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Hemp waste valorization through enzymatic hydrolysis for biofuels and biochemicals production.

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Enzymatic hydrolysis is an essential step in the lignocellulosic biomass treatment for the conversion of cellulose and hemicellulose into fermentable sugars. In the present study, a physical steam explosion (200°C) and chemical alkaline pre-hydrolysis (10% v/v) were applied to hemp (*Cannabis sativa* L.) biomass, to verify the effectiveness of different pretreatment processes, in order to increase enzymatic hydrolysis and the final sugar yield. The enzymatic degradations were carried out also utilizing extremophilic microorganisms, exploited as a source of (hemi)cellulases to be used as components of the enzymatic cocktail applied in the bioconversion into fermentable sugars for production of second generation ethanol and high value products. Alkaline pretreatment showed to be superior over the physical method with respect to the rate of enzymatic hydrolysis, with the obtainment of an hydrolysis yields of 90% xylose and 40% glucose, respectively.

1. Introduction

The long time exploitation of hemp (*Cannabis sativa*) is well known; since 1800s until mid-1930s hemp was grown as a commodity fibers crop and its utilization for fabrics, paper and twine production had great market importance. In 1937, with the Marijuana Tax Act, production and applications of hemp were regulated and it was considered an illegal crop in the U.S. for several decades until 2014, when Section 7606 of the federal Agricultural Act of 2014 allowed the cultivation of industrial hemp (*C. sativa L.*), a cultivar characterized by a content of delta-9 tetrahydrocannabinol (THC) less than 0.3% on dry weight basis. Currently in addressing global challenges linked to environmental issues and energy crisis of fossil fuel based societies, industrial hemp can be considered among the most sustainable and economically feasible lignocellulosic feedstocks for energy and biochemicals production. Industrial hemp is a C3 plant belonging to the family of Cannabaceae comprising the three species of *C. sativa, C. indica* and *C. ruderalis*. The most important features which make industrial hemp a promising non food energy crop comprise the low cost, the elevated dry biomass yield due to high growth rate (50 cm/month), the resistance to different climate conditions, the capability to live on contaminated soil and under drought conditions, the low nutrients requirement and no pesticide demand (Muhammad Saif et al., 2013). The chemical composition of *C. sativa L*., that, despite high variability among different cultivars, is characterized by a huge carbohydrate content (32.6–51.1% cellulose, 10.6–16.6% hemicellulose, 18% pectin) and a reduced lignin presence (3.7–20.0% and in particular 4% in fresh plants), make hemp one of the most promising feedstock for biofuels and bio-based chemical purposes (Viswanathan et al., 2020). After the removal of the conventionally utilized high-quality bast fibers, the cellulosic residues, called hurds, are cheap sources of fermentable sugars that can be subjected to biological conversion processes into biofuels and value-added products (Anqi et al., 2021). Besides high ethanol yields consisting in glucose conversion up to 96.7% by simultaneous saccharification and fermentation (SSF) of pretreated industrial hemp (Zhao et al,. 2020a), also the elevated C5 sugars content of hurds was exploited for the production of value added biochemicals such as succinic acid (Kuglarz et al., 2016) and poly-3-hydroxybutyrate (Khattab and Dahman, 2019). Biorefiney approaches also allowed to obtain succinic acid as a co-product, by following the fermentation of C6 sugars of the saccharified hemp biomass in bioethanol with the conversion of the remaining pentoses in succinic acids by *Actinobacillus succinogenes*. In a similar biorefining fashion (Kreuger et al., 2011), in addition to the methane production by anaerobic digestion (AD) of pretreated biomass, proposed the bioethanol and biogas co-generation by combining SSF followed by AD of the unfermented hemp residues. However, despite its great biotechnological potential, several insights are still

needed to address hurdles related to hemp recalcitrance factors, pretreatments and biomass hydrolysis methodologies that are crucial for the effectivity of bioconversion processes.
The aim of the present work is to improve the saccharification of industrial hemp testing the impact of different pretreatments methods and compositions of the enzymatic cocktails applied for the biomass hydrolysis. Moreover the importance of the arabinofuranosidase, a biocatalyst usually not included in the commercial mix for the saccharification, but relevant for enhancing xylan breakdown, due to its debranching activity, will be evaluated.

