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Research Article

Comparative Study on Liquid Hot Water and Bacteriological Pre-Treatments of Watermelon and Banana Peels

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Abstract

Watermelon (*Citrullus lanatus)* and Banana (*Musa acuminata*) peels are lignocellulosic agricultural waste that has the potential to produce bioethanol as a renewable form of energy. Bioethanol is an alternative liquid fuel produced from biomass that contained large amount of sugar (e.g sugarcane grain or biomass of lignocellulosic). This study was carried out to the process of pretreating *Citrullus lanatus* (watermelon) and *Musa acuminata* (Banana) with liquid hot water and bacteria. Bacteria were isolated from soil using microbiological methods. Pretreatment potentials were identified by determining the structural composition of the sample before and after pretreatment. Occurrence of bacterial isolates from soil, *Bacillus cereus* (3), *Micrococcus* spp (1), *Bacillus subtilis* (8). *A. niger* was isolated from soil which was used for hydrolysis. *Biological* pretreatment produced more lignin (13%) and (10%) for watermelon and banana peels respectively and hemicelluloses (23%) and (21%) for watermelon and banana respectively. The pretreatment with Liquid hot water which yielded less reduction in lignin concentration (11%) and (09%) of watermelon and banana respectively. This research showed that pretreatment of the fruit peels with *Bacillus subtilis* can increase accessibility of plant component during enzymatic saccharification.

Keywords: Liquid, Water, Bacteriological, Watermelon, Banana.

INTRODUCTION

Biofuels are an alternative source of energy which have received special attention worldwide due to depletion of fossil fuels. Banana is one of major constitute the principal food resources in the world and occupy the fourth world rank of the most significant foodstuffs after rice, corn and milk (INIBAP, 2002). Most of the fruit peels/residues are dried, ground, pelletized, and sold to the feed manufacturers at a low price which is not considered a highly viable proposition (Mammal *et al.*, 2008). As per the FAO statistics, Nigeria is among the largest producer of banana in the world and accounts for nearly 30% of the total world production of banana (Emaga *et al*., 2008). Though banana peel is a fruit residue, it accounts for 30 to 40% of the total fruit weight (Emaga *et al.*, 2008) and contains carbohydrates, proteins, and fiber in significant amounts. Banana peels are readily available agricultural waste that is underutilized as potential growth medium for yeast strain, despite their rich carbohydrate content and other basic nutrients that can support yeast growth (Brooks, 2008; Essien *et al*., 2005). Since banana peels contain lignin in low quantities, it could serve as a good substrate for production of value-added products like ethanol (Essien *et al*., 2005).

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Plate 1 and 2: Watermelon waste and Banana peels

Lignocellulose biomass is characterized by its carbohydrate content, serving as a prerequisite for ethanol production. The biomass is composed of cellulose (40–50%), hemicellulose (25–35%) and lignin (15– 20%) with Cellulose been the dominant polymer among the three components. The efficient conversion of lignocellulosic biomass to fermentable sugars is the rate limiting step for efficient ethanol production (Garay *et al*., 2006). Cellulose is a structural polymer of glucose joined together by β-1, 4 glycosidic linkages. Due to this linkage, cellulose is conferred with a high resistant and compacted crystalline structure that is able to sustain some biological and chemical hydrolysis. Hemicellulose is found almost together with cellulose in plant cell wall. It consists of several heteropolymers with main chain xylan backbone (β-1, 4 linkages), glucuronoxylan, arabinoxylan, glucomannan, xyloglucagan and other sugars like mannose, xylose, galactose, rhamnose, and arabinose. It is not as resistant to hydrolysis as cellulose due to its little strength amorphous nature.

Hemicellulose can be hydrolyzed easily by acidic treatment or enzymatic activity (Garay *et al*., 2006). The type of plant greatly influences the content of hemicellulose to be found. The last component in the lignocellulosic biomass is the lignin, a complex polymer of aromatic alcohols known as monolignols that provide support to the plants (Garay *et al*., 2006). Overcoming the compactness and the strength of the lignocellulosic biomass in the production of ethanol is the overall aim of all pre-treatments and hydrolytic processes.

Therefore, this study analyzes the use of bacterial strains from soil as a source of hydrolyzing lignin and other structural composition of the peels that may hinder their utilization as a feedstock in biofuels productions.

