*https://doi.org/10.48047/AFJBS.6.15.2024.11595-11619*

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

# INTEGRAL APPROACH FOR SECOND-GENERATION BIO-ETHANOL PRODUCTION USING BARNYARD MILLET HUSK

**Kaviyashree V[1], Sridhar R[2], Lokeshwar P C[3], Shyam Pranhav D[4], Philip Robinson J[5], Rubavathi S[6]. and Ayyappadasan G*[7].**

[1,2,3,4,5 & 6]Department of Biotechnology, K.S. Rangasamy College of Technology, Tiruchengode – 637 215, Tamil Nadu, India.

***Corresponding author** : Dr. Ayyappadasan G*[7], Associate Professor, Department of Biotechnology, K.S. Rangasamy College of Technology

Email: [7]ayyappadasan07@gmail.com

Volume 6, Issue 15, Sep 2024

Received: 15 July 2024

Accepted: 25 Aug 2024

Published: 25 Sep 2024

*doi: 10.48047/AFJBS.6.15.2024.11595-11619*

**Abstract**

The paper discusses the production process of second-generation bioethanol, focusing on its differentiation from first-generation and subsequent generations of biofuels due to its utilization of lignocellulosic biomass as a raw material. The two different pretreatment methods for barnyard husk were examined by different concentrations of Sulphuric acid and Sodium hydroxide. After hydrolysis, the reducing sugar concentration was determined using Dinitro salicylic acid (DNS) method. Results showed that the best result obtained by the pretreatment was 1% $H_2SO_4$ and 1% NaOH. The concentration of the Bioethanol produced was determined using the Potassium Dichromate method. The results showed that the highest bioethanol yield of 3.098 grams/liter was obtained after 1% $H_2SO_4$ pretreated hydrolysates were fermented with *Saccharomyces cerevisiae* on the Third day. The comparative FESEM image of the pretreated biomass sample showed enhanced roughness as compared to the untreated sample. FTIR showed the peak at the absorbance of 3942.50 $cm^{-1}$ which can show the functional group of O-H stretching of Alcohol. The volatile compounds produced by acid pre-treated samples using Gas Chromatography-Mass Spectroscopy (GCMS) show that Ethanol was the highest volatile compound with a peak area of 41.98%. Therefore, bioethanol production using 1% $H_2SO_4$ pretreated samples produced a large quantity of bioethanol and this study concluded that Barnyard husk is a suitable substrate for bioethanol production and hydrolyzing with *Saccharomyces cerevisiae* and optimization of fermentation yielded higher bioethanol concentration.

**Keywords:** Lignocellulosic biomass, biomass pre-treatment, total sugar, enzymatic hydrolysis, fermentation.

## Introduction

The major concern over the continuous depletion of non-renewable fossil fuels, their negative impact on the atmosphere due to the emission of greenhouse gases, and energy security have led to the research for alternative and sustainable fuels from renewable resources. Population growth and increasing industrial development led to greater demand for energy, but conventional fossil fuels, including petroleum, are both finite resources and emit greenhouse gasses (GHG) when combusted. Initially, bioethanol was produced by the first-generation substrate biomass including starchy materials and sugar crops (sugarcane). Since the first-generation feedstocks are food crops, exploiting them for bioethanol production will directly compete and affect the world's food supply. This has led to serious enterprises regarding large-scale production's socio-profitable and environmental consequences (Jambo et al 2016). Hence, this led to the exploration of the non-edible second-generation feedstocks lignocellulosic biomass including agricultural wastes, and forest wastes. Lignocellulosic feedstocks are cheaper and their utilization in bioethanol production supports the enhancement of local agricultural economy too. Second-generation (2Gen) Lignocellulosic feedstocks are renewable and most energy devoted material for bioethanol production which is very cheap and freely available. Lignocellulosic feedstocks are cheaper as they are plant wastes. Utilization of agro-wastes such as Millet waste component for bioethanol production will boost the local agricultural economy. Even though the technology for bioethanol has been commercialized, the process is complicated due to the complex nature of lignocellulose, unlike first-generation feeds (Robak et al 2018).

Barnyard millet (Echinochloa frumentacea) indeed belongs to the family Poaceae and it is primarily a self-pollinated crop that leads to genetic uniformity. Barnyard millet is the oldest domesticated small millet. It is a good source of protein about 10.5%, highly digestible and an excellent source of dietary fibre Barnyard millet contains about 8.7 – 9.63% moisture. The carbohydrate content in barnyard millet is low and also sluggishly digestible. The nutritional composition of barnyard millet contains Moisture (8.74%), Protein (10.1%), Fat (3.9%), Crude Fibre (6.7%), Total Minerals (2.1%), Total Dietary Fibre (12.5%), Carbohydrate (68.8%), Phosphorus (281 mg), Magnesium (83 mg), Iron (5 mg), Calcium (19 mg) (Ugare et al 2014).

## Composition of lignocellulosic feedstocks for ethanol production

For optimal ethanol production, the biomass needs to have a high amount of cellulose and hemicellulose, which are carbohydrates that can be fermented into ethanol, while having a low

lignin content. Lignin is a complex compound in plant cell walls that can interfere with conversion. (Lange 2007, Anwar et al 2014).

**a) Cellulose**

The primary component of lignocellulosic feedstock is cellulose, which makes up 15-55% of the feedstock. Cellulose is a homo-polysaccharide that contains anhydrous glucose bound by 1-4-glycosidic bonds with repeating small cellobiose units.

**b) Hemicellulose**

Its structure is random and amorphous, making it simple to hydrolyze into monomeric sugar is found in barnyard millet husk. Hemicellulose is mainly enclosed between the lignin and the cellulose which can help to enhance the strength of plant cell wall.

**c) Lignin**

The presence of lignin is the sole cause of feedstock. It acts as a barrier for plant cell wall permeability and microbial attack. Lignin became important due to its diluting effect during the saccharification and fermentation processes.

**Significance of Research**

The study was to evaluate the potential importance of millet husk of second-generation bioethanol from the lignocellulosic biomass. Developing bioethanol from lignocellulosic biomass diversifies the energy sources and reduces the dependence on fossil fuels, contributing the energy security and it can reduce the dependence on non-renewable energy sources. Agricultural non-edible residues generally emit less greenhouse gases compared to fossil fuels, contributing to mitigating climate change.

**Statement of Industrial Relevance**

The authors consider that this research is appropriate for publication because of the sustainable and efficient utilization of agricultural waste to produce biofuels, contributing to both environmental conservation and energy security. An integral approach for second-generation bio-ethanol production using barnyard millet husk addresses the pressing need for sustainability and Resource Optimization. The authors confirm that this work is original and has not been published

elsewhere, nor is it currently under consideration for publication elsewhere. All the authors have no conflicts of interest to disclose.

## Materials and Methods

### Collection of substrate: Barnyard husk

The millet husk is collected from the waste dumping sites in the place of Virudhunagar, Tamil Nadu. The collected sample is air-dried. The dried sample is ground to a fine powder using a mixer grinder to reduce the particle size to 1 mm with a sieve shaker, and then screened to analyze the higher feasibility of the process. The powdered sample will be tightly stored in the saline bottle at room temperature under dry condition until its requirement.

### Determination of physiochemical composition

To study the characteristics of the substrate to conform to its composition in biochemical properties.

