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Studies on process optimization of the *Chenopodium album* demonstrate its substantial potential for the generation of bioenergy

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Abstract

We must reduce our consumption of fossil fuels. Bioethanol is derived from crops, such as annual and perennial dry energy grasses, as well as forestry waste. The hydrolysis of lignocellulosic biomass enzymes yields fermentable sugars, but, this process necessitates costly pretreatment and energy consumption. Utilizing bioethanol derived from cannabis plants could potentially address the issues surrounding the use of lignocellulosic biomass. Weeds are suitable for use as bioenergy feedstock because they grow quickly and have a high concentration of glucose. The work resulted in the creation of Chenopodium album biofuel. The physicochemical analysis revealed elevated levels of volatile solids, minimal ash content, and an augmented presence of cellulosic and hemicellulosic compounds, indicating a promising potential for bioenergy production. Acid pretreatment resulted in a higher amount of fermentable sugars for biohydrogen and biomethane production compared to alkaline pretreatment. Additional preliminary investigations were conducted utilizing a 1% (w/w) solution of sulphuric acid at a temperature of 121 °C for a duration of 15 minutes. Acid-treated materials exhibited perforations and expanded structures on scanning electron microscopy (SEM). Therefore, the cellulose underwent intrinsic structural changes as a result of a 1% acid pretreatment, which made it more compatible with microbial activity. The above studies confirm the potential of *Chenopodium album* for production of biofuel.

Keywords: Biofuel, Chenopodium analysis, lignocellulosic content, SEM, Weed

1. Introduction

Bioethanol is a renewable biofuel derived from plant materials, primarily used as an alternative to fossil fuels in the transportation sector. Produced from various feedstocks such as food crops (e.g., corn and sugarcane), non-food biomass (e.g., agricultural residues and woody crops), and even algae, bioethanol involves several steps including pre-treatment, hydrolysis, fermentation, distillation, and dehydration [1]. Its applications range from being blended with gasoline to create fuel mixtures like E10 and E85, to serving as an industrial solvent and a raw material in chemical production [2]. Bioethanol offers environmental benefits by reducing greenhouse gas emissions and promoting the use of renewable resources. It also supports rural economies by creating new markets for agricultural products [3]. However, challenges such as the food vs. fuel debate, land and water use, energy balance concerns, and technological hurdles for second and third-generation bioethanol production need to be addressed [4]. Despite these challenges, advancements in biotechnology and sustainable practices hold promise for the future of bioethanol as a key player in the transition to greener energy sources.

Quantifying the potential of Chenopodium album as a feedstock for biofuel production involves evaluating its biomass yield, chemical composition, and conversion efficiency. Chenopodium album, known for its rapid growth and adaptability, can produce substantial biomass, with yields reaching up to 10-15 tons per hectare annually under optimal conditions. The plant's biomass primarily consists of lignocellulosic material, with cellulose and hemicellulose comprising about 60-70% of its dry weight, making it a viable candidate for bioethanol production [5]. Efficient pre-treatment methods, such as a steam explosion or alkaline treatment, can release fermentable sugars from this lignocellulosic matrix, achieving conversion efficiencies of around 80-90%. Additionally, thermochemical processes like pyrolysis and gasification can transform Chenopodium album biomass into bio-oil and syngas, respectively, with energy conversion efficiencies ranging from 40-60%. The overall biofuel yield from Chenopodium album can be competitive with other non-food biomass sources, provided that collection, transportation, and processing costs are optimized [6]. This quantification underscores the plant’s potential to contribute significantly to sustainable biofuel production while offering a solution for managing this invasive species.

2. Methodology

2.1 Collection of raw materials

This study utilised the Chenopodium album, a type of weed, as feedstocks for biofuel production [7]. The weed was acquired at Om Sterling Global University, located in Hisar. It was meticulously washed with water, dried under the sun, and ground to a mesh size of 0.4 mm.