MATERIALS AND METHODS

Study Area

The studied samples were obtained at Kasuwan daji market situated adjacent to Pepsi Area and Sokoto modern abattoir Sokoto North local government area, with coordinates $13^0$03N $5^0$14E. Sokoto state is located at 13.0533^{0}N 5.3223^{0}E

Collection of Samples

Twenty Kilograms (20kg) of watermelon and banana fruit peels were collected in a new polythene bag from Kasuwar Daji Market, Sokoto State and was transported to the Laboratories of Microbiology and Biochemistry Departments, Usmanu Danfodiyo University, Sokoto for analyses.

Sample Processing

The banana and watermelon peels were washed with distilled water to remove dirt and other contaminants. The samples were chopped into small pieces using sterile knife and allowed to air dry. It was grounded to powdered form and transported to Microbiology research laboratory, UDUS inside a new polythene bag.

Isolation and Characterization of Bacteria from Soil

One (1g) gram of the soil sample was transferred into 9ml of sterilized distilled water and diluted serially in ten folds according to the method of Oyeleke and Manga (2008). 0.1ml of the sample from the 5th dilution was inoculated and spread throughout the media surface using bent glass rod. The inoculated agar plates were incubated at 37^{0}C for 24 hours. After incubation, colonies were observed, characterized and sub cultured onto a fresh nutrient agar plate.

Grams Staining

Smear was prepared by taking a loopful of a colony and emulsified with water on a grease-free glass slide and allowed to air dry. The slide was passed through the interface between the blue flame and the yellow flame 5 times for a second.

After fixing, the slides were placed on a slide rack. The entire mounting region was flooded with crystal violet. The crystal violet was allowed to stand for 60 seconds. When the time elapsed, the slides were washed for 5 seconds with distilled water. The specimen appeared blue-violet when observed with the naked eyes. The slides were then flooded with the iodine solution; it was allowed to stand for a minute as well, then, the slides were rinsed with distilled water for 5 seconds and immediately decolorizer (ethanol) was added drop wise and allowed to stand for a minute. The slides were rinsed with distilled water for 5 seconds. The counter-stain (Safranin) was added and was allowed for 60 seconds. Again, the slides were rinsed with distilled water for 5 seconds to remove any excess dye and allow drying. The slide was mounted on a stage of microscope and viewed under x100 objective lens under oil immersion. Presences of purple cells indicated Gram-Positive and pink cells indicated Gram-Negative as a result of losing crystal violet colour (Oyeleke and Manga, 2008).

Biochemical Characterization of Bacterial Isolates

Indole Test

A test tube of sterile peptone water, enriched with 1% tryptophan was inoculated with fresh culture of isolates and incubated at 37^0C for 48hrs. About 4 drops of Kovac's reagent was added and shaken gently. A red colour was observed for positive result, and yellow colour at the surface denotes a negative result (Oyeleke and Manga, 2008).

Catalase Test

The container containing 3% hydrogen peroxide solution was shaken to expel the dissolved oxygen. One drop of the solution was placed on a clean glass slide. Presence of gas bubbles indicates a positive test while absence of gas bubbles indicates negative reaction (Cheesbrough, 2006).

Urease Production Test

The test organism was inoculated in test tube contain Christensen's modified urea agar. Incubated for 24hrs at 37^0C. Pink colour: positive urease test, no pink colour: negative urease test (Cheesbrough, 2006).

Citrate Utilization Test

In this test organism has the ability to use citrate as only source of carbon. Using a sterilizedwireloop, a part of isolate to be tested was emulsified in Koser citrate medium and incubated overnight at 37^0C. Positive result was indicated deepblue colour (Cheesbrough, 2006).

Motility Test

Little growth was inoculated in the motility test medium (Sulphide indole motility) by making a fine stab with a needle to a depth of 2cm short of the bottom of the tube. The tubes were incubated at 37°C for 24 hours. A positive test was indicated when a line of inoculation was not sharply defined and the rest of the medium appeared cloudy. If the organism was not motile, growth was restricted to the line of inoculation, which became sharply defined (Oyeleke and Manga, 2008)

Triple Sugar iron (TSI) agar

A speck of the isolate was inoculated by streaking and stabbing into the TSI agar slant and was incubated at 37^0C for 24 hours. Fermentation of any of the sugars was indicated by a change in colour, from red to yellow and crack or raised in the medium indicated gas production (Oyeleke and Manga, 2008).

