

EFFECT OF ACID AND BASE PRE-TREATMENT ON ENZYMATIC HYDROLYSIS OF DESMOSTACHYA BIPINNATA (DAABH)

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Desmostachya bipinnata; lignocellulosic biomass; acid pre-treatment; alkaline pre-treatment; enzymatic hydrolysis; cellulase; *Aspergillus niger*; reducing sugar yield; bioethanol; delignification.

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Abstract

The transformation of lignocellulosic biomass to fermentable sugars in an efficient manner is also a key aspect of challenge in biofuel production in the second generation. This paper examines the relative opportunities of acid and base pre-treatment of the enzymatic hydrolysis of *Desmostachya bipinnata* (Daabh) which is a salty halophytic grass common in South Asia and can grow in both salty and marginal soils. The fractions were diluted with sulfuric acid (1.5 percent w/v, 2.0 percent w/v and 2.5 percent w/v) and sodium hydroxide (1 percent w/v, 1.25 percent w/v and 1.5 percent w/v) and incubated at 100 °C in 15, 30 and 60 minutes to assess the best delignification and hemicellulose solubilization conditions. The amounts of reducing sugars were measured by 3, 5-dinitrosalicylic acid (DNS) assay, and enzymatic hydrolysis of the cellulose under controlled conditions (55°C, 24h) was used to test the presence of cellulase produced by *Aspergillus niger*. It was found that 2.5% H₂SO₄ 30 minutes gave the highest acid pre-treatment yield (84.44%), and 1% NaOH 60 minutes gave the highest yield of base (94.26-percent). Enzymatic hydrolysis, after-facto, showed the total saccharification yields of 90.94 and 96.48% of acid and base-treated biomass, respectively, providing support to the high productivity of alkaline delignification in improving the access section of the enzymes. The experiment provides evidence that mild NaOH pre-treatment is superior to acid hydrolysis to break down the recalcitrant lignocellulosic skeleton of the *D. bipinnata* which makes the plant a promising feedstock in the generation of bioethanol at large scale in arid and salty conditions that require no food to grow.

1. Introduction

The energy situation in the world is experiencing a critical change brought about by the twin issues of increased energy demand and environmental depletion. In recent decades, fossil resources among which are petroleum, natural gas, and coal have been the primary energy sources as they provide almost 80% of the world energy consumption (International Energy Agency [IEA],

2023). Nevertheless, their rapid exhaustion combined with their role in the emission of greenhouse emissions (GHG) has increased the worldwide interest in identifying viable and renewable energy sources (Qin et al., 2021). Fossil burning fuels contribute to roughly two-thirds of anthropogenic CO₂ emissions, which contribute to a speedy climate change, global warming, and

air pollution (Ritchie and Roser, 2023). Therefore, the shift to renewable sources of bioenergy has turned out to be not only an environmental challenge but also an economic one to meet the long-term energy security and carbon neutrality goals.

Biofuels, as one of the renewable energy sources, have become one of the most promising to be an alternative to fossil fuels because they are biodegradable, carbon neutral, and can be used with the current energy infrastructure (Kandasamy et al., 2022). The plants, algae, and organic waste constitute biological materials which can be transformed into biofuels, and they can be divided into three generations depending on the type of feedstock and the maturity of the technology. The production of the first-generation biofuels, using edible crops such as sugarcane, corn, and vegetable oils has been ethically and economically questionable because of their competition with food production and arable land (Ahmad et al., 2020). This food versus fuel discussion has led to the transition to the second-generation biofuels, which will use such lignocellulosic biomass as agricultural residues, non-food crops, and grasses (Xie et al., 2023). These biofuels have a sustainable solution of changing non-edible substances of the plant to fermentable sugars which helps in reducing food insecurity as well as environmental effects.

The lignocellulosic biomass is the richest source of renewable carbon on Earth with almost 200 billion tons of biomass being produced every year (Hassan et al., 2024). It is largely composed of three structural polymers namely cellulose, hemicellulose and lignin which form a complex network which cannot be helped by microbes or enzymes to break. The structural recalcitrance is the central bottleneck of the efficient production of biofuels because it limits the enzyme accessibility and the release of sugars in the hydrolysis process (Zhang et al., 2022). Consequently, this strong lignin-carbohydrate complex must be broken down using proper pre-treatment approaches, enhance the digestibility to enzymes, and extract the fullest numbers of fermentable sugars (Sun et al., 2020). Typical pre-treatment methods comprise physical (mills,

extrusion), chemical (acid, alkali or solvent), and biological (fungal) methods, with common examples of acidic and alkaline chemical pre-treatments being highly considered the most efficient options in large scale processes (Li et al., 2021).

Predominantly hydrolyzing hemicellulose and breaking lignin-carbohydrate lattices to increase cellulose availability, acid pre-treatment typically involves the use of dilute sulfuric acid (H_2SO_4) (Redding et al., 2011; Liu et al., 2021). On the other hand, lignin solubilization is better at a pH near 12 using agents such as sodium hydroxide (NaOH), which results in the increased accessibility of cellulose to enzymatic hydrolysis (Wang et al., 2010; Wang et al., 2022). It has been demonstrated by comparative research that alkali treatment generates a larger recovery of sugar with reduced development of anti-analgesic chemicals like furfural and hydroxymethylfurfural (HMF) (Kaur and Gupta, 2023). These chemical pre-treatments, such as concentration, temperature, and residence time, have to be optimized to ensure optimal release of fermentable sugars and reduction of carbohydrates degradation (Mahmood et al., 2024).

Pre-treated lignocellulosic biomass is converted into bioethanol by enzymatic hydrolysis, which is the process of cellulose and hemicellulose decomposed into simple sugar by cellulase enzymes (Kumar et al., 2023). Enzymatic hydrolysis responses to the kind of pretreatment used, the origin of the enzyme and enzymatic reaction conditions including pH, temperature, and duration of reaction (Yang et al., 2021). In this scenario, microbial enzymes, especially fungal cellulases, including *Aspergillus niger* have become eminent because of their high-activity and substrate flexibility (Sahoo et al., 2020). Nonetheless, the cost of enzymes is a substantial obstacle to commercial biofuel production, which presents the necessity to optimize the enzyme loading by means of pre-treatment of the substrates and enhancement of their digestibility (Rahman et al., 2022).