### Determination of "Moisture" content

A crucible was weighed and, 2 g of the substrate was transferred to the weighted crucible, placed in a hot air oven at a temperature of 105ºC, and heated for 24 hours. The process was repeated until a constant weight was measured. The crucible was removed from the oven, cooled in a desiccator containing silica gel, and weighed (Garba et al 1996). The percentage moisture content was calculated as:

Moisture content = loss in moisture*100 / Initial weight of a sample

### Determination of "Ash" content

The ash of the sample was determined by weighing a crucible and charging it with 2g of the substrate. The crucible and its content were put into a muffle furnace and heated at 600ºc for 3 hours, cooled in a desiccator containing silica gel, and weighed (Gwandu et al 2021). The percentage ash content was calculated as:

Ash content (%) = Amount of ash*100 / weight of sample

### Determination of "Organic matter" content

The organic matter is determined by subtracting the percentages of moisture and ash content (Garba, 1996).

Organic matter = 100% - (% ash + %moisture content)

**Determination of "Percentage carbon" content**

The percentage of Carbon is estimated using the equation: (SE, A 1989)

%C = 0.58 x Organic matter

The 58 % carbon content estimation for millet husk likely comes from empirical data or standard assumptions used for plant-based biomass materials. This percentage is commonly used in biomass analysis based on the consumption of lignocellulosic material like Banyard millet husk.

**Empirical Data:** These data explains that the carbon content of many plant-based materials, especially lignocellulosic biomass like Banyard millet husk, is around 50% to 60% by weight. This estimation in many agricultural residues, the carbon content tends to range between 45% to 58%, depending upon the materials and chemical composition.

**Stoichiometric Assumption:** The 58% could be derived from stoichiometric calculations, where the carbon content is estimate based on the molecular structure of organic components such as cellulose, hemicellulose, and lignin, which are the major components primarily consist of carbon, typically makes up a significant percentage.

**Pre-treatment Methods of lignocellulosic feedstock**

The pretreatment process allows the internal complex structure of the feedstock to be opened, increasing enzyme digestibility. Here the study focuses on the structural changes primarily in Cellulose, Hemicellulose, and Lignin during the chemical pretreatment technologies (Cao et al 2012).

**Acid & Alkaline pre-treatment**

A 30g of millet husk sample was mixed and soaked with 100 mL of 1% - 5% (w/v) $H_2SO_4$ solution and 100 mL of 1-5% (w/v) NaOH aqueous solution in a 250 mL flask, and the samples were treated in an autoclave at 121°C, for one hour. The pre-treated samples were filtered through a Whatman filter paper to separate the solid residue. The residue was washed with distilled water until neutral pH. The sample was oven-dried and stored in a tightly sealed container for further use (Hendriks & Zeeman 2009).

**Structural composition analysis of untreated and pretreated substrate**

The holocellulose content of untreated and pretreated barnyard millet husk (initial biomass, 0.5 g) was determined by following the Browning method. Standard TAPPI protocols were used for the determination of hemicellulose and acid-insoluble lignin (ADL) contents (initial biomass, 1 g). The analysis was carried out with more precision and negligible wastage of biomass to achieve less internal error. Approximate cellulose content was determined by subtracting the hemicellulose from the holocellulose content of each sample.

**Confirmatory test for cellulose**

The iodine solution and sulphuric acid are added to the sample of cellulose. If the cell wall of cellulose turns blue-violet color it indicates the presence of cellulose nitrate. The cellulose is a non-reducing sugar (Beukes et al 2011).

**Determination of total sugar content by DNS method**

A dinitro salicylic acid colorimetric method was used to calculate the reducing sugars of the hydrolyzed residue of the Acid and Alkaline process with glucose as standard. It was assayed by adding 3 ml of DNS reagents into each test tube containing the sample. Each tube's contents were boiled in a water bath for 10 minutes to generate a yellow to red-brown color. Added 1 ml of 40% potassium sodium tartrate solution to stabilize the color and allowed to cool at room temperature. With a UV-visible spectrophotometer, the absorbance was measured at 600 nanometers for growth. The reducing sugar content was determined by referring to a standard curve of known glucose concentration (Miller & Hester 2007).

**Solid-State Fermentation**

The fermentation of lignocellulosic hydrolysates is to achieve efficient conversion of hexose and pentose sugars into ethanol by fermenting microorganisms such as yeasts. Optimization of enzyme production under SSF micro-organism cultivated on moist, solid substrates in the absence of flowing liquid phase. From the hydrolysate of the substrate, add minimal media compositions such as Ammonium sulfate, Di-ammonium hydrogen phosphate, Potassium chloride, Magnesium sulfate, Calcium chloride, and Yeast extract were added. And autoclaved at 121°C then cooled, then the media was inoculated by the yeast strain

(Saccharomyces cerevisiae) for culturing into the fermentation broth medium (Xayasene et al 2018).

Researchers have engineered strains of *Saccharomyces cerevisiae* to better handle pentose sugars, but they still face challenges due to a shortage of xylose-specific transporters. Pentose specific transporter proteins and enzymatic reactions determining the metabolism of pentoses such as l-arabinose and d-xylose have not been found in naturally occurring baker's yeast. There is a shortage of xylose-specific transporters in engineered strains, which are required for efficient xylose use without hampering fermentation of combining C5 and C6 sugars. To introducing specific genes, metabolic pathways can be optimized to enhance the efficiency of pentose conversion. *Saccharomyces cerevisiae* can be subjective to selective pressure to evolve strains with improved pentose fermentation capabilities. This process involve growing yeast in environment with pentoses and selecting for strains that exhibit better growth and fermentation on these sugars. In S. cerevisiae, affinity for xylose uptake depends on the extracellular concentration of glucose (Kruckeberg & Dickinson 2004).

**Effect of incubation time on ethanol production**

The flask containing pre-treated substrate and composition of Minimal media are inoculated by inoculums of fungi Saccharomyces cerevisiae and incubated for 7 days at room temperature. Then, the sample was taken every 24 hours at intervals and a confirmatory assay was done for every sample collected (Kruckeberg & Dickinson 2004).

**Procedure for fermentation**

Pretreated barnyard husks are hydrolyzed by enzymes and temperature. From the hydrolyzate of the substrate, minimal media compositions such as Nitrogen source often as Ammonium sulfate to support protein synthesis, Di-ammonium hydrogen phosphate, Potassium chloride for energy transfer and metabolism, Magnesium sulfate, Calcium chloride, and Yeast extract were added. And autoclaved at 121°C then cooled, then the media was inoculated by the yeast strain (*Saccharomyces cerevisiae)* for culturing into the fermentation broth medium (Abu et al 2005).

**Effect of Fermentation Condition**

**a) Temperature**

The fermentation temperature is critical for optimal yeast performance. Here's the optimal range between 30°C to 35°C for most strains of *Saccharomyces cerevisiae.*

**b) Agitation**

Gentle agitation is often applied during fermentation to ensure uniform nutrient distribution and maintain yeast in suspension. Agitation also helps with gas exchange, preventing $CO_2$ accumulation and providing oxygen in early stages for yeast growth. The speed can typically range from 100-200 rpm.