Methyl Red Test (MR)

A speck of the isolate was inoculated into the MR-VP broth, and was incubated at 37^0C for 48 hours. Few drops of methyl red were then added to the culture. M.R positive test indicated red colour while no changes denote negative (Oyeleke and Manga, 2008).

Vogues proskauer Test (VP)

A speck of the isolate was inoculated into MR-VP broth and incubated at 37^0C for 48hrs. Ethanoic solution of 5% α-naphthol (1.2ml) and 0.4ml potassium hydroxide solution was added to 2ml of culture and was shaken vigorously then placed in a slopping position (for maximum exposure of the culture to air) and was examined after 30 to 60 minutes. The evolution of red colour indicated a positive test for Voges- Proskauer (Oyeleke and Manga, 2008).

Sugar Fermentation and Gas Production Test

This was performed as described by Oyeleke and Manga (2008). A little growth of each isolate was inoculated by streaking and stabbing into triple sugar iron agar medium and incubated at 37°C for 24 hours. Fermentation of any of the sugars in the medium was indicated by change in colour from red to yellow and crack or raise in location of the medium, which was indicative of a positive test (Oyeleke and Manga, 2008).

Determination of Structural Composition of Fruit peels

The structural composition of Fruit peels includes; lignin, hemicelluloses and celluloses were determined before and after pretreatment.

Determination of Klason Lignin

The percentage of acid – insoluble lignin was determined according to TAPPI procedure (T224 om-88). Firstly, 2g each of the Samples i.e (Watermelon and Banana) were added into 30 ml beaker, then, 72% sulfuric acid (15 ml) was carefully added for 2 hours. The mixture was stirred frequently. After 2 hours, the sulphuric acid was diluted with distilled water (560 ml) to obtain sulphuric acid of 3 % concentration. The solution was then boiled for 4 hours, maintaining constant volume (575 ml) by frequent addition of hot distilled water. The solution was then filtered through a medium porosity filtering crucible. The filtered residue (acid insoluble lignin) was washed free of acid with hot distilled water. The crucibles and the contents were dried at 103ºC for 3 hours and weighed (Hatfield *et al.*, 1994). The percentage of acid insoluble lignin in feedstock was calculated using the following equation:

$$\text{Lignin Klason} = \{(W2 - W3/W1)\ X\ 100\} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{eqn (7)}$$

Where: W_1 = Initial weight of sample W_3 = Weight of crucible

W_2 = Weight of crucible and acid- insoluble lignin after drying in oven

Determination of Holocellulose

The holocellulose content was determined according to DIN 2403. A flask containing a mixture of 80 ml distilled water, 1 ml acetic acid (98%), 3 g sodium chlorite, and 2 g of feedstock was heated in a water bath at 70ºC for one hour. The mixture was stirred every five minutes during this time. Acetic acid (1 ml) and sodium chlorite (3 g) were added each hour for the next 3 hours. After 4 hours, the samples were cooled and the holocellulose was filtered. The holocellulose was washed with methanol 3 times then air- dried to remove the remaining trace of methanol and then placed in a vacuum oven at 103ºC for 3 hours (Hatfield *et al.*, 1994). The percentage of holocellulose in feedstock was calculated based on dry weight using the following equation:

$$\%\ \text{Holocellulose} = [W2 - W/W1\ X\ 100] \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{eqn}(8)$$

Where: W = Dry weight of crucible W_1 = Initial weight of sample

W_2 = Sample weight plus crucible after drying in oven

Determination of α-Cellulose and Hemicellulose

α -Cellulose is the pure cellulose content of the materials which was extracted from holocellulose using alkali solution. The **α** -Cellulose content of the samples was determined as the residue insoluble in the 17.5% NaOH solutions according to TAPPI 203 om-93 method. Aqueous NaOH solution 17.5% (25 ml) was added to a flask containing a sample of holocellulose (1 g), and the mixture was stirred at 20^0C for 40 minutes, then 25 ml of distilled water was added into the mixture. After 5 minutes, the residue was filtered with a rubber filter, and to the residue, a 10% acetic acid aqueous solution (40 ml) was added, filtered and washed with 1 L of boiling water. The residue (**α** -Cellulose) was filtered, dried at 103^OC for 3 hours in a vacuum oven and weighed (Hatfield *et al.*, 1994).