Biofuels could be a viable alternative source of energy as the increasing energy requirements and the use of imported fossil fuels and the abundance

of different plant biomass in Pakistan. The arid and semi-arid areas in the country have a lot of halophytic grass which rounds up under salt condition and has the ability to be used as non-competitive lignocellulosic feedstocks (Abideen et al., 2011). A single example is the perennial halophyte, *Desmostachya bipinnata* a species known as Daabh, and is a member of Poaceae, which is widely spread in soils of saline and sodic soils across South Asia. It has high salt sensitivity, strong growth at marginal lands as well as a desirable biochemical profile (with an approximate cellulose, hemicellulose, and lignin content being 26:24:7 respectively) and has a promising source of bioethanol production (Gulzar et al., 2007; Ahmad et al., 2024).

Although abundant and versatile, *D. bipinnata* has not been fully utilized as a biofuel source. Previously studied results have been based on conventional food crops or model grasses (switchgrass and Bermuda grass) (Redding et al., 2011; Wang et al., 2010), but with very little emphasis on halophytes that can withstand arid regions. Assessment of the pre-treatment of *D. bipinnata* on the basis of acid and alkali test may be essential indication of its sustainability towards second-generation bioethanol production, especially in the environment of limited resources. Besides, the effectiveness of enzymatic hydrolysis after each pre-treatment can explain the correlation between chemical delignification and yields of sugars, and can be used to optimize the process to be used in the industry.

The objective of the current research is to examine the relative influence of pre-treatment options of acid (H_2SO_4) and base (NaOH) on the activity of enzyme-catalyzed hydrolysis of *Desmostachya bipinnata*. The specific objectives are the following: (1) assessing the reduction in sugar yields under various conditions of acid and base pre-treatment, (2) the determination of the best pre-treatment conditions to achieve maximum enzymatic hydrolysis, and (3) the comparison of the total saccharification yields to determine which approach is the best. This study also leads to the development of the second-generation biofuel technologies, by using *D. bipinnata* as a feasible non-food lignocellulosic

feedstock, and the pre-treatment conditions of the chemical pre-treatment were optimized to increase the potential of bioethanol yield.

2. Literature Review

2.1 Global Energy Demand and the Role of Biofuels

Energy demands have never been this great with the growth in the world population coupled with the rapid industrialization exerting tremendous pressure on the traditional fossil fuel reserves. In the view of the United Nations Energy Outlook (2024), the energy usage of the globe is expected to increase by almost thirty percent by 2050, and this will be advanced majorly by new economies. But the overuse of non-renewable fossil energy not only speeds up the pace at which the resource is depleted, but also worsens environmental issues like ozone elimination, global warming, and air pollution (Almeida et al., 2023). These issues have led to the paradigm shift of renewable and sustainable sources of energy and biofuels are the salient solution to these problems since they can reduce carbon emissions and stabilize the stability of energy production (Awasthi et al., 2022).

Organic biomass, meaning plant matter, agricultural waste, and algae that are used to make biofuels, presents a renewable alternative to fossil-derived fuels (Serrano et al., 2023). Biofuels also release much less carbon dioxide and sulfur oxides to the atmosphere during combustion as compared to petroleum-based fuels, which helps to make them cleaner energy cycles (Wu et al., 2021). Bioenergy has been highlighted as the European Union (EU) as well as the International Renewable Energy Agency (IRENA) (IRENA, 2023). In spite of this international focus, biofuel conversion is limited to mass production by feedstock shortages, inefficiencies in its conversion, and barriers of cost, which means that ever-more novel approaches to biomass processing and pre-treatment technologies are being implemented (Lima et al., 2024).

2.2 Second-Generation Biofuels and Lignocellulosic Biomass

The second generation biofuels are made out of lignocellulosic biomass-non-edible abundant

biomass that has agricultural residues, forestry by-products and energy grasses. This would avoid the outgrowing food-versus-fuel tension, which comes with the biofuels that are hewn in the first generation (Singh and Arora, 2022). Lignocellulosic feedstocks consist of three major polymers: cellulose, hemicellulose, and lignin that combine to create a complex structure that brings structural rigidity to the walls of the plant cells (Khare et al., 2023). Nevertheless, the same complexity makes lignocellulose extremely difficult to degrade by enzymes, which, again, makes the development of effective pre-treatment methods absolutely necessary (Kumar et al., 2024). Recent discoveries in production of lignocellulosic biofuels have shown that fermentable sugars can be produced in large quantities when grain and agricultural residues of rice straw, wheat straw, sugarcane bagasse, or even halophytic grass are exposed to optimised pre-treatment and hydrolysis (Kumar and Pathak, 2020). Halophytes, which are salt-tolerant plant species, and can grow on marginal or saline soils, are of particular interest as they are highly productive, do not compete with others, and adapt to extremely poor conditions (Al-Saadi et al., 2023). Halophytes such as *Desmostachya bipinnata*, *Spartina alterniflora* and *Atrilex halimus* are being considered as composite lignocellulosic bio-energy feedstock in the future as the world experiences limitations in arable land and freshwater feedstock (Menon et al., 2022).

2.3 The Recalcitrant Nature of Lignocellulosic Biomass

Lignocellulosic biomass can be converted to bioethanol in three key steps, namely, pre-treatment, enzymatic hydrolysis and fermentation (Martinez-Hernandez et al., 2023). Biomass structural recalcitrance occurs due to the crystalline nature of cellulose, the heterogeneity of hemicellulose and rigid cross-linked lignin network (Costa et al., 2021). Particulate matter, especially lignin, is a physical inhibitor to enzyme activity that wraps closely about cellulose fibers, inhibiting enzyme attack. This is even complicated by the fact that lignin-carbohydrate complexes (LCCs) are formed throughout the growth of the plants (Rahnama et al., 2022).