**c) Fermentation Time**

The fermentation time depends on the sugar concentration, yeast strain, and fermentation conditions. The process followed is continuous fermentation condition, it lasts from 24 hours, After every 24 hours the medium can be inoculated by the yeast strain (*Saccharomyces cerevisiae).* The production rate of ethanol concentration are monitored to assess fermentation progress.

**Confirmatory Test for Bio-ethanol Produced**

The confirmatory test was carried out on the extracted ethanol sample by using the method of potassium dichromate test. In this method, the ethanol is oxidized to ethanoic acid by reacting it with an excess of potassium dichromate in acid. The ethanol standard is prepared by using absolute alcohol, to each test tube containing the fermented sample add 2ml of dichromate solution. The test tubes will be warmed in the water bath. In the case of primary or secondary alcohols, the orange solution turns blue-green. The absorbance is measured at 600nm using a UV spectrophotometer (Isah et al 2019)**.**

**FTIR and FESEM analyses of untreated and pre-treated substrate**

The untreated substrate and the pre-treated sample from the best pre-treatment (1%$H_2SO_4$) screened from the hydrolysis were used for FTIR (Fourier Transfer Infrared Spectroscopy) and Field Emission Scanning Electron Microscope (FESEM). The samples were separately mixed with KBr in the ratio of 100:1 (KBr: Biomass), ground using mortar and pestle and pelleted using a press. The pellets were scanned by Fourier-transform infrared (FTIR) spectroscope within the

wavenumber range, 4000-450 cm-1. Field emission scanning electron microscopic (FESEM) analysis of the above samples was carried out by placing the biomasses on a carbon tape fixed to a stub, coating them with gold, and scanning by FESEM (Sim et al 2012).

## Results and Discussion

The dried substrate was ground using a mixer grinder to reduce the particle size to 1mm and the feedstock was sieved to analyze the higher feasibility of the process (Figure1). The powdered substrate will be stored in the saline bottle until its requirement.

**Figure 1 The Powdered Barnyard substrate**

Figure 1 shows the before and after grinding the substrate, the husk is typically larger, coarse pieces in before grinding substrate, which have a lower surface area. After grinding, the particle size is reduced, increasing the surface area, the smaller and uniform particles created by grinding allow for better contact between the biomass and the microorganism used in the fermentation process.

The before and after figure 1 show how grinding act as a form of mechanical pretreatment. It helps to show a demonstrate progress in the bioethanol production process, showing how raw materials are transformed step by step into more refined substrates for fermentation.

## Determination of physiochemical composition

The results of the proximate composition of barnyard husk are presented in Table 1. The Moisture content value is 10.5%. The Ash content value is 6%. The Organic matter value ranges from 83.5%. The Carbon value percentage is 48.43%.

**a) Moisture Content**

The Barnyard biomass with a moisture content of 10.5% for bioethanol production. The moisture content of biomass is the key parameter in selecting the appropriate pretreatment process. For bioethanol production, pretreatment methods such as steam explosion, acid hydrolysis or enzymatic hydrolysis. A 10.5% moisture content is low enough to avoid excessive dilution during pretreatment, allowing for more effective chemical treatment such as acid and alkaline hydrolysis. Moisture content affects the overall efficiency of fermentation during bioethanol production. A 10.5% moisture content suggests that most of the biomass is dry matter, providing a higher proportion of fermentable sugar after the hydrolysis. The maximizes ethanol yield because water des not dilute the biomass unnecessarily during fermentation.

**Table 1 Physiochemical composition of Barnyard husk**

| COMPOSITION | AMOUNT |
|---|---|
| **MOISTURE CONTENT** | 10.5% |
| **ASH CONTENT** | 6% |
| **ORGANIC MATTER** | 83.5% |
| **CARBON** | 48.43% |

**b) Ash Content**

Ash is the inorganic, non-combustible residue that remains after the combustion of biomass. It typically consists of minerals such as silica, potassium, calcium, and magnesium. The ash content of 6% contains 6% of inorganic material. The remaining 94% would be organic matter including cellulose, hemicellulose and lignin which are the primary source of fermentable sugars used in bioethanol production. A agricultural residue such as husk can have ash content ranging from 5% to 10%. while 6% content is moderate is more desirable because it contains a higher proportion of fermentable materials, leading to greater ethanol yields.

**c) Organic matter**

Organic matter content such as 83.5% in biomass refers to the carbon based compounds. The organic fraction components such as cellulose, hemi cellulose and lignin that are crucial for bioethanol fermentation. With 83.5% organic matter, there is likely a higher amount of cellulose, which will directly correlate with the amount of glucose produced for fermentation. Lignin is also a part of organic matter, it can be converted into valuable by-products and chemicals. Higher organic matter biomass like barnyard husk also presents economic benefits, as a larger portion of the biomass can be converted into ethanol.

**d) Carbon Content**

Carbon source is key element in the biochemical made up of biomass as it directly linked to the cellulose, hemicellulose, and lignin composition for ethanol production. A carbon composition of 48.43% suggest a substantial amount of organic matter within the barnyard husk that could be fermented into sugars. The cellulose and hemicellulose are the polysaccharides made up of carbon based sugars that can be hydrolysed into fermentable sugars.

**Pretreatment Methods of lignocellulosic feedstock**

(Figure 2a-b) The effect of Acid pretreatment of barnyard husk was carried out by varying the concentration of $H_2SO_4$ (1% - 5%). The purpose of $H_2SO_4$ was to identify the condition to break down the lignin structure and matrix of cellulose. The target was to produce a maximum yield of sugar and hemicellulose which increases the cellulose content.

**Fig.2**


**Fig.3**

**Figure 2 Sulphuric acid pretreatment at varying concentrations (1% - 5%)**

**Figure 2 a)** Before Autoclave **Figure 2. b)** After Autoclave of Acid pretreatment

**Figure 3 Sodium hydroxide pretreatment at varying concentrations (1% - 5%)**

**Figure 3 a)** Before Autoclave **Figure 3 b)** After Autoclave of Alkali pretreatment

(Figure 3a-b) The effect of Alkaline pretreatment of barnyard husk was carried out by varying the concentration of NaOH (1% - 5%). After alkaline hydrolysis, the large increase in cellulose is due to the removal of hemicellulose, lignin of millet husk. The effect of NaOH on substrate results in delignification.

To investigate the effect of concentration of NaOH and $H_2SO_4$ and time on the concentration of released glucose from the pretreated rice husk. The first experiment was carried out with $H_2SO_4$ for the pretreatment of rice husk. The experiments were conducted with 1%, 2% 3%, 4% and 5% $H_2SO_4$ for 121℃, for one hour, after which the solid residue were prepared for hydrolysis. As shown in Figure with various $H_2SO_4$ pretreatment conditions, 1% concentration of $H_2SO_4$ gave higher glucose concentration than other acid concentrations.

**Structural composition analysis of untreated and pretreated substrate**

According to Table 2, there was more cellulose in acid pretreatment ($H_2SO_4$) than in raw husk and alkali pretreated husk (NaOH). The amount of lignin left after pretreatment is a key factor for degrading during chemical hydrolysis. The acid treatment has more xylose concentration in hydrolyzate, than alkaline-treated hydrolyzate, due to the high content of cellulose and low content of lignin.

