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INTEGRATED PRODUCTION OF BIOFUELS AND SUCCINIC ACID FROM BIOMASS AFTER THERMOCHEMICAL PRETREATMENTS

ZINTEGROWANA PRODUKCJA BIOPALIW I KWASU BURSZTYNOWEGO Z BIOMASY PO OBRÓBCE WSTĘPNEJ METODAMI TERMOCHEMICZNYMI

Abstract: The aim of this study was to develop an effective thermochemical method for treatment of industrial hemp, in order to increase its bioconversion to biofuels and bio-products. Industrial hemp was subjected to various thermochemical pretreatments using: alkaline (3 % NaOH), oxidative (3 % H_2O_2 at pH 11.5) and glycerol-based methods (70-90 % of glycerol, 1-3 % NaOH), prior to enzymatic hydrolysis with Cellic® CTec2/Cellic® HTec2 (15 FPU·g^{-1} glucan). Innovative pretreatment with glycerol fraction (80 % glycerol content, 2 % NaOH, 12.5 % biomass loading) showed to be superior over commonly used alkaline and oxidative methods with respect to by-products generation and sugar losses. Integrated process of ethanol production from enriched cellulose fraction (172 kg EtOH·Mg^{-1} of dry hemp) and succinic production from xylose-rich residue after ethanol fermentation (59 kg·Mg^{-1} of dry hemp) allowed to convert about 97 % of sugars released (glucose and xylose) during enzymatic hydrolysis of pre-treated biomass. The present study showed that it is possible to replace 50 % of the costly yeast extract, used during succinic fermentation as nitrogen source, by alternative nitrogen source (rapeseed cakes) without significant deterioration of succinic yield. Pretreatment liquor after lignin precipitation (52 kg·Mg^{-1} of biomass treated) exhibited a high biodegradability (92 %) and allowed to produce 420 m^3 CH_4/Mg VS). Results obtained in this study clearly document the possibility of biofuels (bioethanol, biogas) and bio-chemicals production from industrial hemp, in a biorefinery approach.

Keywords: cellulosic bioethanol, succinic acid, biogas, industrial hemp, glycerol, biomass pretreatment, delignification

Introduction

Industrial hemp (*Cannabis sativa* L.) known as a non-food crop is considered as particularly promising alternative to currently used energy crops, e.g. energetic willow [1, 2]. Currently, an increased interest on hemp research and cultivation has been observed [3, 4]. Besides its traditional usage as isolation material and substrate used in textile industry, hemp has been tested as solid fuel as well as feedstock for biofuels (biogas, biohydrogen, bioethanol) [5, 6] or bio-chemicals (succinic and lactic acid, furfural)

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production [7-9]. Additionally, hemp was tested as feedstock for integrated biofuels (bioethanol and biogas) [10] as well as bioethanol coupled with succinic acid production [11, 12].

Before biofuels and/or bio-chemicals production, it is necessary to release fermentable sugars from lignocellulosic biomass, which is strictly connected with pretreatment and/or subsequent enzymatic hydrolysis [13]. Among various pretreatments, application of alkaline or oxidative pretreatment has proved to be one of the most effective for softening the biomass structure and removing the lignin [14, 15]. In previous studies, industrial hemp was effectively pretreated with application of 1-3 % NaOH (121 °C) and 1-3 % H_2O_2 (90-121 °C). In these cases, lignin solubilization amounted to 42-51 % and 35-47 % after alkaline (NaOH) and oxidative (H_2O_2) pretreatment, respectively [8, 12]. However, hemp pretreatment by alkaline/oxidative method led to simultaneous release of C5 sugars into pretreatment liquors as well as sugar losses. Due to low sugar content in pretreatment liquors after alkaline/oxidative method, such residues are very frequently not utilized for biofuels and chemicals production, which causes significant losses of hemicellulosic fraction, i.e. even 36-41 % and 31-42 % for alkaline and oxidative method, respectively [8, 12]. One of the solution constitutes application of organic solvents (organosolv pretreatment), especially glycerol, which can easily penetrate into the biomass and provides an effective reagent for biomass fractionation, minimizing sugar losses and improving enzymatic hydrolysis of cellulose [14, 15]. Compared to other pretreatments (acid-, alkali- and oxidative-based methods), glycerol has only been used as solvent during pretreatment of a very limited range of agricultural residues, e.g. wheat straw, sugarcane bagasse, oil palm empty fruit bunch fiber as well as a few wood species (*Eucalyptus globulus*, Japanese cedar, beech, Scots pine, Norway spruce) [16-18]. In these cases, applied glycerol-based pretreatments turned out to be very effective in lignin solubilization and resulted in high effectiveness of subsequent enzymatic hydrolysis (74-98 %). According to our best knowledge, glycerol-based pretreatment has never been applied before biofuels and bio-chemicals production from industrial hemp, in a biorefinery approach.

In the present study, biofuels (bioethanol, biogas) and succinic acid (valuable bio-based chemical) production potential of hemp was evaluated in a biorefinery approach. As the cost of lignocellulosic ethanol production is still too high to fully compete with the market price of the ethanol produced from first generation substrates, integrated co-production of other fuels (i.e. biogas) and chemicals (e.g. succinic acid, separated lignin fraction) can reduce the costs of lignocellulosic bioethanol production and maximize the degree of biomass utilization [19]. This is the first study comparing the effectiveness of commonly used alkaline and oxidative pretreatment methods with emerging organosolv methods (based on glycerol), applied before integrated biofuel and biochemical production from industrial hemp. Moreover, by-products generated during biomass processing and ethanol fermentation (pretreatment liquor and residue after ethanol fermentation) were analysed as feedstock for biogas and biosuccinic acid production.

Materials and methods

Feedstock

Industrial hemp (*C. sativa* L., *Felina 32 variety*) was cultivated, at Lönnstorp experimental farm, Swedish University of Agricultural Sciences (55°40'N 13°06'E). Biomass harvested was dried indoors at approx. 18 °C to simulate field-drying. Dry hemp

was chopped using a shredder (2-3 cm length) and ground to particles of 2-3 mm; using a cutting mill. The chemical composition of the untreated hemp is presented in Table 1. Biomass of *hemp* was pretreated with the following methods (each pretreatment was repeated four times): alkali based on sodium hydroxide (NaOH), alkaline hydrogen peroxide treatment (H_2O_2) and glycerol-based methods. After pretreatment, the slurry was separated into solid fraction (insoluble fraction, FIS) and liquid fraction (pretreatment liquor), using a Buchner unit. Liquors after pretreatments were subjected to lignin precipitation at pH to ~ 2. The precipitated lignin was washed twice with 1 dm^3 of hot distilled water (pH = 6.6-6.8) in order to remove impurities. Small amounts of solid fraction recovered were dried (60-65 ^oC) and used for chemical composition analyses. Distribution of carbohydrates and lignin after pretreatments between solid and liquid fractions was determined, while, the difference in cellulose/hemicellulose content between the untreated and treated (solid and liquid fraction) was shown as process loss (Fig. 1).