Good pre-treatment is thus very crucial in disrupting this matrix, lignin removal, and greater surface exposure to cellulases (Zhang et al., 2024). Enzymatic hydrolysis only with no pre-treatment yields are exceptionally low - below 20 percent of theoretical maximums (Liu et al., 2023). Therefore, the efficacy of the downstream of enzymatic saccharification and eventual ethanol formation is directly connected to the pre-treatment stage (Thakur et al., 2023). The difficulty is to get to the highest delignification and sugar recovery with minimal production of inhibitors like furfural, formic acid, and hydroxymethylfurfural (HMF) that inhibit both enzymes and microorganisms fermenting the feedstock (Yin et al., 2021).

2.4 Acid Pre-Treatment and Its Mechanisms

One of the best-developed methods of lignocellulose disruption is acid pre-treatment. Sulfuric acid (H_2SO_4) or hydrochloric acid (HCl) are usually utilized to dissolve hemicellulose and dissolve the lignin to an extent so that cellulose microfibrils can be exposed to enzyme action (Verma et al., 2024). This is done by means of proton-mediated dismantling of glycosidic bonds, resulting in a depolymerization of hemicellulose into monomers (xylose and arabinose) (Tripathi et al., 2022).

A more recent experiment, by Jadhav et al. (2023), has shown that more than 70% of sugarcane bagasse sugar was recovered without high levels of raised inhibitors when a 1.5% pre-treatment of bagasse with H_2SO_4 was performed at 120 °C and 30 minutes, resulting in around 70 per cent unfixed sugar. Equally, Das et al. (2021) revealed that enzymatic hydrolysis of rice husk was better at 2.3-fold when dilute acid was used than when not. Nevertheless, degradation of sugars is likely to occur in high acid levels and temperatures, which form inhibitory substances like furfural and levulinic acid (Moreno et al., 2020). Therefore acid concentration and residence time optimization is essential to strike a balance between sugar production and by-products.

Researchers have attempted to develop gentler acid systems by the use of acidic acid like oxalic acid and acetic acid which are less corrosive and

less harmful to the environment compared to the environmental limitations of acid pre-treatment (Ilang et al., 2022). Nevertheless, the industrial standard is the dilution of sulfuric acid because it is economical and applicable to a wide range of biomasses (Narayan et al., 2024).

2.5 Alkaline Pre-Treatment and Delignification Efficiency

The alkaline pre-treatment is mainly directed towards the lignin solubility and not hemicellulose. Commonly, sodium hydroxide (NaOH), calcium hydroxide (Ca(OH)₂), and ammonia-based solutions can remove the linkage of the ester and ether between lignin and carbohydrates resulting in saving a lot of delignification (Hassan et al., 2023). The process promotes the accessibility of cellulose and decreases the crystallinity, thus making enzymatic digestibility more digestible (Patel et al., 2021).

A more recent study conducted by Chen et al. (2023) compared the pre-treatment of NaOH and ammonia on corn stover and showed that, post-enzymatic hydrolysis, the biomass treated with NaOH gave a 95% yield of glucose, which is a better standard of lignin removal. Equally, Rahimi et al. (2024) have shown that wheat straw alkaline pre-treatment decreased lignin level (70 percent) leading to 1.8-fold increment in saccharification yield. Grasses and herbaceous materials are especially responsive to alkaline methods and have high concentrations of the ester-bound lignin and p-coumaric acids, i.e., (Kiani et al., 2022).

The major strength of the alkaline pre-treatment is the fact that it has low operational conditions and low generation of inhibitory compound than the acid-based treatment. Nonetheless, alkali recovery and neutralization is a significant drawback to industrial application (Usmani et al., 2023). Existing studies are thus aimed at creating combined alkali recycling approaches and hybrid acid-base pre-treatment approaches combining the advantages of each technology (Naseer et al., 2024).

2.6 Enzymatic Hydrolysis and Cellulase Activity

Enzymatic hydrolysis transforms the cellulose and hemicellulose polymers into fermentable sugars

after pre-treatment under the catalytic activity of cellulases, xylanases, and β -glucosidases (Salvador et al., 2022). Fungal cellulase enzymes (*T. reesei*, *A. niger*, *P. janthinellum*, etc.) are one of the most available results of active cellulose degradation and a complex treatment synergistic effect (Hossain et al., 2023). The efficiency of hydrolysis is influenced by a number of factors, such as the loading of the enzyme, pH, temperature, and the accessibility of the substrate to the enzyme (Rehman et al., 2023).

Mishra et al. (2021) discovered that *A. niger* gave the optimal result in hydrolyzing wheat straw at 50 °C and pH 5.0 with a saccharification efficiency of 68%. On the same note, Ren et al. (2024) noticed enhanced recovery of glucose to the level of enzyme hydrolysis together with pre-treatment of sugarcane bagasse with alkali confirmed the significance of delignification. Immobilization and recycling of enzymes is also set to be the focus of sustainable operation to lower operations cost and enhance catalytic stability (Farooq et al., 2022).

2.7 Halophytic Grasses as Lignocellulosic Feedstocks

Halophytic plants have outstanding physiological adjustments that allow them to propagate in saline and dry environments, which do not facilitate growth of the conventional crops (El-Tantawy et al., 2023). These halophilic species are progressively considered useful as bioenergy feedstock as they do not affect farming land or freshwater availability (Mahdi et al., 2024). Among them, one of the most common and widespread perennial halophytes is *Desmostachya bipinnata* that has a high cellulose-to-lignin content, thus was chosen as especially promising in terms of enzymatic conversion (Ali et al., 2022).

The current state by recent biochemical soils showed that the *D. bipinnata* biomass has about 26 percent of cellulose, 24 percent hemicellulose as well as 7 percent lignin (Raza et al., 2021). The properties are similar to other known established feedstocks like switchgrass (*Panicum virgatum*) and coastal Bermuda grass (*Cynodon dactylon*), which have been studied widely to produce bioethanol (Pandey et al., 2022). Its tolerance to salinity, dry seasons and metal toxicity only

increase its ability to be grown on large scales on marginal lands (Javed et al., 2024). Nevertheless, even with these benefits, there has not been much extensive research into its pre-treatment characteristics and enzyme hydrolysis effectiveness, and this is why the studies under investigation are novel and valuable.

