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Microbial production of 2,3-butanediol from rice husk using anaerobic *Clostridium* species

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ABSTRACT

Interest in the area of biomass based-product production is increasing all over the world due to the environmental challenges posed by fossil fuel and fear of its extinction. Production of biofuel and other compounds especially from agricultural waste can reduce these environmental problems because of its sustainability and environmentally friendliness. One of the major petrochemical product widely used in many industries is 2,3-butanediol and was found to be produced from agricultural wastes by microorganisms. Therefore, Microbial production of 2,3-butanediol from rice husk using *Clostridium* species was investigated in this research. Structural composition of the rice husk was determined before and after pretreatment. Hemicellulose and lignin content of rice husk was determined after extraction while cellulose was determined as the difference from the extractives, hemicelluloses and lignin. Dilute (2%) NaOH was used for the pretreatment of rice husk. Hydrolysis was carried out using *Aspergillus niger* and reducing sugar released was determined using standard method with UV-VIS spectrophotometer. *Clostridium* species was isolated from sugarcane bagasse, identified using basic morphological and molecular biology techniques. The fermentation of rice husk was performed using the *Clostridium* species. Fermentation by-product was determined using Gas Chromatography Mass-spectrometry. Cellulose content increased from 32% before pretreatment to 53.3% after pretreatment, lignin increased from 8.4% before pretreatment to 30.7% after pretreatment and hemicellulose decreased from 30% before pretreatment to 8% after pretreatment. A total of 1.05 g/l of reducing sugar was released after enzymatic hydrolysis of the rice husk with *Aspergillus niger*. Alcohol 2,3-butanediol (0.6%) and Furfuryl alcohol (0.45%) were detected in the by-product of fermentation. Other compounds detected are fatty acids that ranges from C₁₆ to C₂₅ with 9,12-Octadecanoic acid as the major fatty acid. From the results of this work, Rice husk was found to have substantial amount of sugar (cellulose and hemicellulose) that can be converted to valuable product including 2,3-butanediol.

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Keywords: Biofuel, Bio-refinery, Cellulose, *Clostridium*, fermentation.

INTRODUCTION

Development of bio-refinery system that integrates biomass conversion process and equipment to produce fuels and chemicals is a worldwide phenomenon due to the experiencing scarcity of crude oil reserves (Ragauskas et al., 2006). In view of this, many chemicals that are produced only from chemical processes can now be generated using biological means from renewable resources; in which microbial production of 2,3-butanediol is the typical example (Hatti-Kaul et al., 2007). There is remarkable interest in this bioprocess, because 2,3-butanediol has diverse industrial applications, and microbial production will relatively reduce the current dependence on oil supply for the production of valuable chemicals (Celinska and Grajek, 2009; Wu et al., 2008).

Some potential applications of 2,3-butanediol include manufacture of printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods, and pharmaceuticals (Koutinas et al., 2014; Ji et al., 2011; Syu, 2001). It can also be used as a precursor in the production of other chemical products, such as solvents methyl ethyl ketone (MEK), gamma-butyrolactone (GBL), and 1,3-butadiene (Celinska and Grayek, 2009). Methyl ethyl ketone is considered to be an effective liquid-fuel additive and 1,3-butadiene plays important role in the production of synthetic rubber (Kopke et al., 2011).

The production of 2,3-butanediol is known to be from various sugar (or citrate)-fermenting microorganisms, including *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Lactococcus lactis*, *Paenibacillus polymyxa*, and *Serratia marcescens* (Perego et al., 2003; Xiu and Zeng, 2008; Celinska and Grayek, 2009; Cheng et al., 2010; Jurchescu et al., 2013; Wang et al., 2014). Considering the health concern, these organisms in the group of *Enterobacter* and *Klebsiella* species (except for *K. oxytoca*) are categorized as Risk Group 2 (RG2) because of their pathogenicity by the World Health Organization (Celinska and Grajek, 2009).

While *Bacillus licheniformis* which is potentially the most promising for industrial production of 2,3-BDO belongs to the class of risk 1 (Perego et al., 2003; Jurchescu et al., 2013). To overcome the challenges of using pathogenic organism, bacteria will be isolated from sugarcane bagasse and used for 2,3-butanediol production in this research using rice husk. The objective of this work was to investigate the possibility of producing 2,3-butanediol from rice husk.