2. Materials and Methods

2.1 Pretreatments and determination of chemical composition

Hemp feedstocks were provided by a local farm situated in Campania region, Italy. The biomass was dried at 50 °C, milled in a homogenizer to obtain a fine powder, and stored in sealed vials at room temperature until use. The raw material was subjected to two different pretreatments. The steam explosion (SE) pretreatment, performed in a batch reactor, was conducted at 200°C for 10 minutes at a 20 bar pressure.
For the Aqueous Ammonia Soaking (AAS) pretreatment, the biomass reduced to a powder was soaked in 10% (v/v) ammonium hydroxide solution at a solid loading of 5% at 70 °C for 22 h in screw-capped 25 mL bottles to reduce the evaporation. The alkaline mixtures were centrifuged at 800 x g, and the residues were extensively washed with 50 mM sodium acetate buffer (pH 5) or in phosphate buffer (pH 6-7) until obtaining the required pH for the subsequent enzymatic saccharification process.
Carbohydrate compositions of the biomass untreated and pretreated samples were determined according to the method of Davis (1998). The procedure involved an acid hydrolysis carried out in two steps which fractionate the polysaccharides into their corresponding monomers. The samples were first soaked in 72% v/v H_2SO_4, at a solid loading of 10%, at 30 °C, for 1 h; then the mixtures were diluted to 4% (v/v) H_2SO_4 with distilled water, fucose was added as an internal standard, and the secondary hydrolysis was performed for 1 h at 120°C. The samples were filtrated through a 0.45 µm Teflon syringe filter (National Scientific, Lawrenceville, GA). The products of hydrolysis were analyzed through a HPLC analysis, as described below.
The acid insoluble lignin (Klason lignin) was determined by weighting the dried residue after total removal of the sugars.

2.2 Enzymatic activities

The following commercial enzymatic preparations: cellulase from *Trichoderma reesei* ATCC26921, cellobiase from *Aspergillus niger*, xylanase from *Trichoderma viride* and a thermostable β-xylosidase X3504 were purchased from Sigma-Aldrich (St. Louis, MO).
The α-L-arabinofuranosidase from *Clostridium thermocellum* (from Megazyme Co., Bray Ireland) was utilized in combination with the enzymatic preparations.
The enzymatic activities were measured at 50°C in 50 mM sodium acetate buffer (pH 5.0). Cellulase activity was determined spectrophotometrically utilizing a soluble chromogenic substrate carboxymethyl cellulose-Remazol Brilliant Blue R (AZOCM- Cellulose) (Megazyme Co., Bray Ireland), following supplier's instructions. The absorbance of the supernatant was read at 590 nm, and the enzyme units were calculated from a standard curve constructed with known amounts of cellulase from *T. viride*.
The xylanase activity was evaluated by the same assay method utilizing methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Azo- Oat Spelt Xylan) (Megazyme Co., Bray Ireland) as substrate (Biely et al., 1985). One unit of xylanase activity was defined as the amount of enzyme required to increase the absorbance at 590 nm of 1 OD min^{-1} under the experimental conditions.
β-xylosidase, β-glucosidase and α-arabinofuranosidase activities were determined by using *p*-nitrophenyl-glycoside substrates as described in (Biely et al., 2000). One unit of β-xylosidase, β-glucosidase or α-arabinofuranosidase, was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol per min under the assay conditions. All the enzymatic measurements were performed in triplicate.

2.3 Enzymatic hydrolysis

The saccharification experiments were carried out at 50 °C for 72 h in a total volume of 10 mL (50 mM sodium acetate buffer pH 5.0 or phosphate buffer pH 6.0 -7.0, plus enzyme cocktail), at a solid loading of 5 - 7 - 10% (w/v). The hydrolysis of pretreated lignocellulosic material was carried out using the following commercial products at the indicated amounts expressed as units per grams of pretreated biomass: 5.4 U g^{-1} of cellulase from *T. reesei* ATCC26921, 145 U g^{-1} of cellobiase from *A. niger*, 80 U g^{-1} of xylanase from *T. viride*, 8 U g^{-1} of thermostable β-xylosidase.
Subsequent experiments were performed utilizing the α-arabinofuranosidase in combination with the enzymatic mixture described.

The saccharification mixtures together with blanks (pretreated lignocellulosic materials without enzyme cocktail) were incubated in a rotary thermoblock (Thermomixer C, from Eppendorf) at 50 °C and 500 rpm for 72 h. Samples were withdrawn at different time intervals and centrifuged at 16,500 x g for 30 min at 4 °C. The supernatants were analyzed to quantify the amount of sugars released. Each saccharification experiment was run in triplicate. The saccharification yield was expressed as percentage of sugar production calculated with respect to the amount of sugars present in the dry weight pretreated materials before the enzymatic hydrolysis treatment.

2.4 Determination of sugar content

For estimation of the sugars released from untreated and pretreated biomasses and from the product of hemp enzymatic hydrolysis, the cleared supernatants were opportunely diluted, and analyzed by a high-performance liquid chromatographic (HPLC) system (Dionex, Sunnyvale, CA, USA), equipped with an anionic exchange column (Carbopac PA-100) and a pulsed electrochemical detector. Glucose and xylose were separated with 16 mM sodium hydroxide at a flow rate of 0.25 mL/min, and identified by the respective standards. Fucose was used as internal standard.

