The Pharma Innovation Journal 2023; SP-12(12): 560-567

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(12): 560-567

www.thepharmajournal.com
Received: 26-10-2023
Accepted: 30-11-2023

Kiran KC
Department of Forestry and Environmental Science, University of Agricultural Sciences, Bangalore, Karnataka, India

Prasanna KT
Department of Forestry and Environmental Science, University of Agricultural Sciences, Bangalore, Karnataka, India

Corresponding Author:
Kiran KC
Department of Forestry and Environmental Science, University of Agricultural Sciences, Bangalore, Karnataka, India

Foxtail millet (*Setaria italica*) husk: A potential source for bioethanol production

Kiran KC and Prasanna KT

Abstract
The current energy landscape, the rising demand for sustainable alternatives has intensified the focus on bioethanol. This study investigates the composition and pretreatment effects on foxtail millet husk (FH) as a potential bioethanol feedstock. FH with 38.44% cellulose and 33.71% hemicellulose, demonstrates promise for bioethanol production. Various pretreatments are evaluated, with the pretreatment N_2P_2 yielding the highest cellulose content (64.29%). Subsequent simultaneous saccharification and fermentation (SSF) with *Saccharomyces cerevisiae* resulted in highest ethanol yields of 13.77% (N_2P_1) and 12.80% (N_2P_2). Additionally, the combination of (N_1P_2) with SSF using *Zymomonas mobilis* exhibited the highest ethanol yield (9.42%), albeit slightly lower than the yields obtained with *Saccharomyces cerevisiae*. These findings highlight the efficacy of different pretreatment strategies, particularly the N_2P_2 combination, in enhancing cellulose content and subsequently maximizing ethanol production.

Keywords: Foxtail millet husk, pretreatment, SSF, *Saccharomyces cerevisiae*, *Zymomonas mobilis*, bioethanol production

Introduction
Due to increase in human population and increasing industrial prosperity in developing countries the global demand for energy continues to grow. The major energy demand is still met from the conventional fossil fuels such as oil, coal and natural gas. Utilization of fossil fuels over the last century and following years has drastically increased the level of greenhouse gases in the earth's atmosphere (Ballesteros *et al*., 2006) [3] so, interest in search of alternative fuels and among them renewable energy sources such as solar energy, biodiesel and bio-ethanol production from biomass are the most appropriate (Saha *et al*., 2014) [28]. The oil demand is expected to increase to 57% from 2002 to 2030. In the total primary energy supply, the contribution of fossil fuels (81%), nuclear energy (5%) and renewable energy sources 14% (of which the contribution of biomass is about 70%) (REN21, 2019) [24].

Today, bioenergy is the largest source of renewable energy globally, accounting for 55% of renewable energy and over 6% of global energy supply. As per the Net Zero Emissions by 2050 (NZE) Scenario, bioenergy is expected to replace fossil fuels at a rapid rate by 2030. The utilization of contemporary bioenergy has exhibited an upward trend, with an average annual growth of approximately 3% between 2010 and 2022. While simultaneously ensuring that the production of bioenergy does not incur negative effects on society or the environment, more work must be done to expedite the deployment of modern bioenergy in order to meet the NZE Scenario, which calls for an 8% annual increase in deployment between 2022 and 2030 (IEA, 2023) [14]. Bioenergy refers to the energy content in solid, liquid and gaseous products derived from biomass (IEA, 2010) [14].

Ethanol is one of the most promising biofuels derived from any material containing simple or complex sugars. Sugar and starch-based materials such as sugarcane and grains are two groups of raw materials currently used as the main resources for ethanol production. The third group is lignocellulosic materials (Second generation) produced from agricultural residues representing the most viable option for production of ethanol.

India's National Policy on Biofuels, 2018 sets ambitious biofuel blending targets and aims to source biofuels only from sustainable feedstocks. Feedstocks are primarily defined as non-food feedstocks that do not threaten food security. Specifically, India intends to build upon its previous ethanol mandate by expanding ethanol blending to 20% by 2030; the policy also adds a supplemental biodiesel mandate of 5%.

Second generation ethanol technologies are complicated and their efficiencies can be influenced by many factors such as the type of lignocellulosic feedstock, pre-treatment methods, the microorganisms used, Therefore, to find an optimum combination of all variables for a particular feedstock is challenging (Eggert and Greaker, 2014) [9].

Agricultural residues are the residues (Leaves and stalks) of plants left over after harvesting and the residues obtained from post-harvest operation (Husk, maize cob). They are generally considered to be a sustainable alternative to food crops purpose-grown for biofuels (Second generation), as they can be collected without expanding cropland. Foxtail millet husk (FH) is one such residue that will be available due to the growing usage of millets in daily diets due to their high nutritional content. In the future, India and world will produce more of these millets and the husk from them will be less expensive when used to produce bioethanol on a large scale.

The present investigation is undertaken to evaluate potential of foxtail millet husk in the generation of bioethanol through different physico-chemical pretreatment and hydrolysis methods using *Saccharomyces cerevisiae* and *Zymomonas mobilis* as fermenting microorganisms.

Materials and Methods

Collection and preparation of raw material

The FH was procured from Centre of excellence nutri-cereals, GKVK, Bengaluru. The collected FH was shade dried and oven dried at 80 °C for 48 hours. Then, they were grounded, sieved through 2 mm sieve and stored in air tight bags at room temperature for further use.