**Table 2 Barnyard husk cellulose composition**

| Composition | Raw husk | Acid pretreatment | Alkaline pretreatment |
|---|---|---|---|
| Cellulose | 10.5 mg/ml | 13.2 mg/ml | 11.6 mg/ml |

**Confirmatory test for cellulose**

**Table 3 Confirmatory test for cellulose**

| Sample | Test | Observation |
|---|---|---|
| Cellulose | Sample + few drops of Iodine solution + drop of diluted sulphuric acid | Cellulose sample turns into blue violet |

**Determination of total sugar Content**

The different Hydrolysis methods with acid and alkaline were used to break down the complex sugars into simple monosaccharide units. The total sugar content of the hydrolyzed sugar was measured by the DNS method.

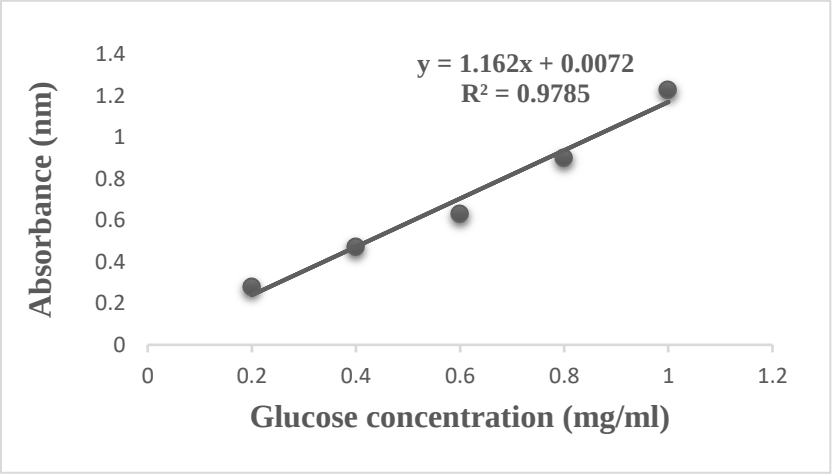



**Figure 4 Standard graph for Glucose of Acid hydrolysis using DNS**

A standard curve for DNS analysis is made to determine the initial concentration of glucose. As the volume of glucose increases and the volume of water decreases.

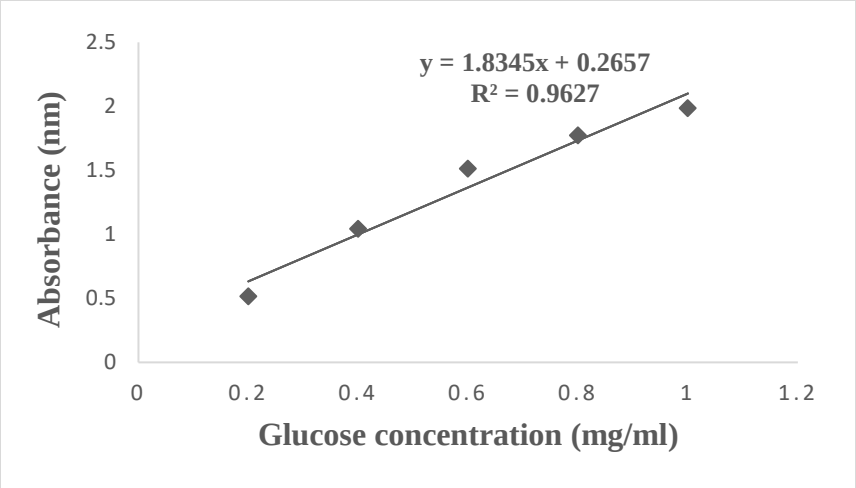



**Figure 5 Standard graph for Glucose of Alkaline hydrolysis using DNS**

The absorbance of the samples was measured at 600 nm using a spectrophotometer is represented in (Fig 4) & (Fig 5). The reducing sugar content was subsequently determined by referring to a standard curve of known glucose concentrations.

The figure shows that in the presence of heat, the reducing sugar gives a hydrogen atom to DNS while simultaneously losing a hydrogen atom. As a result, the reduced sugar generates yellow to yellow-to-orange-red color. A spectrophotometer can then be used to measure the absorbance at 600nm to determine the starting concentration of glucose, the reducing sugar.

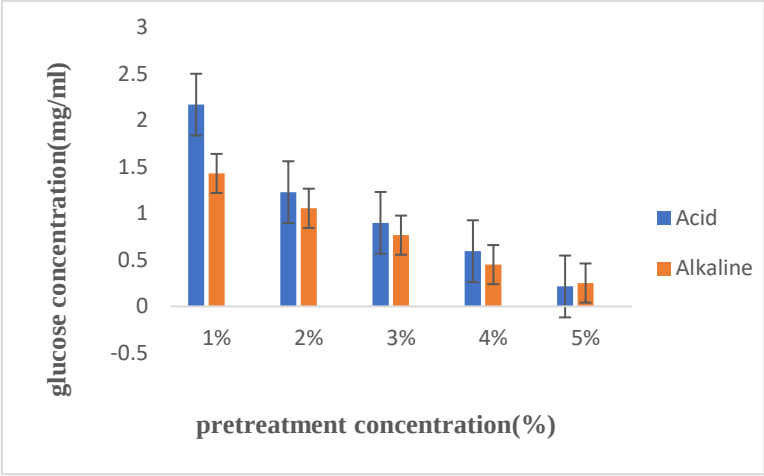



**Figure 6 Effect of different pretreatment methods on glucose yield**

Millet husk hydrolyzed with 1% $H_2SO_4$ and 1% NaOH (Figure 6) released the highest percentage of reducing sugar, which indicates that the best acid concentration for the hydrolysis of the millet husk is 1%.

**Confirmatory Test for Bio-ethanol Produced**

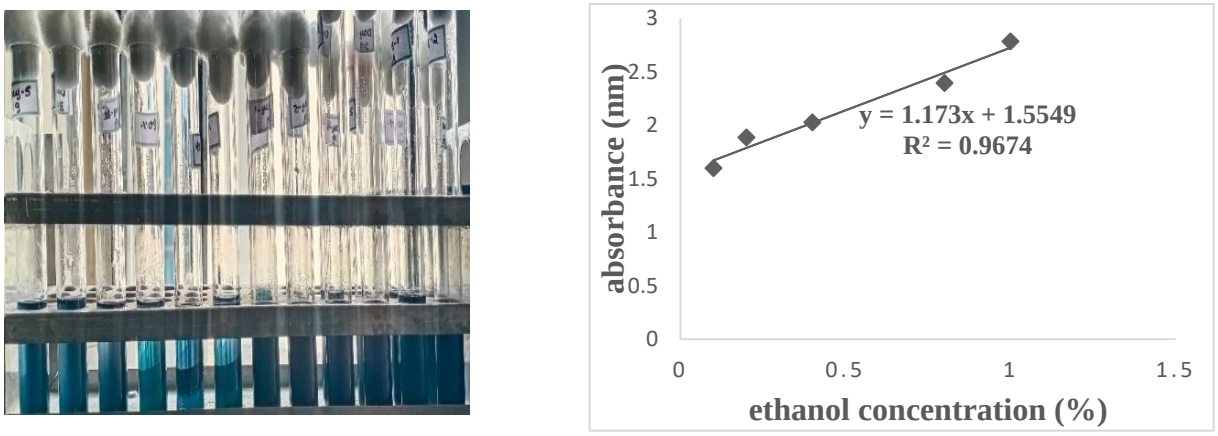



**Figure 7 Ethanol estimation and Standard ethanol by potassium dichromate**

(Figure 7) In the case of primary or secondary alcohols, the orange solution turns blue-green. The absorbance is measured at 600nm using a UV spectrophotometer**.** The formation of blue-green color is strong evidence for the existence of ethanol in the distillate.

