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Bioethanol Production from *Citrus limon* (Citrus) Peel Substrate Using *Aspergillus Niger* and *Saccharomyces Cerevisae*

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Abstract

The consumption of petroleum products is marred by the inconveniences of environmental pollution and the emission of greenhouse gases, which are responsible for global warming. Excessive use of fossil fuel has resulted in climate change, the elevation of the greenhouse gas effect, etc., contributing to the search for renewable sources of energy in harmony with the world's energy needs. Although bioconversion of lignocellulose residue has received much attention, most plant biomass has not been fully exploited to meet human energy needs. Among these alternative energy carriers, ethanol receives great attention. This study aims to determine the optimal conditions for the production of bioethanol from lemon peel using Aspergillus Niger and Saccharomyces cerevisae. Aspergillus niger and Saccharomyces cerevisiae were isolated using the streak plate method and identified macroscopically and microscopically. The cellulose hemicellulose and lignin contents of the substrates were determined, and after that, the substrates were pretreated with 5% sulfuric acid and then subjected to enzymatic hydrolysis using Aspergillus niger. Subsequently, the reducing sugar content of the hydrolysate was determined, followed by fermentation using Saccharomyces cerevisae. The percentage of bioethanol produced, as well as the fermentation efficiency (%), were calculated. Analysis of Variance was employed to determine statistically significant differences among the determined parameters. The cellulose, hemicellulose, and lignin contents of Citrus Limon were found to be 32.00%, 4.99%, and 9.80%. Pretreatment increased the cellulose content from 23.00% to 32.00%. The highest reducing sugar content, 6.63 g/L,, was recorded after hydrolysis for 96 hours. Fermentation using Saccharomyces cerevisae yielded 19.20% of bioethanol from Citrus limon with a fermentation efficiency of 38.42%, respectively. The Optimum fermentation conditions recorded were a time of 96hrs, pH of 3.5, temperature of 30°C and amount of substrate of 15g. The combination of these parameters produced a bioethanol yield of (22.90%) and a fermentation efficiency of 44.44%, respectively. This research revealed the potential of Citrus limon peel waste as a substrate for bioethanol production and optimized the physicochemical parameters for the fermentation.

Keywords; Aspergillus niger, Bioethanol, Citrus limon, Optimization, and Saccharomyces cerevisae.

INTRODUCTION

Access to clean, modern energy services is an enormous challenge facing the African continent because energy is fundamental for socioeconomic development and poverty eradication Broda *et al.* (2022). Today, 60% to 70% of the Nigerian population does not have access to electricity. There is no doubt that the present power crisis afflicting Nigeria will persist unless the government diversifies the energy sources in domestic, commercial, and industrial sectors (Oyedepo,2020). Agricultural wastes such as lemon, pineapple, and other fruit wastes are generated annually in Nigeria when processed into various food products, e.g., Five Alive. These wastes end up in open dumps or drainage systems, threatening both surface and groundwater (Oyedepo,2020). It is, therefore, necessary to convert these wastes into useful end products. Bioethanol is a colourless liquid produced by fermentation. It is an alternative energy source derived from various sources, such as crop biomass, which is converted into fuel for use in engines Broda *et al.*,(2022).

Fruit waste has become a nuisance in the environment, which has resulted in problems of waste management. Excessive use of fossil fuels

has resulted in global warming and climate change. Therefore, plenty of bioethanol would be required to be produced if bioethanol has to replace fossil fuel with a cleaner and renewable fuel and will address the problem of waste management. (Kaur,2017). This research was focused on the production of bioethanol using lignocellulose biomass as substrate, which can make a major contribution to the economic and environmental development of Nigeria.

Biofuels are expected to reduce dependence on imported petroleum and its associated consequences, including political and economic vulnerability, reduce greenhouse gas emissions and other pollutants, and revitalize the economy by increasing the demand and prices of agricultural products (Balat, 2018).

Lemon, *citrus Limon,* also called *lemon tsami* in Hausa, is a small evergreen tree in the family Rutaceae. Lemon juice contains 5% to 6% citric acid with a pH of around 2.2. Its distinct sour taste makes it a key ingredient in drinks and food such as lemonade. In the 19th century, lemons were increasingly planted in Florida and California (Julia, 2015).

