BIOETHANOL PRODUCTION FROM RICE HUSK USING NAOH PRETREATMENT AND ENZYMATIC HYDROLYSIS WITH *PENICILLIUM* AND *ASPERGILLUS FLAVUS*

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Abstract
Bioethanol can be produced from any biomass that contains sugars or starch which are lignocellulosic and do not compete with food crops. This study demonstrated the effectiveness of sodium hydroxide (NaOH) pretreatment followed by enzymatic hydrolysis by comparing enzymes from Penicillium and Aspergillus flavus to convert rice husk (RH) into fermentable sugars and bioethanol. Rice husk, a lignocellulosic biomass, was subjected to NaOH pretreatment at concentrations of 3.3%, 5%, 6.67%, 8.33%, and 10% w/v. Two different enzymes, Penicillium and Aspergillus flavus, were compared for enzymatic hydrolysis to break down cellulose and hemicellulose into fermentable sugars. The hydrolysates were subsequently fermented using Saccharomyces cerevisiae to produce bioethanol. The results revealed that increasing NaOH concentration significantly enhanced the breakdown of the lignocellulosic structure, leading to higher sugar yields. The maximum sugar content recorded was 21.6 °Brix for Aspergillus flavus and 16.0 °Brix for Penicillium at 10% w/v NaOH. Also, the ethanol yield increased with higher NaOH concentrations, reaching a peak of 40.95% for Penicillium and 34.35% for Aspergillus flavus at 10% w/v NaOH. Fourier-transform infrared (FTIR) spectroscopy confirmed the presence of ethanol in the distilled samples, and the confirmatory potassium dichromate test verified the successful production of bioethanol.

Keywords: Bioethanol, Lignocellulosic, Rice husk, Pretreatment, Enzymatic hydrolysis, Fermentation

Introduction

The growing environmental challenges in recent years and the need to reduce dependency on fossil fuels, have intensified interest in bioethanol as a sustainable alternative to vehicle fuel. Bioethanol not only offers the potential to reduce carbon monoxide (CO) emissions but also serves as an octane booster in gasoline, enhancing engine performance and fuel efficiency [1]. In many agricultural regions, rice husk and other lignocellulosic biomass are often burned or used as cattle fodder. However, the burning of these waste materials releases harmful gases into the atmosphere, causing environmental pollution [2]. Therefore, utilizing agro waste like rice husk for bioethanol production presents a more economically and environmentally favourable solution.

Lignocellulosic biomass (LB) is an abundant and cost-effective feedstock that holds significant promise for energy production. Its use can lead to substantial reductions in greenhouse gas emissions, as it is considered a carbon-neutral fuel source [3]. LB is composed of three primary structural polymers:

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[1] Malla, F. A., Bandh, S. A., Wani, S. A., Hoang, A. T., & Sofi, N. A. (2022). Biofuels: Potential Alternatives to Fossil Fuels. In S. A. Bandh & F. A. Malla (Eds.), *Biofuels in Circular Economy* (pp. 1–15). Springer Nature Singapore. https://doi.org/10.1007/978-981-19-5837-3_1.
[2] Moayedi, H., Aghel, B., Abdullahi, M. M., Nguyen, H., & Safuan A Rashid, A. (2019). Applications of rice husk ash as green and sustainable biomass. *Journal of Cleaner Production*, *237*, 117851. https://doi.org/10.1016/j.jclepro.2019.117851.
[3] Sooch, B. S., Mann, M. K., & Kaur, S. (2023). Lignocellulosic biomass: A feedstock to support the circular economy. In *Advances in Lignocellulosic Biofuel Production Systems* (pp. 23–46). Elsevier. https://doi.org/10.1016/B978-0-323-91192-4.00009-2.

cellulose, hemicellulose, and lignin, with cellulose being the most abundant[4]. These polymers can be hydrolysed into fermentable sugars, such as glucose and xylose, which can then be fermented with yeast to produce ethanol. Given its high carbohydrate content (37.1% cellulose, 19.5% hemicellulose, and 17.6% lignin), rice husk, an often unwanted by-product of rice cultivation, represents a valuable resource for bioethanol production. [5]

Alkaline pretreatment, particularly using sodium hydroxide (NaOH), has proven effective in preparing biomass for ethanol production through bioconversion. NaOH pretreatment facilitates the delignification and removal of hemicellulose, significantly increasing the surface area of the fibre, which enhances subsequent enzymatic hydrolysis. This method is both efficient and economical, making it suitable for large-scale bioethanol production [6].