**FTIR and FESEM analyses of untreated and pre-treated substrate**

The comparison of the FTIR spectra of untreated and pretreated (1% $H_2SO_4$ autoclaving at 121 C and 15 psi for 30 min) samples showed that the peaks related to the presence of lignin are absent in the pretreated sample (Figure 9). The peak positions related to different functional groups in the lignocellulosic biomass are shown in Tables 4 & 5. This explains the action of acid on the hemicellulose part of biomass. amorphous cellulose is visible in the pretreated sample, whereas it is faintly present in the untreated sample. This shows that the crystalline part of the cellulose was converted into amorphous cellulose. FESEM image of the pretreated biomass sample showed enhanced roughness as compared to the untreated sample (Figure 8) portraying how the acid delignification led to the exposure of holocellulose. Therefore, the structural composition of

untreated and effectively pretreated barnyard husk is validated well by their respective FTIR spectrum and FESEM image.

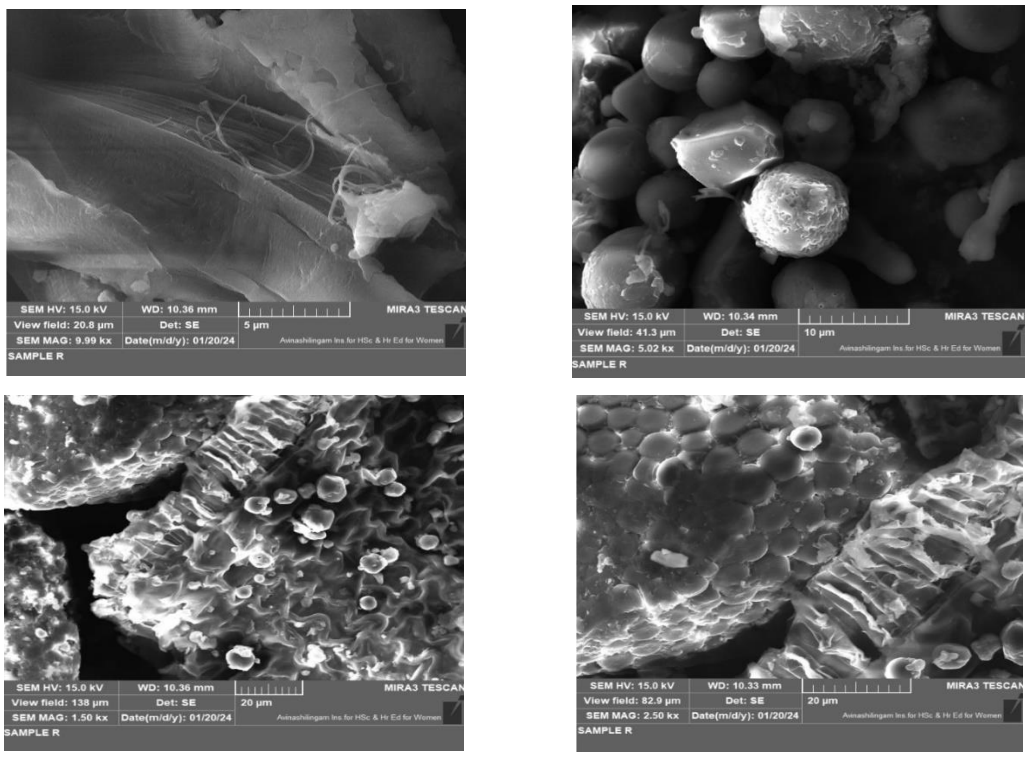
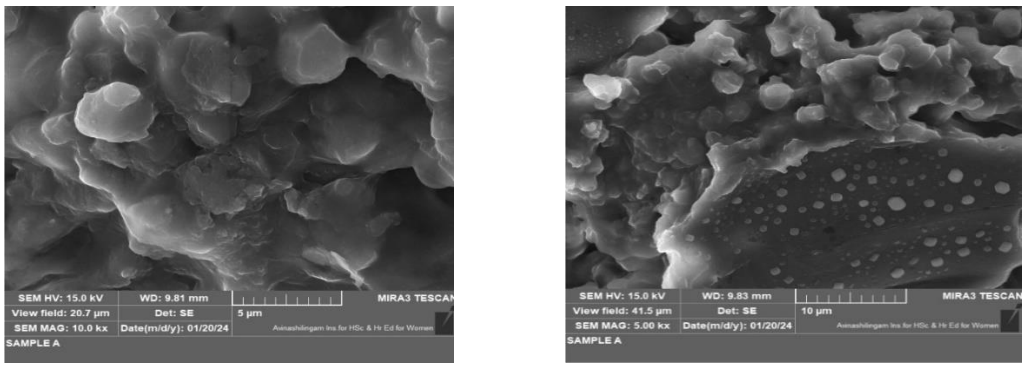



