

Article



Chemical and Enzymatic Treatment of Hemp Biomass for Bioethanol Production

Aleksandra Wawro *, Jolanta Batog and Weronika Gieparda

Department of Innovative Biomaterials and Nanotechnologies, Institute of Natural Fibres and Medicinal Plants, Wojska Polskiego 71B, 60-630 Poznan, Poland; jolanta.batog@iwnirz.pl (J.B.); weronika.gieparda@iwnirz.pl (W.G.)

* Correspondence: aleksandra.wawro@iwnirz.pl; Tel.: +48-61-8455-814

Received: 10 October 2019; Accepted: 3 December 2019; Published: 6 December 2019



Abstract: In this study chemical and enzymatic treatment of hemp biomass were optimized to obtain maximum ethanol production. In the first stage, physical and chemical pretreatment of hemp biomass was carried out. It was found that the Tygra variety is susceptible to alkaline treatment at an optimum concentration of 2% NaOH. Next, the effect of NaOH on the value of reducing sugars and the chemical composition of the solid fraction before and after the treatment was determined. Hemp biomass before and after the chemical treatment was analysed by FTIR spectra and SEM. The effect of enzymatic hydrolysis, i.e., substrate content, temperature, time, pH and dose of enzyme by means of Response Surface Methodology on glucose content was determined. The highest glucose value was observed at 50 °C, in time process between 48 and 72 h, and the dose of enzyme was not less than 20 FPU·g⁻¹. After the optimization of enzymatic hydrolysis two processes of ethanol fermentation from hemp biomass, SHF and SSF, were carried out. In the SHF process a 40% higher concentration of ethanol was obtained (10.51 g/L). In conclusion, hemp biomass was found to be an interesting and promising source to be used for bioethanol production.

Keywords: bioethanol; hemp biomass; lignocellulose; pretreatment; enzymatic hydrolysis; ethanol fermentation

1. Introduction

The increase in consumption of fossil fuels, the environmental pollution and the threat of greenhouse effect have forced a dynamic development of alternative fuel markets.

A promising lignocellulosic raw material for bioethanol (second generation biofuel) is hemp (*Cannabis sativa L.*) biomass. In recent years, there has been a dynamic increase in the area of hemp cultivation in Poland (over 1000 ha). The cultivation of hemp for seed purposes is currently intensively developed, therefore hemp biomass remains unused in the field, which can be a suitable raw material for the production of second generation bioethanol. The hemp dry matter yield is $10-15 \text{ t}\cdot\text{ha}^{-1}$. It is an environmentally friendly plant, with a short vegetation period of 3–4 months and a rapid growth of up to 4 m in height, which improves soil quality and is useful for the reclamation of degraded areas (post-mining heaps). Based on data from 2016, it is estimated that in Poland, the area of devastated and degraded land requiring remediation, constituting a potential area for growing hemp for energy purposes, is about 65,000 ha.

Hemp is also extremely resistant, perfectly adapt to different climatic conditions. They grow on almost any soil and can improve its quality, are not susceptible to various pests and do not require the use of plant protection products, and 1 ha of hemp binds around 2.5 t CO_2 [1,2].

Hemp biomass contains in its structure a polymeric complex called lignocellulose, which is relatively difficult to degrade. The lignocellulosic complex found in cell walls of hemp is composed

of the cellulose, hemicellulose and lignin. Cellulose and hemicellulose, after efficient deconstruction, can become productive substrates in the fermentation process. Lignin, consisting of phenolic alcohol derivates, is an effective obstacle in bioethanol production from plant biomass. The production of biofuel from lignocellulosic material requires deconstruction of the cell wall into individual polymers and the hydrolysis of carbohydrates into monomeric sugars [3–5].

Bioethanol production from lignocellulosic material can be divided into three steps: physical treatment, followed by chemical pretreatment, enzymatic hydrolysis and ethanol fermentation.