Given the increasing demand for clean, renewable fuels and the need to reduce reliance on food crops for bioethanol production, there is a pressing need for sustainable production methods. Additionally, mitigating environmental pollution caused by the burning of lignocellulosic biomass is crucial. This study aims to investigate the effects of varying NaOH concentrations on the pretreatment of rice husk, followed by enzymatic hydrolysis using *Penicillium* and *aspergillus flavus* and batch fermentation with *Saccharomyces cerevisiae* (yeast) for bioethanol production. The goal is to identify the optimal conditions that maximize bioethanol yield while minimizing chemical usage and energy consumption, ultimately contributing to more cost-effective and sustainable bioethanol production methods.

Materials and Methodology

Preparation of samples

The materials used in this study were Rice husk (RH), NaOH, HCl, distilled water, *Penicillium, aspergillus flavus* and *Saccharomyces cerevisiae* (yeast). All chemicals used were of analytical grade. The rice husk samples from African rice (Oryza glaberrima) obtained from the ZIL Rice Processing Mill in Kaduna State, Nigeria, were carefully washed with distilled water to remove impurities. The samples were air-dried, milled using a crusher (Model No. GD-305AD JAPAN), and then screened with a sieve shaker to achieve a particle size of 0.5 mm. The screened materials were stored in a tightly sealed plastic container at room temperature under dry condition to avoid contamination and moisture absorption.

Dilute-NaOH Solution Pretreatment

20g of RH sample was soaked in 150 ml of various concentrations sodium hydroxide (NaOH): 3.3%w/v, 5%w/v, 6.67%w/v, 8.33%w/v and 10%w/v. The samples were subjected to 15 minutes autoclaving at 121°C and 15 psi and allowed to cool at room temperature for 30 minutes, after which the pH was adjusted to 4.5 with a 10% H_2SO_4 solution. The solid residue was separated from the liquid by filtration. The treated samples were used for further studies.

Enzymatic hydrolysis & Fermentation

The samples resulting from the pretreatment with varying concentrations of diluted NaOH were subjected to enzymatic hydrolysis to break down the cellulose and hemicellulose into sugars that the yeast could use (Zhao et al., 2021). Two different enzymes were used for the hydrolysis process: *Penicillium* and *Aspergillus flavus*, each with an enzyme dose of 0.04 mL/g of the sample. This resulted

[4] Shrestha, S., Fonoll, X., Khanal, S. K., & Raskin, L. (2017). Biological strategies for enhanced hydrolysis of lignocellulosic biomass during anaerobic digestion: Current status and future perspectives. *Bioresource Technology*, *245*, 1245–1257. https://doi.org/10.1016/j.biortech.2017.08.089.

[5] Malik, K., Sharma, P., Yang, Y., Zhang, P., Zhang, L., Xing, X., Yue, J., Song, Z., Nan, L., Yujun, S., El-Dalatony, M. M., Salama, E.-S., & Li, X. (2022). Lignocellulosic biomass for bioethanol: Insight into the advanced pretreatment and fermentation approaches. *Industrial Crops and Products*, *188*, 115569. https://doi.org/10.1016/j.indcrop.2022.115569.

[6] Xu, L., Zhang, S.-J., Zhong, C., Li, B.-Z., & Yuan, Y.-J. (2020). Alkali-Based Pretreatment-Facilitated Lignin Valorization: A Review. *Industrial & Engineering Chemistry Research*, *59*(39), 16923–16938. https://doi.org/10.1021/acs.iecr.0c01456.

in a total of 10 samples: 5 samples with *Penicillium* and 5 samples with *Aspergillus flavus*, each corresponding to the different NaOH concentrations.

The fermentation and hydrolysis were conducted simultaneously using the simultaneous saccharification and fermentation (SSF) process, as detailed by[7]. Saccharomyces cerevisiae (yeast) was aseptically added to each flask, and the flasks were incubated at 30°C for 4 days. The flasks were sealed and wrapped with cotton wool and aluminium foil.