MATERIALS AND METHODS

Collection of samples

Rice husk was collected in a new clean polythene bag from Kalambaina rice mills, Wamakko Local Government Area of Sokoto State. Sugarcane bagasse was collected in a sterile specimen bottle from vegetable market, Sokoto. All the samples were taken to laboratory of Usmanu Danfodiyo University Sokoto.

Composition analysis of the rice husk

Extractives

Approximately 2.5 g of the rice husk was loaded into a cellulose thimble. With the Soxhlet extractor set up, 150 ml of acetone was used as solvent for extraction. Temperature was carefully adjusted to 70 °C on the heating mantle for a 4 h run period. After extraction, the sample was air dried at room temperature. Constant weight of the extracted rice husk was achieved in a convection oven at 105 °C. The % (w/w) of the extractives content was calculated using Eq. 1 (Li et al., 2004; and Lin et al., 2010).

$$W1 \text{ (wt.\%)} = \frac{Go - G1}{Go} \times 100 \quad \text{Eq.1}$$

Where: *W1* = Extractives,

Go = Dry weight of the sample,

G1 = Constant weight of the residues after extraction

Hemicellulose

One gram (1 g) of extracted dried biomass (*G1*) was transferred into a 250 mL Erlenmeyer flask. 150 ml of 0.5 mol/dm³ NaOH was added. The mixture was boiled for 3.5 h with distilled water. It was filtered after cooling and washed until neutral pH. The

residue was dried to a constant weight at 105 °C in a convection oven (G2). Hemicellulose content (%w/w) of rice husk was calculated given Eq. 2 (Li et al., 2004; and Lin et al., 2010; Ayeni et al., 2013).

$$W2 \text{ (wt. \%)} = G1 - G2/Go \times 100$$

Eq. 2

Lignin

From the extracted rice husk, 0.3 g (G3) was weighed in glass test tubes and 3 mL of 72% H₂SO₄ was added. The sample was kept at room temperature for 2 h with careful shaking at 30 min intervals to allow for complete hydrolysis. After the initial hydrolysis, 84 mL of distilled water was added. The second step of hydrolysis was made to occur in an autoclave for 1 hour at 121 °C. The slurry was then allowed to cooled to room temperature. Hydrolysate was filtered. Constant weight of the residues was obtained using conventional oven (G4). Lignin content wt. % was determined using Eq. 3 bellow (Li et al., 2004; Sluiter et al., 2008).

$$W3 \text{ (wt. \%)} = G4(1-W1)/G3 \times 100$$

Eq. 3

Cellulose

The cellulose content (wt.%) was calculated by difference, assuming that extractives, hemicellulose, lignin and cellulose are the only components of the entire biomass (Li et al., 2004; Lin et al., 2010).

$$W4 \text{ (wt. \%)} = 100 - (W1 + W2 + W3)$$

Eq. 4

Dilute alkaline pretreatment

In a 1 Litre capacity conical flask, 50 g of rice husk was mixed with 500 ml of 2% NaOH and shake until it homogenized. The solution was covered with cotton wool and aluminum foil and then allowed to suck for 24hrs. The mixture after 24 hrs was autoclaved at 121 °C for 15 mins and then allowed to cooled and filtered using Whatman filter paper No1. The residue was washed with distilled water until neutral pH of 7.0 was obtained and dried (Nikzad et al., 2013).

Hydrolysis of rice husk

Stock culture of *Aspergillus niger* was collected from Mycology laboratory of

Usmanu Danfodiyo University Sokoto. It was sub-cultured in a newly prepared SDA and incubated at room temperature for 5 days. Ten grams of the pretreated rice husk was weighed in 250 ml flask and 100 ml of distilled water was added. The flask was covered with cotton wool and aluminium foil and then sterilized at 121 °C for 15 minutes. The flask was then inoculated with 0.5 ml (1.3×10⁷cfu/ml) suspension of the *Aspergillus niger*. The flask was incubated at 37 °C for 5 days on an orbital shaker, and then the sample was filtered through Whatman filter paper No1. The filtrate was then used for determination of reducing sugar and fermentation (Gupta et al., 2009; Negi and Banerjee, 2006).