Alkali and oxidative method

Alkali pretreatment based on NaOH (3 % w/v) and oxidative based on H_2O_2 (3 % w/v) were conducted at solid content of 10 % (w/v) feedstock/solvent. In case of H_2O_2 pretreatment, pH was adjusted to 11.5 using 4 M NaOH. Detail information about alkali (NaOH) and oxidative (H_2O_2) pretreatment was previously described [7].

Organosolv method

Organosolv pretreatment involved pre-purified glycerol fraction after biodiesel production. Glycerol used in the study was characterized as technical class and its chemical composition was as follows: glycerol 90 ±1.5 %; inorganic components: 2.55 ±0.5 %, methanol: < 0.02 %; non-glycerol organic substances: < 2.0 %. Density of glycerol fraction amounted to 1235 $g \cdot dm^{-3}$ at 20 °C; pH = 6.3-6.4. Three sets of glycerol-based pretreatment were conducted. Firstly, the biomass was treated with glycerol fraction without dilution (including 90 % of glycerol) and glycerol:water mixtures (% $w \cdot w^{-1}$), containing: 80 % and 70 % of glycerol. During this part of the experiment, a solid content of 10 % ($w \cdot w^{-1}$ feedstock/solvent) was applied. Secondly, glycerol-water mixture of 80:20 (% $w \cdot w^{-1}$) was supplemented by an appropriate amounts of solid NaOH in order to achieve 1-3 % solutions. In this set, 10 % (% $w \cdot w^{-1}$) feedstock/solvent ratio was also used. Finally, the influence of biomass loading (12.5-20 % $w \cdot w^{-1}$) on the effectiveness of glycerol-based pretreatment (80 % glycerol content, 2 % NaOH) was analysed. Separated solids were washed with 1 % sodium hydroxide solution to remove the adsorbed lignin from its surface. Next, solid fraction was washed with distilled water until reaching the neutral pH value.

Enzymatic hydrolysis

Biomass glycerol based method in conditions assumed as the most favorable (80% glycerol, 2 % NaOH, 10-15 % $w \cdot w^{-1}$ solid loading) was used as feedstock for enzymatic hydrolysis. Enzymatic hydrolysis was also performed on hemp after alkaline (3 % NaOH), and oxidative (3 % H_2O_2) pretreatment as well as untreated hemp to compare with enzymatic assays of pretreated biomass. Enzymatic hydrolysis using Cellic® CTec2 at the dosage of 15 filter paper unit [FPU]$\cdot g^{-1}$glucan was conducted at a solid loading of 5 % in a 50 mM sodium citrate buffer, pH = 4.8 at 50 ^oC for 48 h. Due to the fact that the feedstock after tested pretreatment methods contained considerable amount of xylan,

20 % of Cellic® CTec2 dosage was also replaced by Cellic® HTec2, reach in endoxylanases. Glucose and xylose yields obtained during enzymatic hydrolysis as well as total hydrolysis yields (pretreatment and enzymatic hydrolysis) were calculated according to formulas presented in our previous studies [6].

Ethanol fermentation

For ethanol production, biomass after pretreatment (80 % of glycerol, 2 % NaOH, 12.5 % $w \cdot w^{-1}$ biomass solid loading) and enzymatic hydrolysis (Cellic® CTec2 with 20 % supplementation of Cellic® HTec2) was used. Hydrolysed biomass was supplemented with 5 % $v \cdot v^{-1}$ of *S. cerevisiae* and minerals as previously described [6]. The fermentation was performed at 30 °C for 48 h in 300 cm^3 Pyrex flasks equipped with air locks. After fermentation, ethanol was removed from the solution by membrane filtration (Osmonics unit, type GH-100-400, 200 $rpm \cdot min^{-1}$) [20]. The device worked in the dead-end mode, on flat sheet membranes (SW30XLE, MWCO 100 Da) using trans-membrane pressure of 2.0 MPa. Almost all xylose present in the solution (> 99 %) was rejected, which resulted in more than 6 times more concentrated solution (45-46 $g \cdot dm^{-3}$) compared with its content in the residue after enzymatic hydrolysis/ethanol production (7.0-7.2 $g \cdot dm^{-3}$). In a present study, membrane fouling analyses were outside the paper scope.

Succinic acid production

The xylose-rich residue after ethanol fermentation was tested as feedstock for succinic acid production, using four identical 3-dm^3 fermenters. Before using as feedstock for succinic acid production, pH value of the residue was adjusted to about 6.7-7.0, using 4 M NaOH and autoclaved at 121 ^oC for 20 min. The start-up of the fermenters was previously described [7]. Yeast extract and/or rapeseed cakes, a residual material from pressing of oil from rapeseed, were used as an alternative nitrogen source. The rapeseed cake dry matter (d.m.) content amounted to 950 ±20 $g \cdot kg^{-1}$ and the main components of this substrate included: total protein 340 ±20 $g \cdot kg^{-1}$, cellulose 148 ±12 $g \cdot kg^{-1}$, hemicellulose 54.0 ±5.2 $g \cdot kg^{-1}$, insoluble lignin 154.0 ±9.3 $g \cdot kg^{-1}$, ash 48.3 ±2.9 $g \cdot kg^{-1}$. The amount of total bound amino acid content in cakes before hydrothermal processing amounted to 305 ±10 $g \cdot kg^{-1}$. The hydrothermolysis of rapeseed cake was performed in a batch reactor (Minotavr-1), at 210-220 °C, which is in consistent with previous studies on effective amino acids conversion from rapeseed cake [21]. The fermentation was conducted using the following ratios of yeast extract and rapeseed cake (% $w \cdot w^{-1}$): 100:0, 75:25, 50:50, 25:75 and 0:100. In all assays, the nitrogen source (yeast extract and/or rapeseed cakes) amounted to 5 and 10 $g \cdot dm^{-3}$, which is in the range commonly used for succinic fermentation, using *A. succinogenes.* 1.1 g of solid $MgCO_3$ per 1 g of total sugar was supplied and acted as an indirect CO_2 source and pH buffer of fermentation medium. 5 % ($v \cdot v^{-1}$) of exponentially growing inoculum (OD_{660} = 4.6) was added. *A. succinogenes* 130Z (ATCC 55618) was used in this study in concentration of 0.625 $g_{DCW\ (dry\ cell\ weight)} \cdot dm^{-3}$.