Fractional distillation

Distillation of the fermented liquid was performed according to the method described by [8]. The round bottom flask was used to collect the fermented liquid and placed on a heating by mantle connected to the distillation setup. The distillate was collected at 78ºC using the second flask attached to the setup.

Analytical Techniques for Bioethanol Production

The bioethanol produced was analyzed at various stages of production using several analytical methods, including pH measurement of the pretreated samples, ethanol yield assessment, total sugar estimation of the hydrolyzed samples, percentage alcohol determination of the distillate, FTIR analysis, and confirmatory tests for ethanol.

Ethanol Yield Estimation

The volumetric ratio of the distillate to the quantity of fermented substrate was recorded as ethanol yield [9].

Total Sugar Estimation

The total sugar content of the hydrolysate sample was determined by dropping 0.5ml on the glass slide of a hand refractometer. The brix value (°Brix) was then recorded, following the procedure outlined in AOAC (2000) [10].

percentage Alcohol Estimation

The alcohol percentage in the produced ethanol was determined using a Brix table in accordance with AOAC (2000) standards.

Potassium Dichromate Test

Ethanol presence in the samples was confirmed using the potassium dichromate method. Two drops of potassium dichromate solution were added to 5 ml of the distillate samples in a test tube, which was then heated for 30 minutes in a water bath [11].

FTIR Test

The functional groups and bond structures of bioethanol produced from rice husk, pretreated with different concentrations of NaOH, were analyzed using Fourier-transform infrared (FTIR) spectroscopy to compare the enzymatic hydrolysis efficiency of Penicillium and Aspergillus flavus. This analysis was performed using a PerkinElmer Spectrum 400 FTIR/FT-FIR spectrometer within the range of 4000–400 cm^{-1}.

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[7] Rastogi, M., & Shrivastava, S. (2017). Recent advances in second generation bioethanol production: An insight to pretreatment, saccharification and fermentation processes. *Renewable and Sustainable Energy Reviews*, *80*, 330–340. https://doi.org/10.1016/j.rser.2017.05.225.

[8] Sivakumar, K., & Sangavai, C. (2019). *BIOETHANOL PRODUCTION BY USING MICROBIAL ENZYME*. *November*.

[9] Bermejo, P. M., Badino, A., Zamberlan, L., Raghavendran, V., Basso, T. O., & Gombert, A. K. (2021). Ethanol yield calculations in biorefineries. *FEMS Yeast Research*, *21*(8), foab065. https://doi.org/10.1093/femsyr/foab065.

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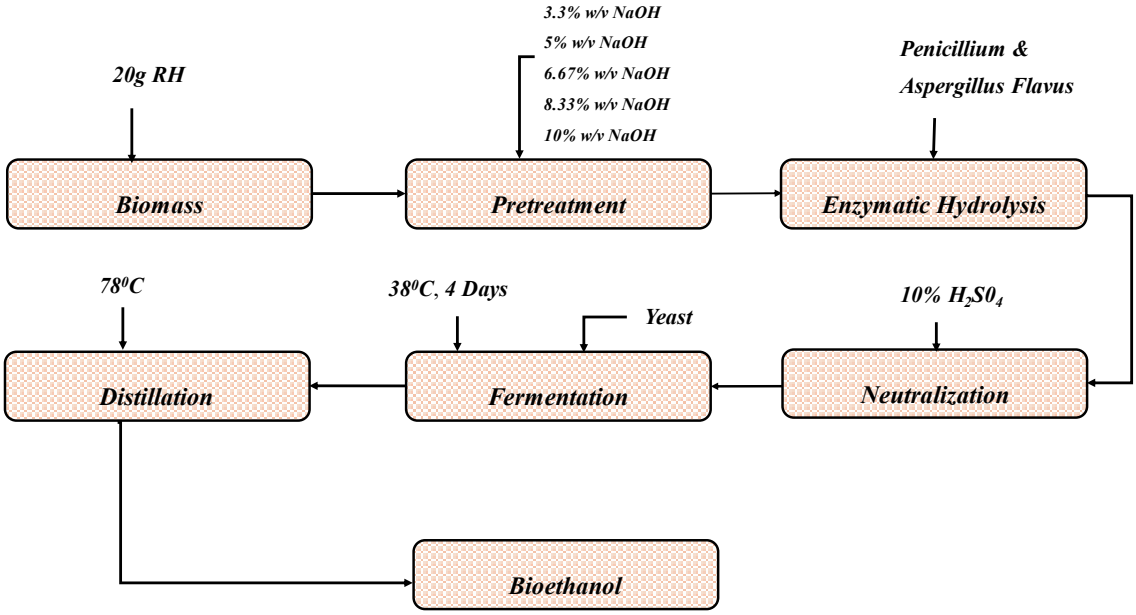

