SEED PODS OF *HETEROPHRAGMA ADENOPHYLLUM* AS POTENTIAL FEEDSTOCK FOR BIOETHANOL PRODUCTION

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In this study, seed pods of *Heterophragma adenophyllum* were used as a cheap feedstock for bioethanol production. The substrate was pretreated chemically (different concentrations of alkali (KOH and NaOH) and acids (H_2SO_4 and HCl), and thermochemically. Results revealed that alkali treatment yielded maximum exposure of cellulosic content (56%) at the concentration of 1.5%. The pretreated substrate, having maximum cellulose content was further utilized in separate hydrolysis and fermentation, and simultaneous saccharification and fermentation using *Saccharomyces cerevisiae*, along with seven different bacterial strains, such as *Bacillus paralichniformis*, *Bacillus megaterium*, *Bascillus flexus*, *Bascillus wiedmanni*, *Pseudomonas stutzeri*, *Bascillus aerius* and *Bascillus subtilis*. The results of both fermentation techniques showed that separate hydrolysis and fermentation demonstrated a higher yield of bioethanol (6.43%) than SSF (5.08%). These results suggested that seed pods of *H. adenophyllum* could be utilized as a potential feedstock for large-scale ethanol production.

Keywords: *H. adenophyllum*, fermentation, bioethanol, pretreatment, saccharification

INTRODUCTION

The demand for non-renewable energy sources like fossil fuels has increased for the last few decades. This ever-growing demand and limited resources, on the other hand, have increased concerns about the increasing global warming and emission of greenhouse gases (GHGs). The level of carbon dioxide gas related to the burning of these sources increased to 409 ppm,[1,2] playing a role in global warming.[3] Energy demands are increasing day by day due to the revolution in industrialization, global population and geopolitical factors.[4,5] The energy supply is one of the major problems faced by many countries in the world due to intensive consumption of energy sources. For this purpose, the use of renewable sources of energy is a good option.[6] Renewable sources, *i.e.*, nuclear, water, solar, wind energy, and biofuels or bio-based energy, have gained more attention owing to their sustainability, some of them being used to produce valuable chemicals and fuels.[7] Fuels obtained from natural sources help in the maintenance of C levels and the reduction of GHGs in the environment.[8]

Bioethanol has recently become one of the most promising biofuels, with useful applications in transportation, industry, and energy sectors. This compound can be obtained from organic matter containing a sufficient amount of carbohydrates and many other plants with high sugar content.[9-12] It can be directly used as pure ethanol or mixed with gasoline to form "gasohol".[13] It can be used as an octane enhancer or gasoline improver, and its combination with diesel helps lower exhaust gas emissions.[14] Bioethanol shows many advantages over gasoline, such as broader flammability limits, higher octane number (108), increased heat of

vaporization, and higher flame speeds.[15] Compared to fossil fuels, bioethanol is easily biodegradable, less toxic and produces low air-borne pollutants.[16,17]

Lignocellulosic biomass (LCB) is the largest renewable source of the world for the production of bioethanol, and it can be classified into three main categories: (i) municipal solid wastes and agricultural residues, (ii) marine algae, (iii) forestry residues. Lignocellulosic biomass consists of three main components: lignin, hemicelluloses and cellulose. Hemicelluloses and cellulose together comprise about 70% of all biomass. Both are closely packed to the lignin component via hydrogen and covalent bonds; this makes their structure more resistant and robust.[18]

There are three main steps involved in converting lignocellulosic biomass to ethanol: pretreatment, saccharification and fermentation. The pretreatment can be achieved by physical, biological, chemical, steam explosion or a combination of methods. Every method has its limitations, which differ from each other, depending on the capital investment, operational cost, waste treatment systems and chemical recycling.[19] Pretreatment causes alteration in the structure of the cellulosic material and exposes a higher cellulose content.[20] Pretreatment imparts both physical and chemical effects. Physically, it causes damage to the lignin structure and enhances the surface area, resulting in the chemical or physical perforation of the plant's cell wall. Chemically, it changes the depolymerization and the solubility of biomass. After pretreatment, hydrolysis or saccharification is performed by enzymes (cellulases or hemicellulases) or acids to hydrolyze the polymeric hemicelluloses or cellulose into fermentable sugars. Pretreatment and hydrolysis are the main contributors to the optimization of the fermentation process.[21] Different techniques can be used for the fermentation of sugars to bioethanol, such as simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), separate hydrolysis and fermentation (SHF), non-isothermal simultaneous saccharification and fermentation, filtration and fermentation, consolidated bioprocessing (CBP). Among these, SHF and SSF are commonly used technologies.[22] In simultaneous saccharification and fermentation (SSF), sugars formed from the cellulase enzyme are simultaneously converted into ethanol by the action of *Saccharomyces cerevisiae*.[23] This causes the neutralization of inhibitory effects produced by the sugars over the cellulase enzyme.[24] This technique has many advantages: high ethanol yield, cost-effectiveness, high efficacy for saccharification, low requirement for enzymes, lower risk of contamination or inhibition, less operational time and no need for large volume reactors.[25-27]