Biogas production

Liquid fractions after lignin recovery were tested as feedstock for methane production. Biochemical methane potential (BMP) tests were determined in batch experiments, in triplicates. The experiments were performed in 540 cm^3 serum glass bottles with working volume of 200 cm^3, at 2 $g_{VS\ (volatile\ solids)} \cdot dm^{-3}$. Digestate from a full scale plant (55 °C)

treating cow manure was used as inoculum. Methane produced from inoculum was subtracted from the assays. Bottles were flushed with pure N_2 for 3-5 min, sealed with rubber stoppers and aluminum crimps and finally incubated at 55 °C until no significant amounts of CH_4 were produced (approx. 30 days). Methane produced was expressed per unit of VS added. The substrates biodegradability was expressed as percentage of theoretical methane yield. The theoretical methane yield [$m^3 \cdot kg_{VS}^{-1}$] ($Y_{CH_4,th}$) at standard temperature and pressure (0 °C, 100 kPa) was based on the average organic composition of the feedstock. The following theoretical yields of compounds were used for calculations [m^3 $CH_4 \cdot kg_{VS}^{-1}$]: carbohydrates (0.415), proteins (0.497), lipids (1.015), acetate (0.375), xylose (0.373), glycerol (0.426), furfural (0.583) [22, 23]. The content of lignin was not taken into account while calculating the theoretical biogas potential, as lignin is inert or very poorly degraded in anaerobic conditions [10].

Analytical methods

Dry matter (d.m.), volatile solids (VS) and ash content were determined according to Standard Methods for Examination of Water and Wastewater [24]. Lipid content was analyzed after extraction using the Soxhlet method. Total amino acids content was analysed using Ninhydrin assays according to detail description presented [25]. The content of cellulose, hemicellulose and lignin (raw material as well as solid residues after pretreatments) were determined according to the National Renewable Energy Laboratory (NREL) analytical methods for biomass characterization. Concentrations of sugars (glucose, xylose, arabinose), ethanol, glycerol, organic acids (acetic, formic, succinic) were measured by high performance liquid chromatography HPLC (Agilent) equipped with a BioRad Aminex HPX-87 H at 63 ºC and refractive index (RI) detector (RID 1362A) using 0.6 $cm^3 \cdot min^{-1}$ of 4 mM H_2SO_4 as an eluent. All chemicals used in this study were of analytical grade. Inhibitors generated during pretreatment (furfural and 5-hydroxymethyl-2-furaldehyde (HMF)) were detected by HPLC fitted with ultraviolet (UV) detector. Cell growth (OD660) during succinic fermentation was monitored by measuring the optical density at 660 nm, using a spectrophotometer (Hach Lange DR 5000). Before OD analyses, insoluble $MgCO_3$ was removed, by mixing the sample with 7 % (w/v) HCl at the ratio of 1:1. The characteristics of raw and pretreated hemp as well as data obtained during enzymatic hydrolysis, ethanol and succinic acid fermentations were compared statistically. One-way ANOVA test followed by Tukey's HSD test were used for multiple comparisons between samples, with the level of significant set at < 0.05. Data significantly equivalent were indicated by the same letters.

Results and discussion

Influence of biomass pretreatment on carbohydrates and lignin content

Depending on the pretreatment type, cellulose content increased by 17-48 % compared to untreated hemp (Table 1). The highest cellulose content was observed after glycerol treatment in alkaline conditions (glycerol content 80 %, 2-3 % NaOH). Furthermore, the biomass loading increase from 10 to 12.5 % did not have a negative influence on cellulose content. Whilst, a gradual decrease of cellulose content was observed as the biomass loading was increased to 15-20 % (Table 1, 3rd set of experiment).

During alkaline and oxidative-based pretreatment, about 23-24 % of hemicellulose was released into xylose. The application of glycerol without water addition (100 % glycerol

fraction) resulted in significantly lower hemicellulose release, i.e. 11 % (Fig. 1). Pretreatment with glycerol containing 20 % of water (1st part of experiment, Table 1) allowed to receive a similar amount of hemicellulose released into pretreatment liquors compared alkaline and oxidative-based method. Further increase of water in reaction mixture did not have a positive influence on the effectiveness of pretreatment (Fig. 1). The highest hemicellulose release into xylose (28-30 %) was obtained in case of hemp pretreated with glycerol method in alkaline conditions (1-2 % NaOH, 10.0-12.5 % biomass loading, Fig. 1). Further increase of NaOH dosage or biomass loading during pretreatment resulted in a significant decrease of hemicellulose fraction or/and increased xylose losses (Fig. 1). Moreover, glycerol treatment at optimized conditions resulted in more than twice lower C5 sugar losses compared to alkaline and oxidative method (Fig. 1). This is an advantage of using glycerol for lignocellulosic biomass pretreatment [14, 15].

The highest lignin solubilization (38-40 %) was obtained during alkaline, oxidative and glycerol pretreatment in alkaline conditions (80 % of glycerol, 2-3 % NaOH, 10-12.5 % biomass loading, Fig. 1). In these cases, pretreated biomass contained between 9-11 % dry matter of lignin (Table 1), which is a level reported for effective biomass delignification [25, 26]. However, pretreatment with NaOH and H_2O_2 resulted in higher lignin losses (21-23 %) compared to glycerol-based methods (6-15 %) (Fig. 1). This constitutes an advantages of glycerol-based methods in relation to alkaline/oxidative pretreatments [14, 15]. Simultaneous losses of hemicellulose and lignin, during alkali-based pretreatment of hemp, have also been previously observed [7] and constitute a major drawback of alkali pretreatments.