Characterization of biomass

Various properties of FH were analyzed prior to the different pretreatment to know the composition of FH. The FH was characterized for both the physico-chemical properties *viz*., cellulose, hemicellulose, lignin, ash content, carbon and nitrogen content. The cellulose and hemicellulose were estimated by the procedure outlined by Fruedenburg (1955) [11], Lignin was estimated through the procedure given by Pandey *et al*., (2007) [22], the ash content determined by AOAC (2000) [2] method and the total carbon and nitrogen were estimated using the CN analyzer (LECO Truspec, USA 2009).

Pretreatment

The FH was pretreated with the following chemicals (Table 1) with solid loading 8% (w/v). Samples were autoclaved at 121 °C (at 15 psi) for 1 h and these pretreated samples were filtered, solid part was collected, oven dried and stored in air tight bags at room temperature.

Table 1: Different pretreatments evaluated for feedstock preparation in bioethanol production

Sl. No.	Pretreatment	Concentration	Code
1	Control	Soaking in water for 24 hours	C
2	Autoclave	15 psi, 121 ℃, 1 hour	A1
Acid			
3	Sulphuric Acid	1% H_2SO_4	H1
4		2% H_2SO_4	H2
5	Hydrogen peroxide	5% H_2O_2	P1
6		10% H_2O_2	P2
Alkali			
7	Sodium hydroxide	2% NaOH	N1
8		4% NaOH	N2
Autoclaved + Acid Combination			
9	Autoclaved with Sulphuric Acid	1% H_2SO_4, 15 psi, 121 ℃, 1 h	A_1H_1
10		2% H_2SO_4, 15 psi, 121 ℃, 1 h	A_1H_2
Autoclaved + Alkali Combination			
11	Autoclaved with Sodium hydroxide	1% NaOH, 15 psi, 121 ℃, 1 h	A_1N_1
12		2% NaOH, 15 psi, 121 ℃, 1 h	A_1N_2
Acid + Acid Combination			
13	Sulphuric Acid and Hydrogen peroxide	1% H_2SO_4 + 5% H_2O_2	H_1P_1
14		1% H_2SO_4 + 10% H_2O_2	H_1P_2
15		2% H_2SO_4 + 5% H_2O_2	H_2P_1
16		2% H_2SO_4 + 10% H_2O_2	H_2P_2
Alkali + Acid Combination			
17	Sodium hydroxide and Sulphuric Acid	2% NaOH + 1% H_2SO_4	N_1H_1
18		2% NaOH + 2% H_2SO_4	N_1H_2
19		4% NaOH + 1% H_2SO_4	N_2H_1
20		4% NaOH + 2% H_2SO_4	N_2H_2
21	Sodium hydroxide and Hydrogen peroxide	2% NaOH + 5% H_2O_2	N_1P_1
22		2% NaOH + 10% H_2O_2	N_1P_2
23		4% NaOH + 5% H_2O_2	N_2P_1
24		4% NaOH + 10% H_2O_2	N_2P_2

Inoculum preparation

The *Saccharomyces cerevisiae* (fungi) culture was obtained from the Department of Agricultural Microbiology, UAS, GKVK, Bengaluru and maintained on MGYP medium (Composition: Malt extract 3 g, Glucose 10 g, Yeast extract 3 g, Peptone 5 g, Agar 20 g, distilled water 1000 mL). The *Zymomonas mobilis* (bacteria) (*Zymomonas mobilis* sub sp. *Mobilis*, MTCC No. 91) culture obtained from MTCC, Chandigarh and maintained on MTCC-recommended medium (3.5 g/L K_2HPO_4, 7.5 g/L $(NH_4)_2SO_4$, 0.75 g/L $MgSO_4.7H_2O$, 1 g/L $CaCl_2.2H_2O$, and 5 g/L yeast extract). The initial pH of microbial cultivation medium was adjusted to 4.5 for fungi and 7 for bacteria using NaOH /HCl.

Simultaneous Saccharification and fermentation (SSF)

The solution containing the citrate buffer (0.05M) and the media (composition: 3.5 gL^{-1} K_2HPO_4, 7.5 gL^{-1} $(NH_4)_2SO_4$, 0.75 gL^{-1} $MgSO_4.7H_2O$, 1 gL^{-1} $CaCl_2.2H_2O$ and 5 gL^{-1} yeast extract) were added in a 1 L fermenting bottle with pretreated sample of 100 g, the initial pH was adjusted to 4.5 for fungi, 7 for bacteria and autoclaved for 20 minutes at 121 °C at 15 psi. These samples were used for fermentation. Along with of commercial enzyme (1% v/v), 10% (v/v) inoculum was added and these bottles were incubated at 30 °C for fermentation.

Estimation of ethanol

The one ml of SSF samples were taken from each bottle and diluted with 35 ml distilled water. Each sample was distilled at 70 °C and the distillate containing alcohol was collected in 25 ml of 0.23 N $K_2Cr_2O_7$ solution, till total volume of 45 ml was obtained. Similarly, ethanol standards (0 – 100 mg concentration) were prepared separately using absolute ethanol. These samples and standards were kept in water bath at 60 °C for 30 min and were cooled, volume was made up to 50 ml with distilled water and optical density was measured at 600 nm using spectrophotometer (Multiskan Sky, Thermo scientific). The standard curve was plotted considering the known concentration against absorbance. From the standard graph, the amount of ethanol in the sample was calculated (Caputi *et al*., 1968) [6].

