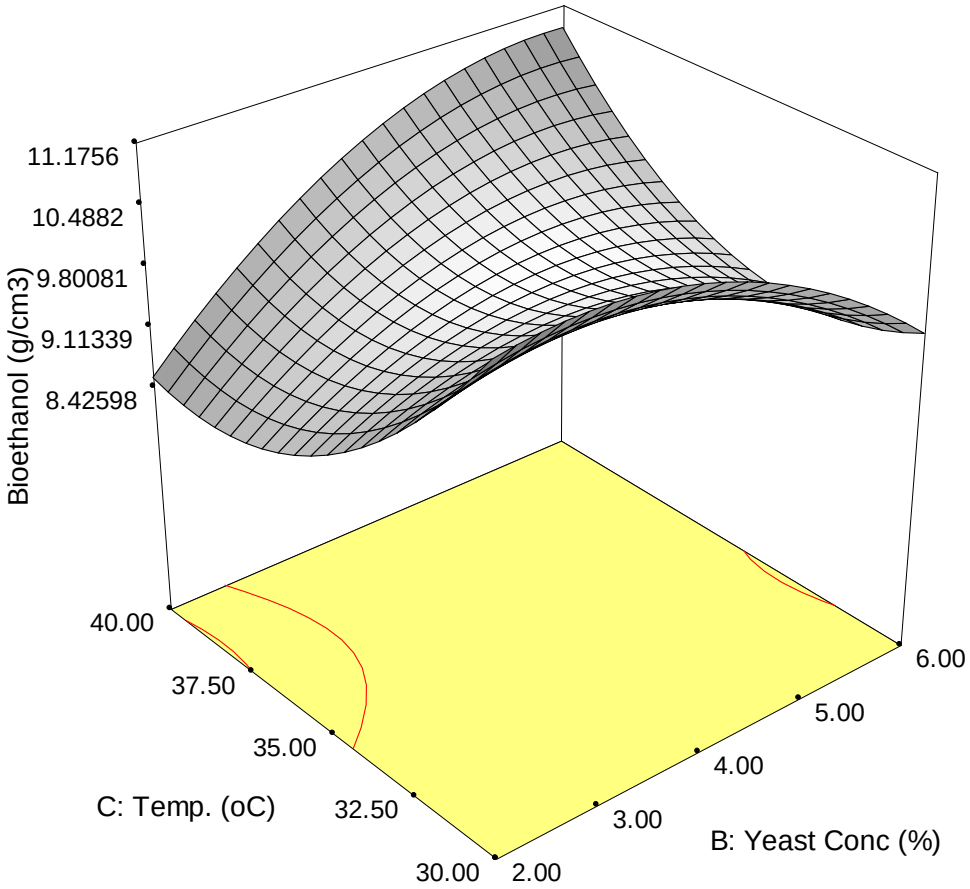

Figure 4: Response surface plots for the interaction between (B) Yeast conc and Temperature (C)

The surface plot (Fig.4) indicated that at a temperature of 30°C, the yeast convert the sugar faster than at any other temperature, the ethanol yield was observed to be low at high temperature greater than 30^{o}C. Similar trend was also observed by Ravi Kant Singh *et al*, (2014) in the fermentation of four different fruits (*Vitis vinifera* (grapes), Sugarcane (*Saccharum officinarum*), *Citrus cimetta* (Mosambi) and *Citrullus canatus* (Watermelon) using *Saccharomyces cerevisia*. The lower yield at high temperature may be due to poor organism growth and thermal decomposition of ethanol (Pimpakan *et al.,* 2012). The plot also depicts that the high the yeast concentration, the higher the bioethanol obtained. This may be due to increasing metabolic activity of microorganisms thereby converting the sugar to more ethanol. But as the yeast concentration exceeds 4.5 %, the ethanol yield was observed to decline. At high enzyme concentrations, more substrate is converted, but too much concentration can affect the rate of enzymatic reactions (Chairul and Azis, 2016). This is consistent with the pattern observed in this study, that high yeast concentration at certain level inhibits the conversion rate and also agreed with observation made by Suleiman *et al*, (2016) who report that, the more the fungi concentration the more ethanol produced.

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FT-IR Spectra of the Produced Bioethanol