With an increase in the consumption of fruits and industrial production of fruit juices and other value-added products from fruits such as *Citrus limon* (lemon), sweet orange (*Citrus paradisis*), and other fruits, a great problem of disposal of waste is faced by the fruit processing industries (Kaur, 2017). These wastes have adverse effects on the ecological condition of the environment, and companies spend to dispose of these wastes (Gashaw, 2015).

Lemon, *citrus limon* peel is a potential feedstock for bioethanol production due to its high carbohydrate content, which is similar to that of other studied feedstock (Boluda-Aguilar, 2015). Lemon fruit waste can Be used to generate bioethanol, which will probably address the problem of fuel crisis. The study aimed to produce bioethanol from lemon using *A. niger* and *S. cerevisae*.

MATERIALS AND METHODS

Sample Collection and Processing

Lemons were purchased from the Yan Lemo market at Yankaba, Kano State, Nigeria, stored in a polythene bag, and then transported to the Herbarium Department of Plant Biology Bayero University Kano for identification by a plant taxonomist. The samples were given voucher number 338 and thereafter transported to the laboratory for further analysis.

Cellulose hemicellulose and lignin content of the lemon substrate

Cellulose hemicellulose and lignin content of the lemon peels were determined using the method of AOAC (2012).

Mechanical Pretreatment of the lemon substrate

The substrate (lemon) was washed thoroughly to avoid excess sand and was allowed to air dry at room temperature before milling using a ball mill. Then, it was sieved using a sieve to reduce the particle size and increase the surface area and volume ratio (Jaisamut *et al.*, 2014).

Isolation of *Aspergillus Niger* and *Saccharomyces cerevisae*

A. niger was from soil isolated by adding 10g of soil to 90mL distilled water, which was shaken vigorously. An aliquot was then spread on the plate on PDA potato dextrose agar media using the streak plate method. The plates were stored in an incubator for 5 days at room temperature. *A. niger* colonies were sub-cultured in slants and stored in a refrigerator at 4°C Zakpa *et al.*, (2009).

On the other hand, *S. cerevisiae* was isolated from rotten papaya fruit, and the fruit was homogenized in approximately 10g of the fruit in 90 mL distilled water. Thereafter, it was shaken and then streaked onto PDA plates, which were kept at 37°C for 5 days. Subsequently, the cultural characteristics of the microbial growth on the plates were observed, as described by Zakpa *et al.* (2009).

Standardization of Inoculum

Aspergillus niger culture was inoculated into potato dextrose broth (PDA) medium. After 5 days, spores were dispersed in the desired quantity in sterile distilled water containing 0.1% tween 80. Spores were counted using a haemocytometer Ajeet *et al.*,(2014). *Aspergillus niger*

Twenty milliliters (20mL of 0.1% tween 80 were added to the cultures, it was homogenized, and the fungal spore was counted such that10^6 spores mL were contained in 1mLof the suspension Ajeet *et al.*,(2014).

Acid Pretreatment of the lemon Substrate

Acid pretreatment was applied to further

degrade the polysaccharide present in the biomass. Five grams (5g) of the substrates were placed in a conical flask containing 100mL of 5% sulfuric acid and was autoclaved at 121°C for 30 minutes. The autoclaved sample was filtered through man filter paper, and the residue was washed with distilled water and oven-dried at 60°C (Asma *et al.*, 2019).

Enzymatic Hydrolysis of the lemon Substrate

Enzyme hydrolysis was carried out according to the method described by Gupta *et al.* (2014) in a 500 mL conical flask using 5g, 10g, and 15g of lemon peel as the substrate to which 100mL of distilled water was added the flask was plugged with cotton wool and aluminum foil and then sterilized at 121°C for 30minutes each flask was inoculated with 0.5mL suspension of theinocular (*A. niger*) and an uninoculated flask was used as a negative control. The flask was incubated at 37°C for 5 days with frequent shaking and agitation to provide agitation and aeration. The hydrolyzed samples were afterward filtered through what man NO 1 filter paper, and the filtrate was analyzed for reducing sugar content daily for 5 days.

The reducing sugar content of the sample (hydrolysate) was determined using the dinitrosalicyclic method described by (Rabah *et al.*, 2011) using a glucose standard curve as a reference.