Statistical Analysis

The data were analyzed statistically for ethanol yield by complete randomized design using R software (Version 4.2.2). The significance level for determining the statistical significance of the means for different pretreatment methods and SSF using different microorganisms was set at $p<0.05$.

Results and Discussion

Raw material characterization

The FH was characterized to determine the composition of raw material were given in table 2. Cellulose accounts for 38.44±0.17% to the dry weight of raw material and the hemicellulose content was found to be 33.71±0.29% of dry biomass. The presence of 72.15±0.46% holocellulose content in the cell wall of FH, which provides a potential feedstock for bioethanol production. FH contains 20.84±0.17% lignin, 12.59±0.30% ash content, 42.14±0.04% of total carbon, 1.23±0.02% of nitrogen content and 34.30±0.51% of CN ratio. The similar outcomes were reported by Cao *et al*. (2015) [5] in millet husk having cellulose content (38.9%), hemicellulose content (16.8%) and lignin content (15.1%), Zeenat *et al*. (2021) [33] reported cellulose content 37.81% in millet husk and Hammajam *et al*. (2017) [12] reported hemicellulose content 23.17% and lignin 13.19% in millet husk. The difference in the cell wall composition was due to heterogeneity in raw material, geographical location, season, processing methods and analytical methods used for chemical composition (Silverstein *et al*., 2007 and Binod *et al*., 2012) [30, 4].

Table 2: Composition analysis of FH

Parameters	Composition (%)
Cellulose	38.44±0.17
Hemicellulose	33.71±0.29
Holocellulose	72.15±0.23
Lignin	20.84±0.17
Ash	12.59±0.30
Total carbon	42.14±0.04
Nitrogen	1.23±0.02
C/N ratio	34.30±0.51

Effect of pretreatments on the feedstock composition of FH

Pre-treatment is an important tool for cellulose conversion processes and is essential to change the structure of cellulosic biomass to make cellulose more available to the enzymes that convert the complex carbohydrate molecules to simpler sugars increasing the accessibility of enzymatic saccharification (Anita and Pavithra, 2019) [1]. The pretreatments were imposed on the FH for the removal or to breakdown the lignin and hemicellulose, to reduce the crystallinity of cellulose. The suitable pretreatment and condition usually depend on the type of the lignocellulosic content present in the raw material (Taherzadeh and Karimi, 2008) [31].

The utilization of various concentrations of acid, alkali and combinations for the pretreatment of FH shown in Table 3. The pretreatment combination of 4% NaOH + 10% H_2O_2 (N_2P_2) resulted in yielding the highest cellulose content (64.29%), which is 67.25% higher than the control (38.44%). Among the various treatments, alkali and acid combination found to be the highest cellulose content (57.17%) and within this group, NaOH with H_2O_2 showed the highest. NaOH induces swelling in foxtail straw, resulting in an increased internal surface area and the rupture of lignin structures (Kiran and Prasanna, 2022) [16]. This swelling effect enhances the vulnerability of cellulose to enzymes, leading to improved glucose yields (Huzir *et al*., 2018) [13]. Subsequently, in the same process, hydrogen peroxide (H_2O_2) plays a crucial role. The breakdown of H_2O_2 under alkaline conditions generates hydroxyl radicals, which are instrumental in the deconstruction of hemicellulose and lignin (Rabetafika *et al*., 2014) [23]. H_2O_2 itself promotes delignification through oxidative reactions (Lai, 2001) [18]. However, caution must be exercised, as higher concentrations of H_2O_2 can lead to a rapid increase in the rate of oxygen evolution, reducing its incorporation at lignin sites and diminishing delignification efficiency (Rojith and Singh, 2013) [25]. The concentration of H_2O_2 used in delignification processes is adjusted based on the lignin content of the biomass (Ross *et al*., 2008) [26]. In summary, the procedure begins with the swelling effect induced by NaOH, followed by the generation of hydroxyl radicals and oxidative reactions facilitated by H_2O_2 in the subsequent delignification process. The order of treatments based on their effectiveness in increasing cellulose content in the present study was: Autoclaved + Alkali Combination > Alkali + Acid Combination > Alkali > Acid + Acid Combination > Autoclaved + Acid Combination > Acid > Autoclaved > Control.

The control had the highest hemicellulose content (33.71%) and the highest loss was observed in the pretreatment combination of 2% H_2SO_4 + 5% H_2O_2 (H_2P_1) and 2% H_2SO_4 + 10% H_2O_2 (H_2P_2) (16.54% and 16.90%, respectively) were statistically on par with each other, which is 50.93% lower than the control (Table 3). Chemical pretreatment in bioethanol production induces a decrease in hemicellulose content through several mechanisms. The application of acids or alkalis during pretreatment initiates hydrolysis, breaking down complex hemicellulose structures into monomeric sugars. Simultaneously, chemical agents solubilize hemicellulose, allowing its extraction into the liquid fraction for subsequent processing. Acid-catalyzed cleavage reactions further fragmentize hemicellulose polymers, facilitating their conversion into fermentable sugars. Importantly, the modification or removal of hemicellulose enhances the accessibility of cellulose to enzymatic hydrolysis, a crucial step in ethanol production. The order of treatments based on their effectiveness in reducing hemicellulose content in the present study was: Control > Autoclaved > Alkali + Acid Combination > Autoclaved + Alkali Combination > Alkali > Acid > Acid + Acid Combination > Autoclaved + Acid Combination.