Determination of reducing sugar

The reducing sugar content following hydrolysis of the rice husk was determined using the dinitrosalicylic acid (DNS) colorimetric method according to Miller (1959) with glucose as standard. It was assayed by adding 2 ml of 3, 5-DNS reagents to 1ml of the sample. The mixture was heated in bath for 10 min to develop a red-brown colour. Then 1ml of 40% potassium sodium tartrate solutions was added to stabilize the colour, it was then cooled to room temperature. The absorbance of the sample was measured at 540 nm using ultraviolet (UV-VIS) spectrophotometer. The reducing sugar content was determined by making reference to a standard curve of known glucose (Rabah et al., 2011).

$$\% \text{Reducing sugar (mg/dl)} = \text{Abs. of sample/Abs. of Std} \times \text{Conc. of Std} \quad \text{Eq. 5}$$

Keys: Abs. = Absorbance, Std. = Standard, Conc. = Concentration

Fermentation of the hydrolysate

Isolation and identification of *Clostridium* species

A gram of the sugarcane bagasse was transferred in 10 ml of distilled water and heat shocked in water bath for 10 min at 70 °C. An aliquot was transferred in to already prepared plate of Nutrient Agar and incubated in an anaerobic incubator at 37 °C. The isolates after 24hrs were sub cultured based on physical characteristics and incubated. After 24 hrs of

anaerobic incubation, Gram's staining was carried out to find the morphological characteristics of the isolates. Spore staining technique was employed to determine the organisms' ability to produce spores. Gram positive rod that produced spores was subjected to further biochemical tests for characterization. The tests include: glucose, lactose, sucrose, catalase, indole, H₂S motility and urease tests (Owuna et al., 2018; Nata'ala, 2018). PCR and agarose gel electrophoresis were also carried out.

Fermentation

Fermentation was carried out with *Clostridium* species according to procedure followed by Owuna et al. (2018) with some modification. The hydrolysate obtained from hydrolysis was used as the fermentation media. Ten milligrams of the hydrolysates were transferred in to 100 ml capacity conical flasks, covered with cotton wool and aluminium foil and autoclaved at 121°C for 15 min. and then cooled to room temperature. The pH of the fermentation medium was adjusted to 6.5 and then 1ml of prepared suspension of the organism prepared (0.5% McFerland Standard) was added in the hydrolysate. The fermentation was allowed to occur in an anaerobic incubator for 3 days with frequent shaking. The fermentation by-product was taken for Gas Chromatography Mass-Spectrophotometer analysis.

Determination of fermentation products

After the fermentation, the by- products were subjected to analysis using GC-MS to detect and identify the products.

RESULTS

Structural compositions of the rice husk

Structural compositions of rice husk were investigated to determine the extractives, hemicellulose, lignin and cellulose. The results (Table 1) shows increase in cellulose and lignin content of the rice husk 32% to 53.3% and 8.4 to 30.7 after pretreatment respectively. While extractives and hemicellulose reduces after

pretreatment from 29.6% to 8% and 30% to 8% respectively.

Reducing sugar released after hydrolysis of rice husk using *Aspergillus niger*

The results of reducing sugar show increase the amount of sugar daily with the highest production at day 4. Table 2 shows the results of mean reducing sugar released from hydrolysates with a total of 1.05 g/l of sugar. There was significant difference in the daily release of sugar as $P = 0.01$ ($P < 0.05$).

Isolation and Identification *Clostridium* species used for fermentation of rice husk

Basic Microbiological techniques were employed for identification of *Clostridium*. The macroscopic observations revealed that the colony is white, large, raised and smooth edged. Microscopically, the isolate was found to be purple rod shaped, hence, is Gram positive. The isolate was further subjected to biochemical test for identification. Some of the tests include; spore detection, lactose, glucose, sucrose, catalase, Motility, indole, gas formation, urease, citrate and H₂S. Table 3 shows the results of characterization and identification of *Clostridium*.

Molecular biology

Colony PCR was carried out after series of repeated subculture and obtained a pure culture. The PCR product amplified was run on 1% agarose gel electrophoresis to visualize the products. Figure 1 shows a product of 1000bp size.

Analysis of fermentation products using gas chromatography-mass spectroscopy (GC-MS)

Characterization of compounds present after fermentation was carried out using Gas Chromatography-Mass Spectroscopy (GC-MS). Figure 2 shows various spectra of about 15 compounds which was identified and presented in Table 4. Alcohol 2,3-butanediol (0.6%), Furfuryl alcohol (0.45%) were found in the fermentation by-product.

Table 1: Mean structural compositions of rice husk.

Composition	Before pretreatment (%)	After pretreatment (%)
Extractives	29.6 ± 0.05	8.0 ± 0.06
Hemicellulose	30.0 ± 0.1	8.0 ± 0.03
Lignin	8.4 ± 0.11	30.7 ± 0.35
Cellulose	32.0 ± 0.06	53.3 ± 0.32

Table 2: Mean Reducing Sugar released from hydrolysates.