In separate hydrolysis and fermentation (SHF), hydrolysis and fermentation are carried out in separate units at their optimum conditions. The main benefit of this process is that hydrolysis is carried out at 50 °C, and fermentation is performed at 30 or 37 °C.[28] Hence, it is a more time-consuming process with more chances of contamination or inhibition.[23,26]

Haplopharagma adenophyllum is a flowering plant that belongs to family of Bignoniaceae. Its seeds are enclosed in seed pods that fall on ripening. Seeds are spread, leaving the pods as residual biomass. Many fallen pods are found around the *H. adenophyllum* as waste. The main reason for the selection of this substrate is that it is easily available, nature-friendly and inexpensive. The main aim of this study was to optimize the pretreatment condition for maximum exposure of cellulose and saccharification using indigenous and commercial enzymes to obtain maximum sugar content and to obtain the optimized production of bioethanol via SSF and SHF.

EXPERIMENTAL

Substrate

Heterophragma adenophyllum (HA) seed pods were obtained from the University of Sargodha (main campus) District Sargodha, Punjab, Pakistan. The collected material was thoroughly washed with tap water and then with distilled water to remove dust particles or impurities, followed by oven-drying at 60 °C temperature. Afterwards, it was ground into fine powder and kept in an air-tight container for further use and characterization.

Substrate pretreatment

The substrate was pretreated thermo-chemically and chemically by using different concentrations of alkali (KOH and NaOH) and acid (HCl and H_2SO_4), as reported by Ghazanfar *et al*.[29] The fine powder of the sample was dissolved in the different concentrations of acids and alkalis ranging from 0.1-1.5% at the ratio of 1:10, and kept at room temperature for 2 h. Then, the samples were autoclaved at 121 °C, 15 psi pressure for 15 minutes. After autoclaving, samples were filtered with muslin cloth and solid residues were washed until they reached neutrality.

Saccharification and fermentation

The raw substrate and the samples pretreated by each pretreatment with maximum cellulose (%) (1.5% KOH-steam-treated and 1.5% NaOH-steam-treated), were employed for the production of ethanol via simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF).

Separate hydrolysis and fermentation

This process was done by the use of indigenous as well as commercial enzymes separately. 40 FPU/mL concentration of commercial cellulase enzyme was used for enzymatic hydrolysis. This was taken in a 250 mL Erlenmeyer flask along with 100 mL citrate buffer at 5 pH. 2% substrate (treated as well as untreated) was also dissolved in it and incubated in a water bath at a temperature of 50 °C until the maximum concentration of sugars obtained. About 100 mL of the indigenous enzyme was taken in another 250 mL Erlenmeyer flask. 2% substrate (treated and untreated) was dissolved and incubated in a water bath at 50 °C until the maximum concentration of sugars was obtained. Samples were taken out after different time intervals and centrifuged at 10,000 rpm speed for almost 10 minutes. The supernatant was used to further analyze the reducing sugar content.

The following formula was used for the calculation of saccharification (%), as described by Irfan *et al*.:[30]

$$\text{Saccharification (\%)} = \frac{\text{Reducing Sugars released (mg/ml)}}{\text{Substrate used (mg/ml)}} \times 100 \quad (1)$$

Inoculum preparation of *Saccharomyces cerevisiae*

Different media components, *i.e*., $(NH_4)_2SO_4$ 0.25%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, and yeast extract 0.25%, were dissolved in distilled water for the inoculum preparation. Afterwards, the media was sterilized at 121 °C for 15 minutes before being inoculated aseptically with a loopful from the yeast slant and incubated at 35 °C temperature, 120 rpm speed for 24 hours. The produced cells were used as a source of inoculum.

Bioethanol production

Fermentation was done as described by Irfan *et al*.[30] The hydrolysate obtained after the process of saccharification was dissolved in the fermentation medium consisting of KH_2PO_4 0.1%, ammonium sulphate 0.25%, yeast extract 0.25 and $MgSO_4$ 0.05%. Then, it was sterilized at a temperature of 121 °C for 15 minutes. The medium was allowed to cool at room temperature and inoculated with 1% suspension of *S. cerevisiae*. Then, it was incubated in a rotary shaker for 96 hours at 30 °C. After every 24 hours, samples were taken to estimate ethanol.