**Figure 8 SEM images of Untreated sample with the scale of 5µm, 10 µm, and 20 µm**



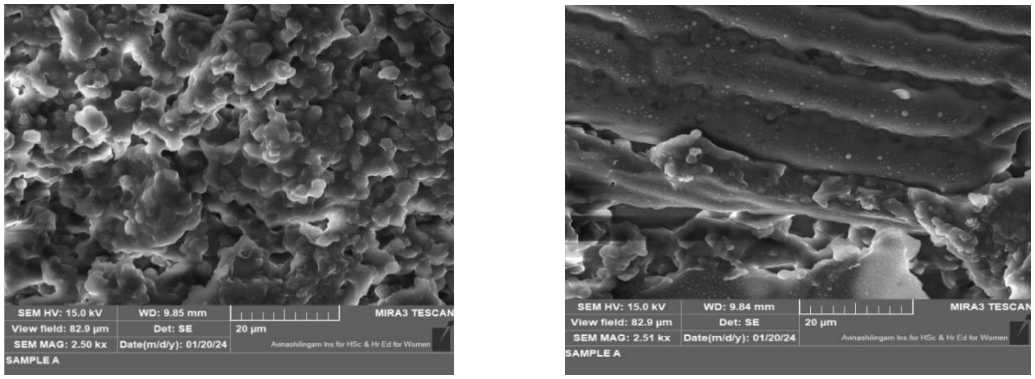



**Figure 9 SEM images of Pretreated sample (1%$H_2SO_4$) with the scale of 5µm, 10 µm and 20 µm**

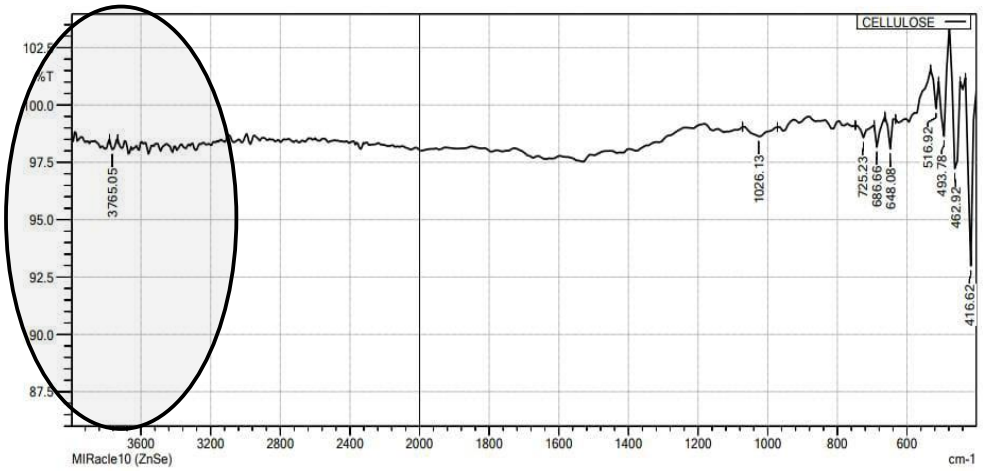



**Figure 10 FTIR spectra of Cellulose**

**(**Figure 10) FTIR analysis confirmed the presence of cellulose, with an absorbance peak at 3765.05 $cm^{-1}$ assigned to O-H stretching and at 1026.13 $cm^{-1}$ corresponding to C-O stretching among other peaks.

**Table 4 Peak assignment for different functional groups of (a) untreated sample in the FTIR spectrum**

| a) | **Absorbance** | **Bonds** | **Functional group** |
|---|---|---|---|
| | 3417.86 $cm^{-1}$ | C-H / N-H stretching | Amine & Amide |
| | 686.66 $cm^{-1}$ | O-H out of plane | Amorphous Cellulose |
| | 648.08 $cm^{-1}$ | O-H out of plane | Amorphous Cellulose |
| | 594.08 $cm^{-1}$ | C-C-N | Nitriles |
| | 493.78 $cm^{-1}$ | C-C | Cycloalkanes |

**Table 5 Peak assignment for different functional groups of (b) pretreated sample in FTIR spectrum**

| b) | Absorbance | Bonds | Functional group |
|---|---|---|---|
| | 3942.50 $cm^{-1}$ | O-H Stretching | Alcohol |
| | 3811.34 $cm^{-1}$ | O-H Stretching | Cellulose |
| | 725.23 $cm^{-1}$ | C-Cl | Alkyl halide |
| | 563.21 $cm^{-1}$ | C-C-N | Nitriles |
| | 493.78 $cm^{-1}$ | C-C | Cycloalkane |
| | 439.77 $cm^{-1}$ | C-C | Cycloalkane |

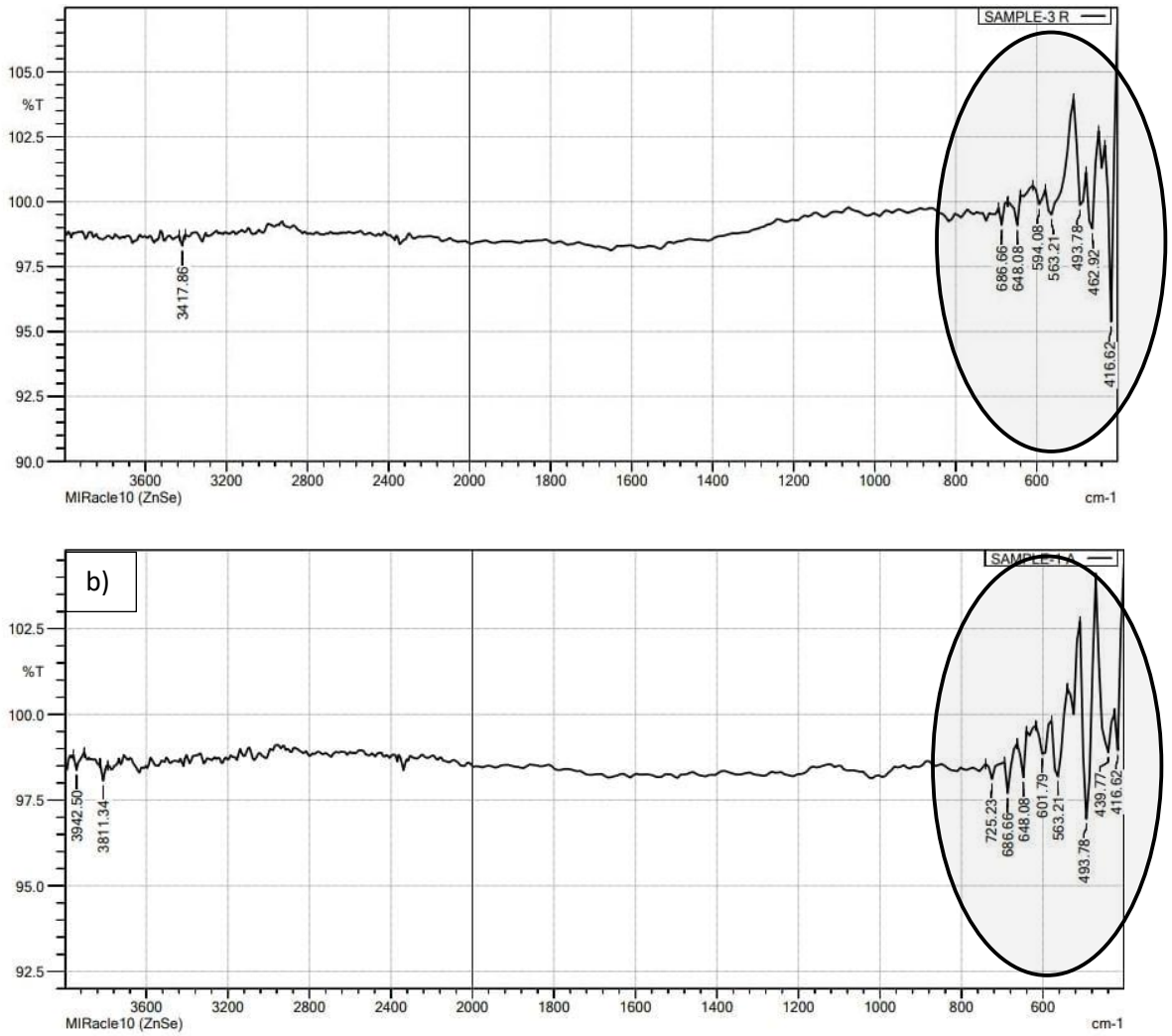



**Figure11 FTIR spectra of (a) untreated and (b) pretreated (1% $H_2SO_4$)**

The FTIR spectrum ranging between 3800 and 3000 cm−1 is related to the O-H stretching of cellulose in the lignocellulosic biomass. The peak at 1732 cm−1 is assigned to the ester linkage between hemicellulose and lignin. the peaks at 1513 cm−1 and 1247 cm−1 were absent in the millet husk sample pretreated by 1% $H_2SO_4$ when compared with the untreated barnyard husk (Figure 11 a and b). this showed that the pretreatment involving $H_2SO_4$ heating helps in delignification and hemicellulose breakdown in the barnyard husk. FESEM images of untreated and pretreated samples showed significant structure disruption, leading to roughness on the surface (Figure 9) as compared with the untreated biomass (Figure 8) displaying the effectiveness of the pretreatment.