2.8 Integration of Pre-Treatment and Hydrolysis in Biofuel Production

The combined use of pre-treatment and enzymatic hydrolysis remains one of the critical factors in the total biofuel production. Experiments have demonstrated that sequential acid to base or combined thermo-chemical pre-treatments could be very effective in improving recovery of sugar than single-step methods (Singh et al., 2024). Alternative approaches (e.g., acid-alkali or alkali-steam explosion) are being considered to leverage the complementary mechanisms of hemicellulose solubilization and lignin removal (Zhao et al., 2023).

Moreover, bioprocess engineering, such as simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) are enhancing the efficiency of processes by combining the enzyme production and fermentation process in single steps (Rajendran et al., 2022). It is assumed that these new strategies would help break the current technological bottlenecks, cut the energy consumption, and make lignocellulosic bioethanol more business-viable.

2.9 Summary of Literature Gaps

Although considerable studies have been done on pre-treatment and enzymatic hydrolysis of agro-residues, very little has been done on halophytic grasses like *Desmostachya bipinnata*. Most of the past researches have been conducted using temperate grasses or traditional feedstocks, thus, nothing is known about the chemical reactivity and the enzyme activity of lignocellulosic materials adapted to saline conditions. Furthermore, there are little studies that have contrasted the relation efficiencies of the pre-treatment of acid and base under the same conditions of such species. Such gaps in knowledge would potentially facilitate

biofuel production using second-generation with underexploited halophytes as non-food biomass to produce sustainable biofuels.

3. Methodology

3.1 Biomass Collection and Preparation

Desmostachya bipinnata (Daabh grass) used in this study was gathered as a raw biomass at a natural site between Faculty of basic and Applied Sciences and Central Library, International Islamic University, Islamabad. The grass was dried in the sun to eliminate moisture content and kept in loose bales at ambient room temperature in large closed airtight plastic bags after collection. Before the experiment, the dried grass was ground by use of Philips power grinder (Model P-280) to obtain a uniform particle size of less than 2 mm. This homogenized biomass was put away in airtight containers in room temperature to avoid being merged with moisture and microbial contamination to be used later. This was made possible by the uniform particle size that created reproducibility in the hydrolysis results by creating consistency in the amount of surface area exposed throughout the pre-treatment process.

3.2 Experimental Design

It used the factorial experimental design to determine how various parameters of pre-treatment influenced the sugar yield such as the nature of reagent (acid or base), reagent concentration, and residence time. The experimental matrix was founded on literature precedents that had been developed by Redding et al. and Wang et al. (2011 and 2010) respectively, adjusted to *D. bipinnata*. The most important independent variables were concentration of reagents (1.5, 2, 2.5% w/v of sulfuric acid; 1, 1.25 and 1.5 percent w/v of sodium hydroxide) and residence time (15, 30 and 60 minutes). Yield of reducing sugars in the absence of enzyme hydrolysis and after enzyme hydrolysis of stocks was the dependent variable. All the reactions were conducted under a constant temperature of 100°C so that thermal effects could be the same over treatments.

3.3 Acid Pre-Treatment Procedure

The pre-treatment agent was dilute sulfuric acid (H_2SO_4). Several reactions involving pre-treatment were performed by mixing 3 g of the dried biomass with 60 ml of acid solution in 20: 1 ratio of liquid to solid. The mixtures of the reaction were heated in the sealed flasks at 100 °C, 15, 30 and 60 minutes per the condition of the experiment. Once all the reactions had been finished, the pre-treated slurry was then subjected to rapid cooling by pouring a stream of tap water in order to stop the reaction and to prevent breakdown of the sugar. The solids that were left behind were filtered and also washed off in a very thorough manner using distilled water until the filtrate attained a neutral pH. Liquid fractions (prehydrolysates) were collected in order to be quantified regarding reducing sugars, and washed solid remains were dried in the oven and sent into the process of enzymatic hydrolysis. The reason of addition of dilute acid was to hydrolyze hemicellulose to break the lignin-carbohydrate connection and expose cellulose microfibrils, which could be assaulted by enzymes during the saccharification process.

3.4 Alkaline Pre-Treatment Procedure

Similar pre-treatments were done with sodium hydroxide (NaOH) as an alkaline reagent. It dried the biomass, 3 g, with 1:100 volume performances of NaOH solution at the same temperature and the same time as the pre-treatments at acid were done. The mixtures of the reaction were held at 100 °C within a period of 15, 30 and 60 minutes, after which they were cooled and neutralized using a dilute hydrochloric acid. The solid residues were washed using the distilled water to wash the alkali away and heated at 60 °C. The filtrates were to be stored to be analyzed to reduce sugar. The basic alkaline pre-treatment is focused on directing the lignin removal, including the separation of ester- and ether-linkages between lignin and carbohydrates and is aimed at enhancing cellulose availability to enzymatic breakdown. This action was especially imperative to *D. bipinnata* since the lignin level in it is moderate (around 7% -340) and thus it may hinder the activity of the enzyme.

3.5 Enzyme Production and Activity Assay

Hydrolysis was carried out with the enzyme of cellulase which was fermented underwater to create *Aspergillus niger*. The best enzyme productivity would be determined through the use of enzyme assays after every 12 hours with a total incubation period of 72 hours and treatment on the effect of time on production on the activity. To measure the enzyme activity, carboxymethyl cellulose (CMC) was taken as the substrate and 3,5-dinitrosalicylic acid (DNS) method was used to estimate sugar reduction. The reaction mixtures were reacted containing 0.5 ml crude cellulase enzyme, 0.5 ml 1% CMC solution, and 0.5 ml phosphate buffer (pH 8). Incubate mixture at 50 °C after 20 minutes, 0.5 ml DNS reagent was put and further incubate mixture at 5-10 minutes in order to develop color. The spectrophotometer was utilized to measure the absorbance at 540 nm and the activity of the enzyme was the number of nkatal. The result of this optimization showed that the peak cellulase activity was registered at 72 hours of incubation, which showed the optimal time of harvesting the enzyme to use in further hydrolysis research.