Simultaneous saccharification and fermentation (SSF)

For the production of bioethanol, SSF was performed as described by Afzal *et al*.[31] For this purpose, different bacterial strains were used individually, *i.e*., *Bacillus megaterium* (MG597037), *Bacillus paralichniformis* (MG597036), *Bascillus flexus* (MG597039), *Pseudomonas stutzeri* (MG597035), *Bascillus subtilis* (MW5906771), *Bascillus aerius* (MG597041) and *Bascillus wiedmanni* (MG597040), along with *Saccharomyces cerevisiae* with each strain. Medium composed of $(NH_4)_2SO_4$ 0.25%, KH_2PO_4 0.1%, $MgSO_4$ 0.05% and yeast extract 0.25%, as described in Irfan *et al*.,[32] along with 2% substrate (untreated and pretreated), was taken in different Erlenmeyer flasks (500 mL). Then, it was sterilized at 121 °C temperature for 15 minutes. After that, it was inoculated with 1% bacterial strain and 1% *S. cerevisiae* separately in each flask and incubated in a rotary shaker at 35 °C temperature, 120 rpm shaking speed for 96 hours. Samples were taken out every 24 hours until 96 hours. Then, centrifugation was done for 10 minutes at 10,000 rpm speed, and supernatants were used for further analysis.

Analytical methods

The estimation of total sugars was done using the method described by Dubois *et al*.,[33] while reducing sugars were analyzed using Miller's 3,5-dinitrosalicylic acid method.[34] The estimation of the cellulosic content and CMCase was done using the method described by Irfan *et al*.[35] To estimate the cellulosic content, oven-dried residue and raw substrate were used. 0.5 g of all treated and raw samples (W1) were taken in round bottom flasks with 30 mL of 80% acetic acid and 1.5 mL of conc. HNO_3 and refluxed for half an hour. Then, the resulting material was filtered through Whatman filter paper no. 1 and washed with distilled water. After that, the digested material was placed in crucibles, oven-dried overnight at 50 °C, and then weighed (W2). A few drops of conc. HNO_3 were added to crucibles containing the dried samples. Then, these crucibles were put on the flame through a tong, and the material turned black upon burning. It was incinerated in a muffle furnace at 550 °C temperature for 5 hours. The material turned into ash, and was weighed again (W3). The following formula was used for the calculation of cellulosic percentage (on the basis of dry matter):

$$\text{Cellulose (\%)} = \frac{\text{Weight of digested material (W2) - Weight of Ash (W3)}}{\text{Weight of material on dry basis (W1)}} \times 100 \quad (2)$$

Total phenolic compounds were estimated using the method reported by Sanz *et al*.[36] and Tsao and Deng.[37] Ethanol was estimated spectrophotometrically by the method reported by Irfan *et al*.[32] For this purpose, three test tubes (control, experimental and standard) were taken. 1 mL of $K_2Cr_2O_7$ was added to the three test tubes and incubated in a water bath at 60 °C for 20 minutes. Optical density (OD) was measured at 600 nm via a spectrophotometer. The following formula was used for the calculation of ethanol (%):

$$\text{Ethanol (\%)} = \text{Optical density} \times \text{Dilution factor} \times \text{Standard factor} \quad (3)$$

Cell biomass estimation

For this purpose, a 2 mL sample was taken out in an Eppendorf tube after every 24 h of incubation and centrifuged at 10,000 rpm speed for almost 10 minutes. The supernatant was discarded, and the remaining biomass was oven-dried at 60 °C temperature till constant mass and then weighed (W2). The empty Eppendorf tube was also weighed and labelled as W1. The following formula was used for the calculation of cell biomass (%):

$$\text{Cell biomass (\%)} = (W2 - W1) \times 100 \quad (4)$$

Statistical analysis

For statistical analysis, we determined standard deviation and standard error. Also, the analysis of variance (ANOVA) was used for assessing the treatment effects, and Tukey test for pairwise comparison (p-value<0.05). For data analysis, Minitab 19 software was used.