2.2 Inoculum

A laboratory-scale anaerobic digester was utilized to create a mixed microbial anaerobic culture, which was then employed to generate biomethane through methanogenesis [8].The electrochemically active bacteria (EAB) obtained from a functioning microbial fuel cell (MFC) were utilized for the generation of hydrogen by electrohydrogenesis [9].To produce hydrogen, a weed together with an anaerobic culture, was subjected to heat at a temperature of 80 °C for a duration of 2 hours. This heat treatment was carried out to eliminate methanogens and facilitate the growth of spore-forming bacteria such as Clostridium and Bacillus, which are capable of producing hydrogen [10]. The mixed culture was subjected to heat treatment and then underwent numerous cycles of enrichment in anaerobic media, as detailed in Table 1.

Table 1: Composition of media used for cultivation of anaerobic microbes

Anaerobic media	g/dL
Potassium Chloride (KCl)	0.034
Magnesium Chloride ($MgCl_2.6H_2O$)	0.4
Magnesium Sulphate ($MgSO_4$)	0.345
Ammonium Chloride (NH_4Cl)	0.025
Calcium Chloride ($CaCl_2.6H_2O$)	0.14
(Potassium dihydrogen phosphate) KH_2PO_4	0.14
Sodium Chloride (NaCl)	1.8
Sodium acetate (CH_3COONa)	0.1
Yeast Extract	0.2
Trypticase peptone	0.2
L-Cysteine-HCl	0.050
Sodium Sulphide	0.050
Distilled Water	100ml

The *Saccharomyces cerevisiae* strain was utilized as the inoculum for the manufacture of alcohol. The specimen was cultivated in YEPD (Yeast Extract Peptone Dextrose) broth media with a pH of 6.0 at a temperature of 30 °C for 24 hours in an incubator-shaker [11]. The formulation of YEPD media is provided in Table 2.

Table 2: Composition of media used for cultivation of yeast

Yeast Extract Peptone Dextrose Media	g/dL
Yeast Extract	1
Peptone	2
Dextrose	2
Distilled water	100ml

2.3 Characterization of the studied weeds as a biofuel feedstock

Biomass characterization include proximal analysis, ultimate analysis, and compositional analysis [12]. The analysis was completed using the conventional methods, which are briefly stated below.

2.3.1 Proximate analysis

Proximate analysis refers to the process of determining the basic composition of a substance or material. Proximate analysis is a crucial method for assessing the appropriateness of biomass as a feedstock for biofuel production. The analysis encompasses the measurement of moisture content, ash content, volatile matter content, and fixed carbon content of the raw biomass [13]. The moisture content of aquatic biomass was measured using a moisture analyzer, namely the Hal. Moisture Analyzer HE53 (230V) by Mettler Toledo. The volatile solids (VS) content of aquatic biomass was determined using the methodology outlined in ASTM D3175. In summary, a crucible containing a 1 g sample was heated in a furnace at a temperature of 550 °C for 6 hours. The weight of the sample after ignition was subtracted from the original weight to determine the total volatile solids (TVS). The ash content of biomass was determined using the methodology developed by NREL [14]. Ash refers to the weight of the remaining residue after completely burning 1 gram of biomass at a temperature of 575 ± 25 °C. This weight was determined through calculation.

$$\text{Ash (\%A)} = \left[\frac{(W1-W2)}{W3}\right] \text{ x } 100$$

W1- weight of the crucible and oven dried biomass; W2- weight of the crucible and residue left post combustion; W3- weight of dried biomass taken (g)

The fixed carbon content was computed

Fixed Carbon= 100%- (PVM-PAC)

PVM denotes percentage volatile matter and PAC denotes percentage ash content

2.3.2 Ultimate Analysis

Ultimate analysis involves determining the crucial chemical constituents present in biomass, specifically carbon, hydrogen, nitrogen, and sulphur. An elemental study of the weed biomass was conducted using a CHNS/O analyzer [15]. The aquatic biomass was incinerated at a high temperature of 1000 °C in an oxygen-rich furnace, resulting in the conversion of the biomass into carbon dioxide, water, and nitrogen oxides. The items that were released were subsequently identified using thermal conductivity detection [16].