Days	NaOH (g/l)
1	0.256±0.004
2	0.2891±0.001
3	0.2516±0.003
4	0.2546±0.002

Table 3: Biochemical Identification of *Clostridium* species.

Biochemical Test	1	2	3	4	5	6	7	8	9	10	11	Isolate
Observation	Gram+ Rod	+	-	+	-	-	-	-	-	weak	-	<i>Clostridium</i> spp

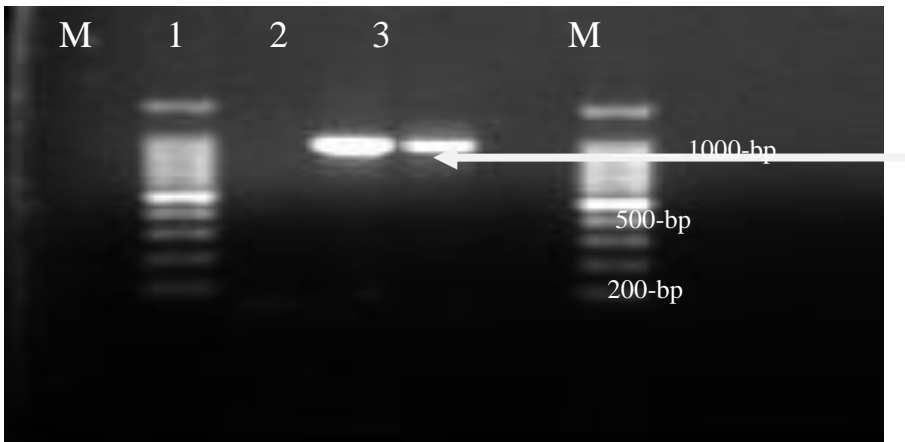


Figure 1: Agarose gel image showing the PCR product amplified from the suspected *Clostridium* isolate.

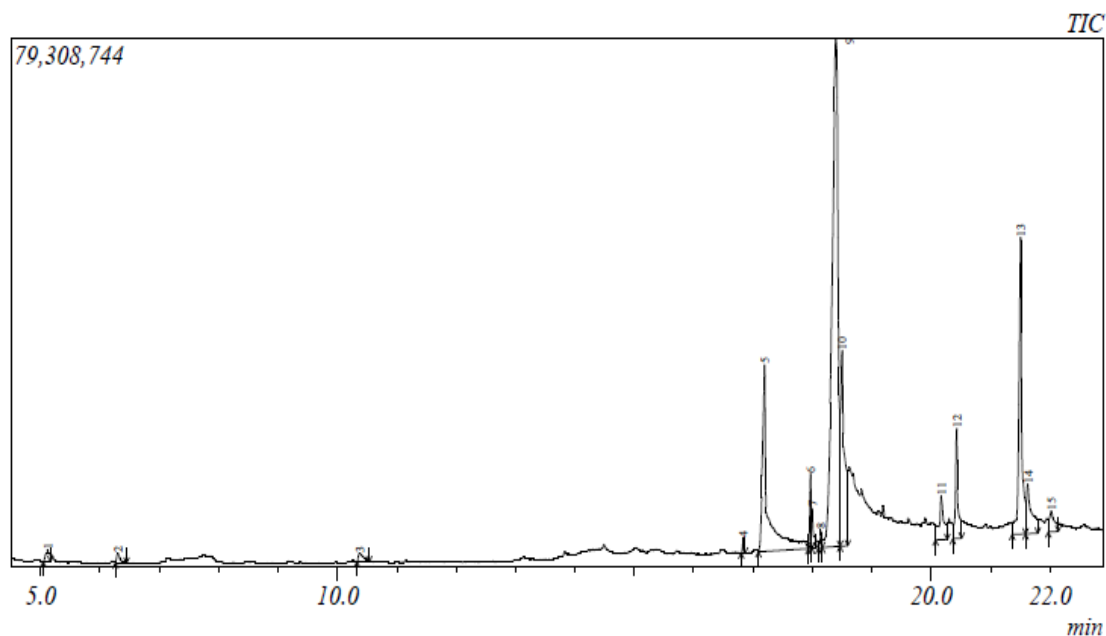


Figure 2: GC Spectrum showing various peaks of the fermentation by-product.

Table 4: Volatile organic compounds identified from the fermentation broth product using GC-MS.