Figure 1: Process flow diagram to produce bioethanol

Result and Discussion

Sugar Content

The sugar content after pretreatment of the samples with NaOH at varying concentrations was estimated using a Brix refractometer. The results, presented in Figure 2, indicate that the highest total sugar content estimated was 16.00 °Brix and 21.6 °Brix for *Penicillium* and *Aspergillus flavus* hydrolysed samples, respectively, at 10% w/v NaOH. The lowest sugar content was recorded at 2% w/v NaOH, with *Penicillium* hydrolysed samples at 3.15 °Brix and *Aspergillus flavus* hydrolysed samples at 5.1 °Brix. These results suggest that a significant portion of cellulose and hemicellulose was successfully exposed during the pretreatment, making them more accessible for hydrolysis into fermentable sugars. The higher NaOH concentration effectively disrupts the lignocellulosic structure, enhancing the exposure of cellulosic and hemicellulosic components. This leads to an increased yield of fermentable sugars, which are essential for bioethanol production.

The observed sugar content is consistent with findings from similar studies on alkaline pretreatment of lignocellulosic biomass. For example, a study by [12] investigated the pretreatment of olive stone (OS) biomass using NaOH in a reactive extrusion process. Their study demonstrated that a NaOH/biomass ratio of 15% (dry weight basis) at 125 °C significantly enhanced sugar release, achieving carbohydrate conversion rates of 55.5% for cellulose and 57.7% for xylan.

[12] Doménech, P., Duque, A., Higueras, I., Iglesias, R., & Manzanares, P. (2020). Biorefinery of the Olive Tree—Production of Sugars from Enzymatic Hydrolysis of Olive Stone Pretreated by Alkaline Extrusion. *Energies*, *13*(17), 4517. https://doi.org/10.3390/en13174517.

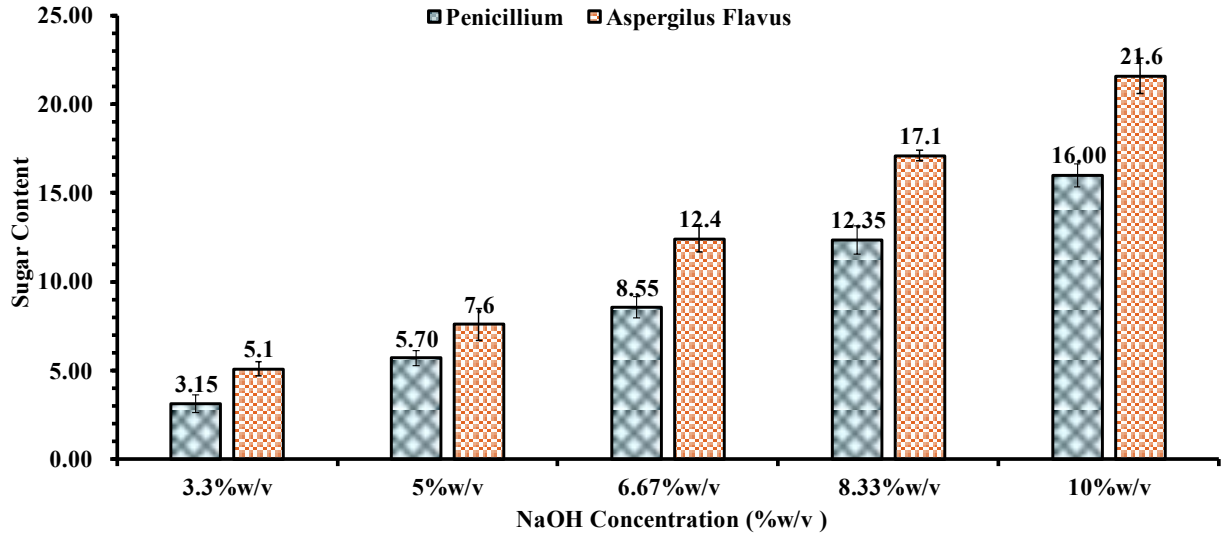

Figure 2 Sugar Content of Penicillium and Aspergillus flavus hydrolysed samples treated with Varying NaOH concentrations