2.3.3 Compositional Analysis

The composition of weed was assessed in terms of cellulose, hemicelluloses, and lignin using the procedure established by the National Renewable Energy Laboratory (NREL) in 2008. In summary, this procedure involves two steps of hydrolysis. Initially, 300±10 mg of dehydrated aquatic biomass is hydrolyzed in 72% H_2SO_4 for one hour at 30 °C [17]. This is then followed by dilution to a 4% acid concentration and hydrolysis at 121 °C for one hour. Throughout this procedure, the intricate polysaccharides underwent hydrolysis, resulting in the formation of simple reducing sugars such as glucose and xylose. These sugars were subsequently quantified using High-Performance Liquid Chromatography (HPLC). The lignin underwent decomposition, resulting in the formation of acid insoluble lignin (AIL) and acid soluble lignin (ASL). The AIL was examined using the gravimetric technique, whereas the acid soluble lignin was quantified using a UV-Vis spectrophotometer [18].

The lipid content in weed biomass was assessed. A total of 6 grams of biomass (dry weight) was combined with 20 milliliters of methanol, 20 milliliters of chloroform, and 20 milliliters of distilled water [19]. The mixture was vigorously agitated using a vortex mixer for 10 minutes, and thereafter subjected to centrifugation to separate and isolate the lipids.

The protein content of biomass was quantified following protein extraction with hot Trichloroacetic acid (TCA) using the extraction procedure [20]. To extract the protein content,

5 mg of dried aquatic biomass was initially mixed vigorously with 250 µl of 25% (w/v) TCA. The resulting mixture was thereafter subjected to incubation at a temperature of 95 °C for 15 minutes, within centrifuge tubes, followed by cooling to the ambient room temperature. The solution was made less concentrated by adding 600 µl of distilled water and then subjected to centrifugation at 10000 g for 15 minutes at 4 °C [21]. The supernatant obtained was discarded, and the pellet was resuspended in 0.5 ml of Lowry reagent by repeatedly pipetting or vortexing. The resuspended pellet was then incubated at a temperature of 55 °C for a duration of 3 hours. The sample was subsequently cooled to ambient temperature, then subjected to centrifugation at 15000 g for 20 minutes [22]. In summary, the Lowry reagent, consisting of Na_2CO_3, Na-K Tartrate, and CuSO4, was introduced to the sample. The mixture was then allowed to incubate for 10 minutes before the addition of the Folin-Ciocalteu phenol reagent. The solution was thereafter placed in an incubator for a duration of 30 minutes at a temperature of 35 °C [23]. The absorbance of the solution was then determined at a wavelength of 600 nm using a spectrophotometer. The starch concentration was measured using the anthrone technique, as described by Hodge and Hofreiter in 1962 [24]. In summary, a 0.1 mg sample was well mixed and subjected to centrifugation using 80% hot ethanol multiple times to eliminate sugars. The residue was dehydrated and combined with 6 ml of water and 7 ml of 50% perchloric acid. Subsequently, the mixture underwent centrifugation to separate and obtain the starch. The supernatant was combined with 5 ml of anthrone reagent and incubated at a temperature of 90 °C in a water bath until a dark green colour was observed [25]. Once the substance had reached the same temperature as the room, its absorbance was measured at a wavelength of 630 nm using a UV-Vis spectrophotometer.

Similarly, the pectin content was determined using the gravimetric method. In this method, 50 g of dried biomass was boiled with HCl at varying concentrations (0.01, 0.05, and 0.3 N) in a stepwise manner, with the filtrate being retained at each step [26]. Following neutralisation using a 1 N NaOH solution, it was combined with 25 ml of a 1 N CaCl2 solution and 5 ml of a 1 N acetic acid solution. The liquid was heated to its boiling point for a duration of 1 to 2 minutes and then left undisturbed for a period of 1 hour. The mixture was filtered via Whatman filter paper to collect calcium pectate [27].