R.T ⁻¹ (min)	Compound	Structural Formula	Area (%)
5.12	2,3-Butanediol	C ₄ H ₁₀ O ₂	0.63
6.30	2(3H)-Furanone,5-methyl (Furfuryl alcohol)	C ₅ H ₆ O ₂	0.45
10.37	Thiophene,2-propyl (2-Propylthiophene)	C ₇ H ₁₀ S	0.63
16.83	Cyclopentanetridecanoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	0.25
17.19	n-hexadecanoic acid (palmitic acid)	C ₁₆ H ₃₂ O ₂	14.73
17.97	9,12-Octadecadienoic acid, methyl ester	C ₁₉ H ₃₄ O ₂	1.34
18.00	9-Octadecenoic acid (Z)-, methyl ester	C ₁₉ H ₃₆ O ₂	1.04
18.14	Unknown	C ₂₅ H ₄₂ O ₂	0.49
19.18	17-Octadecynoic acid	C ₁₈ H ₃₂ O ₂	10.69
20.17	9,12-Octadecadienoic acid (Z,Z)-	C ₁₈ H ₃₂ O ₂	47.26
20.42	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₁₉ H ₃₈ O ₄	4.06
21.50	(R)-(-)-14-methyl-8-hexadecyn-1-ol	C ₁₇ H ₃₂ O	13.17
21.62	13-Tetradecenal	C ₁₄ H ₂₆ O	3.66
22.02	Z,Z-3,13-Octadecadien-1-ol	C ₁₈ H ₃₄ O	1.61

DISCUSSION

The result of the structural composition of rice husk shows a high proportion of cellulose accounting for about 32% and 53.3% before and after pretreatment, respectively (Table 1). This might be attributed to the fact that rice is herbaceous plant known to have less rigid cell wall as such is expected to have more cellulose content. Sticlen (2007) reported that lignocellulosic biomass contains about 30% to 50% cellulose, for which rice husk is characterized as one of the lignocellulosic biomass. This is similar with the studies by Noha (2015) who reported a cellulose content of 31.01%, 32.23%, 30.80% and 33.65% from rice straw collected from Egypt, Murcia, Valencia and Andalusia respectively. The study also showed similarity with finding of Anwar et al. (2017) who reported a cellulose content of rice husk of 32.67% in their study. Adamafio et al. (2009) reported decrease in lignin, cellulose and hemicelluloses in their work. Increase in the cellulose content of rice husk was observed after pretreatment. This implies that cellulose was released from the compacted structures after pretreatment. The finding implies that rice husk contained substantial sugar that can be converted to product such as alcohols or biofuels.

Hemicelluloses content of rice husk in this research work was found to be 30% and 8% before and after pretreatment, respectively. The result falls within the range (5% to 30%) of hemicellulose content reported for lignocellulosic biomass (Jie, 2004). The result of this work differs from the values of 26.47%, 25.26%, 24.79% and 26.68% previously reported for rice straw collected from Egypt, Murcia, Valencia and Andalusia respectively (Noha, 2015). However, the study shows similar finding of Williams and Nugranad (2000), who reported a hemicelluloses content of 29.3% from rice husk in their work. When compared with the hemicelluloses content of 11.96% and 17.7% reported by Saha et al. (2005) and Park et al. (2004) respectively, the reported content of this work revealed higher

value, which might be as a result of difference in geographic location, methods of harvesting or processing methods. The finding implies that rice husk is a good substrate for biofuel production and other valuable alcohols.

The lignin content found in this research work was found to lower with the value of 8.4%. The result is lower than the value (18.81%) reported by Anwar et al. (2004) who conducted their research using rice husk in Indonesia. Moreover, another study on rice husk by Noha (2015) at Egypt shows higher content of lignin compared the value found in this work. The finding implies that rice husk could be easily hydrolyzed and produces valuable compounds.

Extractives account for about 29.6% and are mainly a group of cell wall chemicals comprising fats, fatty acids, phenols and many other organic compounds. Extractives are non-structural components of plant cell walls responsible for colour, smell and durability (Rowell, 2012). The value of extractives in this work is higher compared to the value (11%) reported by Mansor et al. (2019) from Pineapple leaves. The results is also higher than the values found in sugar cane bagasse and Shea tree sawdust reported by Ayeni et al. (2015) which account for 2.14% and 1.9%, respectively. The differences in the values of extractives might be attributed to the difference in the biomass substrates.