3. Results and Discussion

3.1 Biomass composition and pretreatments

The use of agricultural residues and non-food crops such as hemp (*Cannabis sativa* L.) as alternative cheap substrates has considerable potentialities for the cost-effective biofuel and bio-products productions. Even more important for the use of *Cannabis sativa* L. in the bioenergetic field is its composition characterized of about 65% woody core and 35% bast fiber, with a relatively large variation in composition compared to wood fibers (Gumuskaya et al., 2007). Although the differences in chemical composition between bast fiber and woody core fraction, the whole hemp composition consisted of about 70% total carbohydrate (55% cellulose, 16% hemicellulose, 18% pectin) and a low content of lignin (only 4% in fresh and green fibres) (Andre et al., 2016).

In order to improve the cellulose and hemicellulose digestibility, the biomass was subjected to pretreatment using steam explosion (SE) or aqueous ammonia soaking (AAS) methods. These kinds of pretreatments modify the structure and composition of lignocelluloses. In Table 1 macromolecular composition of hemp biomass before and after the pretreatments, in terms of percentage of glucans, xylans and Klason lignin, was reported. Glucan content in solid fractions' dry pretreated matter increased, reaching 40.6 and 58.2% compared to its initial content. The data highlighted that the SE pretreatment removed the major part of hemicelluloses that from 21.5% reached 6.3% and was predominantly solubilized from the material making cellulose more susceptible to chemical or enzymatic digestion. However, one of the major drawbacks of SE is that the steam pretreatment lead to an incomplete separation of lignin and cellulose (Hendriks and Zeeman, 2009). Moreover it was clear that the AAS mainly caused the removal of lignin with a consequent enrichment of glucans and xylans that accounted to 58.2% and 22.0% after the pretreatment. Usually a higher-temperature ammonium hydroxide soaking pretreatment resulted in greater removal of ammonium hydroxide-soluble components. Alkali pretreatment prevents unwanted sugar degradation by neutralizing released acids during the decomposition of cellulose and hemicellulose (Gunnarsson et al., 2015) with the obtainment of high glucan and xylan recoveries without any inhibitors formation during pretreatment of hemp biomass. Lignin removal represents the major factor responsible for the efficiency of the alkali pretreatment. The lignin content in the AAS pretreated hemp biomass was significantly lower (10.7%) compared to SE pretreated biomass (25.2 %) that can be attributed to the unlocking of small lignin units during the alkali pretreatment (Zhao et al., 2020b). The conditions used for SE and AAS treatments as identified by previous experimental tests, as well as reported in literature, allowed to obtain good yields in the subsequent biotransformation process.

Table 1: Macromolecular composition of untreated and pretreated Hemp. Chemical composition (% total dry weight ± standard deviations)

	Hemp composition (from literature)[1]	Hemp (untreated)	Hemp (after AAS)	Hemp (after SE)
Glucan	36.5 ± 0.6	35.3 ± 0.9	58.2 ± 1.7	40.6 ± 1.8
Xylan	17.0 ± 0.2	21.5 ± 1.2	22.0 ± 1.5	6.3 ± 0.6
Klason lignin	21.9 ± 0.2	19.7 ± 2.1	10.7 ± 0.8	25.2 ± 1.3

[1] (Dasa et al., 2017)

3.2 Effect of enzymatic hydrolysis on pretreated hemp.

Influence of the applied pretreatment methods on the effectiveness of enzymatic hydrolysis, with the use of the commercial enzymatic cocktail previously described, was analyzed.
The conditions of temperature and agitation adopted in saccharification experiments were those at which all the tested enzymes retained almost 80% of their initial activities. The following main effects were revealed in the different examined conditions.
Several tests were conducted on *Cannabis sativ*a L. SE and AAS pretreated to identify the optimal pH conditions for obtaining high enzymatic hydrolytic yield. In particular, in the saccharification experiments were tested sodium acetate buffer at pH 5.0 and phosphate buffer at pH 6.0 and 7.0. The best results were obtained at pH 5.0 utilizing buffer sodium acetate, reaching a glucose yield of 62.0% after 72 h of biotransformation of hemp SE treated, whereas at pH 6.0 and 7.0 the glucose yields were respectively of 59.1 and 53.2 %, at the same experimental conditions.
The results indicated that the cocktail of commercial enzymes resulted more tolerant to acidic conditions than to the alkaline ones, using hemp as substrate as also reported by Phummala et al. (2015).
The solid loading of biomass (3 - 5 - 7% w/v) in the saccharification tests was also explored, and it was chosen to adopt a 5% (w/v) solid loading, at which the better result in terms of sugars released (data not shown) was obtained in accordance with previous experiments whose better hydrolysis yields were reached with this biomass percentage (Liguori et al., 2015).
In order to identify which pretreatment methods and enzyme combinations could improve the hydrolysis yield, other trials were performed using a cocktail of commercial enzymes (EC) from Sigma including cellulolytic and xylanolytic activities.
Analyzing the sugars recovery utilizing hemp SE pretreated as substrate, it was obtained a high yield of glucose (62.0%) and xylose (95.8 %), after 72 h of biotransformation (pH 5.0, T 50 °C and 500 rpm). The addition of the α-L-arabinofuranosidase (ara) as auxiliary enzyme did not substantially influence the xylose recovery (97.1 %) (Table 2).