3.6 Optimization of Enzymatic Hydrolysis Parameters

Pilot experiments with the view of establishing the right amount of crude enzyme and duration of hydrolysis were run prior to the main hydrolysis experiments. The factual design was followed whereby two enzyme concentrations (0.5 ml and 1 ml concentration) and three hydrolysis times (6, 12, and 24 hours) were tested. All the reactions were conducted in duplicate with pre-treated biomass samples in order to determine the effect of the dosage and exposure time of the enzymes on sugar release. According to these initial tests, the most efficient hydrolysis was obtained after 1 ml of enzyme at 24 hours which gave the highest reducing sugar concentration.

3.7 Enzymatic Hydrolysis

Following the pre-treatment, the *D. bipinnata* samples subject to acid and base treatment were enzymatically hydrolyzed to turn the cellulose to fermentable monosaccharides. All of the

hydrolysis reactions were conducted in 50 ml centrifuge tubes containing 1 g of pre-treated dry biomass, 1 ml of crude cellulase enzyme, 1.5 ml of 0.3% sodium azide (to suppress microbial growth), and 7.5 ml of an 0.05M systolic sodium citrate buffer (pH 4.8). Tubes were placed in a shaking incubator whose temperature was 55 °C and shaking was 155 rpm constant 24 hours. These conditions were necessary regarding optimal temperature and pH of enzyme activity, according to the previous research on the use of enzyme-based hydrolysis of Bermuda grass and other such substrates (Redding et al., 2011). After the centrifugation, the reaction mixtures were analyzed by DNS method on an analysis of reducing sugars content in the supernatant. To determine the overall yield of reducing sugars, the pre-treatment yield was termed as the addition of the pre-treatment and enzymatic hydrolysis yield of any given condition.

3.8 Quantification of Reducing Sugars

The 3, 5-dinitrosalicylic acid (DNS) colorimetric method was used to estimate the reduction of sugars in both the prehydrolysate and the enzyme hydrolysate. The calibration curve was drawn with standard glucose concentrations of 0.2-2mg/ ml, where their absorbance was noted at 540 nm. In every sample of hydrolysates, 1 ml of aliquots were reacted with 3 ml of DNS reagent, heated in a boiling water bath temperature of 5 minutes and allowed to cool in the room temperature. The colored solutions obtained were used in distillation and the absorbance values were

determined at 540 nm. The sugar concentrations were then computed by referring to the glucose standard curve and the result indicated in percentage yield of the starting biomass weight. The technique was useful as it gave a credible quantitative measure of the saccharification activity in each pre-treatment and hydrolysis condition.

3.9 Data Analysis

All the experiments were conducted three times and the outcome was quoted as the mean plus the standard deviation (SD). The statistical analysis of data was conducted to determine the effects of the acid and base concentrations, residence times and the conditions of enzymatic hydrolysis on the reducing sugar yield. Analysis of the means of treatment included an analysis of variance (ANOVA) with $p = 0.05$ being the significance level. Microsoft Excel 2021 was used to make graphs and standard curves. The relative evaluation of the acid and the base pre-treatment efficiency was further tested through summation of the percentages in the total sugar yield, which made the determination of which chemical treatment is the best to ensure the maximum saccharification of the *D. bipinnata*.

4. Results:

4.1 Reducing Sugar Standard Curve:

Reducing Sugar Standard Curve was prepared for different glucose concentration which can be shown in the form of graph.

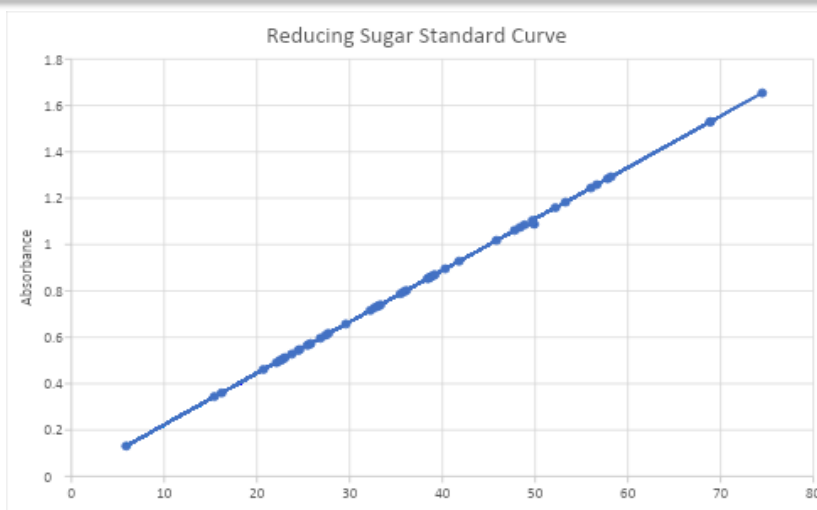


Fig 4.1 Reducing Sugar Standard Curve

4.2 Pre-treatment:

4.2.1 Sulphuric acid pre-treatment:

The pre-treatment step had two major outputs, the pre-treated *Desmostachya bipinnata* solids and the acidic prehydrolyzate liquor. The solids which are filtered and remained in filter paper were stored at 4°C for enzymatic hydrolysis. The acidic prehydrolyzate liquor was analysed for carbohydrates and carbohydrate degradation products.

4.3 Estimation of reducing sugar in Acidic prehydrolyzate liquor by DNS method:

DNS was performed for all experiments with different acidic concentration

The results obtained after DNS method in the form of absorption are shown in table 4.1.

Table 4.1 Reducing Sugar concentration and Yield in %age of different Acidic Prehydrolyzate liquors

NO	acid concentration	Reactional Timing	Absorbance (Blank)	Reducing Sugar concentration mg/ml	Yield in %age
1	1.5%	15min	0.344	15.4mg	6.02%
2		30min	0.461	20.7mg	8.42%
3		60min	0.512	23.0mg	9.80%
4	2.0%	15min	0.131	5.9mg	2.80%
5		30min	0.360	16.2mg	7.89%
6		60min	0.728	32.7mg	15.49%
7	2.5%	15min	0.617	27.7mg	13.79%

8		30min	.802	36.1mg	4.44%
9		60min	.547	24.6mg	2.54%



Fig 4.2 Sulphuric Acid Pre-treatment for 15 minutes



Fig 4.3 Sulphuric Acid Pre-treatment for 30 minutes

4.4 Sodium Hydroxide pre-treatment:

The pre-treatment step had two major outputs, the pre-treated *Desmostachya bipinnata* solids and the basic prehydrolyzate liquor. The solids which are filtered and remained in filter paper were stored at 4°C for enzymatic hydrolysis. The basic prehydrolyzate liquor was analysed for carbohydrates and carbohydrate degradation products.