The pretreatment with 4% NaOH + 10% H_2O_2 (N_2P_2) led to the lowest ash content (3.96%) due to the effective removal of mineral impurities during the alkaline conditions of NaOH treatment. This resulted in a substantial reduction of 68.55% compared to the control (12.59%), emphasizing the efficiency of this pretreatment in minimizing ash content in the biomass. The order of treatments based on their effectiveness in reducing ash content was: Alkali + Acid Combination > Autoclaved + Alkali Combination > Alkali > Acid > Autoclaved + Acid Combination > Acid + Acid Combination > Autoclaved > Control (Table 3).

The highest solid loss (65.26%, 65.13%, 68.03%, and 67.38%, respectively) and the lowest solid recovery (31.97%, 32.62%, 34.74%, and 34.87%, respectively) were observed in the pretreatment with 4% NaOH (N2), autoclaved with 4% NaOH (N2), 4% NaOH with 10% H_2O_2 (N_2P_2), and 4% NaOH with 5% H_2O_2 (N_2P_1) were statistically on par with each other, while the lowest solid loss was observed in the control (Table 3). Solid loss during the pretreatment of raw materials in bioethanol production occurs due to multiple factors. Mechanical pretreatment methods, such as milling, may physically break down biomass, leading to the loss of fine particles. The breakdown of complex lignocellulosic structures leads to the solubilization of hemicellulose and leaching of soluble sugars, contributing to the loss of biomass solids. Additionally, the dissolution of cellulose and lignin, especially under severe pretreatment conditions, can further diminish the solid fraction. The alkali pretreated feedstock found to be the highest solid loss and lowest solid recovery (64.02% and 35.98%, respectively). The order of treatments based on the high solid loss was: Alkali > Autoclaved + Alkali Combination > Alkali + Acid Combination > Autoclaved + Acid Combination > Acid + Acid Combination > Acid > Autoclaved > Control.

The NaOH pretreatment substantially boosts ethanol yield in wheat straw by enhancing cellulose accessibility to enzymes (Shao *et al*., 2014) [29]. Combining alkali and acid pretreatment, particularly NaOH and H_2O_2, yields the highest ethanol concentration, consistent with Maurya and Gnansounou's (2019) [19] findings on rice straw. Ethanol production from rice husk using palm wine yeast resulted in a 6.60±0.48% yield, surpassing baker's yeast at 5.60±0.42% (Chukwuma *et al*., 2014) [7]. However, Oyeleke and Jibrin (2009) [21] reported higher ethanol yields in guinea corn husk (26.83 g/l) and millet husk (18.31 g/l) compared to the present study. Similarly, Nachaiwieng *et al*. (2015) [20] explored factors influencing SSF for ethanol production from rice husk cellulose, achieving an actual ethanol yield of 15.63 g/L under optimal conditions, a 1.44-fold increase compared to separate hydrolysis and fermentation. These diverse outcomes underscore the nuanced interplay of pretreatment methods, yeast strains and biomass sources in optimizing bioethanol production.

Similarly, Copur *et al*. (2013) [8] investigated the effect of different pretreatment methods on the composition of hazelnut husk. They found that after steam explosion, the holocellulose, lignin, and ash content increased from 41.10%, 39%, and 5.43%, respectively, to 45.04%, 37.4%, and 5.92%, respectively. Additionally, they found that chemical pretreatment with sulfuric acid, sodium hydroxide, hydrogen peroxide and sodium borohydrate resulted in solid recovery rates ranging from 63.2% to 79.1% and solid loss rates ranging from 18.3% to 36.8%. Lignin reduction rates ranged from 1.67% to 42.5%, with the highest reduction observed with hydrogen peroxide pretreatment. Overall, these studies demonstrate that different pretreatment methods can significantly alter the chemical composition of lignocellulosic materials, and highlights the need for further research to optimize pretreatment methods for specific applications.

The combination of 4% NaOH + 10% H_2O_2 (N_2P_2) pretreatment was found to be effective in improving the cellulose content while reducing the hemicellulose, lignin, and ash contents. However, the solid loss was found to be high, especially in the alkali-treated feedstock. These findings can help in selecting the appropriate pretreatment conditions for producing cellulose-rich feedstock for various applications. These results suggest that the combination of NaOH with H_2O_2 is the most effective pretreatment for increasing cellulose content, reducing lignin and ash content. These findings can guide the selection of appropriate pretreatment methods for enhancing bioconversion efficiency while minimizing solid loss.

Ethanol yield from simultaneous saccharification and fermentation of pretreated feedstock of FH using *Saccharomyces cerevisiae* and *Zymomonas mobilis*

The pretreated FH was subjected to simultaneous saccharification and fermentation using commercial enzyme and *Saccharomyces cerevisiae*. The highest pretreated feedstock to ethanol conversion was observed in 4% NaOH with 5% H_2O_2 (N_2P_1) (0.14 g/g, 13.77%) and 4% NaOH with 10% H_2O_2 (N_2P_2) (0.13 g/g, 12.80%) pretreated feedstock, which were statistically on par with each other and four times higher compared to the control (0.03 g/g, 3.16%) (Table 4). The highest conversion was observed in the alkali with acid combination pretreated feedstock (0.10 g/g, 9.84%). The order of conversion from highest to the lowest was: Alkali + Acid Combination > Autoclaved + Alkali Combination > Autoclaved + Acid Combination > Alkali > Acid + Acid Combination > Acid > Autoclaved > Control.