In order to disintegrate the biomass and remove lignin, several pretreatment methods often used including - physical, chemical and biological methods [6]. The physical methods of the pretreatment of lignocellulosic biomass, whose aim is to reduce the size of the substrate as well as to facilitate the access of bioactive substances to the surface, reduction of polymerization and crystallization degree of lignocellulose, include: milling, an extrusion method, and an ultrasound pretreatment. The chemical processes include treatment with acid (typically sulfuric or hydrochloric acid), alkali (sodium hydroxide, calcium carbonate, ammonia) or neutral (ionic liquids, liquid hot water LHW), the organosolv process, steam explosion, SO₂ or ammonia (AFEX), the ammonia recycle percolation (ARP), ozonolysis [6–8]. Depending on the method used, different changes occur within the lignocellulosic complex. The alkali pretreatment's function is mainly delignification, while the acid pretreatment process degrades most hemicellulose. A neutral solvent mainly depolymerises lignin, while the application of hot liquid or steam induce the degradation of hemicellulose and a small quantity of lignin. The non-specificity of the acidic treatment leads to the formation of complex sugars and compounds inhibitory to the functioning of microorganisms utilized for ethanol production [9]. An effective pretreatment process should solves the following issues: decrystallize the cellulose without causing its hydrolysis; depolymerize hemicellulose; restrict the formation of inhibitors which impede the hydrolysis of carbohydrates; require low energy input; allow the value added products such as lignin to recover; and, finally, it should be cost-effective [10]. Unfortunately, none of the lignocellulosic biomass pretreatment methods described above meets all these criteria at the same time.

It is therefore necessary to subject the lignocelullosic biomass to pretreatment, which significantly affects the course of the further stages of bioethanol production i.e., enzymatic hydrolysis and fermentation process [11].

The alkaline pretreatment removes the acetyl and uronic acid groups present on the hemicellulose, thus increasing access for hemicellulases. This process can significantly improve in solubilizing lignin, showing less solubility of cellulose and hemicellulose. This pretreatment allows to increase the internal surface of cellulose, reduce the degree of polymerization and crystallinity and disrupt the structure of lignin [12].

The next stage is enzymatic hydrolysis, which determines the amount of simple sugars metabolized by yeast in the fermentation process. The decomposition of cellulose to simple sugars requires synergistic action of three types of cellulases: endoglucanases, cellobiohydrolases and ß-glucosidase. The action of enzymes involves the attack on the cellulose by bonding with cellulose fibres in amorphous places, the cleavage of cellulosic chains, cutting off their considerable fragments, and then degrading them until the glucose polymer is obtained [13].

The last stage of bioethanol production is an ethanol fermentation. The ethanol fermentation process can be carried out in two ways; the first is separated hydrolysis and fermentation (SHF) while the other is simultaneous saccharification and fermentation (SSF). In SHF process the enzymes obtain the optimum at 50–60 °C and the pretreated biomass is first converted into fermentable sugar by cellulase. In SSF process the enzymes must be adapted to the temperature of the fermentation process 30–40 °C (the optimum temperature for *Saccharomyces cerevisiae*) and the pretreated biomass is converted into bioethanol in the presence of both enzymes and yeast in one bioreactor [14–16].

The aim of the study was to evaluate the pretreatment, enzymatic hydrolysis and fermentation process of hemp biomass during the preparation of the material for the production of bioethanol. The main goal is to increase the digestibility of maximum available sugars. Each chemical pretreatment

and enzymatic hydrolysis has a specific effect on the cellulose, hemicellulose and lignin fraction. Thus, the most efficient methods and conditions should be chosen, allowing maximum cost reduction of the entire process.

2. Materials and Methods

2.1. Strain

The *Saccharomyces cerevisiae* strain yeast Ethanol Red was obtained from the French company Lesaffre. This strains are resistant to elevated concentrations of ethanol (12–14%) and temperatures above 35 °C. The microorganisms was stored on YPD medium with the addition of 2% w/v agar-agar kept at temperature 4–8 °C.

2.2. Biomass Preparation

The raw material used in the study was Tygra biomass hemp (*Cannabis sativa* L.) from the Experimental Farm of INF & MP in Petkowo, Poland. The raw material was subjected to preliminary crushing to particles of size 20–40 mm and then dried in 50–55 °C for 24 h. Next, the material was disintegrated on knife mill (Retsch SM-200, Haan, Germany) with a sieve of the mesh size of 2 mm.