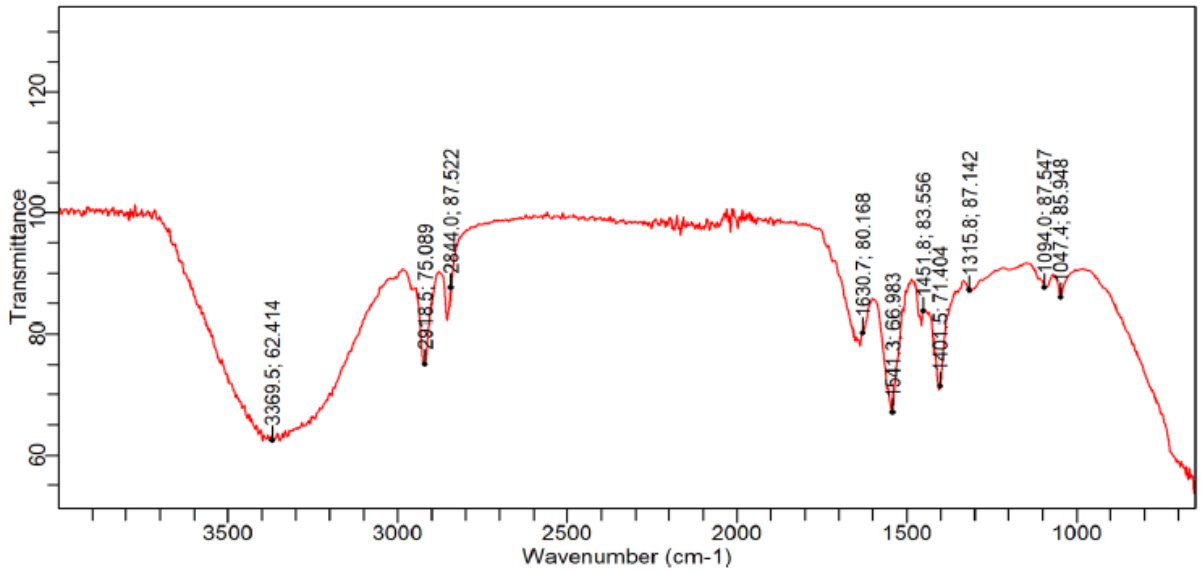

Figure 5: FTIR Spectrum of the Produced Ethanol

The most prominent feature in the IR spectrum of alcohol is the broad absorption band centered on 3400-3230 cm^{-1}, which is attributed to O-H stretching vibrations. This can be observed between wavenumbers from 3500 to 3000 cm^{-1} at 3369.52 cm^{-1} in the resulting ethanol spectrum. The absorption peaks between 2700 and 3000 cm^{-1} identified at 2918.5 and 2844.0 cm^{-1} may be attributed to the C-H- stretching of alkanes.These absorption peaks are similar to those of bioethanol produced from plantain leaves (Ogunsuyi and Olawale, (2021). Peaks observed at regions of 1630.7 cm^{-1}, 1641.66 cm^{-1}, 1451.8 cm^{-1}, 1401.71 cm^{-1} and 1315.8 cm^{-1}represents the C-O stretching vibration of alcohol.

Physicochemical Properties of the Bioethanol Produced

The results of some physicochemical properties of the produced bioethanol were presented in Table 5.

Table 5: Physicochemical Properties of the Bioethanol Produced

Properties	Units	Values obtained	ASTM Standard
Concentration	%	26.5±0.20	
Moisture content	%	0.22± 0.10	1max
Density	g/cm^3	0.9933±0.00	0.99
Ash content	%	0.10±0.0	30
Viscosity	cSt	4.46±0.29	1.20
Flash point	^oC	24.90	18.60
Octane number	-	109.1	110
Sulphur content	%	0.033	-
Cloud point	^oC	2.90	23.00

Reducing Sugar of the seed cake: 62.32±1.82%

The reducing sugar content of the seed cake was found to be 62.32±1.82%. The value obtained is lower than 68.00 % for Jatropa seed cake (Mohit *et al.*, 2011). Literatures have revealed that bioethanol yield of a feedstock is a function of its reducing sugar concentration (Ye and Jiang, 2004). The reducing sugar content obtained for the cake indicates it good tendency for bioethanol production. The water content of the produced bioethanol is 0.22 %. The water content in fuel is one of the factors in determining its fuel quality. The lesser the water content, the better the ethanol. The water content more than 1% in bioethanol can cause damage during the burning process (Muhaji and Sutjahjo, (2018). The low water content of the ethanol implies that it will not

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cause filter plugging and injectors wear when used directly as fuel. Density is the mass to volume ratio of a fuel at 150 ^{o}C. It greatly affects the ignition quality of the fuel. The density of the produced bioethanol was determined to be 0.993gcm^{-3}.The value exceeds the 0.789 gcm^{-3} specified for bioethanol. The high value recorded may be due to the presence of trace amounts of water in the ethanol. The viscosity of bioethanol was recorded as 4.46 cSt, which was found to exceed 1.2 cSt specified for bioethanol. Viscosity measures the resistance of a fluid to flow. It decreases with increasing temperature. It is an essential property of fuel storage and use.If the fuel has a high viscosity, it makes it difficult for the engine to pump, ignite the burner, and flow, however, high viscosity in the fuel can worsen atomization and cause carbon deposits (Muhaji and Sutjahjo, (2018).Flash point is the temperature at which a fuel will ignite when exposed to an ignition source. The resulting bioethanol has a flash point of 24.90^{o}C. This value is higher than the 12^{o}Cspecified for standard bioethanol, but lower than the 38^{o}C specified for standard conventional ethanol.This suggests that the ethanol produced will be less flammable than conventional ethanol. Ash content gives inorganic minerals present in a fuel. The content of the ash was found to be 0.1 %. High ash content in a fuel leads to after burn residues and wear of the engine's injection system. Octane number is a measure of a fuel's ability to prevent knocking and detonation during combustion (Sharmiladevi *et al.,* 2019).The octane number obtained does not exceed the specified value for bioethanol.The mutagenic potential is attributed to sulfur dioxide and particulate matter emitted by vehicles using high-sulfur fuels. For these reasons, international restrictions are now severely tightened (Suleiman *et al.,* 2016). Biofuels have been known to have less toxic emissions, one of their good advantages over fossil fuel. The sulphur content determined for the ethanol was 0.033. Cloud point is the temperature at which crystals appear in a liquid cooled under specified test conditions. It provides information on the low temperature availability of fuels in extremely cold conditions (Suleiman *et al.,* 2016). The cloud point recorded for the bioethanol is 2.90°C, which is lower than the 23 specified by ASTM (Graeme and Walker 2010). The low cloud point for the ethanol signifies it's used in cold temperate environment with no crystals formation.

4. CONCLUSION

The present study demonstrates that the seeds cake of *Rothmannia longiflora* could be used as raw materials for bioethanol production. The production of the ethanol was confirmed by the FT-IR result. Our findings reveal that pH and temperature exert significant effects on the bioethanol production yield. The fuel properties analyzed reveals that moisture content, ash, density, octane number and cloud point compared favorably with ASTM standard.

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