RESULTS AND DISCUSSION

In this study, seed pods of *H. adenophyllum* were treated with different concentrations of alkali and acids, using chemical and thermochemical pretreatment. The results of the pretreatment illustrated that the percentage of cellulose increased after the treatment with bases (KOH and NaOH) and acids (H_2SO_4 and HCl), compared to the raw substrate containing 38% cellulose content. The release of total and reducing sugars indicates the hydrolysis of cellulose and hemicelluloses, while the liberation of phenolic compounds revealed the degradation of lignin content in biomass. So, we also measured the total phenolic compounds, total and reducing sugars, and cellulose content.

Acidic pretreatment normally causes the degradation of glycosidic linkage among hemicelluloses. This causes the release of cellulose from the polymer matrix and enhances the porosity of biomass and efficiency of enzymatic hydrolysis, resulting in the direct yield of fermentable sugars.[38,39] Among acid treatments, the maximum cellulose content was 54% at 1% H_2SO_4 concentration, while 1.5% KOH treatment yields 56% cellulose content, which is statistically significant as compared to the untreated or raw sample (Fig. 1).

During the pretreatment, the maximum amounts of total phenolic compounds released of 0.83%, of reducing sugars – 2.11%, and of total sugars – 11.72% were obtained from 1% H_2SO_4-steam-pretreated substrates, as compared to 1% HCl-steam-pretreated substrates, which led to values of 0.74%, 1.67% and 11.35%, respectively, as shown in Figure 2. These results were statistically significant within the group, while insignificant between the groups.

Bera *et al*.[40] used acidic pretreatment techniques to generate ethanol using rice straw as a substrate. The maximum amount of reducing sugars (135.2 ± 0.45 mg/g) was obtained after 240 hours of hydrolysis. Tahir *et al*.[41] demonstrated that the release of glucose from *Quercus infectoria* leaves depends on the temperature and concentration of the acid (H_2SO_4) used for pretreatment. 2% acid at a temperature of 120 °C for 2 hours is the best condition for the release of the maximum concentration of glucose from the substrate. Kaur and Singh[42] also used acids to produce ethanol from rice husk. Dagnino *et al*.[43] also optimized the acidic pretreatment of rice hulls for ethanol production. They established that 0.3% v/w H_2SO_4 for 33 minutes were suitable conditions for production.

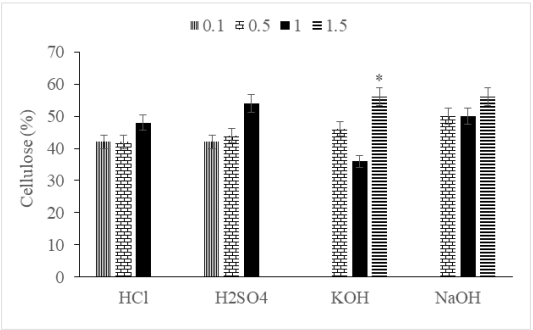

Figure 1: Effect of thermochemically pretreated substrates with different concentrations of acids (HCl, H_2SO_4) and alkalis (KOH, NaOH) on cellulose content (%). The asterisk indicates statistical difference using Tukey's test ($p < 0.05$)

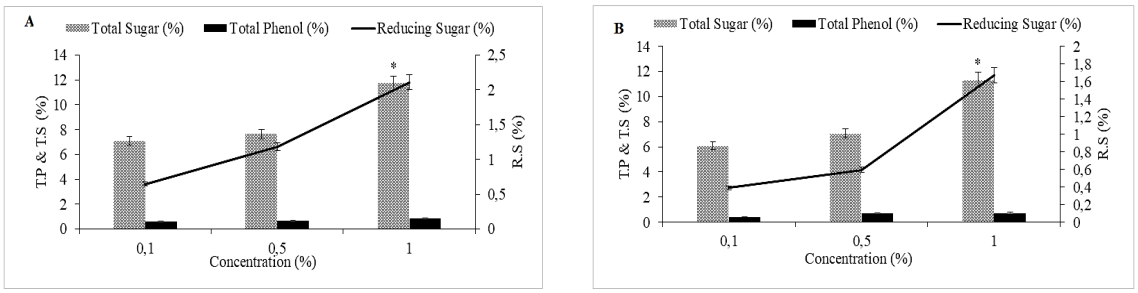

Figure 2: Effects of different concentrations of (A) H_2SO_4 and (B) HCl as thermochemical pretreatment on total sugars, total phenols and reducing sugars of the substrate. The asterisk indicates statistical difference using Tukey's test ($p < 0.05$)