2.3. Chemical (Alkaline) Pretreatment of Hemp Biomass

The evaluation of pretreatment conditions for hemp biomass was carried out at 5 h treatment with 1.5–3% sodium hydroxide in 90 °C. The alkali effect on the content of the released reducing sugars was determined by Miller's method with 3,5-dinitrosalicylic acid (DNS) in the enzymatic test [17]. The test was performed with the use of Celluclast 1.5 L (Novozymes) enzymatic preparation at the dose of 10 FPU·g⁻¹. The raw material was incubated in 55 °C in 0.05 M citrate buffer of pH 4.8 for 24 h. Then, after the enzymatic test supernatant was respectively diluted, DNS reagent was added and the mixture was incubated in a boiling water bath for 10 min. After cooling to room temperature, the absorbance of the supernatant at 530 nm was measured (UV-VIS Spectrophotometer, Jasco V-630, Pfungstadt, Germany).

2.4. Enzymatic Hydrolysis Process

The selection of the enzyme complex for the enzymatic hydrolysis process was made by conducting enzymatic tests using selected enzymes, their mixtures (compositions — 30/70, 50/50 and 70/30%) and by supplementing them with glucosidase, xylanase and their mixture (50/50%). The enzyme test was carried out under the following conditions: substrate concentration 5%, enzyme dose 10 FPU/g, temperature 55 °C (SHF process)/38 °C (SSF process), pH 4.8, time 24 h. The selection parameter was the content of released reducing sugars determined by the Miller method with 3,5-dinitrosalicylic acid (DNS).

The optimization of the enzymatic hydrolysis of hemp biomass as the SHF process was carried out acc. to the Response Surface Methodology (RSM) using the parameters: biomass content 5–10% w/v, temp. 50–70 °C, time 24–72 h, pH 4.2–5.4, dose of Flashzyme (AB Enzymes) 10–30 FPU·g⁻¹. Also, the Flashzyme enzyme supplementation by using glucosidase 20 CBU·g⁻¹ and xylanase 500 XU·g⁻¹ (Sigma-Aldrich, St. Louis, MI, USA) was tested. Next, the fermentation process was carried out in 100 mL Erlenmeyer flasks containing 40 mL of medium with added *S. cerevisiae* (1 g dry matter/L) in the following process conditions: 37 °C, pH 4.8, 120 h.

To optimize the SSF process according to the RSM, the ranges of process parameters were selected: substrate content 5–7% w/v, dose of Flashzyme/Celluclast 1.5 L enzymes 10–30 FPU g⁻¹ using *S. cerevisiae* yeast (1 g dry matter/L) at 37 °C, pH 4.8 and 120 h.

2.5. Ethanol Fermentation

The ethanol fermentation was carried out in bioreactor Biostat B Plus (Sartorius) with 2 L vessel equipped with pH, temperature and agitation controls. The temperature was maintained at 37 °C and agitation at 900 rpm, pH was controlled at 4.8 by adding 1 N NaOH or 1 N HCl. The fermentation process was used not hydrated freeze-dried yeast *S. cerevisiae* at a dose of 1 g/L, which corresponded to cell concentration after inoculation of about 1×10^7 cfu/mL. After inoculation, a 96 h-fermentation was carried out and samples were taken every 24 h.

2.6. Analytical Methods

The chemical composition of hemp biomass before the pretreatment was determined, i.e., cellulose acc. to TAPPI T17 m-55 [18], hemicellulose as the difference holocellulose according to TAPPI T9 m-54 and cellulose [19], and lignin acc. to TAPPI T13 m-54 [20].

In order to provide a more complete picture of the molecular structure of hemp biomass before and after the chemical pretreatment the analysis of FTIR spectroscopy was performed using a Fourier Transform Infrared Spectrometer (FTIR, Bruker ISS 66v/S, Karlsruhe, Germany) at infrared wavenumbers of 400–4000 cm⁻¹ [21].

The physical morphologies of hemp biomass before and after the chemical treatment were performed by using Scanning Electron Microscope (SEM, S-3400N, Hitachi, Tokyo, Japan) in high vacuum conditions. The samples were covered with gold dust.

The content of glucose and ethanol was determined by High Performance Liquid Chromatography on Elite LaChrom by VWR-Hitachi using an RI L-2490 detector, Rezex ROA 300×7.80 mm column from Phenomenex, at a flow rate of 0.6 mL/min, at 40 °C.

2.7. Statistical Analysis

The experiments of ethanol fermentation were carried out in triplicates. Standard deviation was calculated using the analysis of variance ANOVA, Statistica 13.0 software (p < 0.05).