Ethanol Yield

The volumetric ratio of the distillate to the quantity of fermented substrate recorded, demonstrated that ethanol yield increased with increase in concentrations of sodium hydroxide (NaOH). As illustrated in Figure 3, there was a significant increase in ethanol yield from 17.30% to 40.95% as the concentration of NaOH increased from 3.3% w/v to 10% w/v for samples hydrolysed with *Penicillium*. Similarly, ethanol yield increased from 11.25% to 34.35% for samples hydrolysed with *Aspergillus flavus* under the same NaOH concentration range.

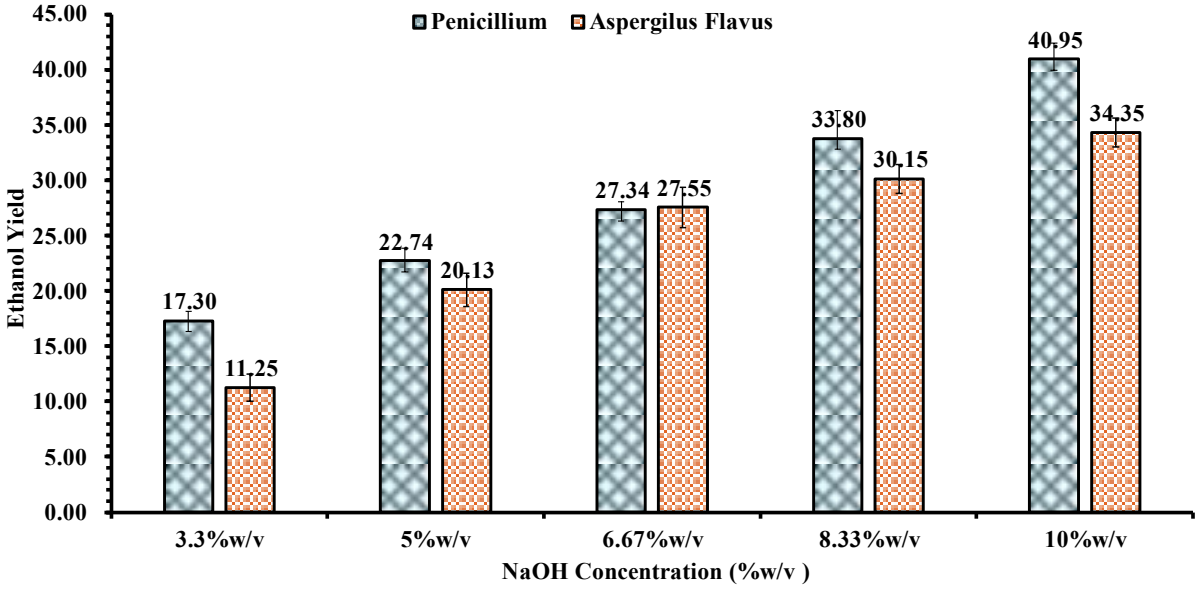

Figure 3: Ethanol Yield of Penicillium and Aspergillus flavus hydrolysed samples treated with Varying NaOH concentrations

These results demonstrate a clear correlation between NaOH concentration during pretreatment and ethanol yield from rice husk. The observed increase in ethanol production with increasing NaOH

concentration aligns with findings from previous research. For example, [13] showed that NaOH pretreatment significantly enhanced cellulose conversion in sorghum straw, resulting in improved enzymatic hydrolysis and increased ethanol yield. Similarly, [14] observed that an optimal NaOH concentration maximized ethanol yields from pine needles. These findings, along with the results from our study, suggest that NaOH pretreatment is an effective strategy for improving the conversion of lignocellulosic biomass, such as rice husk, into ethanol.

Percentage Alcohol

The percentage of alcohol in the distillate samples was used determined using Brix table, revealing that the percentage alcohol increases with increase in the sugar content of the samples. The maximum alcohol percentages recorded were 8.75% for *Penicillium* hydrolysed samples and 12.6% for *Aspergillus flavus* hydrolysed samples, both at 10% w/v NaOH.