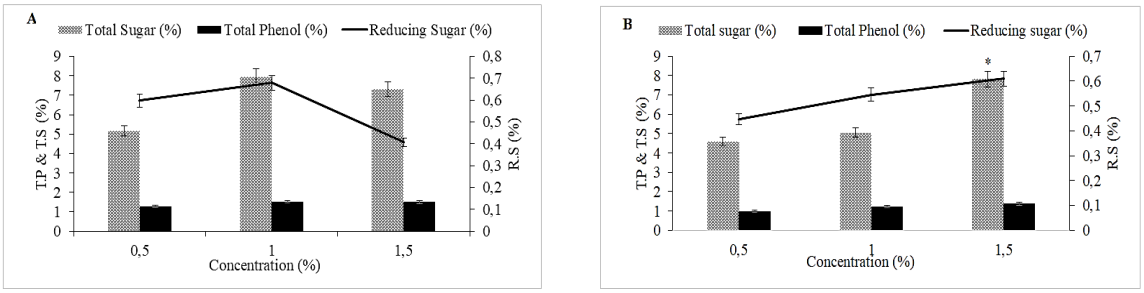

Figure 3: Effects of different concentrations of (A) NaOH and (B) KOH as thermochemical pretreatment on total sugars, total phenols and reducing sugars of the substrate. The asterisk indicates statistical difference using Tukey's test ($p < 0.05$)

On the other hand, alkali pretreatment is an effective technique for breaking down and solubilizing hemicelluloses and lignin. In this treatment, a maximum % cellulose content of 56% was obtained from 1.5% KOH-steam-pretreated and 1.5% NaOH-steam-pretreated substrates, as illustrated in Figure 1. Also, the maximum amounts of total phenolic compounds of 1.52%, of reducing sugars – 0.68% and of total sugars – 7.98% were obtained from 1% NaOH-steam-pretreated substrates, as compared to KOH-steam-pretreated substrates, with 1.37%, 0.61% and 7.81%, respectively, as illustrated in Figure 3. All these maximum values were obtained from 1.5% KOH-steam-pretreated substrates. In both treatments the results were insignificant between groups, while significant in the case of the KOH treatment within the group.

Compared to acidic hydrolysis, the saccharification of complicated lignocellulosic biomass in the presence of bases increased ethanol production. In strong acids, many inhibitors produced in the fermentation medium cause harmful effects on the fermentation organism and the final output. Acid pretreatment also showed many drawbacks like dangerous, corrosive and poisonous nature, along with the production of inhibitory compounds (hydroxymethylfurfural; HMF) and by-products,[44,45] which lessen the efficiency of the medium by dropping the ethanol productivity. Ghazanfar *et al*.[46] used the seed pods of *Bombax ceiba* as a substrate and performed KOH and KOH steam pretreatment for the removal of the lignin content. Maximum ethanol production was obtained from KOH-steam pretreated biomass. Tsegaye *et al*.[47] pretreated rice straw with NaOH to remove a significant amount of lignin, releasing the maximum amount of cellulose. Afzal *et al*.[31] discovered that alkali pretreatment was more effective than acid pretreatment for sawdust. Similarly, Goriwale and Khan[48] worked on Neem tree leaves (*Azadirachta indica*). Different sodium hydroxide and sulfuric acid concentrations were used to hydrolyze the dried leaves powder. It was observed that 1% NaOH-treated Neem leaves would produce 6% more ethanol by using *Saccharomyces spp*. as compared to *Bacillus spp*.

After the pretreatment step, the samples with maximum cellulose (%) were further utilized in simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) for bioethanol production. The findings of SSF indicate a decrease in the level of sugar content and an increase in ethanol production after every 24 h owing to the consumption of sugars by the yeast. In SSF, seven different bacterial strains named *Bacillus megaterium* (MG597037), *Bacillus paralichniformis* (MG597036), *Bacillus*

flexus (MG597039), *Pseudomonas stutzeri* (MG597035), *Bacillus subtilis* (MW5906771), *Bacillus aerius* (MG597041) and *Bascillus wiedmanni* (MG597040), along with 1% *S. cerevisiae*, were co-cultured in this research, which concluded that strain *Bacillus megaterium* (MG597037) gave the maximum concentration of ethanol 5.39%, which was statistically significant among all others. From this strain, the maximum yield of ethanol, *i.e*., 3.38% and 5.39%, was obtained from thermochemically 1.5% KOH pretreated and untreated substrates, respectively, after 96 h of fermentation at 35 °C temperature. In comparison, 2.19% was obtained from the NaOH-steam-pretreated substrate after 72 h of fermentation, as shown in Figure 4. Maximum total sugar (from the untreated substrate) and reducing sugar (from KOH steam-pretreated substrate) contents were also observed in the strain *Bacillus paralichniformis* (MG597036), *i.e*., 2.92% and 0.49%, respectively. Meanwhile, the maximum CMCase activity of 4.087 IU/mL/min was shown by the strain *Bacillus megaterium* (MG597037).