Table 1

Characteristics of raw and pretreated biomass
(average values n = 4, ± standard deviations, the same letters represent data equivalent statistically $p > 0.05$)

Pretreatment	Pretreated solid fraction				Pretreatment liquor	
	Glucan [%] d.m.	Xylan [%] d.m.	Lignin [%] d.m.	FIS [%]	AcA [g·dm^{-3}]	TPC [g·dm^{-3}]
Untreated	40.1 ±1.6^{f}	16.0 ±0.5^{a}	14.8 ±1.0^{ab}	-	-	-
Alkali and oxidative method						
3% NaOH/10%L	56.6 ±1.5^{ab}	12.9 ±1.2^{bc}	9.17 ±0.6^{fg}	67 ±1^{cd}	4.0 ±0.2^{c}	2.8 ±0.3^{a}
3% H_2O_2, pH = 11.5/10%L	58.7 ±2.2^{ab}	10.4 ±1.1^{d}	8.50 ±0.5^{g}	64 ±2^{d}	5.1 ±0.4^{b}	3.4 ±0.4^{a}
Glycerol-based method (1st set of experiment)						
GL (90%)/10%L	46.9 ±1.7^{e}	14.5 ±1.4^{abc}	15.2 ±0.8^{a}	84 ±2^{a}	2.6 ±0.2^{d}	1.0 ±0.2^{d}
GL (80%)/10%L	49.8 ±1.1^{de}	12.7 ±0.8^{bc}	12.9 ±0.5^{cd}	80 ±2^{ab}	2.1 ±0.2^{de}	0.9 ±0.1^{d}
GL (70%)/10%L	50.3 ±1.7^{de}	10.9 ±0.9^{cd}	13.8 ±0.6^{bc}	78 ±2^{b}	2.8 ±0.1^{d}	1.0 ±0.1^{d}
Glycerol-based method (2nd set of experiment)						
GL (80%)/1%NaOH/10%L	54.9 ±1.8^{bc}	13.2 ±1.1^{bc}	12.0 ±0.3^{de}	71 ±2^{c}	1.5 ±0.2^{f}	1.2 ±0.2^{cd}
GL (80%)/2%NaOH/10%L	58.0 ±1.5^{ab}	13.5 ±0.6^{bc}	9.87 ±0.3^{fg}	67 ±2^{cd}	1.8 ±0.1^{ef}	1.5 ±0.2^{bc}
GL (80%)/3%NaOH/10%L	59.4 ±1.6^{a}	13.6 ±1.0^{bc}	10.5 ±0.5^{ef}	64 ±2^{d}	2.2 ±0.1^{ef}	1.6 ±0.2^{bc}
Glycerol-based method (3rd set of experiment)						
GL(80%)/2%NaOH/12.5%L	58.0 ±1.5^{ab}	13.3 ±0.7^{bc}	10.2 ±0.4^{fg}	68 ±2^{cd}	2.2 ±0.2^{ef}	1.6 ±0.2^{bc}
GL(80%)/2%NaOH/15%L	54.2 ±1.8^{bc}	15.0 ±1.0^{ab}	13.4 ±0.4^{bc}	70 ±2^{c}	2.8 ±0.1^{d}	1.5 ±0.3^{bc}
GL (80%)/2%NaOH/20%L	48.6 ±1.3^{e}	16.1 ±0.8^{a}	15.1 ±0.8^{a}	79 ±2^{ab}	7.1 ±0.9^{a}	1.9 ±0.2^{c}

FIS - water insoluble fraction, AcA - acetic acid, TPC - total phenolic compounds, d.m. - dry matter, GL - glycerol, %L - biomass loading used during pretreatment

The effectiveness of pretreatment methods used was also evaluated by formation of sugar and lignin degradation products. Acetic acid was observed as the main degradation

product, which is associated with hydrolysis of acetylated hemicellulose fraction (Table 1). At biomass loading not exceeding 15 %, significantly higher content of acetic acid was released after alkaline pretreatment (NaOH) and oxidative method (H_2O_2) compared to all tested glycerol-based methods. Increasing biomass loading above 15% during glycerol-based methods resulted in a significant increase of acetic acid (Table 1). Besides acetic acid, soluble phenolic compounds were generated, most probably due to partial breakdown of lignin, which is usually observed in this type of biomass pretreatment [27]. As regards to other glucan- and xylan-based by-products, low concentrations of furfural (< 0.5 $g \cdot dm^{-3}$, data not shown) and no HMF (5-hydroxymethyl-2-furaldehyde) were recorded.