3. Results and Discussion

3.1. Chemical Pretreatment of Hemp Biomass

The alkaline pretreatment is a helpful method of removing lignin from materials with lignocelullose and it leads to an increase in the accessibility of biomass structure. The main advantages of the alkaline treatment are high efficiency in lignin removal as well as effective removal of acetyl groups and uronic substitutions from hemicellulose. The type of reagent used has a significant effect on the performance of the alkaline pretreatment. Sodium hydroxide is one of the most popular alkaline reagents used in this process. The reagent concentration, reaction time and temperature also affect the biomass delignification [22].

The optimization of alkaline treatment conditions for hemp biomass ground on a knife mill with a mesh size of 2 mm was carried out with 1.5–3% sodium hydroxide. In order to determine the efficiency of the chemical processing of the hemp biomass, the content of reducing sugars was measured by Miller's method in the enzymatic test. The results allow to conclude that the Tygra variety is susceptible to alkaline treatment at an optimum concentration of 2% NaOH. The content of reducing sugars for 2% NaOH was about 13% higher than for 1.5%. In turn, for 3% sodium hydroxide, the content of reducing sugars was at a similar level. Thus, the choice of time and temperature treatment proved to be effective. Kumar et al. [23] showed a similar result and they stated that the sodium hydroxide pretreatment of waste plant biomass resulted in the highest level of delignification at 2% NaOH.

Subsequently, determination of the chemical composition of hemp biomass after NaOH treatment was performed and compared to the chemical composition of the biomass before pretreatment (Table 1).

Samples	Cellulose (%)	Hemicellulose (%)	Lignin (%)
BP	50.82 ± 0.12	27.79 ± 0.33	14.68 ± 0.46
AP	62.70 ± 0.09	20.16 ± 0.16	15.12 ± 0.22

Table 1. Chemical composition of hemp biomass (% of dry matter); BP—before pretreatment; AP—after pretreatment.

The analysis of chemical composition confirmed an increase in cellulose content and partial degradation of hemicellulose.

The results obtained during the analysis of the chemical composition of hemp biomass were confirmed by FTIR spectra. The major bands observed in the FTIR spectra of hemp biomass and their assignments to vibrations of chemical group and molecules are summarized in Table 2. FTIR spectroscopy is the most useful tool in providing information about molecular fragments, the presence or absence of specific functional groups.

Table 2. Characteristic absorption bands in infrared (A-amorphous; C-crystalline) according to Stevulova et al. [21].

Vibration of Function Group	Source	Wavenumber (cm ⁻¹)
OH stretching	polysaccharides	3340
C-H symmetrical stretching	polysaccharides	2897
C = O unconjugated stretching	hemicellulose	1733
OH (water)	cellulose	1636
C = C symmetrical stretching of the aromatic ring	lignin	1507
CH ₂ bending	cellulose	1422
glycosidic bonds symmetric ring-stretching mode	polysaccharides	896

Figure 1 shows the changes in FTIR spectra after alkali treatment of hemp biomass in between 600 cm^{-1} and 4000 cm^{-1} .



Figure 1. FTIR spectra of hemp biomass before and after the chemical treatment.

The spectrum shows typical cellulose absorption peaks at 3300 cm^{-1} , 2900 cm^{-1} , 1610 cm^{-1} and 1420 cm^{-1} . At the wave number of 1420 cm^{-1} , stretching vibrations of methylene functional groups (CH2) occur. In addition, the band in the $3300-3400 \text{ cm}^{-1}$ region corresponds to bending vibrations of the O-H groups, and the 2900 cm^{-1} band corresponds to the stretching vibrations of the C-H groups. The peak at 1610 cm^{-1} is generated by the stretching vibrations of O-H bonds, derived from absorbed water or moisture in the sample, was reduced, which can be attributed to the loss of water due to drying the sample [24]. Absorption bands in the $1500-890 \text{ cm}^{-1}$ region show a reduction in intensity, indicating lower crystallinity and an increase in the amorphous form of cellulose as a result of alkaline treatment [25]. A slight decrease was observed at 1730 cm^{-1} , a characteristic band for carbonyl groups contained in hemicellulose. This demonstrates the effectiveness of alkaline treatment. Many researchers describe the problem that lignin can't be completely removed by the alkaline process (peak at 1510 cm^{-1}). The process of degradation or fragmentation of lignin is complicated due to the presence of strong C-C bonds and other functional groups, such as aromatic groups [21]. The obtained results are consistent with the existing data found in literature describing studies of lignocellulosic biomass by infrared spectroscopy [26,27].