The results of SHF with indigenous cellulase showed maximum saccharification, *i.e*., 34.06% after 29 h in KOH-steam-pretreated substrate, followed by NaOH-steam-pretreated (24.36%) and untreated substrate (31.79%) after 29 h and 45 h, respectively. Meanwhile, maximum saccharification of 40.12% with commercial cellulase enzyme was reported in NaOH-steam-pretreated seed pods of *H. adenophyllum* after 54 h, followed by KOH-steam-pretreated (36.86%) and untreated substrate (34.67%) after 50 h and 52 h, respectively (Fig. 5).

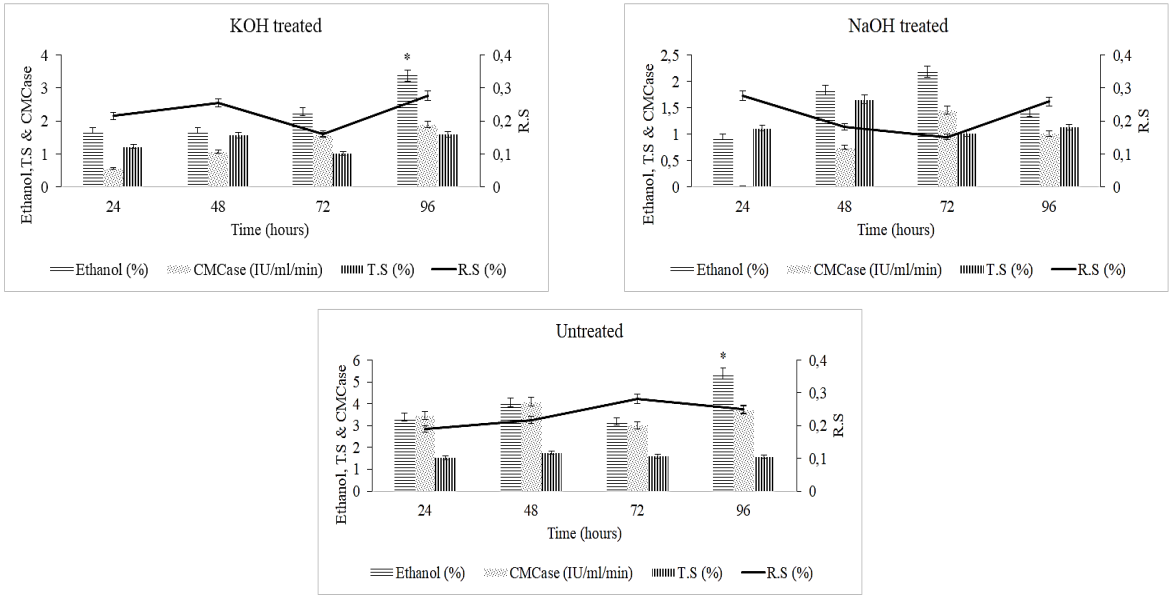

Figure 4: Simultaneous saccharification and fermentation (SSF) of *H. adenophyllum* for the production of ethanol using *Bacillus megaterium* (MG597037) and *S. cerevisiae.* The asterisk indicates statistical difference using Tukey's test ($p < 0.05$)

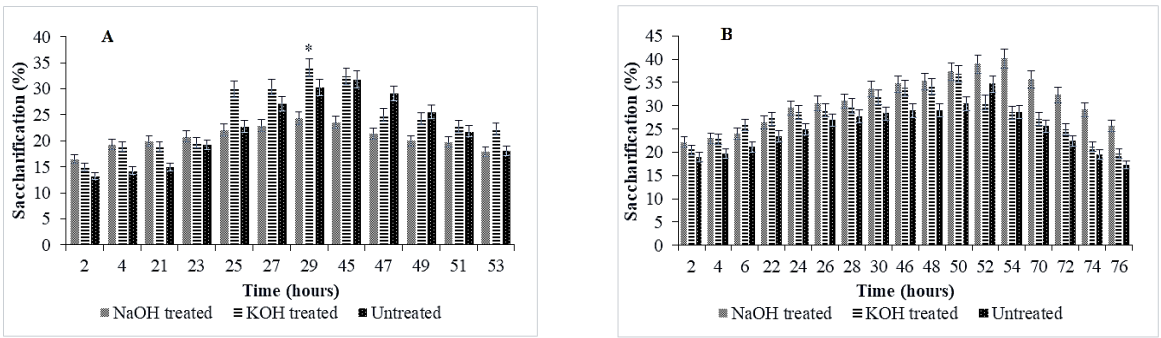

Figure 5: Saccharification (%) of untreated and treated substrates at different time intervals using (A) indigenous cellulase enzyme, and (B) commercial cellulase enzyme. The asterisk indicates statistical difference using Tukey's test ($p < 0.05$)