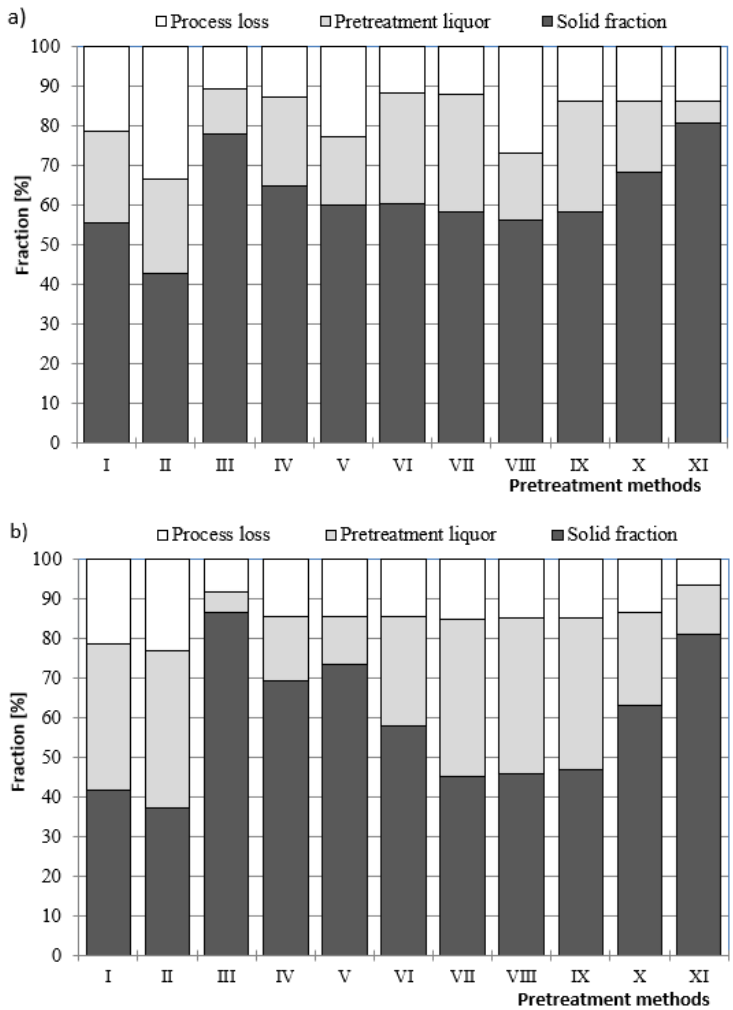

Fig. 1. Influence of biomass pretreatment on: a) hemicellulose and b) lignin (I - 3%NaOH/10%L, II - 3%H_2O_2/pH = 11.5/10%L, III - 90%GL/10%L, IV - 80%GL/10%L, V - 70%GL/10%L, VI - 80%GL/1%NaOH/10%L, VII - 80% GL/2%NaOH/10%L, VIII - 80%GL/3%NaOH/10%L, IX - 80%GL/2%NaOH/12.5%L, X - 80%GL/2%NaOH/15%L, XI - 80%GL/2%NaOH/20%L)

Considering effectiveness of the pretreatment, hemp pretreated with glycerol in alkaline conditions (2 % NaOH, 80 % of glycerol content, 10-15 % biomass loading) was selected as feedstock for enzymatic hydrolysis (Table 1, Fig. 1). Higher biomass loading did not have a positive influence on the effectiveness of glycerol-based pretreatment (Table 1), which can be connected with lower accessibility of the pretreatment agent and reduced steam diffusion at higher solid loadings. High biomass loading during pretreatment is also associated with difficulties in mixing. This is in agreement with previous studies, e.g. [28].

Enzymatic hydrolysis of pretreated biomass

The glucose yield of untreated feedstock amounted to 33 % after 48 h of enzymatic hydrolysis, which proves that hemp without pretreatment is difficult to hydrolyze. Using Cellic® CTec2 as the only enzymatic cocktail, there was no significant difference in process effectiveness between biomasses pretreated with alkaline, oxidative or glycerol-based methods (glucose yield: 84-90 %, xylose yield: 80-83 %) (Table 2, data equivalent statistically, $p > 0.05$). Taking into account the fact that the analysed pretreatment methods (alkaline, oxidative- and glycerol-based) are not very effective in hemicellulosic fraction hydrolysis compared to acid-based methods [14, 15], it is recommended to supplement Cellic® CTec2 with reach in endoxylanases - Cellic® HTec2. Finally, application of Cellic® HTec2 did not have a significant impact on the final concentration of glucose in reaction mixtures and glucose yields compared to hydrolysis with Cellic® CTec2 cocktail (Table 2). Due to endoxylanases supplementation, xylose yields increased by 12-14 % compared to process with Cellic® CTec2 used as the only enzymatic cocktail (Table 2).

Table 2

Effectiveness of the enzymatic hydrolysis and overall sugar yields after pretreatment and enzymatic hydrolysis (± standard deviation, $n = 3$, the same letters represent data equivalent statistically $p > 0.05$, GL - glycerol, %L - biomass loading used during pretreatment)

Pretreatment	Glucose yield [%]	Xylose yield [%]	Overall yield[a] [%]	Overall yield[b]$_{(T)}$ [%]
Untreated/ CTec2	33.0 ±1.3^{c}	20.9 ±1.4^{c}	29.8 ±1.2^{e}	29.8 ±1.2^{e}
3% NaOH/10%L/CTec2	85.5 ±2.2^{b}	80.9 ±1.6^{b}	84.6 ±1.7^{cd}	79.1 ±0.7^{cd}
3% H_2O_2, pH = 11.5/10%L/CTec2	84.4 ±2.7^{b}	79.9 ±2.5^{b}	83.7 ±1.9^{cd}	74.8 ±1.7^{d}
GL (80%)/2%NaOH/10%L/CTec2	87.6 ±1.8^{ab}	80.1 ±3.3^{b}	89.5 ±2.0^{ab}	85.4 ±1.4^{b}
GL(80%)/2%NaOH/12.5%L/CTec2	89.5 ±2.4^{ab}	83.3 ±2.7^{b}	88.1 ±2.3^{ab}	87.1 ±1.4^{ab}
GL(80%)/2%NaOH/15%L/CTec2	84.1 ±1.9^{b}	79.6 ±2.4^{b}	83.1 ±2.0^{d}	79.7 ±1.7^{c}
GL (80%)/2%NaOH/10%L//CTec2+HTec2	89.7 ±1.6^{ab}	96.9 ±2.6^{a}	91.1 ±1.7^{a}	89.7 ±1.2^{ab}
GL(80%)/2%NaOH/12.5%L/CTec2+HTec2	91.9 ±2.5^{a}	94.3 ±2.7^{a}	91.8 ±1.7^{a}	90.9 ±1.2^{a}
GL(80%)/2%NaOH/15%L/CTec2+HTec2	88.1 ±1.9^{ab}	91.9 ±1.8^{a}	89.0 ±2.1^{ab}	80.9 ±1.7^{c}

[a] - total amount of glucose and xylose released during enzymatic hydrolysis, [b] - total amount of glucose and xylose released during both biomass pretreatment into liquid fraction and enzymatic treatment of solid fraction after pretreatment

Considering the total effectiveness of the biomass hydrolysis (pretreatment + enzymatic hydrolysis), hemp pre-treated by glycerol method (80 % glycerol, 2 % NaOH) at loading of 10-12.5 % and hydrolyzed mixture of Cellic® CTec2+HTec2 allowed to reach the highest overall sugar yields (Table 2). In general, glycerol-based method turned out to be superior over alkaline and oxidative-based methods and these results are most likely due to effective lignin solubilization and lower sugar losses compared to alkaline and

oxidative-based methods (Fig. 1). Due to the fact that higher biomass loading during pretreatment is more beneficial due to reduced size of equipment and lower energy consumption for processing a unit mass of biomass, biomass pretreated by glycerol-based method at 12.5 % loading was selected as feedstock for ethanol and succinic acid production.