These findings are consistent with existing literature on the relationship between sugar content and alcohol yield in lignocellulosic biomass conversion. For instance, a study by Mustapha et al. (2019) demonstrated that higher sugar concentrations after pretreatment directly correlate with increased alcohol yields during fermentation. In their study, rice husk treated with 10% H_2S0_4 resulted in the highest ethanol yield, due to the enhanced availability of fermentable sugars. The alcohol percentages obtained in this study align with these findings, further emphasizing the importance of optimizing pretreatment conditions, such as NaOH concentration, to maximize both sugar release and ethanol production in lignocellulosic biomass processes.

The confirmatory test for bioethanol was conducted adding 2 drops of potassium dichromate, heated in a water bath for 30mins to each distilled sample. Formation of green color was observed for all samples there by indicating presence of bioethanol.

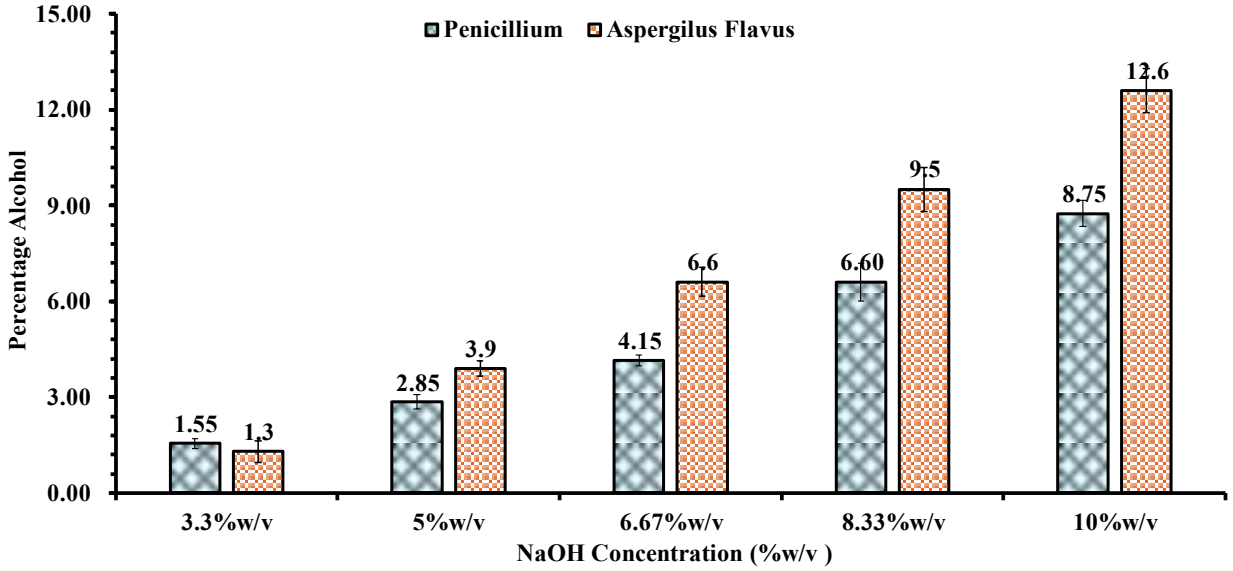

Figure 4: Percentage Alcohol of Penicillium and Aspergillus flavus hydrolysed samples treated with Varying NaOH concentrations

[13] Bhati, N., & Sharma, A. K. (2023). Comparative study of different chemical pretreatments for enhanced enzymatic hydrolysis of sorghum straw. *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-023-05185-7.

[14] Irfan, M., Nadeem, M., Syed, Q., & Qazi, J. I. (2016). Statistical optimization of dilute acid pretreatment of pinus needles to be used as substrate for biofuel production. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, *38*(14), 1983–1992. https://doi.org/10.1080/15567036.2015.1037973.

Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Several common peaks were observed across all samples. The transmittance spectra for the Penicillium and Aspergillus flavus hydrolyzed samples are illustrated in Figures 4 and 5. Notable peaks included those in the 3400–3200 cm^{-1} range, indicating the presence of hydroxyl groups; 2356–2322 cm^{-1} and 1658–1638 cm^{-1}, suggesting the presence of alkene groups; 1384–1377 cm^{-1} and 1060–1001 cm^{-1}, corresponding to ethanol and glucose, respectively; and a broad band around 3336 cm^{-1}, attributed to water. Additionally, an intense peak around 1045 cm^{-1} was observed, which is commonly used to identify ethanol, as noted by Zhengcai Pu [15] and Veale et al[16]. Comparing the FTIR results from this study with those from other research, Kassim and Bhattacharya[17] reported that the peak between 3400 and 3200 cm^{-1} corresponds to the hydroxyl (OH) group in the samples. Furthermore, the absorption band between 1658 and 1638 cm^{-1} indicates the presence of an alkene group with variable C=C bonds, exhibiting medium intensity.

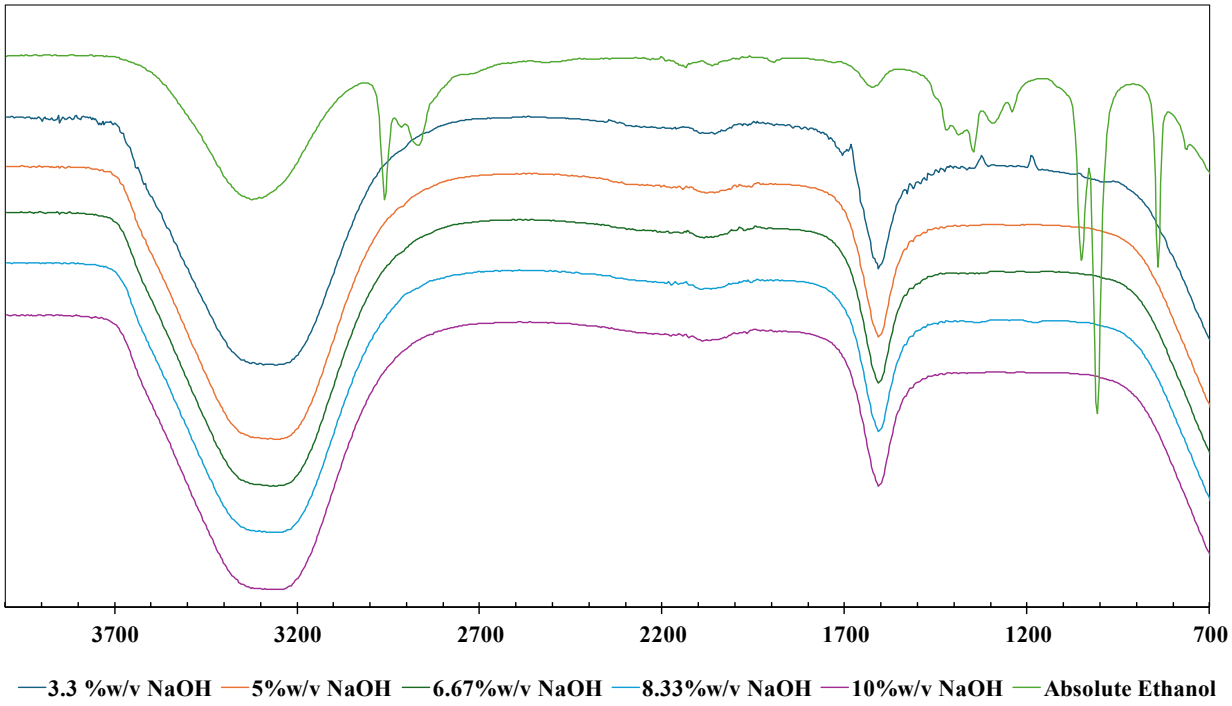

Figure 5: FT-IR spectra of Penicillium hydrolysed samples treated with varying NaOH concentrations

[15] Zhengcai Pu, Van Ooij, W. J., & Mark, J. E. (1997). Hydrolysis kinetics and stability of bis(triethoxysilyl)ethane in water-ethanol solution by FTIR spectroscopy. *Journal of Adhesion Science and Technology*, *11*(1), 29–47. https://doi.org/10.1163/156856197X01001.

[16] Veale, E. L., Irudayaraj, J., & Demirci, A. (2007). An On-Line Approach To Monitor Ethanol Fermentation Using FTIR Spectroscopy. *Biotechnology Progress*, *23*(2), 494–500. https://doi.org/10.1021/bp060306v.