Table 3: Feedstock composition of FH after pretreatment

FH	(Per cent dry weight)				
Treatment	**Cellulose**	**Hemicellulose**	**Lignin**	**Ash**	**Solid loss**
Control	38.44 ^{r}	33.71 ^{a}	20.84 ^{l}	12.59 ^{a}	0.00 ^{k}
Autoclaved (A1)	37.85 ^{s}	31.65 ^{b}	20.87 ^{l}	11.92 ^{ab}	20.13 ^{i}
Acid					
H1	43.10 ^{o}	22.42 ^{jk}	19.68 ^{k}	11.76 ^{b}	46.61 $^{f-h}$
H2	44.40 ^{n}	22.27 ^{jk}	19.50 ^{k}	11.97 ^{ab}	49.73 $^{e-h}$
P1	41.21 ^{q}	22.53 ^{jk}	16.77 ^{f}	9.58 ^{d}	11.09 ^{j}
P2	41.80 ^{p}	22.69 ^{j}	16.49 ^{f}	9.57 ^{d}	8.50 ^{j}
Average	42.63	22.48	18.11	10.72	28.98
Alkali					
N1	48.52 ^{i}	22.89 ^{j}	17.56 ^{g}	9.79 ^{d}	60.02 $^{a-d}$
N2	50.45 ^{h}	23.80 ^{i}	16.61 ^{f}	8.33 ^{e}	68.03 ^{a}
Average	49.49	23.35	17.09	9.06	64.02
Autoclaved + Acid Combination					
A_1H_1	46.20 ^{kl}	19.42 ^{m}	18.65 ^{ij}	11.22 ^{bc}	49.46 $^{e-h}$
A_1H_2	46.67 ^{k}	17.56 ^{n}	17.97 ^{hi}	10.69 ^{c}	51.66 $^{e-g}$
Average	46.44	18.49	18.31	10.96	50.56
Autoclaved + Alkali Combination					
A_1N_1	59.65 ^{cd}	25.97 ^{ef}	15.55 ^{e}	5.91 ^{g}	59.97 $^{a-d}$
A_1N_2	59.24 ^{d}	25.31 ^{fg}	14.44 ^{d}	4.98 ^{h}	67.38 ^{ab}
Average	59.44	25.64	15.00	5.45	63.67
Acid + Acid Combination					
H_1P_1	45.87 ^{l}	20.92 ^{l}	19.15 ^{jk}	11.74 ^{b}	43.51 ^{h}
H_1P_2	45.28 ^{m}	21.82 ^{k}	18.17 $^{h-j}$	10.92 ^{c}	44.39 ^{gh}
H_2P_1	47.70 ^{j}	16.54 ^{o}	18.50 ^{ih}	11.25 ^{bc}	45.58 $^{f-h}$
H_2P_2	47.50 ^{j}	16.90 ^{no}	16.18 ^{f}	10.86 ^{c}	46.14 $^{f-h}$
Average	46.59	19.05	18.00	11.20	44.91
Alkali + Acid Combination					
N_1H_1	53.42 ^{g}	24.32 ^{hi}	13.67 ^{bc}	6.10 ^{g}	52.80 $^{d-f}$
N_1H_2	55.56 ^{e}	24.59 ^{gh}	12.87 ^{a}	5.07 ^{h}	53.12 $^{d-f}$
N_2H_1	59.54 ^{d}	29.00 ^{d}	14.24 ^{cd}	5.03 ^{h}	62.75 $^{a-c}$
N_2H_2	61.32 ^{b}	26.58 ^{e}	13.51 ^{b}	4.75 ^{h}	59.77 $^{b-d}$
N_1P_1	49.09 ^{i}	22.98 ^{j}	15.24 ^{e}	7.08 ^{f}	56.29 $^{c-e}$
N_1P_2	54.01 ^{f}	24.50 ^{hi}	14.28 ^{cd}	5.13 ^{h}	56.59 $^{c-e}$
N_2P_1	60.15 ^{c}	29.57 ^{cd}	13.95 $^{b-d}$	4.89 ^{h}	65.13 ^{ab}
N_2P_2	64.29 ^{a}	30.06 ^{c}	12.88 ^{a}	3.96 ^{i}	65.26 ^{ab}
Average	57.17	26.45	13.83	5.25	58.97
SEM±	1.56	0.91	0.51	0.61	3.87
CD (p<0.05)	0.58	0.74	0.62	0.77	8.10

Similarly, pretreated FH was subjected to simultaneous saccharification and fermentation using commercial enzyme and fermentation with *Zymomonas mobilis*. The highest pretreated feedstock to ethanol conversion was observed for 2% NaOH with 10% H_2O_2 (N_1P_2) (0.09 g/g, 9.42%) pretreated feedstock, which was five times higher than the control (0.02 g/g, 1.85%) (Table 4). The second highest conversion rate was observed for autoclaved with alkali combination pretreated feedstock (0.08 g/g, 8.13%), which were statistically on par with each other. The order of effectiveness for the pre-treatments, from most effective to least effective was: Autoclaved + Alkali Combination > Alkali + Acid Combination > Alkali > Autoclaved + Acid Combination > Acid + Acid Combination > Acid > Autoclaved > Control.