The morphological changes of hemp biomass before and after the alkali pretreatment was investigated by using Scanning Electron Microscope, as illustrated in Figure 2.



Figure 2. SEM analysis of hemp biomass (a) before pretreatment and (b) after alkali pretreatment.

The untreated hemp biomass was observed to have a sedimentary layer on the surface area. The SEM images of hemp biomass after pretreatment showed that the surface area of the biomass was partially purified, was cleaner and smoother. The morphological changes were observed that indicated damage to the structure of biomass. It can be stated that 2% NaOH degrades the linkage between lignin and hemicellulose, increasing the surface area and making it more accessible to the cellulolytic enzymes [26,28,29].

3.2. Enzymatic Hydrolysis Process

The complex with the composition—Flashzyme Plus 200, glucosidase and xylanase was selected for the SHF process (1653 mg/g), and the complex with the composition—Flashzyme Plus 200/Celluclast 1.5 L (70/30%) and xylanase for the SSF process (2311 mg/g) according to the enzyme test (Table 3).

The optimization of the enzymatic hydrolysis of hemp biomass as the SHF process was carried out according to the RSM (Figure 3). This method is an effective optimization tool which consists of mathematical and statistical techniques, and it is used for the process of optimization [10,30].

Enzymes	Reducing Sugars (mg/g)	
	55 °C (SHF)	38 °C (SSF)
Flashzyme Plus 200	1100	1214
Celluclast 1.5 L	875	909
Flashzyme/Celluclast 1.5 L (30/70)	1197	1306
Flashzyme/Celluclast 1.5 L (50/50)	1265	1295
Flashzyme/Celluclast 1.5 L (70/30)	1258	1315
Flashzyme/glucosidase	1547	1905
Flashzyme/xylanase	1518	1914
Flashzyme/glucosidase/xylanase	1653	2005
Flashzyme/Celluclast 1.5 L (70/30)/glucosidase	1488	2035
Flashzyme/Celluclast 1.5 L (70/30)/xylanase	1507	2311
Flashzyme/Celluclast 1.5 L (70/30)/glucosidase/xylanase	1431	2289

Table 3. The content of the reducing sugars after the enzyme test.



Figure 3. Enzymatic hydrolysis process of hemp biomass (RSM). Response surface representing the interaction effect of temperature, time, dose, pH and substrate content on glucose yield.

Figure 3 presents the interaction effect of variables on the glucose yield. The highest was observed at 50 °C, and in time process between 48 and 72 h, in turn the dose of enzyme was not less than 20 FPU·g⁻¹. The higher the temperature and the pH of the enzymatic hydrolysis were, the lower the glucose yield occurred. It was found that in the SHF process optimum enzymatic hydrolysis conditions were obtained for the substrate content of 10%, using the following enzymes: Flashzyme 30 FPU g⁻¹, glucosidase 20 CBU·g⁻¹ and xylanase 500 XU g⁻¹, for the process parameters: 50 °C, pH 4.2, 48 h. These parameters gave the opportunity to obtain a maximum glucose yield which was 36.9 ± 0.64 (g/L). Similar research was conducted by Abraham [31], during biomass hydrolysis at 50 °C and 18 FPU g⁻¹ he obtained the highest glucose yield. Salimi et al. 2017 [32] optimized the enzymatic hydrolysis of lignocellulosic biomass using the RSM method. They used a temperature range of 45–60 °C and a pH of 4.5–6.0. They obtained the highest content of monosaccharides at 45 °C and pH 6.0. Next, the fermentation process was carried out with *S. cerevisiae* and the following process conditions: 37 °C, pH 4.8, 120 h.

In turn, the hydrolysis and fermentation process for SSF must be carried out under conditions that ensure optimal synergy of enzymes and distillery yeast. To optimize the SSF process acc. to

the RSM, the ranges of process parameters were selected: substrate content 5–7% w/v, the dose of Flashzyme/Celluclast 1.5 L enzymes 10–30 FPU·g⁻¹ using *S. cerevisiae* yeast at 37 °C, pH 4.8 and 120 h. Then, the tests were carried out using the selected parameters and the amount of ethanol (HPLC) was determined. The optimal conditions of the SSF process for Tygra hemp biomass were selected. The highest ethanol concentration for SSF was observed at substrate content 5% w/v and the dose of enzyme not lower than 20 FPU·g⁻¹. Fojas and Rosario [33] optimized the enzymatic saccharification of lignocellulosic biomass and SSF process was carried out with the following parameters: 3–6% the amount of substrate, 20–25 FPU·g⁻¹ dose of enzyme and at the temperature of 37 °C for 120 h.