**Ethanol production by Potassium dichromate**

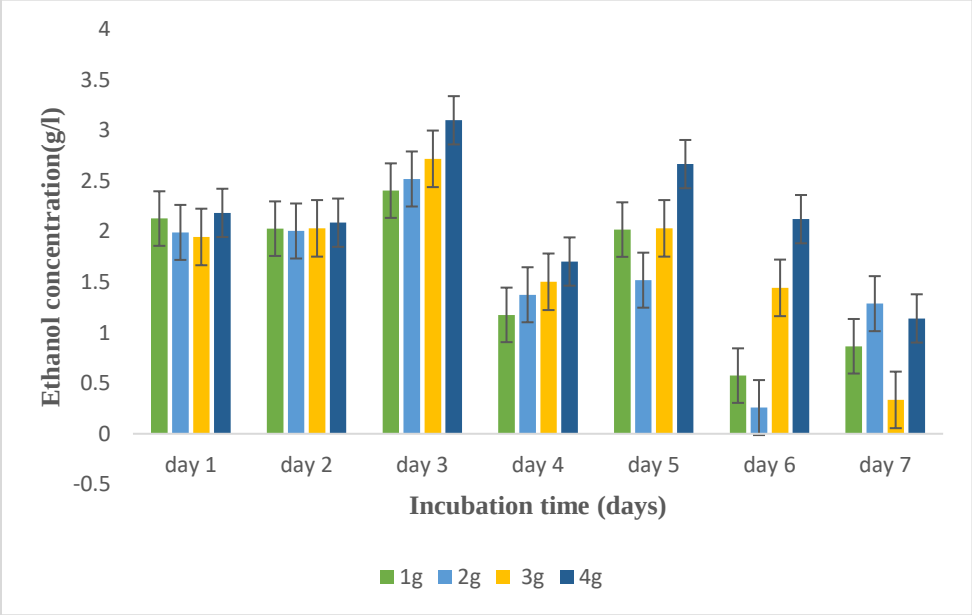



**Figure 10 Graph showing ethanol production by potassium dichromate**

Utilization of glucose and production of bioethanol were calculated at regular intervals of every 24hrs of 7 days of Saccharomyces cerevisiae cultivation. The incubation period of ethanol production was investigated and solid-state fermentation was carried out for 7 days. As shown in

Figure 10 it was reported that production of ethanol from hydrolysate of (1% $H_2SO_4$) treated millet husk was enhanced with an increase in the fermentation period till the 3rd day of incubation 4grams of hydrolysate and beyond the 3rd day, production of ethanol decreased. The decline in ethanol production beyond 3rd day is due to the utilization of sugar molecules by microbes for the production of ethanol.

The maximum amount of ethanol estimated using a potassium dichromate was 3.098 grams/liter which was produced from 4 grams of 1% $H_2SO_4$ on the Third day. 1grams produced 2.403 grams/liter of ethanol on the third day when hydrolyzed using 1% $H_2SO_4$. 2 grams and 3 grams which from lignocellulosic waste produced ethanol was about 2.518 gm/L, 2.717gm/L respectively when hydrolyzed by 1% $H_2SO_4$. The results indicated that maximum ethanol production was observed by 4 grams of 1% $H_2SO_4$ pretreated barnyard millet husk with an ethanol yield of 3.098 grams/liter. 1gm/L, 2gms/L, 3gms/L also produced ethanol but their production is not good as compared to 4 grams of 1% $H_2SO_4$.

**Comparison and Discussion**

The present study results of Barnyard millet husk were comparable to Finger millet husk for showing the potentiality of the present study. By comparing the pretreatment method of the present barnyard husk substrate showing the best results on (1% $H_2SO_4$) causing enhancement in cellulose and hemicellulose content compared to the finger millet substrate treated with 1% NaOH. Another comparison with the Rice husk with the present study for estimation of sugar produced. Millet husk hydrolyzed with (1% $H_2SO_4$) released the highest percentage of reducing sugar while rice husk showed the highest percentage of reducing sugar with (10% $H_2SO_4$). This indicates at a lower concentration of acid pretreatment the millet husk was able to break the lignocellulosic component. For the agro wastes, the combination of saccharomyces cerevisiae and zymomonas mobilis may serve as a best-mixed culture for the production of ethanol but the present study of millet husk showed the best result by using the saccharomyces cerevisiae produced the highest bioethanol concentration from barnyard millet husk after 48 hours of fermentation.

**Conclusion**

Based on the above result, it is recommended that biomass feedstock such as barnyard millet husk should be enhanced towards Bioethanol production industries that will serve as a

source of income and also overcome the effect of global warming and environmental pollution. Among 2 pretreated barnyard samples, 1% $H_2SO_4$ and 1% NaOH were aimed at increasing the holocellulose content and reducing the lignin content of the substrate showing that 1% $H_2SO_4$ was the most effective pretreatment method. Significant improvements in cellulose content, partial removal of lignin, and total glucose yield were obtained. Pretreatment aimed to produce more glucose and xylose for ethanol fermentation. Ethanol fermentation with Saccharomyces cerevisiae was successfully carried out with hydrolyzed sugar. Solid-state fermentation reported that acid-treated husk gave better ethanol production compared to untreated husk. The results indicated that maximum ethanol production was observed by 4 grams of 1% $H_2SO_4$ pretreated barnyard millet husk with an ethanol yield of 3.098 grams/liter. 1gm/L, 2gms/L, 3gms/L also produced ethanol but their production is not good as compared to 4 grams of 1% $H_2SO_4$. The FTIR spectra of pretreated biomass confirmed lignin removal. The enhanced surface roughness of the pre-treated 1% $H_2SO_4$ barnyard sample was visible in the FESEM image.

## AUTHOR CONTRIBUTION

Kaviyashree V, Lokeshwar P C. – Formal analysis, writing, and editing; Sridhar R, Shyam Pranhav D - Construction of figures; Philip Robinson J – Supervision; Rubavathi Subbaiyan – Review and editing, Construction Of Tables, Ayyappadasan Ganesan – original drafting of the work

## ACKNOWLEDGMENT

The authors express their gratitude to the Management, Principal, and Department of Biotechnology at K.S. Rangasamy College of Technology, Tiruchengode for their provision of Review and editing essential facilities and assistance.