Biofuels and chemicals production from hydrolyzed biomass

Ethanol production from enriched in cellulose biomass

Ethanol production started immediately without any lag phase until the glucose was completely consumed. The absence of significant lag phase additionally suggests that the level of inhibitors in un-detoxified hydrolysates was low [28]. An average ethanol yield amounted to 90% (0.46 g EtOH·g^{-1} glucose) of theoretical yield, which was equivalent to about 176 kg EtOH·Mg^{-1} of tested biomass (Fig. 2). The ethanol yield of "pure glucose" was also analyzed and the values amounted to 0.49-0.50 g EtOH·g^{-1} glucose (data not shown). This shows that biomass pretreatment by glycerol-based method has no negative influence on ethanol fermentation by yeasts. This is in compliance with recent studies, e.g. Zhang et al. [29, 30] showing that solution containing up to 5 wt.% of glycerol had no negative effect on yeast fermentation. Insignificant glycerol concentrations were also noticed in current studies (< 1 g·dm^{-3}) (data not shown), most probably as a by-product of ethanol fermentation by *yeasts* [31]. The xylose was not consumed during ethanol fermentation by non-engineered yeast. This is in compliance with current knowledge, as native *S. cerevisiae* (non-engineered) does not consume sugar C5.

Succinic fermentation of xylose-rich residues

The initial sugar (xylose) content before succinic acid fermentation amounted to 40.2 ±1.7 g·dm^{-3}, which is about 10 % lower than xylose concentration after ethanol separation by membrane filtration. Sugar losses during feedstock neutralization, sterilization and dilution (nutrients addition) amounted to 10 %, which is in the range previously reported [6, 7, 11, 12]. In case of fermentation without medium addition, a lag phase was observed during the 24 h of the process and about 50 % of the xylose was still present in the fermentation broth, which shows that that nutrients (nitrogen, minerals) availability could be a limiting factor in succinic acid production from xylose-rich residues, leading to lower sugar utilization (Table 3). In our previous studies, succinic acid was effectively produced from thin stillage after ethanol production without nutrients addition (sugar utilization: 86-90 %). However, sugar (xylose) content before fermentation was between 20-50 % lower compared to present study [11, 12]. Fermentation with yeast extract as nitrogen source started without any lag phase and higher values of sugar utilization and succinic acid titer were obtained after application of higher yeast extract dosage (i.e. 10 g·dm^{-3}) compared to dosage of 5 g·dm^{-3} (Table 3). Assays containing mixtures of yeast extract/rapeseed cakes, in which at least 50 % of yeast extract was present, also started without any lag phase. In such cases, sugar utilization and succinic yields amounted to 86-89 % and 64-71 % (Table 3). These values are within the upper range of succinic yields reported for xylose-based feedstock [7, 31]. A slightly higher succinic yields obtained for assays after addition of 25-50 % of nitrogen, originated from rapeseed cakes, can be connected with carbohydrates content of this substrate (i.e. 20 % of dry matter). This is in accordance with previous studies stating that rapeseed meal (e.g. pretreated by dilute

acid method and hydrolysed with pectinase) can be used as carbon and nitrogen source, for production of succinic acid by *A. succinogenes* [32]. Significantly higher residual sugar contents and lower succinic yields were obtained after application of rapeseed cakes as the only nitrogen source (Table 3), which proves that it is difficult to completely replace yeast extract in the original fermentation medium, by rapeseed cakes as alternative nitrogen sources. Yeast extract, being rich in amino acids, vitamins and trace elements is considered as favorable nitrogen source in fermentation processes. Other researchers obtained similar conclusions, e.g. Shen et al. [9] showed that corn steep liquor (CLS) could not completely replace enriched yeast extract without adding additional ingredients in order to meet physiological demands of *A. succinogenes.*

Depending on fermentation conditions e.g. type of feedstock, nitrogen source used; other metabolites (such as acetic, formic, lactic acid, ethanol or glycerol) can be produced in different amounts [33]. As long as the fermentation medium contained at least 50 % of nitrogen from yeast extract, by-products (acetic + formic) did not exceed 30 % of total acid production. Whilst, fermentation with lower yeast extract content resulted in significant increase of by-products concentration (based on results presented in Table 3). Decreased ratio of succinic acid to by-products increases the costs of succinic acid recovery from the broth. The aim of the present study was to show the possibility of rapeseed cakes usage as an alternative nitrogen source in order to decrease the usage of yeast extract, which is considered as an expensive medium ingredient. Further research on influence of particular components of rapeseed cakes on the succinate production and metabolite profile are being conducted.

Table 3

Influence of nitrogen source (yeast extract and rapeseed cakes) on the course and effectiveness of succinic fermentation (results after 48 h), using *A. succinogenes* (average values $n = 4$, ± represent standard deviations, the same letter represent data statistically equivalent $p > 0.05$)

Yeast extract: rapeseed cakes [% w·w^{-1}]	**Xylose [g·dm^{-3}]**	**Sugar utiliz. [%]***	**SA [g·dm^{-3}]**	**Succinic yield [%]****	**AcA [g·dm^{-3}]**	**FA [g·dm^{-3}]**
-	15.60 ±0.80^{a}	48.2 ±2.5^{c}	9.10 ±0.6^{d}	62.9 ±2.8^{bc}	3.50 ±0.41^{bc}	2.04 ±0.30^{a}
100:0*	9.25 ±0.72^{bc}	69.3 ±2.1^{b}	12.4 ±0.8^{bc}	59.2 ±1.9^{cd}	3.25 ±0.32^{c}	2.05 ±0.11^{a}
100:0**	3.25 ±0.52^{d}	89.2 ±1.7^{a}	17.4 ±0.8^{a}	64.2 ±2.0^{b}	4.11 ±0.31^{bc}	2.54 ±0.12^{a}
75:25**	3.50 ±0.45^{d}	88.4 ±1.4^{a}	17.9 ±0.9^{a}	67.1 ±2.6^{ab}	4.05 ±0.12^{bc}	2.67 ±0.20^{a}
50:50**	4.25 ±0.52^{d}	85.9 ±1.7^{a}	18.3 ±1.0^{a}	70.5 ±2.6^{a}	4.20 ±0.50^{bc}	2.73 ±0.51^{a}
25:75**	8.25 ±0.53^{c}	72.6 ±1.7^{b}	13.8 ±0.5^{b}	62.8 ±0.9^{bc}	4.45 ±0.51^{ab}	2.43 ±0.42^{a}
0:100**	9.75 ±0.91^{b}	67.7 ±2.9^{b}	11.5 ±0.7^{c}	56.4 ±2.1^{d}	5.43 ±0.70^{a}	2.49 ±0.45^{a}

*- total nitrogen source of 5 g·dm^{-3}; ** - total nitrogen source of 10 g·dm^{-3}, sugar utiliz. (%) = [(initial sugar content - sugar content after succinic acid production)/initial sugar content)]·100; SA - succinic acid, AcA - acetic acid, FA - formic acid

Anaerobic digestion (AD) of pretreatment liquors

The methane production started immediately and the maximum values were recorded after 25-30 d of incubation (Table 4). All assays displayed a high initial rate of methane formation, which was accounted for the presence of easily biodegradable compounds (data not shown). This in consistence with the fact that rapid bio-conversion of dissolved components (sugars, organic acids etc., Table 4) is usually observed [34]. The mean methane yields for the studied liquors amounted to between 420 and 436 dm^3 CH_4·kg$_{VS}^{-1}$.