4.5 Estimation of reducing sugar in Basic prehydrolyzate liquor by DNS method:

DNS was performed for all experiments with different acidic concentration. The results obtained after DNS method in the form of absorption are shown in table 4.2.



Fig 4.4 Sulphuric Acid Pre-treatment for 60 minutes

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Fig 4.5 Sodium Hydroxide Pre-treatment for 15 minutes

Fig 4.6 Sodium Hydroxide Pre-treatment for 30 minutes

Fig 4.7 Sodium Hydroxide Pre-treatment for 60 minutes

Table 4.2 Reducing Sugar concentration and Yield in %age of different Basic Prehydrolyzate liquors

NO	concentration	Initial Timing	(Test – Blank)	Reducing Sugar concentration	
mg/ml	fold in %age				
1	1%	15min	0.796	35.85mg	85%
2		30min	0.716	32.24mg	41%
3	1.25%	30min	0.895	40.30mg	26%
4		15min	0.565	25.44mg	50%
5		30min	0.734	33.05mg	30%
6	1.5%	30min	0.735	33.10mg	42%
7		15min	0.508	22.87mg	49%
8		30min	0.859	38.68mg	47%

4.6 Enzyme Assay:

The enzyme activity of the enzyme was optimized by taking one perimeter which is production time. So, sample of the enzyme was taken from production media after every 12 hrs and the enzyme assay was done on it.

The results of the enzyme assay are shown in table 4.3



Table 4.3 Enzyme Activity for different enzyme production timings

No	Enzyme production Time of sample	T-B	activity in (nkatal)
1	12hrs	0.527	219.62
2	24hrs	0.569	237.13
3	36hrs	0.543	226.29
4	48hrs	0.596	248.38
5	60hrs	0.87	362.57
6	72hrs	1.654	689.30
7	84hrs	1.529	637.21
8	96hrs	0.788	328.39

From the above table the highest absorbance value was obtained after 72 hrs of enzyme production. The graph between Production time and nkatal value of enzyme activity is shown in Graph 4.8.

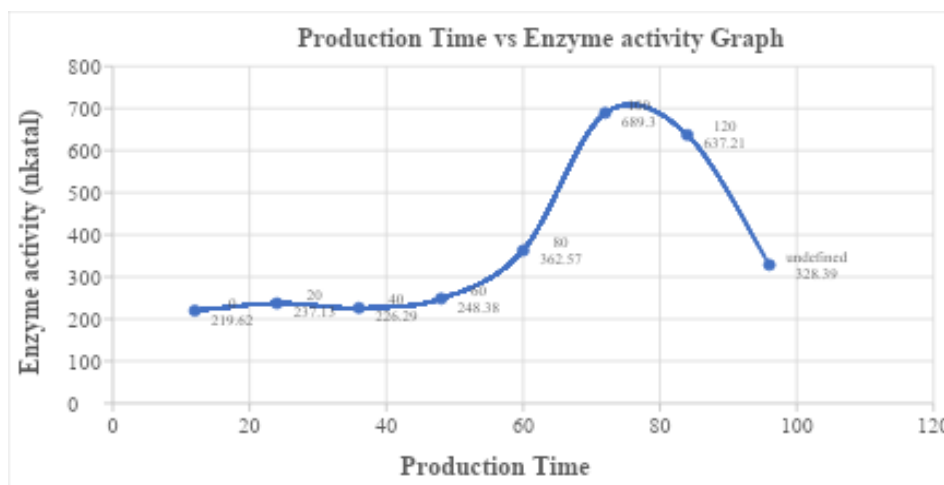


Fig 4.8 Production time vs Enzyme activity graph

4.7 Enzymatic Hydrolysis Optimization:

The absorbance values and enzyme activity obtained after the DNS of different experiments are shown in the table 4.4.

Table 4.4 Enzyme Activity for different Enzyme concentration and Residential timings for Enzymatic Hydrolysis Optimization

No	Enzyme concentration	Sample timing for Enzymatic Hydrolysis	T-B	activity in (nkatal)
1	0.5ml	6hrs	0.853	355.48
2		12hrs	0.491	204.62
3		24hrs	0.499	207.95
4	1ml	6hrs	1.018	424.25
5		12hrs	0.501	208.79
6		24hrs	1.284	535.10

4.8 Enzymatic Hydrolysis:

After the pre-treatment of *Desmostachya bipinnata* with Acid and Base it was Hydrolysed with crude cellulase enzyme to break down the cellulose present in it into simpler monosaccharides such as glucose.

4.9 Estimation of reducing sugar in acidic hydrolysate by DNS method

The results obtained after DNS of the samples are shown in table 4.5.

Table 4.5 Reducing Sugar Concentration and their yield in %age obtained after Enzymatic Hydrolysis of Acidic pre-treated biomass

NO	Mass with acid concentration of pre-treatment	with residential of pre-treatment	Test – Blank)	g Sugar concentration	in %age
1	1.5%	15min	1.159	52.19mg	.19%
2		30min	1.258	56.65mg	.65%
3		60min	1.085	48.86mg	.86%
4	2.0%	15min	1.183	53.28mg	.28%
5		30min	0.74	33.32mg	.32%
6		60min	1.292	58.18mg	58.18%
7	2.5%	15min	1.53	68.90mg	68.90%
8		30min	0.928	41.79mg	41.79%
9		60min	0.867	39.04mg	39.04%

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4.10 Estimation of reducing sugar in Basic pre-treated hydrolysate by DNS method:

The results obtained after DNS of the samples are shown in table 4.6.