## CONFLICT OF INTEREST

No conflict of interest

## References

1. A. Abba1, U. Z. Faruq et al, Study on Production of Biogas and Bioethanol from Millet husk 4(5): 817-827, 2014.
2. Anwar, Z., Gulfraz, M., & Irshad, M. (2014). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. *Journal of radiation research and applied sciences*, *7*(2), 163-173.
3. Balat M, Balat H, Öz C. Progress in bioethanol processing. Prog Energy Combust Sci. 2008;34(5):551–73. https://doi.org/10.1016/j.pecs.2007.11.001.
4. Beukes, N., & Pletschke, B. I. (2011). Effect of alkaline pre-treatment on enzyme synergy for efficient hemicellulose hydrolysis in sugarcane bagasse. *Bioresource Technology*, *102*(8), 5207-5213.
5. Cao, W., Sun, C., Liu, R., Yin, R., & Wu, X. (2012). Comparison of the effects of five pretreatment methods on enhancing the enzymatic digestibility and ethanol production from sweet sorghum bagasse. *Bioresource Technology*, *111*, 215-221.
6. Choudhary J, Singh S, Nain L. Bioprospecting thermotolerant ethanologenic yeasts for simultaneous saccharification and fermentation from diverse environments. J Biosci Bioeng. 2017;123(3):342–6. https://doi.org/10.1016/j.jbiosc.2016.10.007.
7. Domínguez-Bocanegra, A. R., Torres-Muñoz, J. A., & López, R. A. (2015). Production of bioethanol from agro-industrial wastes. *Fuel*, *149*, 85-89.
8. Etalema Desta Tulu, Ramesh Duraisamy, Belay Haile Kebede, Alemu Mekonnen Tura, Anchote (Coccinia abyssinica) starch extraction, characterization and bioethanol generation from its pulp/waste,https://doi.org/10.1016/j.heliyon.2023.e14320.
9. Garba B. Challenges in energy biotechnology with special Reference to biogas technology. A paper presented at the 12th annual conference of the Biotechnology Society of Nigeria at the national institute for fresh water fisheries research institute (NIFFRI), New Bussa; 1999.
10. Garba, B., Zuru, A. A., & Sambo, A. S. (1996). Effect of slurry concentration on biogas production from cattle dung. *Nigerian Journal of Renewable Energy*, *4*(2), 38-43.
11. Hendriks, A. T. W. M., & Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource technology*, *100*(1), 10-18.
12. Jambo, S. A., Abdulla, R., Azhar, S. H. M., Marbawi, H., Gansau, J. A., & Ravindra, P. (2016). A review on third generation bioethanol feedstock. *Renewable and sustainable*

*energy reviews*, *65*, 756-769.

13. John, R. P., Anisha, G. S., Nampoothiri, K. M., & Pandey, A. (2011). Micro and macroalgal biomass: a renewable source for bioethanol. *Bioresource technology*, *102*(1), 186-193.
14. Lange, J. P. (2007). Lignocellulose conversion: an introduction to chemistry, process and economics. *Biofuels, Bioproducts and Biorefining: Innovation for a sustainable economy*, *1*(1), 39-48.
15. Lu, Q., Yang, X. C., Dong, C. Q., Zhang, Z. F., Zhang, X. M., & Zhu, X. F. (2011). Influence of pyrolysis temperature and time on the cellulose fast pyrolysis products: Analytical Py-GC/MS study. *Journal of Analytical and Applied Pyrolysis*, *92*(2), 430-438.
16. M. Nikzad, K. Movagharnejad, Comparative Studies on the Effect of Pretreatment of Rice Husk on Enzymatic Digestibility and Bioethanol Production doi: 10.5829/dose.ije.2013.26.05b.01.
17. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, *31*(3), 426-428.
18. Miller, S., & Hester, R. (2007). Concentrated acid conversion of pine sawdust to sugars. Part II: High-temperature batch reactor kinetics of pretreated pine sawdust. *Chemical Engineering Communications*, *194*(1), 103-116.
19. Moravvej, Z., Makarem, M. A., & Rahimpour, M. R. (2019). The fourth generation of biofuel. In *Second and third generation of feedstocks* (pp. 557-597). Elsevier.
20. Mustafa Hauwa M, Bashir AbdulRahman et al, Production of Bio-Ethanol From Sulfuric Acid Pre-Treated Rice Husk Using Co-Culture Of Saccharomyces Cerevisae And bacteria www.scienceworldjournal.org ISSN 1597-6343.
21. N. Shinde, Iqra Ansari et al, Extraction and Characterization of Bioethanol as Future Fuel www.jetir.org, ISSN-2349-5162.
22. Oyeleke, S. B., Dauda, B. E. N., Oyewole, O. A., Okoliegbe, I. N., & Ojebode, T. (2012). Production of bioethanol from cassava and sweet potato peels.
23. Pooja, N. S., Sajeev, M. S., Jeeva, M. L., & Padmaja, G. (2018). Bioethanol production from microwave-assisted acid or alkali-pretreated agricultural residues of cassava using separate hydrolysis and fermentation (SHF). *3 Biotech*, *8*, 1-12.

24. Prasad, R. K., Chatterjee, S., Mazumder, P. B., Gupta, S. K., Sharma, S., Vairale, M. G., ... & Gupta, D. K. (2019). Bioethanol production from waste lignocelluloses: A review on microbial degradation potential. *Chemosphere*, *231*, 588-606.
25. Rabah, A. B et al, Dilute acid pretreatment of millet husks for bioethanol production International Research Journal of Microbiology (IRJM) (ISSN: 2141-5463) Vol. 2(11) pp. 460- 465, December 2011.
26. Rabah, A. B., Oyeleke, S. B., Manga, S. B., & Hassan, L. G. (2011). Dilute acid pretreatment of millet and guinea corn husks for bioethanol production. *International Journal of Microbiology*, *2*(11), 460-465.
27. Rasaq S. Abolore, Swarna Jaiswal, Amit K. Jaiswal, Green and sustainable pretreatment
28. Ren, H., Zong, M. H., Wu, H., & Li, N. (2016). Efficient pretreatment of wheat straw using novel renewable cholinium ionic liquids to improve enzymatic saccharification. *Industrial & Engineering Chemistry Research*, *55*(6), 1788-1795.
29. Robak, K., & Balcerek, M. (2018). Review of second generation bioethanol production from residual biomass. *Food technology and biotechnology*, *56*(2), 174.
30. Schuerch, C. (1968). Methods of wood chemistry. vol. II. BL Browning, Ed., Wiley, New York, 1967. 498 pp. $18.75.
31. SE, A. (1989). Chemical analysis of ecological materials. *Analysis of vegetation and other organic materials*.
32. Sim, S. F., Mohamed, M., Lu, N. A. L. M. I., Sarman, N. S. P., & Samsudin, S. N. S. (2012). Computer-assisted analysis of fourier transform infrared (FTIR) spectra for characterization of various treated and untreated agriculture biomass. *BioResources*, *7*(4), 5367-5380.
33. Singh, P., & Raghuvanshi, R. S. (2012). Finger millet for food and nutritional security. *African Journal of Food Science*, *6*(4), 77-84.
34. Sumitha Banu J. et al., Saccharification and fermentation of Finger millet straw using recombinant hydrolytic enzymes and fermentative microbe for bioethanol production.
35. Sumitha Banu Jamaldheen et al., Comparative analysis of pretreatment methods on sorghum (Sorghum durra) stalk agro-waste for holocellulose content from its https://doi.org/10.1080/10826068.2018.1466148.
36. TAPPI. Technical Association of Pulp and Paper Industry, Atlanta, GA, 1992.

37. Tolan, J. S. (2005). Iogen's demonstration process for producing ethanol from cellulosic biomass. *Biorefineries-Industrial Processes and Products: Status Quo and Future Directions*, 193-208.
38. Ugare, R., Chimmad, B., Naik, R., Bharati, P., & Itagi, S. (2014). Glycemic index and significance of barnyard millet (Echinochloa frumentacae) in type II diabetics. *Journal of food science and technology*, *51*, 392-395.
39. USDA NCRS- United State Department of Agriculture Natural Resources Conservation Service. USDA NRCS Plant Materials Program, 2002. Plant Materials Program Web site: http://plant-materials.nrcs.usda.gov.