2.4 Scanning Electron Microscopy (SEM)

The JEOl-JAPAN, JSM-6610LV model of scanning electron microscopy was employed to analyse the alterations in the morphological characteristics of both pretreated aquatic biomass

and raw aquatic biomass [28]. The desiccated samples affixed to a carbon tape were covered with a layer of platinum film using a sputtercoating technique, and subsequently placed into a vacuum chamber. The scanning electron microscope (JSM-6610LV) was used to capture SEM images at a magnification of 1000X [29].

2.5 Optimising the pre-treatment process

The pretreatment of biomass is an essential process that enhances the effective breakdown of biomass into easily convertible sugars such as glucose, xylose, and arabinose [30]. Pretreatment disrupts the hemicellulosic structure and exposes the cellulosic portion to microbial activity. The biomass is chemically pretreated using either acid (H_2SO_4) or alkali (NaOH) [31]. Therefore, in this investigation, the aquatic weeds were subjected to various concentrations (ranging from 0.5% to 4%) of acid and alkali at a temperature of 121 °C for 15 minutes to determine the most effective pretreatment condition [32]. The slurries that were treated beforehand and obtained after the acid hydrolysis process were neutralized using a 10% volume/volume solution of NH_3 and a 1% solution of orthophosphoric acid, respectively. The slurries were further filtered using Whatman filter paper discs.

The filtrates that were collected were analyzed for reducing sugars using the dinitrosalicylic acid test (Sinegani, and Emtiazi, 2006) with a glucose standard [33]. The cellulose, hemicellulose, and lignin content in the pretreated solids were examined using the NREL procedure, while the sugars (glucose and xylose) and inhibitory chemicals (HMF, furfurals) in the hydrolysates were measured using HPLC [34]. The rate of hemicellulose and cellulose hydrolysis was determined by analyzing the quantities of xylose and glucose in the hydrolysates, as well as the initial hemicellulose and cellulose content in the raw weeds.

3. Result

Currently, due to the increasing costs of crude oil and its harmful impact on the environment, natural gas is being utilized as a fuel for transportation [35]. Natural gas, predominantly composed of methane, is an environmentally beneficial alternative due to its lower greenhouse gas emissions and cost-effectiveness compared to petrol and diesel [36]. However, similar to other types of fuel, natural gas is a non-renewable fossil fuel that takes billions of years to create. This extended production process renders its use unsustainable. Therefore, it is imperative to investigate alternative sustainable replacements for natural gas [37].

A distinct microbial culture was employed for each biochemical conversion activity conducted in the experiment. Table 3 provides the physicochemical properties of the microbial inoculum.

Table 3: Physicochemical characteristics of the microbial inoculum

Parameters	Inoculum
Total solid (%)	4.2±0.01
Volatile Solid	95.6 ± 0.03
pH	7.0 ± 0.01
Cfu/ml	5.6 x 10^6
TOC (g/l)	12.8 ± 0.05
Carbohydrates (g/l)	4.0 ± 0.03
Proteins (g/l)	5.4 ± 0.02
Lipids (g/l)	0.02 ± 0.01

3.1 Weed Characterization

Chenopodium was used as feedstock in this study for the generation of biofuel. Weeds were characterized using proximate, ultimate, and compositional investigation [38]. The Tables below present the physicochemical features of the weed. The analysis revealed that the sample had a high concentration of volatile solids and a low amount of ash, indicating its potential for use in bioenergy generation. Further, enhanced cellulosic and hemicellulosic content was revealed in the present weed [39].