[17] Kassim, M. A., & Bhattacharya, S. (2016). Dilute alkaline pretreatment for reducing sugar production from *Tetraselmis suecica* and *Chlorella* sp. Biomass. *Process Biochemistry*, *51*(11), 1757–1766. https://doi.org/10.1016/j.procbio.2015.11.027.

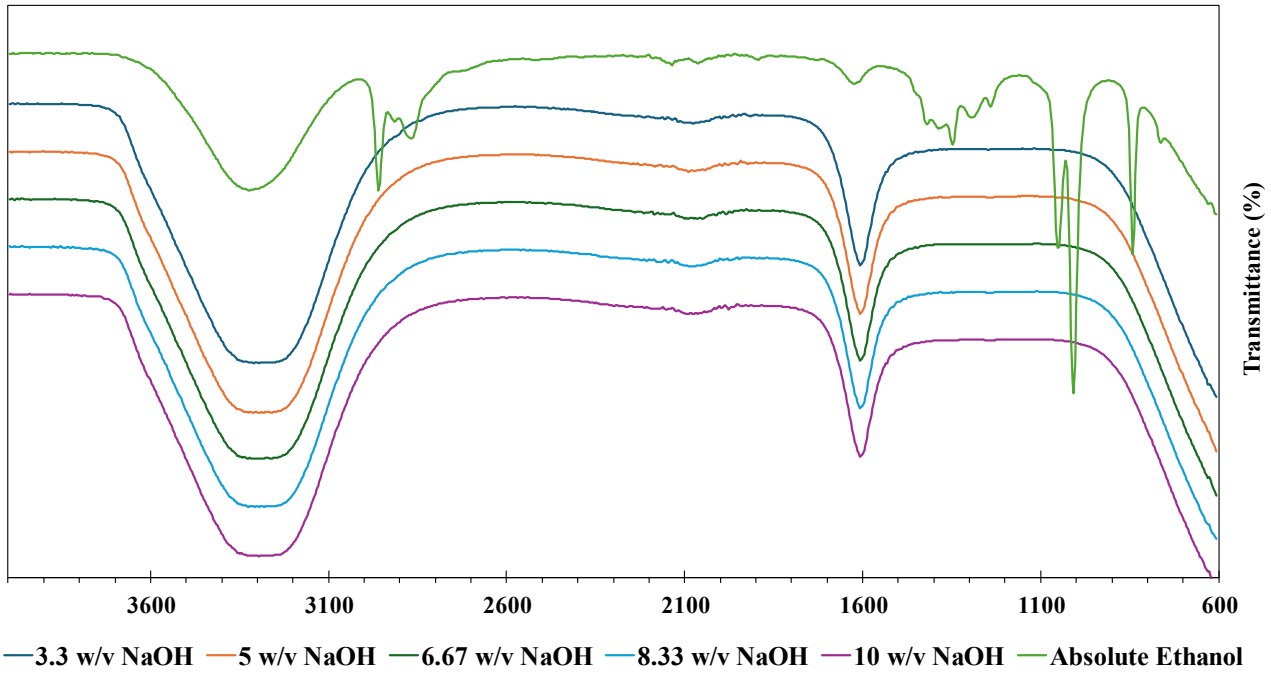

Figure 6: FT-IR spectra of Aspergillus flavus hydrolysed samples treated with varying NaOH concentrations.

CONCLUSION

This study demonstrated the effectiveness of sodium hydroxide (NaOH) pretreatment followed by enzymatic hydrolysis by comparing enzymes from *Penicillium* and *Aspergillus flavus* to convert rice husk (RH) into fermentable sugars and bioethanol. The results showed that increasing the NaOH concentration significantly enhanced the breakdown of lignocellulosic structures, leading to higher sugar release and, consequently, increased ethanol yield. Specifically, at 10% w/v NaOH, *Aspergillus flavus* outperformed *Penicillium* in terms of sugar conversion and ethanol production, achieving a maximum sugar content of 21.6 °Brix and an ethanol yield of 34.35%, compared to 16.00 °Brix and 40.95% for *Penicillium*. The study further confirmed that the percentage of alcohol produced is related to the sugar content available after enzymatic hydrolysis, with the maximum alcohol percentage recorded at 10% w/v NaOH. FTIR analysis provided additional confirmation of the successful production of bioethanol, with characteristic peaks corresponding to the presence of ethanol and other functional groups.