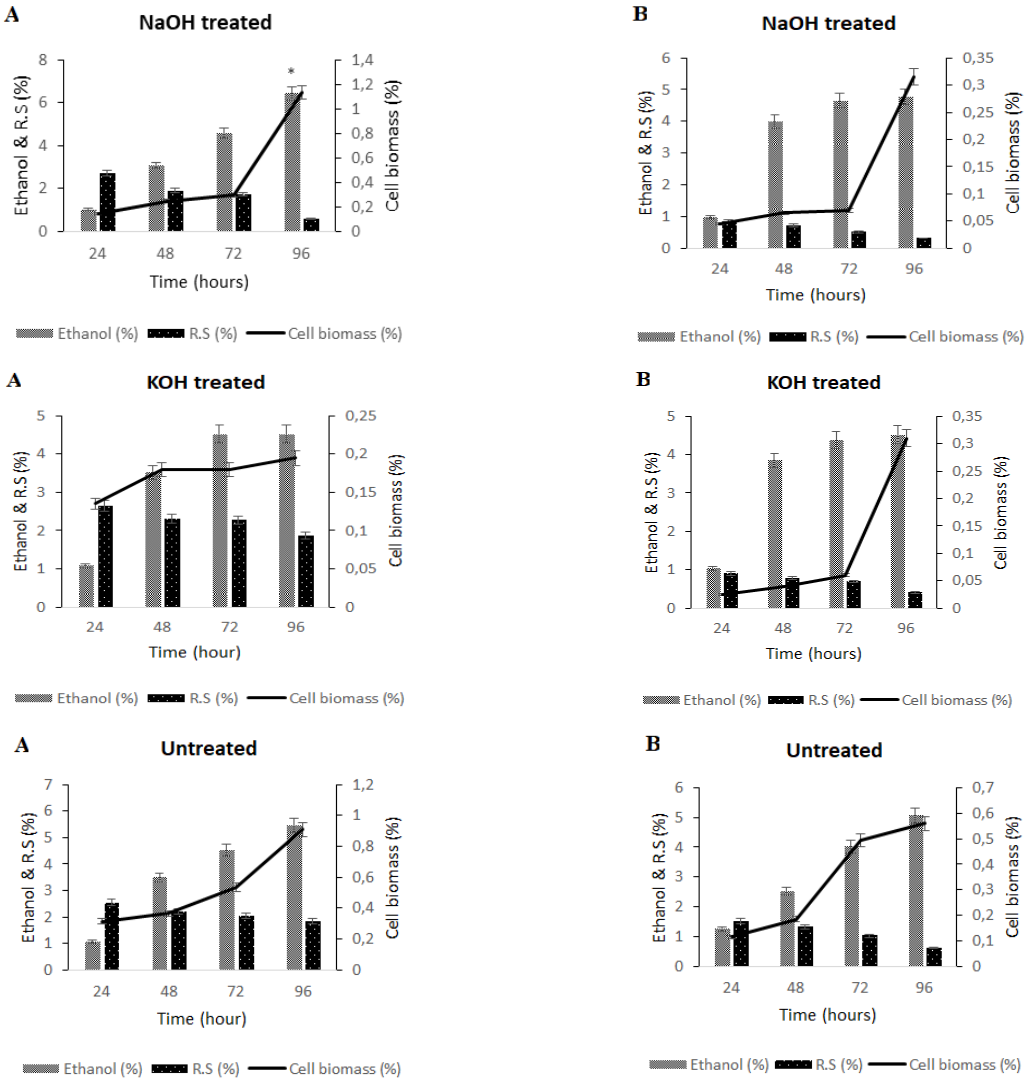

Figure 6: Amount of ethanol, reducing sugars and cell biomass obtained from fermentation using (A) indigenous enzyme, (B) commercial enzyme. The asterisk indicates statistical difference using Tukey's test ($p < 0.05$)

These hydrolysates were further fermented for ethanol production (Fig. 6). Sugars obtained with indigenous cellulase enzyme offered maximum production of ethanol, *i.e.*, 6.43% in NaOH-steam-pretreated (after 96 h of fermentation), 4.52% in KOH-steam-pretreated (after 72 and 96 h of fermentation) and 5.47% in untreated seed pods (after 96 h of fermentation). Hydrolysates obtained with the commercial cellulase enzyme presented a maximum yield of ethanol, that is, 4.77% in NaOH-steam-pretreated and 4.52% in KOH-steam-pretreated substrates after the 96 h of fermentation, while 5.08% in untreated biomass after 96 h of fermentation.