Table 4: Proximate Analysis of weed Chenopodium album

Proximate Analysis	Weed (%)
Moisture	12.44
TVS	80.32
TS	94.21
Fixed carbon	18.7
Ash	8.91

Table 5: Ultimate Analysis of weed Chenopodium album

Ultimate Analysis	Weed (%)
C	71
H	6.6
O	36.21
N	2.1

S	0.11
C/N ratio	20.98

Table 6: Compositional Analysis of weed Chenopodium album

Compositional Analysis	Weed (%)
Starch	2.01
Lipid	10.4
Protein	19.55
Lignin	4.09
Cellulose	36.77
Hemicellulose	25.01
Other Extractives	7.88

3.2 Pre-treatment of Chenopodium album

3.2.1 Pre-treatment with alkali

The efficacy of pretreatment in enhancing the utilization of aquatic weeds was assessed by subjecting them to various concentrations of NaOH [40]. The objective was to identify the most effective approach based on the yield of reducing sugars (mg/g biomass) obtained under different conditions.

Table 7: Pre-treatment of Chenopodium album with Alkali

	Alkali Concentration (% w/w) at temp 121°C	Solid Recovered (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Total Reducing sugars (mg/g biomass)
Chenopodium album	0.5	80.1	37	23.1	3.22	142.08
	1	76.21	37.22	22.03	3.01	151.22
	2	60.98	36.81	21.09	2.43	176.38
	3	43.22	37.32	19.5	2.01	243.55
	4	38.76	38.01	18.76	2.0	160.77

From the table, it is clear that increasing the concentration of alkali increased the solid loss. A slight increase in cellulosic fraction and a significant reduction in the lignin fraction of weed was noticed with increased concentration of alkali [41]. The highest reducing sugar was obtained at 3% w/w alkali concentration while, on further increment in alkali concentration, a drastic a

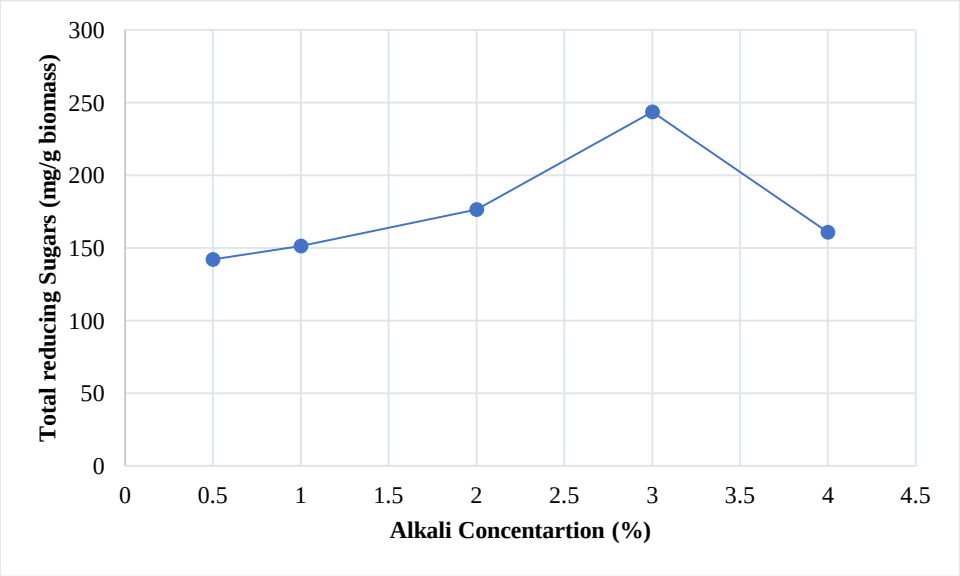

Figure 1: Alkali pre-treatment on release of reducing sugar from *Chenopodium album*

3.2.2. Pre-treatment with acid

The efficacy of pretreatment in enhancing the utilization of aquatic weeds was assessed by subjecting them to various concentrations of H_2SO_4. The objective was to identify the most effective approach based on the yield of reducing sugars (mg/g biomass) obtained under different conditions [42].