Table 2: Cannabis sativa L. pretreated by steam explosion. Biotransformation in 50 mM buffer acetate pH 5.0 T = 50 °C, for 24, 48 and 72 h incubation

Composition	Glucose (%) 24 h	Glucose (%) 48 h	Glucose (%) 72 h	Xylose (%) 24 h	Xylose (%) 48 h	Xylose (%) 72 h
EC	27.2 ± 1.3	43.2 ± 2.7	62.0 ± 2.1	47.3 ± 1.5	71.5 ± 2.6	95.8 ± 4.2
EC + ara	30.3 ± 0.9	48.5 ± 1.9	64.1 ± 3.4	50.1 ± 2.2	73.6 ± 1.1	97.1 ± 2.9

The results of conversion of *Cannabis sativa* L., pretreated with AAS using the commercial enzymatic cocktail in combination with the arabinofuranosidase, highlighted that the yield of glucose after 72 h of bioconversion remained substantially unchanged (Table 3) whilst a higher yield in xylose was obtained with the use of the auxiliary enzyme.

Table 3: Cannabis sativa L. pretreated by AAS. Biotransformation in 50 mM buffer acetate pH 5.0 T = 50 °C, for 24, 48 and 72 h incubation

Composition	Glucose (%) 24 h	Glucose (%) 48 h	Glucose (%) 72 h	Xylose (%) 24 h	Xylose (%) 48 h	Xylose (%) 72 h
EC	19.6 ± 0.6	28.4 ± 1.3	50.7 ± 3.2	41.8 ± 2.0	54.7 ± 1.9	75.3 ± 3.5
EC + ara	23.2 ± 2.1	34.7 ± 1.7	53.1 ± 2.7	43.3 ± 3.4	60.7 ± 1.6	84.6 ± 4.1

Comparing the results of conversion of *C. sativa* L pretreated with SE and AAS it is noticed that the saccharification of the biomass pretreated with AAS led to a lower yield of xylose and glucose expressed as percentage of the amount of sugars present before the hydrolysis (97.1% instead of 84.6% of xylose and 53.1% instead of 64.1 % of glucose, after 72 h of conversion).
The explanation of this behavior could be given considering that the pretreatment with AAS, due to the removal of a larger fraction of lignin, leads to an enrichment of glucans and xylans in the pretreated biomass,

and consequently to a lower xylose and glucose recovery expressed in percentage on the total amount of these polysaccharides.
Moreover, if the absolute amounts of glucose and xylose are compared, the values after AAS pretreatment resulted higher than those achieved by steam explosion (i.e., 9.3 instead of 4.6 g L^{-1} of xylose, after 72 h of conversion with the enzymatic cocktail containing the α-L-arabinofuranosidase).
For these reasons it can be assumed that the pretreatment with AAS reduce the content of lignin and the absorption of hydrolytic enzymes on it, facilitating the biodegradation of polysaccharides (Marcolongo et al., 2014).
Pretreatments of lignocellulosic materials, together with the hyper-production of increasingly efficient hydrolytic biocatalysts, will contain the still high costs for biomass saccharification. The application of advanced enzymatic methodologies will be of big impact to prompt cost-effective green treatments for fermentable sugars recovery from biomass residues with undoubtedly low environmental impact.

4. Conclusions

The study conducted on *Cannabis sativa* L. highlights the relevance of the pretreatments that, decreasing the complexity of the biomass, enable the enzyme to penetrate into the rigid lignocellulose structure causing an exposure of cellulose and hemicellulose which were broken down during the enzymatic hydrolysis. In particular, aqueous ammonia soaking pretreatment was found as an effective method to be used before enzymatic degradation. The addition of auxiliary enzymes such as arabinofuranosidases, improved the hydrolytic efficiency of the enzymatic cocktails allowing to obtain an interesting recovery of reducing sugar from hemp biomass, suitable for biofuels and biochemicals development.

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