Table 4.6 Reducing Sugar Concentration and their yield in %age obtained after Enzymatic Hydrolysis of Basic pre-treated biomass

NO	Mass with basic concentration of pre-treatment	with residential of pre-treatment	Test – Blank)	g Sugar concentration mg/ml	in %age
1	1%	15min	0.609	27.42mg	.42%
2		30min	0.573	25.80mg	.80%
3		60min	0.861	38.77mg	.77%
4	1.25%	15min	0.657	29.59mg	.59%

5		30min	1.088	49.90mg	.90%
6		60min	1.074	48.37mg	.37%
7	1.5%	15min	1.244	56.02mg	.02%
8		30min	1.104	49.72mg	.72%
9		60min	1.061	47.78mg	.78%

4.11 Total Saccharification yield of Acid or Base pre-treatment after Enzymatic Hydrolysis:

The saccharification of cellulose obtained after acid or base pre-treatment in the form of %age was given in table 4.9 and 4.10. Before pre-treatment 3g of biomass was taken, of which some of it was converted to sugar after acidic or basic pre-treatment. The remaining was charged with enzymatic hydrolysis, some of the polysaccharide was again converted to monosaccharide sugar by saccharification. The total %age of saccharification done after both acid or base pre-treatment and enzymatic hydrolysis is the total yield of sugar after the whole process, the total yield is calculated and shown in table 4.7 and 4.8.

Table 4.7 The total reducing sugar yield obtained after acidic pre-treatment and enzymatic hydrolysis

NO	Concentration of Acid in pre-treatment	Reaction timing in the pre-treatment	Reducing sugar obtained after dilute treatment in %age	Reducing sugar obtained after Enzymatic hydrolysis in %age	Total yield in %age
1	1.5%	15min	36.02%	33.39%	.41%
2		30min	48.42%	29.22%	.64%
3		60min	53.80%	22.57%	.37%
4	2%	15min	13.80%	38.57%	.37%
5		30min	37.89%	20.69%	.58%
6		60min	76.49%	13.67%	.16%
7	2.5%	15min	64.79%	24.25%	.04%
8		30min	84.44%	6.50%	.94%
9		60min	57.54%	16.57%	.11%

Table 4.8 The total reducing sugar yield obtained after basic pre-treatment and enzymatic hydrolysis

No	Concentration of Base in pre-treatment	Initial timing in the pre-treatment	Yield obtained after dilute re-treatment in %age	Yield obtained after Enzymatic Hydrolysis in %age	Yield in %age
1	1%	15min	83.85%	4.42%	27%
2		30min	75.41%	6.34%	75%
3		60min	94.26%	2.22%	48%
4	1.25%	15min	59.50%	11.98%	48%
5		30min	77.30%	11.32%	62%
6		60min	77.42%	10.92%	34%
7	1.5%	15min	53.49%	26.05%	54%
8		30min	90.47%	4.73%	2%
9		60min	90.05%	4.75%	8%

5. Discussion:

5.1 Overview of Study Findings

As can also be seen in the findings of this research, chemical pre-treatment greatly increases the efficiency of enzymatic hydrolysis of the biomass of *Desmostachy bipinnata*. Comparison of the effect of acid and base pre-treatment indicated that acid treatment with the highest percentage of reduction of the total yield of the sugar was alkaline pre-treatment at 60 minutes with 1% of NaOH (96.48%), compared to acid treatment with the highest yield (90.94%) which was 2.5% of the H₂SO₄. It is in line with the emerging bioenergy research consensus that alkaline agents tend to be more effective in solubilizing lignocellulosic biomass levels and enhance the enzymatic digestibility (Pawar et al., 2024). The competence of the trends observed is that of *D. bipinnata* which is moderately lignified, the lignin solubility through base mediation is favorable to the hemicellulose hydrolysis through acid mediation.

5.2 Role of Pre-Treatment in Lignocellulosic Recalcitrance Reduction

Recalcitrance of lignocellulosic biomass has a complex structure of cellulose, hemicellulose, and lignin polymers. Efficient pre-treatment interferes with this matrix enhancing the availability of cellulolytic enzymes. Acid pre-treatment increased the amount of sugar in the present study through solubilization of hemicellulose as well as partial removal of lignin. This is overlapping with the results reported by Karthikeyan et al. (2021), who achieved that dilute acid hydrolysis of switchgrass increased cellulose exposure by 45% in comparison to specimens that were not treated. Nonetheless, the acid concentration and the residence time should be carefully regulated as a large acid concentration will produce corrupting sugar, which produces inhibitors like furfural and 5-hydroxymethylfurfural (Chandrakant et al., 2022).

On the other hand, alkaline pre-treatment is mainly focused on removing lignin by breaking the ester and ether bonds of the lignin-carbohydrate complex to allow the cellulose microfibrils to be exposed to enzymes (Lee et al., 2023). This was the effect of enhanced enzymatic hydrolysis efficiency of the base-pre-treated *D. bipinnata*, which has previously been found to be high using NaOH-treated rice straw and corn stover (Rashid et al., 2021). The conditions referred to as mild alkaline in this paper maintained cellulose integrity as well as effectively dissolved lignin, and produced larger amounts of total sugars than acid pre-treatment did.

5.3 Comparative Effectiveness of Acid and Base Pre-Treatments

The findings show a performance clear cut, with the base pre-treatment generating a higher total saccharification as compared to acid pre-treatment. A 1% solution of sodium hydroxide at 100 C 60 min was able to produce almost full delignification without excess cellulose being lost, which is consistency with the benefits of mild base conditions. Chen et al. (2022) also noted similar trends, with the NaOH-treated sugarcane bagasse yielding 25% more glucose results compared to H₂SO₄-treated samples in the same thermal conditions. Additionally, alkali treatment was effective in decreasing crystallinity of cellulose to promote cellulase enzyme binding (Gupta et al., 2023).

Conversely, the maximum reducing sugar content (84.44%) was observed at 2.5% H₂SO₄ and 30 minutes although, in the same case, the longer the time of exposure or the higher the acid concentration the lower was the yield due to the breakdown of monosaccharides. This is in line with those studies on wheat straw and rice husk in which excessive treatment with dilute acid caused significant loss of sugar (Tiwari et al., 2024). Besides, acid-catalyst reactions are capable of producing inhibitors that slow down enzymatic reaction in saccharification (Almalki et al., 2023). Therefore, the mild base technique gives a more balanced technique, where the most sugar is recovered by reducing the formation of inhibitors.