These results were in consistent with the study by Shao *et al*. (2014) [29], which reported that NaOH pretreatment significantly improved the ethanol yield of wheat straw by increasing the accessibility of cellulose to enzymes. Additionally, the combination of alkali and acid pretreatment showed the highest ethanol concentration among the pretreatment methods, and NaOH and H_2O_2 combination showed the maximum ethanol concentration within this combination. This result is consistent with the study by Maurya and Gnansounou (2019) [19], which reported that combined pretreatment methods significantly improved the ethanol yield of rice straw by increasing the solubility and accessibility of cellulose to enzymes.

Similar results were observed in ethanol produced in rice husk from palm wine yeast yield was 6.60±0.48% while baker's yeast yielded 5.60±0.42% ethanol (Ezeonu *et al*., 2014) [10]. Oyeleke and Jibril (2009) [21] using guinea corn husk and millet husk, which revealed ethanol yield for guinea corn husk 26.83 g/l and millet husk 18.31 g/l which is higher the ethanol yield than the present study.

Table 4: Ethanol yield from simultaneous saccharification and fermentation of pretreated feedstock (PF) of FH using *Saccharomyces cerevisiae* and *Zymomonas mobilis*

FH	Ethanol Yield (*Saccharomyces cerevisiae*)		Ethanol Yield (*Zymomonas mobilis*)	
Treatment SSF	g/g PF	%	g/g PF	%
Control	0.03 ^{h}	3.16 ^{h}	0.02 ^{i}	1.85 ^{i}
Autoclaved (A1)	0.03 ^{h}	3.35 ^{h}	0.03 ^{hi}	3.15 ^{hi}
Acid				
H1	0.04 ^{gh}	4.13 ^{gh}	0.06 ^{fg}	5.50 ^{fg}
H2	0.04 ^{gh}	4.13 ^{gh}	0.03 ^{i}	2.65 ^{i}
P1	0.06 $^{d-f}$	6.35 $^{d-f}$	0.06 ^{ef}	6.35 ^{ef}
P2	0.05 ^{g}	4.94 ^{g}	0.06 $^{d-f}$	6.38 $^{d-f}$
Average	0.05	4.89	0.05	5.22
Alkali				
N1	0.08 ^{c}	7.94 ^{c}	0.07 $^{b-f}$	6.81 $^{b-f}$
N2	0.08 ^{c}	8.01 ^{c}	0.07 $^{c-f}$	6.55 $^{c-f}$
Average	0.08	7.98	0.07	6.68
Autoclaved + Acid Combination				
A1H1	0.09 ^{bc}	8.56 ^{bc}	0.07 $^{c-f}$	6.52 $^{c-f}$
A1H2	0.08 ^{cd}	7.65 ^{cd}	0.06 $^{d-f}$	6.38 $^{d-f}$
Average	0.08	8.10	0.06	6.45
Autoclaved + Alkali Combination				
A1N1	0.10 ^{b}	9.50 ^{b}	0.08 $^{a-e}$	7.98 $^{a-e}$
A1N2	0.10 ^{b}	9.65 ^{b}	0.08 ^{ab}	8.29 ^{ab}
Average	0.10	9.58	0.08	8.13
Acid + Acid Combination				
H1P1	0.05 ^{fg}	4.96 ^{fg}	0.07 $^{c-f}$	6.55 $^{c-f}$
H1P2	0.05 $^{e-g}$	5.16 $^{e-g}$	0.03 ^{hi}	3.41 ^{hi}
H2P1	0.07 ^{cd}	7.25 ^{cd}	0.05 ^{gh}	4.65 ^{gh}
H2P2	0.06 ^{de}	6.48 ^{de}	0.07 $^{b-f}$	6.88 $^{b-f}$
Average	0.06	5.96	0.05	5.37
Alkali + Acid Combination				
N1H1	0.07 ^{cd}	7.29 ^{cd}	0.08 $^{a-c}$	8.12 $^{a-c}$
N1H2	0.08 ^{cd}	7.52 ^{cd}	0.08 $^{a-d}$	8.03 $^{a-d}$
N2H1	0.10 ^{b}	9.77 ^{b}	0.08 $^{a-c}$	8.13 $^{a-c}$
N2H2	0.10 ^{b}	9.54 ^{b}	0.08 $^{b-e}$	7.69 $^{b-e}$
N1P1	0.08 ^{bc}	8.41 ^{bc}	0.08 $^{a-c}$	8.10 $^{a-c}$
N_1P_2	0.10 ^{b}	9.60 ^{b}	0.09 ^{a}	9.42 ^{a}
N_2P_1	0.14 ^{a}	13.77 ^{a}	0.07 $^{b-e}$	7.31 $^{b-e}$
N_2P_2	0.13 ^{a}	12.80 ^{a}	0.07 $^{b-e}$	7.45 $^{b-e}$
Average	0.10	9.84	0.08	8.03
SEM (±)	0.01	0.56	0.00	0.40
CD (p<0.05)	0.01	1.40	0.02	1.66

In similar with the present study, Nachaiwieng *et al*. (2015) [20] investigated the factors influencing the SSF process for ethanol production from rice husk cellulose at an elevated temperature of 43 °C. The study used *Kluyveromyces marxianus* CK8, a thermotolerant yeast capable of ethanol fermentation at 45 °C, as the fermenting yeast, along with a commercial cellulolytic enzyme in the SSF on rice husk. The study found that under optimal SSF conditions, an actual ethanol yield of 15.63 g/L was obtained, which was a 1.44-fold increase compared to the separate hydrolysis and fermentation process in rice husk.