Bioethanol production

The hydrolyzed sample was fermented to ethanol using *Saccharomyces cerevisae* by adding 100mL of the hydrolysate into a 300 mL capacity conical flask. The flask was covered with cotton wool and aluminum foil and autoclaved at 121°C for 15 minutes before cooling at room temperature from the prepared 0.5 McFarland standard of S. cerevisiae (1.5 x $10^{8)}$ cells /mL, 2mL was added to the flask which was after that incubated at 30°C for 5days. After 5 days of fermentation, the bioethanol produced was distilled (Wong and Sanggari, 2014) through a fractional distillation setup in the laboratory, as described by (Oyeleke and Jibrin, 2017). The fractioning column has a large surface area so that the vapor passing up the column meets and mixes through with liquid falling. The fermented broth was dispensed into a round button flask fixed to a distillation column enclosed in a running tap watch. A conical flask was fixed to the other end of the distillation column to collect the distillate.

Estimation of Bioethanol Production

The percentage bioethanol yield was calculated based on the concentration of ethanol produced (g) from the concentration of the amount of fermentable sugar (g) x 100 (Hamed *et al.*, 2015). The fermentation efficiency was calculated using the formula described by (Sharma *et al.*, 2004).

$$\text{Fermentation efficiency} = \frac{actual\ yield}{theoritical\ yield}\ x\ 100$$

Statistical Analysis

The data generated were subjected to statistical analysis using analysis of variance (ANOVA) at $p < 0.05$ significance.

RESULTS

Isolation and Characterization of Isolates

The *Aspergillus Niger* isolates were identified using morphological and cultural characteristics. These characteristics include possession of dark brown spores, conidiophores, and conidia, which were found to be brown. Similarly, the yeast isolated was identified as *S. cerevisae* using morphological and cultural characteristics, which include the possession of a creamy, moist texture with a watery consistency.

Cellulose Hemicellulose and Lignin Content of the Lemon Peel

The cellulose hemicellulose and lignin content of the treated and untreated substrates were 32.0% vs. 23%, 5.43% vs4.99%, 19% vs. 9.80% respectively. (Table 1)

Table 1: The cellulose hemicellulose and lignin content of the treated and untreated substrates

Composition	Pretreated citrus (lemon) peel (%)	Untreated citrus (Lemon) peel (%)
Cellulose	32.00	23.00
Hemicellulose	4.99	5.43
Lignin	9.80	19.0

Reducing Sugar Concentration after Hydrolysis of Lemon Peel with A. Niger

The reducing sugar concentration of the hydrolysate of the lemon peel sample was presented in Table 2 the highest concentration of reducing sugar was achieved at a substrate concentration of 15g. Afterward, the reduced sugar concentration or production increased gradually with an increase in substrate concentration. A maximum concentration of 6.63g/L reducing sugar was obtained from the lemon peel. A minimum of 5.94g/L was obtained from the lemon peel sample at a substrate concentration of 5g after 5 days of hydrolysis. The highest production occurred at 96 hours at 15g each (Table 3).

Table 2: Reducing Sugar Concentration (g/L) after Hydrolysis of *Citrus lemon* Peel with *Aspergillus Niger* after 5 days

Amount of Substrates (g)	Reducing Sugar content(g/L
5	5.94
10	6.58
15	6.63
Control	0.22

Table 3: Effect of varying amounts of substrate (g) and Time (hr.) on Percentage (%) Ethanol Produced from Hydroysate of *Citrus* lemon Peel a Temperature of 35°C and pH 4.5

Time (hrs.) substrate Concentration(g)	0	24 Ethanol Yield(%+_SE)_	48	72	96	120
5	0.00	8.98+0.12^{a} (17.02)	11.92+0.17^{b} (23.00)	14.70+0.28^{b} (29.32)	16.52+0.17^{b} (33.52)	11.83+0.10^{b} (25.60)
10	0.00	9.30+0.17^{a} (18.86)	14.72+0.14^{b} (25.35)	16.20+0.11^{b} (33.62)	18.32+0.18^{b} (35.52)	14.20+0.12^{b} (27.77)
15	0.00	9.96+0.16^{a} (19.66)	14.62+0.12^{b} (28.55)	16.72+0.39^{b} (37.53)	19.20+0.11^{a} (38.42)	15.20+0.10^{b} (30.02)
Control	0.00	0.00	0.00	0.00	0.00	0.00