The percentage of **α** -Cellulose in the substrate was calculated using the following equation:

$$\%\ \alpha - \text{Cellulose} = \{(W2/W1\ x\ \%\ \text{holocellulose}/100\%)\ x\ 100\} \ldots\ldots\ldots\ldots\ldots \text{eqn (9)}$$

Where: W1 = Initial weight of holocellulose W2 = Weight of residue (**α** -Cellulose)

The percentage hemicellulose was calculated by subtracting the percentage a-Cellulose from the percentage holocellulose determined (Hatfield *et al.*, 1994).

Pretreatment of the watermelon and banana peels

Two different methods of pretreatment were used in this study namely:

Liquid Hot Water Pretreatment (LHW)

In liquid hot water pretreatment of the samples (Watermelon, Banana, Mixture), the grounded powdered of the samples were slurried with distilled water using a solid to liquid ratio of 10% (w/w) and autoclaved at 121^0C for 15 min. After autoclaving, the samples were filtered, and the solid residue was air-dried and stored for further use (Arumugam and Manikandan, 2011).

Biological Pre-treatment

The samples pretreatment was performed using two conditions. In the initial place, the samples quantities (1 g, 2 g, 3 g and 4 g) were pretreated by adding 2 x 10^8 CFU/ml of *Bacillus subtilis obtained from soil*. The pretreatment was allowed

for seven days, and then the samples were then autoclaved to stop the activities of the bacteria and stored for further analyses. Secondly, inocula size of $3x10^8$, $6x10^8$, $9x10^8$ and $1.2x10^9$ CFU/ml were used to pretreat 2 g of the samples for seven days and then autoclave and stored for further use (Essien *et al*., 2005).

RESULTS

Biochemical Characterization of the Isolates

Biochemical characteristics of the bacterial isolates are presented in table 1. The percentage of occurrence of the isolated bacteria from soil were presented in figure 1.

Table 1:Biochemical Characteristics of the Bacterial Isolates

Isolate	Gram reaction	Lactose	Glucose	Sucrose	Citrate	Motility	Indole	Urease	H_2S	Gas	Methyl red	VP	Catalase	Coagulase	Spore formation	Starch	Organism identified
A	+ Rod	-	+	+	+	-	-	-	-	-	+	-	+	-	+	+	*Bacillus cereus*
B	+Cocci	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	*Micrococcus* spp
C	+ Rod	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	*Bacillus subtilis*

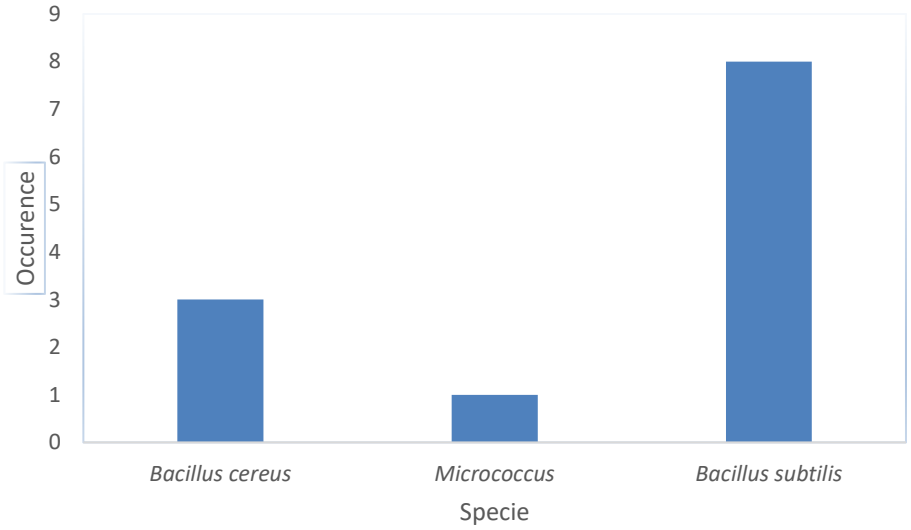

Figure 1: Occurrence of bacterial isolate from soil sample

Structural Composition of the Fruit Peels before Pre-treatments

Structural compositions of the fruit peels before pre-treatment which include Sample A (Watermelon) Extractive (33%), Lignin (17%), Holocellulose (55%), Cellulose (28%), Hemicellulose (27%). Sample B (Banana) Extractive (29%), Lignin (19%), Holocellulose (49%), Cellulose (25%), Hemicellulose (24%) are presented in table 2.