3.3. Ethanol Fermentation

After optimizing both SHF and SSF processes, ethanol fermentation in the bioreactor was performed, as presented in Figure 4.



Figure 4. Ethanol yield after fermentation process for separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

In the SHF process the ethanol concentration achieve higher values than it was noted in the case of the SSF process, while the increasing the fermentation time does not change the ethanol yield. The highest concentration of ethanol was observed at 48 h of the SHF process and it was 10.51 \pm 0.08 g/L, which is 21.02 g/100 g of hemp biomass. In the SSF process the highest concentration of ethanol was observed at 96 h and it was 6.5 ± 0.02 g/L, which is 13g/100 g of hemp biomass. The similar study presented Kusmiyati et al. [34]. In their work conversion lignocelullosic biomass to bioethanol was carried out through pretreatment, saccharification and fermentation processes. Their results showed that the SHF process gave a higher concentration of ethanol (8.11 g/L) compared to the SSF process (3.95 g/L). SHF as a process alternative in an industrial bioethanol plant has both potential and limitations. The main advantage of SHF is the possibility to optimize the process steps separately, especially to be able to run the enzymatic hydrolysis at an optimal temperature, with respect to enzymes. In addition, the SHF process brings the possibility of removing the insoluble solids after enzymatic hydrolysis, which allows to perform liquid fermentation, facilitating the reutilization of the fermentative microorganisms [35]. The SSF process, however, consists in lower energy consumption and thus lower costs of bioethanol production. Due to this advantage, it is often the preferred method of obtaining lignocellulosic bioethanol [11].

4. Conclusions

In this study it is suggested that the hemp biomass is a proper source for second-generation bioethanol, as an alternative to petroleum-oil based fossil fuels. Optimal pretreatment, enzymatic hydrolysis and ethanol fermentation were showed. The use of sodium hydroxide is an efficient pretreatment method of hemp biomass which allows to obtain an increase in cellulose content and partial degradation of hemicellulose. Optimization of enzymatic hydrolysis by the RSM method allowed to achieve glucose yield at the level 36.9 g/L. The ethanol fermentation using *S. cerevisiae* in the present work resulted in the production of ethanol at 10.51 g/L.

Further research needs to focus on achieving a cost-effective process for the production of bioethanol, e.g., by using the genome shuffling technique, which improves the phenotypic traits of *S. cerevisiae* yeast, i.e., an increase in fermentation activity and resistance to temperature as well as acidic and osmotic stress.

Author Contributions: Conceptualization, A.W., J.B., and W.G.; methodology, A.W., J.B., and W.G.; software, A.W., W.G.; validation, J.B.; formal analysis, J.B., A.W.; investigation, A.W., W.G.; resources, A.W., J.B.; data curation, J.B., A.W., and W.G.; writing—original draft preparation, A.W., J.B.; writing—review and editing, A.W., J.B., and W.G.; visualization, A.W.; supervision, J.B.; project administration, J.B.; funding acquisition, J.B.

Funding: This research was funded by the Ministry of Agriculture and Rural Development, Poland, Multiannual Program (2017–2020) and by National Centre for Research and Development, Poland, grant ERA-NET CO-FOUND FACCE SURPLUS 2 and the APC was funded by the Ministry of Agriculture and Rural Development, Poland, and by National Centre for Research and Development, Poland.

Acknowledgments: The study was conducted as research project- Multiannual Program (2017–2020): Reconstruction and sustainable development of production and processing of natural fibre raw materials for the needs of agriculture and the economy and financed by the Ministry of Agriculture and Rural Development, Poland, research project ERA-NET CO-FOUND FACCE SURPLUS 2 financed by National Centre for Research and Development, Poland.

Conflicts of Interest: The authors declare no conflict of interest.