These yields amounted to 90-96 % of theoretical methane yields, showing that the by-products generated during glycerol-based pretreatment were suitable for methane production by anaerobic digestion (Table 4). These methane yields correlates to 0.297-0.312 dm^3 $CH_4 \cdot g^{-1}$ COD (calculated based on results presented in Table 4), which constitutes about 85-89 % of theoretical value (based on methane yield of 0.350 dm^3 $CH_4 \cdot g^{-1}$ COD).

Biorefinery approach

Using ordinary baker's yeast, *S. cerevisiae*, about 172 kg EtOH·Mg^{-1} of dry hemp was produced (retentate after ethanol recovery) from biomass pretreated conditions, considered as the most optimal (80 % glycerol, 2 % NaOH, 12.5 % biomass loading). Ethanol losses amounted to 2.5 % of its initial amount after fermentation (Fig. 2). This value is significantly higher than previously reported for hemp pretreated by dilute acid method (149 kg EtOH·Mg^{-1}) [12]. Due to the fact that xylose is not consumed by non-engineered yeasts, the residue after ethanol recovery was converted into succinic acid (59 kg·Mg^{-1}), using *A. Succinogenes* (Table 2). This value is about 60-68 % higher compared to succinic production from residues after fermentation of biomass pretreated with dilute acid methods [8].

Table 4

Characteristics of pretreatment liquors and assessment of liquors biodegradability (average values $n = 4$, ± standard deviations, the same letters represent data equivalent statistically $p > 0.05$)

Parameter	GL (90%)/ 10%L	GL (80%)/ 10%L	GL (80%)/ 2% NaOH/ 10%L	GL (80%)/ 2% NaOH/ 12.5%L	GL (80%)/ 2%NaOH/ 15%L
Characteristics of pretreatment liquors					
TS [%]	77.3 ±1.8^{a}	61.8 ±1.6^{b}	63.6 ±2.1^{b}	64.9 ±1.8^{b}	63.2 ±2.5^{b}
VS [%]	75.0 ±1.4^{a}	59.2 ±1.7^{b}	56.0 ±1.3^{bc}	56.4 ±1.9^{bc}	54.2 ±2.2^{cd}
Acetic acid [g·dm^{-3}]	2.6 ±0.2^{a}	2.1 ±0.2^{ab}	1.8 ±0.1^{b}	2.2 ±0.2^{ab}	2.8 ±0.2^{a}
Glycerol [g·dm^{-3}]	691 ±22^{a}	544 ±15^{b}	517 ±21^{bc}	512 ±16^{bc}	495 ±21^{c}
Lipids [g·dm^{-3}]	42.5 ±2.7^{a}	30.9 ±2.4^{b}	28.0 ±1.8^{bc}	29.3 ±2.4^{bc}	29.2 ±2.1^{bc}
Proteins [g·dm^{-3}]	1.6 ±0.2^{a}	1.3 ±0.1^{a}	1.4 ±0.1^{a}	1.4 ±0.2^{a}	1.4 ±0.2^{a}
Carbohydrates [g·dm^{-3}]	11.7 ±1.5^{bc}	14.2 ±1.7^{b}	11.3 ±1.1^{bc}	18.3 ±1.3^{a}	13.1 ±1.6^{bc}
Methane production					
$Y_{CH4,th}$ [dm^3 $CH_4 \cdot$ kg_{VS}^{-1}]^{a}	459 ±12^{a}	456 ±15^{a}	455 ±20^{a}	456 ±20^{a}	458 ±22^{a}
Specific CH_4 yield [dm^3 $CH_4 \cdot kg_{VS}^{-1}$]^{b}	421 ±21^{a}	436 ±19^{a}	436 ±16^{a}	420 ±21^{a}	420 ±23^{a}
Biodegradability [%]^{c}	90.7 ±2.8^{a}	95.6 ±2.4^{a}	95.9 ±1.8^{a}	91.8 ±2.7^{a}	91.8 ±2.2^{a}

GL - glycerol; %L - biomass loading used during pretreatment; [a] - theoretical methane yield based on the average organic composition of the feedstock; [b] - biochemical methane potential (BMP) tests determined in batch experiments; [c] - [biochemical methane potential (BMP)/theoretical methane yield]·100; [d] - amount of methane produced from biomass, including biomass pretreatment and sugar losses

Pretreatment liquors containing glycerol added as solvent during biomass treatment, after lignin precipitation, was used as feedstock for anaerobic digestion (AD). This fraction exhibited a high biodegradability (92 %) and allowed to produce 420 dm^3 $CH_4 \cdot$ kg_{VS}^{-1}, which is equivalent to about 1071 m^3 or 765 kg $CH_4 \cdot Mg^{-1}$ biomass (1013 hPa; 0 °C) (Fig. 2).