Key:
Values in the bracket represent fermentation efficiency
SE; Values are Mean + standard error of three determinations
Superscripts with the same alphabet indicate no significant difference at $P < 0.05$ significance

Ethanol Yield from Fermentation of Products of Hydrolyzed Substrates

The 15g substrate setup gave the highest ethanol yield (19.2%) with a fermentation efficiency of 38.42% as compared to an ethanol yield of 18.32% and a fermentation efficiency of 35.52%in the 10g setup. Finally, the 5g setup produced an ethanol yield of 16.52% with a fermentation efficiency of 33.52%.

The ethanol yield from fermentation of hydrolyzed peel is presented in Table 3 as a percentage. As the concentration of substrate increases, the amount of ethanol yield also increases, which implies that ethanol content is directly proportional to the concentration of the substrate. The percentage yield of ethanol from lemon peels was obtained at the fermentation. Conditions of time of 96 hours, pH, and a temperature of 35°C.

DISCUSSION

Aspergillus niger is a haploid filamentous fungi used in industrial fermentation for production in several industrial fields, including the food industry. It is used to produce ethanol, extracellular (food) enzymes, citric acid, and waste treatment (Madika *et al.*, 2020). However, *A. niger* is used for commercial amylase production, producing mycotoxin, and it is found in mesophilic environments such as soil and plant Madika *et al.*, (2020). *S. cerevisae* is the most popular microorganism used for ethanol production due to its high ethanol tolerance. It is also an important microorganism used by industries for the production of many food products. The yeast was isolated and identified as *Saccharomyces cerevisae* from rotten papaya using morphological and cultural characteristics. Its characteristics include a creamy white texture, an ovoid shape, and the ability to ferment glucose and sucrose. This work is in line with the work of Zakpa *et al.*

(2009), who reported the isolation of S. *cerevisae* from the rotten papaya fruit.

The pretreatment of the substrates using dilute sulfuric acid reduced the lignin contents and crystallinity of cellulose while increasing surface area, making the cellulose more accessible and available for enzyme action, as stated by Khokhar *et al.* (2020).

The reducing sugar concentration of the substrate was found to increase gradually with an increase in time and concentration of the substrate. *A niger* was among the three fungi that were found to elaborate higher exoglucanase and end endoglucanase activities than those of fungal strains of the genius *Trichoderma* Madika *et al.,(* 2020).

The fermentation of hydrolyzed substrate to ethanol and carbon dioxide showed maximum ethanolof 18.9% and 19.20% in 15 g of substrate.

Hydrolysates of the substrate with high sugar yielded the highest concentration of ethanol. This indicates that the yield of ethanol is directly proportional to the concentration of sugar in the fermented broth. During the fermentation, the ethanol yield was found to gradually increase before decreasing. This could be due to the utilization of sugars as the carbon source for the growth of microorganisms *(S. cerevisae)* and ethanol production. This result corresponds to the work of Ado *et al.* (2019), who reported a decrease in ethanol after 96 hours of inoculation. After 96 hours, the maximum ethanol (19.20%) was obtained. This corresponds to the work of Rouchas *et al.* (2017), who reported a maximum ethanol concentration of 19.14% from the totapuri mango Kernel. This is also in line with the work of Zakpa *et al.* (2009), who reported maximum ethanol from corn cobs after 6days of fermentation.

CONCLUSION

Findings from the study showed that lemon peel can be a good substrate for bioethanol production. Moreover, *A. niger and S. cerevisiae* have been shown to have high fermentation efficiency and produce bioethanol using the substrate. The reducing sugar content of 6.63 g/L with a bioethanol production of 22.09%, and a fermentation efficiency of 44.44%. The optimum conditions for fermentation were found to be 30 °C, a pH of 3.5, 15g of substrate, and an incubation time of 96 hours.

RECOMMENDATIONS

The study recommends the following:

1. Use of different microorganisms to ferment the same substrates using various fermentation methods to produce higher yields.
2. Other substrate (starch and sugar) should be fermented with the same microorganisms, and the yield should be compared.
3. Other lignocellulose biomass should be exploited for cleaner, renewable energy.

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