References

- 1. Burczyk, H.; Grabowska, L.; Kołodziej, J.; Strybe, M. Industrial hemp as a raw material for energy production. *J. Ind. Hemp.* **2008**, 37–48. [CrossRef]
- Żuk-Gołaszewska, K.; Gołaszewski, J. *Cannabis sativa* L.-cultivation and quality of raw material. *J. Elem.* 2018, 23, 971–984. [CrossRef]
- 3. Pareek, N.; Gillgren, T.; Jönsson, L.J. Adsorption of proteins involved in hydrolysis of lignocellulose on lignins and hemicelluloses. *Bioresour. Technol.* **2013**, *148*, 70–77. [CrossRef] [PubMed]
- Rahikainen, J.L.; Martin-Sampedro, R.; Heikkinen, H.; Rovio, S.; Marjamaa, K.; Tamminen, T.; Rojas, O.J.; Kruus, K. Inhibitory effect of lignin during cellulose bioconversion: The effect of lignin chemistry on non-productive enzyme adsorption. *Bioresour. Technol.* 2013, *9*, 270–278. [CrossRef]
- Kim, D. Physico-chemical conversion of lignocellulose: Inhibitor effects and detoxification strategies. *Molecules* 2018, 23, 309. [CrossRef]
- 6. Kumar, A.K.; Sharma, S. Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review. *Bioresour. Bioprocess* **2017**, *4*, 7. [CrossRef]
- Jedrzejczyk, M.; Soszka, E.; Czapnik, M.; Ruppert, A.M.; Grams, J. Physical and chemical pretreatment of lignocellulosic biomass. In *Second and Third Generation of Feedstocks. The Evolution of Biofuels*; Basile, A., Dalena, F., Eds.; Elsevier: Amsterdam, The Netherlands, 2019; pp. 143–196. [CrossRef]
- Asgher, M.; Ahmad, Z.; Iqbal, H.M.N. Alkali and enzymatic delignification of sugarcane bagasse to expose cellulose polymers for saccharification and bioethanol production. *Ind. Crop. Prod.* 2013, 44, 488–495. [CrossRef]
- 9. Parawira, W.; Tekere, M. Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: Review. *Crit. Rev. Biotechnol.* **2011**, *31*, 20–31. [CrossRef]
- 10. Jaisamut, K.; Paulová, L.; Patáková, P.; Rychtera, M.; Melzoch, K. Optimization of alkali pretreatment of wheat straw to be used as substrate for biofuels production. *Plant Soil Environ.* **2013**, *59*, 537–542. [CrossRef]