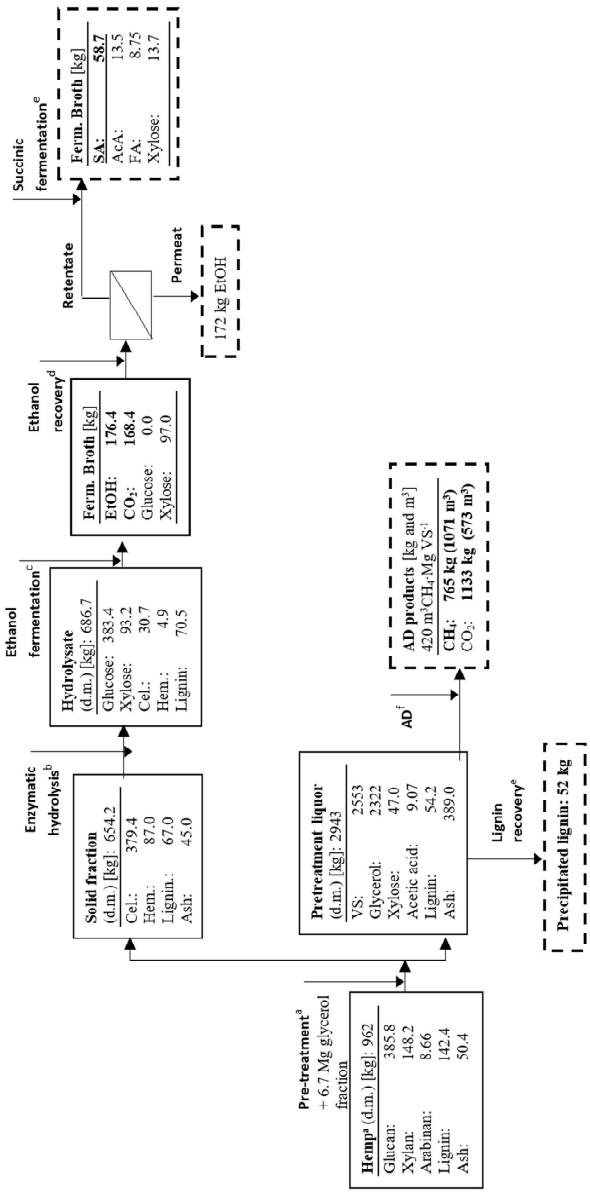

Fig. 2. Simplified mass balance of hemp pretreated at optimized conditions and aimed at maximizing the products in a biorefinery approach (a - biomass pretreated with 80 % glycerol in alkaline conditions 2% NaOH, 12.5 % biomass loading, b - enzymatic hydrolysis with using Cellic® CTec2 at the dosage of 15 FPU·g^{-1} glucan and replaced of 20 % mixture of CTec2 by Cellic® HTec2, c - ethanol production in Pyrex flasks, using non-engineered *S. cerevisiae*, d - ethanol and xylose recovery via membrane module, e - succinic acid production from residue after ethanol fermentation with yeast extract: rapeseed cakes (50:50 % w·w^{-1}) used as nitrogen source (total dosage of 10 g·dm^{-3}), f - anaerobic digestion (AD) of pretreatment liquor after biomass processing at optimal conditions (80 % glycerol, 2 % NaOH, 12.5 % biomass loading), d.m. - dry matter, SA - succinic acid; AcA - acetic acid; FA - formic acid; EtOH - ethanol)

Usage of pretreatment liquors after organosolv biomass processing constitutes a new and renewable feedstock for biogas production compared to commonly used such as: sewage sludge. In the present biorefinery approach, pretreated biomass (enriched in cellulose) and pretreatment liquor after biomass processing were used as feedstocks for bioethanol and biogas production, i.e. processes which generate carbon dioxide as by-products (Fig. 2).

Whilst CO_2 is used during succinic acid fermentation, using *A. succinogenes*, it is estimated that 1 Mg (tone) of biosuccinic produced can save 4.5-5 Mg of CO_2 [7]. Therefore, it is evident that the biorefinery concept presented has a real chance to contribute to the latest trends connected with abatement of CO_2 emissions from biofuels/bio-chemicals production from biomass. Moreover, excessive amounts of CO_2 can be used in numerous industrial applications, e.g. during carbonated soft drinks and soda water production, solid form known as "dry ice" can be used as refrigerants in food industry etc. [35-38]. Alternatively, used for biomass pretreatment can be recovered using distillation and recycled back to biomass pretreatment. The usage of solvent after glycerol-based pretreatments is usually ignored, while, this compounds can be used as feedstock for biotechnological production of various high value-added products. In our study, we also used rich in glycerol, pretreatment liquor after lignin precipitation as feedstock for succinic acid production, using *A. succinogenes*. However, batch fermentations in conditions applied for fermentation of residues after ethanol fermentation were unsuccessful (data not shown). This difficulty in using glycerol is associated with a redox imbalance during cell growth, impairing glycerol conversion. More specifically, ability to ferment glycerol is not a ubiquitous trait of most succinogenes, since this metabolic process requires the recycling of one extra reducing equivalent (i.e. NADH) compared to sugars C5 and C6. Glycerol-fermenting organisms recycle this extra NADH by producing 1,3-propanediol (1,3-PD). *A. succinogenes* is unable to ferment glycerol, since it lacks the complete enzymatic pathway to synthesize 1,3-PD. Most bacteria producing succinic acid (including *A. succinogenes*), which do not generate 1,3-PD, require either an external electron acceptor, or e.g. spontaneous mutation occurring by gradual adaptation to glycerol, in order to produce high concentrations of succinic acid [38, 39]. Additionally, dissolved lignin was precipitated after the acidification of pretreatment liquor (52 $kg \cdot Mg^{-1}$ biomass). Separated lignin can be used for heat or heat and power generation and integrated with lignocellulosic biorefinery for energy-intensive processes, such as: biomass pretreatment, ethanol distillation, heating of AD reactors.

Conclusions

The results obtained clearly showed that industrial hemp can be used for biofuels (ethanol, biogas) and bio-products (succinic acid) production in a biorefinery approach. Results obtained in this study clearly showed that glycerol pretreatment of hemp (2 % NaOH, 80% of glycerol content) was superior compared to alkaline and oxidative methods, due to generation of significantly lower amounts of degradation products and lower sugar losses. Due to the fact that xylose was not consumed by non-engineered yeasts, the residues after ethanol recovery was successfully converted into succinic acid. Integrated process of ethanol (glucose conversion) and succinic fermentation (xylose conversion) allowed to convert about 97 % of sugars released (glucose and xylose) released during enzymatic hydrolysis of pretreated biomass, using Cellic® CTec2 with 20 % content of

Cellic® HTec2. It was found that 50 % of costly yeast extract used during succinic fermentation as nitrogen source can be successfully replaced by rapeseed cakes, i.e. a residual material from pressing of oil from rapeseed. The pretreatment liquor containing glycerol showed a high biodegradability (< 90 %) during anaerobic digestion (AD). Separated lignin can also be treated as hemp biorefinery product. It should be taken into account that the present study is a simplified analysis, which is focused on products (lignocellulosic ethanol, bio-methane and succinic acid). However, results obtained can be used as an important input for extended environmental and economic analyses to prove the industrial hemp as a suitable crop for biorefinery.

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