Table 8: Pre-treatment of *Chenopodium album* with Acid

	Acid Concentration (% w/w) at temp 121°C	Solid Recovered (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Total Reducing sugars (mg/g biomass)
Chenopodium album	0.5	97.66	38.7	16.3	7.09	200.02
	1	94.02	43.12	7.8	6.55	289.98
	2	83.12	33.03	7.0	5.67	301.03

	3	61.23	30.14	6.5	5.55	217.20
	4	55.12	30.01	6.1	5.21	101.11

From the table, it is clear that increasing the concentration of acid increased the solid loss. Slight increase in cellulosic fraction and a significant reduction in the lignin fraction of weed was noticed with increase concentration of acid [43]. The highest reducing sugar was obtained at 2% w/w acid concentration while, on further increment in acid concentration, a drastic reduction in RS yield was observed as depicted in Table. In conclusion, acid treatment was found to be efficient for delignification.

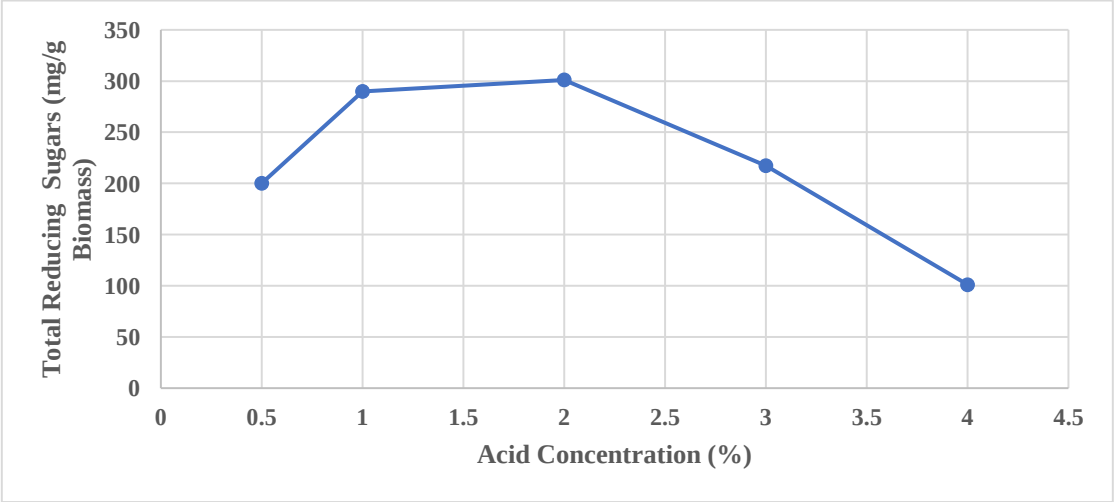

Figure 2: Acid pre-treatment on release of reducing sugar from *Chenopodium album*

3.3 SEM analysis

The effect of acid pre-treatment on the structural integrity of weed biomass was analyzed by scanning electron microscopy by comparing the SEM images of weed treated with H_2SO_4 with the untreated weed [44].

Figure 3 SEM image of Untreated weeds (A) and H_2SO_4 pre-treated weed (B)

Scanning electron microscopy (SEM) research revealed that the raw biomass had a sleek, inflexible, and well-organized structure. This is likely due to the presence of lignin coating on hemicellulose and cellulose fibers [45]. Nevertheless, subjecting weed to pre-treatment with a 1% acid solution resulted in alterations to their structures, leading to the formation of porous and disordered structures. The findings indicate that pre-treatment enhanced the hydrolysis of the hemicellulosic fraction. According to scanning electron microscope (SEM) pictures, the application of a 1% pre-treatment successfully damaged the wall of the weed [46]. This resulted in the formation of pores and an enlarged structure in the treated samples. The cellulose accessibility towards microbial action was improved by the intrinsic structural alterations seen after 1% acid pretreatment [47].