Table 2: Structural composition of the watermelon and banana peels before pre treatment

Parameters	Sample A	Sample B
Extractives	$33 \pm 1.10^{a(x)}$	$29 \pm 1.15^{b(y)}$
Lignin	$17 \pm 1.10^{a(x)}$	$19 \pm 1.11^{a(y)}$
Holocellulose	$55 \pm 1.89^{a(y)}$	$49 \pm 1.19^{b(z)}$
Cellulose	$28 \pm 1.13^{a(x)}$	$25 \pm 1.10^{a(x)}$
Hemicellulose	$27 \pm 1.10^{a(x)}$	$24 \pm 1.10^{a(x)}$

a,b, means values within a row with different superscript are significantly different at ($p<0.05$)

x,y,z within a column with different superscript are significantly different at ($p<0.05$)

Key: Sample A Watermelon
Sample B Banana

Structural Composition of the Fruit Peels after Pre-treatments

Structural composition of the samples after biological pretreatment using *Bacillus subtilis*. Watermelon is composed of Extractives (39%), Lignin (13%), Holocellulose (53%), cellulose (30%), Hemicellulose (23%) and banana is composed of Extractives (33%), Lignin (10%), Holocellulose (49%), cellulose (28%), Hemicellulose (21%) is presented in table 3.

Table 3: Structural Composition of the watermelon and banana peels after biological pre- treatment using *Bacillus subtilis*

Parameters	Sample A	Sample B
Extractives	$39 \pm 1.41^{a(x)}$	$33 \pm 1.28^{b(y)}$
Lignin	$13 \pm 1.11^{a(y)}$	$10 \pm 1.19^{c(z)}$
Holocellulose	$53 \pm 1.76^{b(z)}$	$49 \pm 1.23^{a(x)}$
Cellulose	$30 \pm 1.17^{a(x)}$	$28 \pm 1.13^{b(y)}$
Hemicellulose	$23 \pm 1.08^{b(y)}$	$21 \pm 1.13^{c(y)}$

a,b,c means within a row with different superscript are significantly different at ($p<0.05$)
x,y,z within a column with different superscript are significantly different at ($p<0.05$)
Key: Sample A Watermelon
Sample B Banana

Structural Composition of the Fruit Peels after Liquid Hot Water Pre-treatments

Structural composition of samples after LHW pretreatment. Watermelon is composed of Extractives (38%), Lignin (11%), Holocellulose (52%), cellulose (31%), Hemicellulose (21%) and banana is composed of Extractives (32%), Lignin (09%), Holocellulose (45%), cellulose (29%), Hemicellulose (16%) is presented in table 4.

Table 4: Structural Composition of the watermelon and banana peels after LHW Pre-treatment

Parameters	Sample A	Sample B
Extractives	$38 \pm 1.16^{a(x)}$	$32 \pm 1.35^{b(y)}$
Lignin	$11 \pm 1.10^{c(z)}$	$09 \pm 1.06^{c(x)}$
Holocellulose	$52 \pm 1.22^{a(y)}$	$45 \pm 1.23^{a(z)}$
Cellulose	$31 \pm 1.10^{b(x)}$	$29 \pm 1.10^{a(y)}$
Hemicellulose	$21 \pm 1.00^{a(y)}$	$16 \pm 1.08^{b(x)}$

a,b,c means within a row with different superscript are significantly different at ($p<0.05$)
x,y,z within a column with different superscript are significantly different at ($p<0.05$)
Key: Sample A Watermelon
Sample B Banana

DISCUSSION

From the result of the study, *Bacillus cereus*, *Micrococcus sp*, *Bacillus subtilis* were isolated from the soil samples. *Bacillus cereus*, *Bacillus subtilis* and are *Bacillus* species which adapt more to the environment they inhabit than other bacteria (Park *et al.*, 2003). The presence and abundance of various species of *Bacillus* may not be surprising as *Bacilli* reside in soils and some plants which have antibacterial and antifungal activities (Pan *et al.*, 2004). Pannerselvam and Elvarasi (2015) reported the isolation of α-amylase producing *Bacillus subtilis* from garden soil. *Bacillus subtilis* has been well known as producer of α-amylase and was tested using solid state fermentation for 48 hours at 37^0C with wheat bran as substrate.