After pretreatment, it was observed that the composition of rice husk differs. There was decrease in the amount of extractives, increase in the cellulose content, increase in lignin content and decrease in the content of hemicellulose. The result implies that, the lignin barrier in the rice husk was removed, hemicellulose was also removed and cellulose was released to be accessible by hydrolytic organism.

Reducing sugar was determined daily during hydrolysis with *Aspergillus niger* to determine the amount of reducing sugar released. The hydrolysis was carried out for a period of 5 days. The analysis of sugar was

started at day 2 to allow for acclimatization of organism in the substrate. The results showed increase in the amount of sugars released across the days with peak release at day 4. This might be attributed to the fact that NaOH have the ability to attack lignin in biomass to make cellulose readily available for hydrolytic organism. There was significant difference in the daily release of sugar as $P = 0.01$ ($P < 0.05$). Adejimo et al. (2019) reported similar results of peak release of sugar at 79th hour (day 4). The variation of sugar released across the days is similar with the work by Nata'ala (2018) who reported highest concentration in 5th day and lowest concentration in 2nd day. The result implies that *Aspergillus niger* used in the hydrolysis produced extracellular enzymes that hydrolyzed the cellulose in the rice husk.

Fermentation by-product was analyzed using Gas Chromatography Mass-spectrometry. A close examination of the Spectra from the chromatogram on mass spectrometry revealed that the compounds include alcohols, fatty acids, fatty aldehydes and thiopene with fatty acids as the major compounds. The alcohol 2,3-butanediol (0.6%), Furfuryl alcohol (0.45%) were found in the fermentation by-product. The 2,3-butanediol was formed from the microbial fermentation of sugar in the substrate. Glucose which is hexose sugar (cellulose) and Xylose which is pentose sugar (hemicelluloses) was fermented to produce 2,3-butanediol as previously reported (Wang et al., 2010). 2,3-butanediol is a precursor compound for the production of 2-butanol during anaerobic fermentation of sugar, 1,3-butadiene and methyl Ethyl Ketone (Hannes et al., 2019; Koutins et al., 2014). Moreover, the compound is a promising alcohol that itself can be use as liquid fuel. It has other uses in foods industry, cosmetics and pharmaceutical industry. Furfuryl alcohol (2(3H)-furanone, 5-methyl) is also an alcohol produced from the fermentation of rice husk. The compound was known to be a product from biocatalytic reduction of furfural which could be produced by microbial

conversion of pentosan, a pentose sugar from hemicellulose of rice husk substrate as reported from another research (Xue-Ying et al., 2019; Elisandra et al., 2019). The compound has been in use by foods industries, pharmaceutical industries as fragrance agent as well as in the production of other valuable chemicals such as ethyl furfuryl ether, tetrahydrofurfuryl alcohol (Maris et al., 2016). The results imply that rice husk can be fermented in an anaerobic condition to produce alcohols.

Fatty acids which are the major compounds identified from the fermentation products ranges from C₁₆ to C₂₅ compounds with 9,12-Octadecanoic acid as the major fatty acid found in the fermentation product. The fatty acids might have come from the fermentative organism as fatty acids are constituent of microbial cell wall. Another source of fatty acids could be the rice husk because rice was known to contain oil as previously reported (Fajriyat et al., 2017; Xin-Ping et al., 2015). Fatty acids are also known as important compounds to play role as antioxidants, antimicrobial and anti-inflammatory agents (Zubair et al., 2013). The result implies that rice husk contains essential oil that can be exploited and use in various forms such as biodiesel, foods industries, pharmaceutical industries, perfumes and aromatherapy.

Conclusion

At the end of this research, rice husk was found to have contained lignin (8.4%), cellulose (32%) and hemicellulose (30%) before pretreatment and lignin (30.7%), cellulose (53.3%) and hemicellulose (8.0%) after pretreatment. A total amount of sugar released was 1.05g/l from the rice husk. Rice husk was also found to contain fatty acids that can be exploited in the production of biodiesel. The fatty acids play vital role in bioenergy sector, foods and pharmaceutical industries. This research showed that rice husk has potential in the production of alcohols that can be used as

biofuel and may reduce the burden of fossil fuels.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

AAF and ADI brought the idea of the study. AS, AAF, AYB and ADI design the experiment. AS and AYB, AYB helped with related literatures and reviewed the write-up. AS conducted the lab experiment. AS, ADI, MHU and IM analysed the results. All the authors contributed equally in writing and reviewing the manuscript.

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