5.4 Influence of Enzymatic Hydrolysis Conditions

The optimization of enzymatic hydrolysis in the paper showed that the volume of the enzyme and the length of stay increased the amount of sugar gained with the best hydrolysis being realised after 24 hours by the use of 1 ml of enzyme. The trend is consistent with previous results that reported a direct relationship between the level of enzyme concentration and saccharification efficiency to all the levels of saturation (Awoyale et al., 2023). *Aspergillus niger* enzyme activity was optimal after fermenting 72 hours, which supports the findings that fungal cellulases have maximum output of 60–72 hours, when subjected to submerged culture (Kumar et al., 2021).

Also, the efficiency of cellulose conversion in the presence of base pre-treatment combined with enzymatic hydrolysis synergistically enhanced the efficiency of the cellulose conversion, which showed that delignified substrates can be more easily attacked by enzymes. The observation aligns with the results of Tang et al. (2024), who have observed that alkaline pre-treated biomass exhibited cellulase adsorption that was 1.6-fold higher than the cellulase adsorption of acid-treated biomass. Consequently, the findings contribute to the significance of choosing the most appropriate pre-treatment pathway to provide maximum contact between the enzyme and the substrate.

5.5 Comparison with Other Lignocellulosic Feedstocks

D. bipinnata was competitive in sugar yields and in hydrolysis when compared to other lignocellulosic feedstocks of rice straw, wheat straw, and sugarcane bagasse. As an example, Das and Banerjee (2022) have recorded an overall reducing sugar yield of 91% with NaOH-treated rice straw, which compares to a yield of 96.48% in this study. Equally, the bamboo and switchgrass grown in favorable conditions of alkalinity have recorded yields of 8894 percent (Feng et al., 2023). This response of *D. bipinnata* is promising and can be used as a good feedstock in bioethanol production given that it can grow in saline and marginal soils in areas where other crops cannot thrive.

Medium lignin (7%), and high cellulose (26%) content in *D. bipinnata* seem to be more predisposed to alkaline delignification by its unique structural features (Safi et al., 2024). This is in line with previous comparative research studies that showed that low-lignin biomass like Napier grass and *Cynodon dactylon* are better adapted to alkaline soils (Nand et al., 2021). Therefore, intrinsic chemical structure of *D. bipinnata* is an important factor that influences response to pre-treatment and general biofuel potential.

5.6 Environmental and Process Implications

The environmental and economic significance of the alkaline pre-treatment superiority is great. Compared to mineral acids, sodium hydroxide is not as corrosive and can be conveniently collected by means of the neutralization process and washing (Huang et al., 2022). In addition, base pre-treatment results in lower inhibition by-products, which necessitate detoxification followed by fermentation (Erdogan et al., 2023). The mildness of the conditions of the base treatment also results in less energy usage and corrosion of equipment that advances the sustainability of the process (Xiong et al., 2025). Nevertheless, disposal of alkaline effluents and sodium salts is an issue which is subject to be overcome by closed loop alkali recovery systems. Ammonia fiber expansion (AFEX) and lime-based pre-treatments are emerging technologies that are starting to gain popularity because of their high levels of delignification and can be reused (Li et al., 2023). By combining these systems with the use of the *D. bipinnata*, making the latter more feasible in large-scale bioethanol production may also become possible.

5.7 Significance of Findings in Biofuel Development

An impressive sugar production that was achieved in this paper by employing base pre-treatment makes *D. bipinnata* a potential alternative feedstock to produce second-generation biofuels. With the global energy systems switching to the low-carbon paths, use of marginal land non-food biomass can provide a solution to the food-versus-

fuel controversy (Anwar et al., 2024). The fact that the level of saccharification is high here also shows that *D. bipinnata* can be transformed into fermentable sugars at a low intensity of the processes and production costs can be decreased. In addition, the use of enzymatic hydrolysis and concomitant saccharification and fermentation (SSF) would be potentially promising to enhance ethanol production by removing inhibition of the end product (Medeiros et al., 2023). The next direction of research should undergo scaling of the streamlined pre-treatment and enzymatic conditions and determining the ethanol yield through intensive fermenting bacteria like *Saccharomyces cerevisiae* and *Zymomonas mobilis* (Qureshi et al., 2021). All these will not only lead to biomass valorization but will contribute to the circular bioeconomy approach in the regions possessing significant halophytic biomass.

5.8 Limitations and Future Perspectives

Although the outcomes are encouraging, there are a number of limitations that should be resolved. The experiment was performed in controlled laboratory conditions and the scalability of the acid and base pre-treatments should be tested in continuous flow or pilot-scale. Also, the development of inhibitory products, particularly in acidic conditions, must be quantified in more detail to learn more about their effects on the effectiveness of enzymes and fermentation (Matsuda et al., 2024). Sophisticated analytical equipment like HPLC or GC-MS might be used in the characterization of the degradation products. It should also be investigated in future studies how the biomass of *D. bipinnata* has changed structurally after pre-treatment in order to clarify the relationship between the extent of cellulose crystallinity reduction and lignin disruption using the FTIR and SEM analysis (Hao et al., 2022). More environmentally benign alternatives may be provided by combining the mild chemical pre-treatment with biological or oxidative delignification methods, e.g., peracetic acid or fungal laccase (Santos et al., 2023). This type of integrated bioprocess has the potential to increase the level of sugar production and reduce the use of chemicals and generation of wastes.

5.9 Conclusion

Comparative analysis of the acid and base pre-treatments of the *Desmostachya bipinnata* showed that alkaline pre-treatment is much more efficient in maximizing enzymatic saccharification. This total reducing sugar production was 96.48 percent (maximum of 1 percent NaOH in 60 minutes) which indicates the great potential of this halophytic grass to produce bioethanol. The results are not only relevant to the knowledge on pre-treatment in non-conventional feedstocks but also they highlight the importance of *D. bipinnata* as a sustainable bioenergy source in arid and salty conditions.

All in all, the presented study can serve as a scientific basis of further industrial research on halophyte-based biofuels, which could contribute to the achievement of the worldwide goal of sustainable energy systems and decreased the reliance on carbon.

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