However, structural composition of both the fruit peels before pretreatment yielded higher celluloses composition of 28.0% and 25.0% while hemicellulose has a composition of 27.0% and 24.0% respectively, and lignin has least composition of 17.0% and 19.0% respectively in the fruit peels collected from Kasuwar Daji. The result of structural composition of hemicelluloses and lignin is in conformity with the report of Bajpai (2016) who reported that hemicelluloses compose between 35-50% and lignin compose 10-30% of watermelon fruit peels. However, the result of the current study is in agreement with his report that α-celluloses compose of 25-40%. This study showed that Watermelon peels has higher structural composition than Banana peels.

After pretreatment of the fruit peels with *Bacillus subtilis I*, the lignin content has reduced to as low as 13.0%, and 10.0% of watermelon and banana respectively hemicelluloses was reduced to 23.0% and 21% of watermelon and banana respectively while cellulose content was 30% and 28% respectively. Also, after pretreatment with LHW the lignin content was reduced to 11% and 09% of watermelon and banana respectively, hemicelluloses content was reduced to 21%, and 16% respectively and cellulose content was 31% and 21%, respectively, although, the lowest lignin content of 10% and 09% obtained from both biological and LHW was not the same as 7.54% reported by Ahmadu *et al.* (2017) when pretreatment with bacteria, but it is in close range. Also, the finding was in accordance with the previous reports of Lechner and Papinutti (2006) and Sherief *et al.* (2010) where lignolytic activities of fermenting microorganisms were found during biodegradation of rice straw, saw dust, wheat straw, coffee pulp and banana leaves. The hemicelluloses content obtained was in conformity with a similar work of Sindhu *et al.* (2016) who said *Aspergillus niger, Trichoderma reesei, Bacillus* species and *Humicola insolens* can be good microbial sources of xylanases. Since, hemicelluloses composes of pentoses like xylan, xylanases produced by *Bacillus* specie can degraded it. The cellulose content has little increase in the samples. This can be due to the fact that cellulose degradation is a usual activity of*Bacillus* species as reported by Bisaria *et al.* (1997), Sherief *et al.* (2010) and Jahromi *et al.* (2011).

Conclusion

The study concludes that pretreatment of fruit peels with *Bacillus subtilis* is more effective than liquid hot water treatment in enhancing the accessibility of enzymatic saccharification and reducing lignin content. This microbial pretreatment method offers a promising alternative for improving the efficiency of bioethanol production from lignocellulosic biomass, suggesting that biological methods may be superior to thermal methods for certain substrates.

Future Directions

Future directions for bioethanol production using biological and thermal pretreatment methods could focus on several key areas to enhance efficiency, sustainability, and scalability:

1. Optimization of Pretreatment Conditions

- Biological Pretreatment: Further research could aim to optimize the conditions under which microbial pretreatment occurs, such as temperature, pH, and incubation time. This could involve exploring different microbial strains or consortia that work synergistically to enhance lignin degradation and cellulose accessibility.
- Thermal Pretreatment: Efforts could focus on fine-tuning thermal pretreatment methods to minimize energy consumption while maximizing the breakdown of lignocellulosic structures. This might involve combining thermal methods with chemical catalysts or co-pretreatment strategies.

2. Integration of Pretreatment Methods

- Exploring hybrid approaches that combine biological and thermal pretreatment methods to take advantage of the strengths of each. For example, a thermal pretreatment could be used to initially break down the biomass structure, followed by a microbial treatment to further degrade lignin and hemicellulose.
- By focusing on these areas, future research can contribute to making bioethanol production more sustainable, efficient, and commercially viable, thus playing a significant role in the global transition to renewable energy sources.

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