- 11. Sharma, V.; Sharma, S.; Kuila, A. A review on current technological advancement of lignocellulosic bioethanol production. *J. Appl. Biotechnol. Bioeng.* **2016**, *1*, 61–66. [CrossRef]
- 12. Maurya, D.P.; Singla, A.; Negi, S. An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. *3 Biotech.* **2015**, *5*, 597–609. [CrossRef]
- Lee, C.-R.; Sung, B.H.; Lim, K.-M.; Kim, M.-J.; Sohn, M.J.; Bae, J.-H.; Sohn, J.-H. Co-fermentation using recombinant *Saccharomyces cerevisiae* yeast strains hyper-secreting different cellulases for the production of cellulosic bioethanol. *Sci. Rep.* 2017, 7, 4428. [CrossRef]
- 14. Dong, J.-J.; Ding, J.-C.; Zhang, Y.; Ma, L.; Xu, G.-C.; Han, R.-Z.; Ni, Y. Simultaneous saccharification and fermentation of dilute alkaline-pretreated corn stover for enhanced butanol production by *Clostridium saccharobutylicum* DSM 13864. *FEMS Microbiol. Lett.* **2016**, *3*63, 1–6. [CrossRef]
- 15. El-Naggar, N.E.; Deraz, S.; Khalil, A. Bioethanol production from lignocellulosic feedstocks based on enzymatic hydrolysis: Current status and recent developments. *Biotechnology* **2014**, *13*, 1–21. [CrossRef]
- 16. Suttikul, S.; Srinorakutara, T.; Butivate, E.; Orasoon, K. Comparison of SHF and SSF processes for ethanol production from alkali-acid pretreated sugarcane trash. *KKU Res. J.* **2016**, *21*, 229–235. [CrossRef]
- 17. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
- 18. TAPPI 17 m-55. In Cellulose in Wood; TAPPI Press: Atlanta, GA, USA, 1955.
- 19. TAPPI T9 m-54. In Holocellulose in Wood; TAPPI Press: Atlanta, GA, USA, 1998.
- 20. Bagby, M.O.; Nelson, G.H.; Helman, E.G.; Clark, T.F. Determination of lignin non-wood plant fiber sources. *TAPPI J.* **1971**, *54*, 11.
- 21. Stevulova, N.; Cigasova, J.; Estokova, A.; Terpakova, E.; Geffert, A.; Kacik, F.; Singovszka, E.; Holub, M. Properties characterization of chemically modified hemp hurds. *Materials* **2014**, *7*, 8131–8150. [CrossRef]
- 22. Łukajtis, R.; Rybarczyk, P.; Kucharska, K.; Konopacka-Łyskawa, D.; Słupek, E.; Wychodnik, K.; Kamiński, M. Optimization of saccharification conditions of lignocellulosic biomass under alkaline pretreatment and enzymatic hydrolysis. *Energies* **2018**, *11*, 886. [CrossRef]
- 23. Kumar, P.; Barrett, D.M.; Delwiche, M.J.; Stroeve, P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind. Eng. Chem. Res.* **2009**, *488*, 3713–3729. [CrossRef]
- Gupta, A.D.; Pandey, S.; Kumar Jaiswal, V.; Bhadauria, V.; Singh, H. Simultaneous oxidation and esterification of cellulose for use in treatment of water containing Cu (II) ions. *Carbohydr. Polym.* 2019, 222, 114964. [CrossRef] [PubMed]
- Bregado, J.L.; Secchi, A.R.; Tavares, F.W.; de Sousa Rodrigues, D.; Gambetta, R. Amorphous paracrystalline structures from native crystalline cellulose: A molecular dynamics protocol. *Fluid Phase Equilibr.* 2019, 491, 56. [CrossRef]
- 26. Šutka, A.; Kukle, S.; Grāvītis, J. STEX treated and untreated hemp fiber comparative structural analysis. Material Science. *Text. Cloth. Technol.* **2011**, *6*, 36–42.
- Plácido, J.; Capareda, S. Analysis of alkali ultrasonication pretreatment in bioethanol production from cotton gin trash using FT-IR spectroscopy and principal component analysis. *Bioresour. Bioprocess.* 2014, 1, 23. [CrossRef]
- 28. Fang, G.; Chen, H.; Chen, A.; Mao, K.; Wang, Q. An efficient method of bio-chemical combined treatment for obtaining high-quality hemp fiber. *BioResources* **2017**, *12*, 1566–1578. [CrossRef]
- Abraham, R.E.; Barrow, C.J.; Puri, M. Relationship to reducing sugar production and scanning electron microscope structure to pretreated hemp hurd biomass (*Cannabis sativa*). *Biomass Bioenerg.* 2013, *58*, 180–187. [CrossRef]
- 30. Izmirlioglu, G.; Demirci, A. Ethanol production from waste potato mash by using *Saccharomyces cerevisiae*. *Appl. Sci.* **2012**, *2*, 738–753. [CrossRef]
- 31. Abraham, R.E. Bioprocessing of Hemp Hurd (*Cannabis sativa*) for Biofuel Production. Ph.D. Thesis, Deakin University, Deakin, Australia, 30 October 2014.
- 32. Salimi, M.N.; Lim, S.E.; Yusoff, A.H.M.; Jamlos, M.F. Conversion of rice husk into fermentable sugar by two stage hydrolysis. *J. Phys. Conf. Ser.* 2017, *908*, 012056. [CrossRef]
- 33. Fojas, J.J.R.; Rosario, E.J.D. Optimization of pretreatment and enzymatic saccharification of cogon grass prior ethanol production. *Chem. Mol. Eng.* **2013**, *7*, 296–299.

- 34. Maryanto, D.; Sonifa, R.; Kurniawan, S.A.; Hadiyanto, H. Pretreatment of Starch-Free Sugar Palm Trunk (*Arenga pinnata*) to Enhance Saccharification in Bioethanol Production. *MATEC Web Conf.* **2018**, *156*, 6. [CrossRef]
- Moreno, A.D.; Alvira, P.; Ibarra, D.; Tomas-Pejo, E. Production of ethanol from lignocellulosic biomass. In *Production of Platform Chemicals from Sustainable Resources*; Biofuels and Biorefineries; Feng, Z., Smith, R.L., Jr., Qi, X., Eds.; Springer: Singapore, 2017; pp. 375–410. [CrossRef]



© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).