Similar results were reported in the studies by Kumar *et al*. (2020) [17], *Zymomonas mobilis* was employed for SSF of rice husk pretreated with dilute acid, which led to the production of 4.28 g/L of ethanol. Similarly, another study by Yuan *et al*. (2018) [32] utilized *Zymomonas mobilis* for SSF of maize straw pretreated with dilute acid, resulting in the production of 6.2 g/L of ethanol. In contrast to present study, according to Saha and Cotta (2007) [27], rice hulls (15%, w/v) were pretreated with 7.5% H_2O_2 (v/v) at 35 °C for 24 h. Subsequently, for SSF, cellulase and xylanase were used for enzymatic hydrolysis, and *Escherichia coli* FBR5 was used as the fermenting microorganism. The resulting ethanol production was 8.0 g/L, with a conversion rate of 0.48 g/g sugar and 0.20 g/g rice hulls, which was higher than the results obtained in the present study.

In this study, a combination of 4% NaOH with 5% or 10% H_2O_2 pretreatment of FH was found to significantly enhance ethanol concentration and pretreated feedstock to ethanol conversion in SSF using *Saccharomyces cerevisiae*. These results highlight the critical role of pretreatment methods in improving the efficiency of bioethanol production. Furthermore, the potential of FH as a bioethanol feedstock was demonstrated through SSF with *Zymomonas mobilis*, with the most effective pretreatment being 2% NaOH and 10% H_2O_2. However, variations in ethanol concentration were noted, emphasizing the need for further studies to optimize SSF processes, considering factors such as biomass type, pretreatment method, and fermentation conditions. Additionally, scalability and strategies to address challenges associated with varying ethanol concentrations require further investigation for the successful implementation of bioethanol production from lignocellulosic biomass.

Conclusion

In conclusion, the study on FH identified the combination of

4% NaOH with 10% H_2O_2 (N_2P_2) was considered as the most suitable pretreatment, resulting in the highest cellulose content (64.29%). The SSF method of hydrolysis using *Saccharomyces cerevisiae* exhibited the highest ethanol yields at 13.77% and 12.80% for the combinations of 4% NaOH with 5% H_2O_2 (N_2P_1) and 4% NaOH with 10% H_2O_2 (N_2P_2), respectively. Further, the combination of 2% NaOH with 10% H_2O_2 (N_1P_2) with SSF method of hydrolysis using *Zymomonas mobilis* yields highest ethanol yield (9.42%), which is lower compare to *Saccharomyces cerevisiae*. This is because of the significant structure and chemical bonds changes in the feedstock after pretreatment, which is utilized by the microorganism for ethanol conversion with simultaneous enzymatic hydrolysis. The findings underscore the importance of further research to enhance delignification, hydrolysis and fermentation processes, providing a foundational framework for future studies aimed at optimizing bioethanol production process using foxtail millet husk as a potential feedstock.