In this study, two approaches, SSF and SHF, were used for the saccharification and fermentation of pretreated and untreated substrates. In this work, the maximum amounts of reducing sugars (0.49%) and total sugars (2.92%) were reported after 24 h (using KOH-steam pretreated substrate) and after 96 h (using untreated substrate) of fermentation, respectively, using the *Bacillus paralichniformis* strain during SSF; also, the maximum ethanol titer of 5.39% (after 96 h of fermentation) was obtained from *Bacillus megaterium* and *S. cerevisiae.* However, the highest amount of fermentable sugars in terms of saccharification (%), *e.g.*, 34.06%, after 29 h of fermentation, and the maximum ethanol titer of 6.43% (after 96 h) were observed during SHF in the presence of indigenous cellulase enzyme and *S. cerevisiae.* SHF with commercial cellulase enzyme gave low production. Hence, in the case of SHF, better results were obtained from indigenous cellulase

enzyme than from commercial cellulase, and the overall highest yield was also obtained from SHF.

According to the conclusions drawn by Ghazanfar *et al*.,[29] Sukhang *et al*.[49] and Afzal *et al*.,[31] higher ethanol yield was obtained from SSF as compared to SHF by using lignocellulosic biomass, which is in contradiction to this research. In this work, SHF gave better results than SSF in the presence of an indigenous cellulase enzyme. This may be due to the period, temperature, substrate, nutrients provided, and the nature of the strain used during work. Triwahyuni[50] observed a maximum ethanol yield from separate hydrolysis and fermentation of oil palm empty fruit bunch. 78.95% ethanol yield was generated from 75.48% glucose, which was produced after 96 hours of hydrolysis at a temperature of 50 ºC, 4.8 pH, and 150 rpm speed. Barron *et al*.[51] observed the maximum yield of ethanol by employing other yeast strains to produce ethanol. They observed 11.8 g/L ethanol yield in the presence of *Pachysolen trannophylus* from wheat straw hydrolysate, and 10 g/L yield was obtained from *Kluveromyces marxianus* after 60 h. Sindhu *et al*.[52] reported the ethanol titer of 1.76% v/v from pretreated bamboo biomass in SHF with *S. cerevisiae*. Higher yield, content (g/L) and productivity (g/L.h) of ethanol were obtained from dilute sulphuric acid pretreated vegetable wastes (peels from pumpkin, ash gourd and vegetable banana) through the F-SHF by Mithra *et al*.[53] The current findings demonstrate that with the passage of fermentation time, ethanol yield increased, but the amount of glucose declined in separate hydrolysis and fermentation. However, after 48 hours of fermentation, the ethanol yield decreased using the indigenous cellulase enzyme.

Maximum cell biomass, *i.e*., 0.56%, was obtained from the untreated substrate in the presence of commercial cellulase enzyme and 0.91% by using an indigenous enzyme (after 96 h). Meanwhile, from the KOH pretreated substrate, 0.2% was obtained in the presence of the indigenous enzyme and 0.31% from the commercial enzyme after 96 h of fermentation. Likewise, from the NaOH pretreated substrate, 1.14% was obtained from the indigenous enzyme and 0.32% from the commercial enzyme after 96 h (Fig. 5). According to Chukwuemeka *et al*,[54] this increase in biomass and ethanol concentration with time is related to the *S. cerevisiae* metabolic activities and fermentation. A gradual increase in biomass (%) is also correlated with the definition of fermentation. Our research shows consistency with Irfan *et al*.,[30] who found that ethanol production increases with the fermentation time, while glucose content declines. The growth rate also improved with time.

CONCLUSION

From this study, it was concluded that seed pods of *Heterophragma adenophyllum* could be a potential feedstock for various applications. The maximum ethanol titer, *i.e*., 6.43%, was obtained with 1.5% NaOH treated substrate, saccharified with indigenous cellulase enzyme and fermented with *Saccharomyces cerviseae* for 96 hours at 30 ºC and 120 rpm speed in separate hydrolysis and fermentation. The present research results indicated that this cheap biomass could be a promising feedstock for bioethanol production.

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