4. Discussion

Addressing the world's heavy reliance on non-renewable fossil fuels for energy production has become a pressing concern [48]. Given the detrimental effects of fossil fuel usage, such as global warming, environmental degradation, economic instability, and health hazards, scientists are now actively researching eco-friendly, sustainable, and cost-effective alternative energy sources [49]. Bioethanol is a renewable energy resource that has a higher-octane number than petrol, resulting in reduced engine knocking and improved engine performance. Currently, researchers are using lignocellulosic biomass, such as agricultural wastes, annual and perennial dry energy grasses, and forestry waste, to produce bioethanol [50]. Nevertheless, the challenge lies in the recalcitrant structure of lignocellulosic biomass, which necessitates

expensive pretreatment and substantial energy input for the release of fermentable sugars through enzyme hydrolysis. On the other hand, bioethanol made from weeds could potentially address the challenges associated with lignocellulosic biomass [51]. Considering the rapid growth rate and rich carbohydrate content, weeds have been identified as a promising source of bioenergy feedstock [52]. They have significantly greater productivity than agricultural or terrestrial crops and do not need arable land. They contain high amounts of cellulose, hemicellulose, and starch, which make them a highly efficient and affordable source of biofuel when compared to other types of biomass. In addition, the low lignin content of weed makes it easier and more efficient to reduce energy consumption [53]. Conversion processes are instrumental in enabling the transformation of weed biomass into cost-effective biofuels.

In this study, *Chenopodium album* was chosen as a potential substrate for bioethanol production based on its compositional characteristics [54]. The physicochemical analysis revealed that the sample had a high concentration of volatile solids a low amount of ash and enhanced cellulosic and hemicellulosic content., indicating its potential for use in bioenergy generation. The efficacy of pretreatment in enhancing the utilization of aquatic weeds was assessed by subjecting them to various concentrations of NaOH. A slight increase in cellulosic fraction and a significant reduction in the lignin fraction of weed was noticed with increased concentration of alkali. alkali treatment was found to be efficient for delignification [55]. This agrees with other literature reports that have reported that alkali treatment is more effective in degrading lignin by cleaving ester bonds and has weak impact on hemicellulose solubilisation. In addition to this, increasing the concentration of acid increased the solid loss [56]. A slight increase in cellulosic fraction and a significant reduction ($p<0.05$) in the lignin fraction of weed was noticed with increased concentration of acid. The ultimate examination revealed that the weed exhibited elevated carbon content and diminished nitrogen content, indicating the potential for enhanced substrate conversion efficiency [57]. Hence, during pretreatment optimisation, acid pretreatment proved more effective than alkaline pretreatment for hydrolysis, releasing maximal fermentable sugars for biohydrogen and biomethane generation. Further pretreatment studies were conducted using 1% (w/w) sulphuric acid at 121 °C for 15 min.

SEM analysis showed that the raw biomass was sleek, rigid, and well-organized. This is presumably owing to lignin coating on hemicellulose and cellulose fibers. However, pre-treatment with 1% acid solution caused porous and disorganized weed structures. Pre-treatment increased hemicellulosic hydrolysis. SEM images show that a 1% pre-treatment destroyed the

weed wall [58].The treated samples developed holes and expanded structures. The intrinsic structural changes following 1% acid pretreatment made cellulose more microbial-friendlier.

Conclusion

Annual and perennial dry energy grasses and forestry debris make bioethanol. Fermentable sugars come from lignocellulosic biomass hydrolysis. This procedure needs costly preparations and energy. Weed-based bioethanol may solve lignocellulosic biomass issues. Weeds' quick growth and high glucose content make them biofuel. Bioenergy generation was suggested by high volatile solids, low ash, and high cellulosic and hemicellulosic compounds in the current study. Acid pretreatment produced more fermentable carbohydrates for biohydrogen and biomethane than alkaline. Some early studies used 1% (w/w) sulphuric acid at 121 °C for 15 minutes. Under scanning electron microscopy, acid-treated materials developed holes and expanded structures. Adding 1% acid rendered cellulose microbial-friendly. Thus, *Chenopodium album* can produce biofuel, according to studies.

Author Contribution

Neeraj Sethi and Vivek Srivastava proposed the concept of the manuscript; Vivek and Bhawna investigated the study, collected data, and prepared the initial draft of the manuscript; Sushila Kaura contributed to manuscript writing and data analysis; Ikbal Shah refined the drafts of manuscripts. All authors have read and agreed to the published version of the manuscript.

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