References

1. Anita N, Pavithra R. Pre-Treatment of Sorghum and Pearl Millet Husk for Enhanced Biofuel Production. SOUVENIR 11th National Symposium Eco fest 2019, 2019, 3-6.
2. AOAC. Official Methods of Analysis of AOAC International (17th edition), AOAC International, Gaithersburg, MD; c2000.
3. Ballesteros I, Negro MJ, Oliva JM, Cabanas A, Manzanares P, Ballesteros M. Ethanol Production from Steam-Explosion Pretreated Wheat Straw. Applied Biochemistry and Biotechnology 2006;129(132):496-508.
4. Binod P, Kuttiraja M, Archana M, Janu KU, Sindhu R, Sukumaran RK, *et al*. High temperature pretreatment and hydrolysis of cotton stalk for producing sugars for bioethanol production. Fuel 2012;92:340-345.
5. Cao BB, Wang R, Yang HJ, Jiang LS. In situ ruminal degradation of phenolic acid, cellulose and hemicellulose in crop brans and husks differing in ferulic and p-coumaric acid patterns. The Journal of Agricultural Science. 2015;153:1312-1320.
6. Caputi A, Ueda M, Brown T. Spectrophotometric determination of ethanol in wine. American Journal of Enology and Viticulture. 1968;19(3):160-165.
7. Chukwuma SE, Olawale O, Ikechukwu NEO, Chigozie ME, Ositadinma CU, Ephraim NA. Enhanced availability of biofuel and biomass components in *Aspergillus niger* and *Aspergillus fumigatus* treated rice husk. European Scientific Journal. 2014;10(18):96-117.
8. Copur YA, Tozluoglu A, Ozkan M. Evaluating pretreatment techniques for converting hazelnut husks to bioethanol. Bioresource Technology. 2013;129:182-190.
9. Eggert H, Greaker M. Promoting Second Generation Biofuels: Does the First Generation Pave the Road? Energies. 2014;7:4430-4445.
10. Ezeonu CS, Olawale O, Onwurah INE, Ugbogu OC, Anike EN. Enhanced availability of biofuel and biomass components in *Aspergillus Niger* and *Aspergillus fumigatus* treated rice husk. European Scientific Journal. 2014;10:1857-7881.
11. Fruedenburg K. Lignin. In: Peach, K., Tracey, M.V. (Eds.), Modern Methods of Plant Analysis, Springer-Verlag, Berlin, 1955, 3.
12. Hammajam AA, Zahari IN, Sapuan SM, Leman Z. Characterization of Millet (Pennisetum glaucum) Husk Fiber (MHF) and its use as filler for High Density Polyethylene (HDPE) composites. Bioresources, 2017;12(4):9287-9301.
13. Huzir NM, Aziz MMA, Ismail SB, Abdullah B, Mahmood NAN, Umor NA, *et al*. Agro-industrial waste to biobutanol production: Eco-friendly biofuels for next generation. Renewable and Sustainable Energy Reviews 2018;94:476-485.
14. IEA. Sustainable production of second-generation biofuels: potential and perspectives in major economies and developing countries. International Energy Agency. IEA/OECD, Paris; c2010.
15. IEA. Tracking Bioenergy. https://www.iea.org/ energy-system/renewables/bioenergy (Visited on 03 December, 2023)
16. Kiran KC, Prasanna KT. Bioethanol Production from Foxtail Straw. Mysore Journal of Agricultural Sciences 2022;56(4):244-250.
17. Kumar A, Thakur M, Choudhary P, Shankar V. Comparative evaluation of simultaneous saccharification and fermentation for ethanol production using pretreated rice husk and wheat straw. Renewable Energy 2020;152:223-230.
18. Lai YZ. Chemical degradation. In: Hon DNS, Shiraishi N, editors. Wood and cellulosic chemistry. New York: Basel, 2001, 443-512.
19. Maurya DP, Gnansounou E. Combination of thermal, alkaline and enzymatic pretreatments for enhancing the efficiency of bioethanol production from rice straw. Bioresource Technology. 2019;274:104-112.
20. Nachaiwieng W, Lumyong S, Yoshioka K, Watanabe T, Khanongnuch C. Bioethanol production from rice husk under elevated temperature simultaneous saccharification and fermentation using *Kluyveromyces marxianus* CK8. Biocatalysis and Agricultural Biotechnology. 2015;4(4):543-549.
21. Oyeleke SB, Jibril NM. Production of bioethanol from guinea corn husk and millet husk. African Journal of Microbiology Research. 2009;3(4):147-152.
22. Pandey RR, Sharma G, Tripathi SK, Singh AK. Litter fall, litter decomposition and nutrient dynamics in a subtropical natural oak forest and managed plantation in North-Eastern India. Forest Ecology and Management. 2007;240:96-110.
23. Rabetafika HN, Bchir B, Blecker C, Paquot M, Wathelet B. Comparative study of alkaline extraction process of hemicelluloses from pear pomace. Biomass and Bioenergy. 2014;61:254-264.
24. REN21. Renewables 2018, global status report; Renewable Energy Policy Network for the 21st century (REN21), 2019, 1-324.
25. Rojith G, Singh B. Hydrogen peroxide pretreatment efficiency comparison and characterisation of lignin recovered from coir pith black liquor. Journal of Environmental Research And Development. 2013;7:1333-1339.
26. Ross AB, Jones JM, Kubacki ML, Bridgeman T. Classification of macroalgae as fuel and its thermochemical behavior. Bioresource Technology. 2008;99: 6494-6504.
27. Saha BC, Cotta MA. Enzymatic saccharification and fermentation of alkaline peroxide pretreated rice hulls to

ethanol. Enzyme and Microbial Technology. 2007;41:528-532.
28. Saha P, Baishanab AC, Alam F, Khan MR, Islam A. Production of bio-fuel (Bioethanol) from Biomass (Pteris) by fermentation process with yeast. Procedia Engineering. 2014;90:504-509.
29. Shao Q, Li J, Li X, Xu J, Lu X. Effects of alkaline and acid pretreatments on the composition, structure and enzymatic hydrolysis of wheat straw. BioResources, 2014;9(3):5109-5123.
30. Silverstein RA, Chen Y, Sharma-Shivappa RR, Boyette MD, Osborne J. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresource Technology. 2007;98:3000-3011.
31. Taherzadeh MJ, Karimi K. Pretreatment of lignocellulosic waster to improve ethanol and biogas production: A review. International Journal of Molecular Sciences. 2008;9:1621–1651.
32. Yuan X, Zhang Y, Liu X, Han R, Wang X, Wang J. Production of bioethanol from maize straw via simultaneous saccharification and fermentation using a novel strain of *Zymomonas mobilis*. Recovery, Utilization and Environmental Effects. 2018;40:1594-1604.
33. Zeenat IS, Lawal N, Muntari B, Abdullahi AI. Pretreatment of millet husk using alkaline hydrogen peroxide to enhance enzymatic Hydrolysis for reducing sugar production. FUDMA Journal of Sciences. 2021;5(2):289-294.