Hemp hurds biorefining: A path to green L-(+)-lactic acid production

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Abstract

Sugars streams generated by organosolv pretreatment of hemp hurds, cellulose (C6) and hemicellulose (C5) fractions, were fermented to lactic acid (LA) by Bacillus coagulans strains XZL4 and DSM1. Pretreatment conditions and enzymatic hydrolysis were optimized and B. coagulans aptness to use lignocellulosic-derived sugars as a carbon source was evaluated. Methanolic organosolv pretreatment with 2.5% (w/w) H_2SO_4 gave the best results in terms of glucan recovery (98%), enzymatic hydrolysis of pretreated biomass (70%) and hemicellulosic sugars recovery (61%). C6 and C5 sugars fermentation by strain XZL4 gave, high LA yields (0.90 and 0.84 g/g), high titers (141 and 109 g/L), and high enantiomeric excess (>99%). Overall, 42 g of L-LA were obtained from 100 g of raw hemp hurds. These results can be considered promising for lignocellulosic feedstock valorization toward the production of polymer-grade LA.

Introduction

Replacement of current fossil oil-based economy with the so-called sugar platform route will require some breakthrough changes in the today's production of goods. Within this context, new synergies between biological and chemical sciences are required to exploit the potential of lignocellulosic biomasses (Cherubini 2010). Industrial hemp (Cannabis sativa L.) is a fast growing and a high yielding annual crop that provides a wide range of valuable products. High land use efficiency as well as improvement of soil health are some of the main feature of this species, supporting its use also as an energy crop (Karus and Vogt, 2004). Hemp hurds are the industrial by-products from fiber production, which is currently discarded as solid waste. Due to the high content of carbohydrates (about 70% dry weight), mostly glucose and xylose (Gandolfi et al., 2013), hemp hurds can be considered a potential source of inexpensive fermentable sugars. Aim of the biorefinery approach is to depolymerize and deoxygenate all the biomass components, that is cellulose, hemicellulose and lignin, to obtain a number of platform chemicals via fermentations or chemical synthesis. However, due to the compact and rigid structure of lignocellulosic materials, known as biomass recalcitrance, the release of fermentable sugars has become a bottleneck for industrialization of lignocellulosic biorefinery. In this respect, considerable research has been carried out to enhance cellulolytic enzymes performance and several factors (e.g., lignin content, accessible surface area, pore volume and cellulose crystallinity) are considered to determine the saccharification rate, thus necessitating the use of a pretreatment step prior to enzymatic hydrolysis (Zhao et al., 2012). Even though a variety of pretreatment protocols have been reviewed (Galbe and Zacchi, 2012), the selective fractionation of all lignocellulosic components still remains one of the main open issues in biorefinery. Among them, the OS process can be considered the preferred one to obtain simultaneously valuable hemicellulosic monomers, low-crystalline cellulose and unaltered pure lignin (Zhao et al., 2009). Even though OS is a more expensive technology, process variables optimization and use of recyclable solvents would reduce the overall process cost and abide by the green process principles. Specifically, low boiling points solvents (e.g., methanol, ethanol and acetone) are the most used. OS can be performed with or without catalyst additions, in the latter case temperature over 180 C should be reached to generate organic acids, from the hemicellulose hydrolysis, which acts as catalyst for the process (Zhao et al., 2009). However the addition of mineral acids (e.g., sulfuric, hydrochloric and phosphoric acid) as well as organic acids, has been proved to enhance the delignification process and hemicellulose dissolution, leading to a pretreated biomass that can be easily hydrolyzed by cellulolytic enzymes (Del Rio et al., 2010). OS is commonly considered a flexible pretreatment technology and different target chemicals, such as furan-type compounds, organic acids or bio-oils, can be

obtained by changing the process harshness (Wettstein et al., 2012). Beyond conversion of sugar to furans and organic acids, fermentation of carbohydrates can lead to wide range of products. Among them, LA is a versatile chemical used in food, cosmetic, pharmaceutical, textile and chemical industries, which could be produced by fermentation of lignocellulosic-derived sugars. Over the past few years, its application has been extended also to biodegradable plastics, to synthesize PLA polymers (Abdel-Rahman et al., 2013). The properties of PLA are similar to those of petroleum-derived polymers, so that it can replace them in several instances (e.g., packaging, fiber and foam materials). Because the physical properties of PLA depend on the isomeric composition of its monomers, production of enantiomerically pure LA is highly desirable. Currently, LA is obtained on an industrial scale by fermentation of pure sugars or edible crops by lactic acid bacteria (LAB), which typically have complex nutritional requirements (Wee et al., 2006) Bacillus coagulans, a homolactic bacterium, has been reported to possess many valuable fermentation features, such as thermophilic trait, simple nutrition requirements and high carbon-efficiency (Patel et al., 2006). Moreover, it produces enantiomerically pure LA. To improve the economy of LA production, different authors have proposed the use of low-cost and renewable raw materials as carbon source for fermentations, such as corncomb molasses (Wang et al., 2010) and paper sludge (Budhavaram and Fan, 2009). In the present study, the feasibility of L-(+)-LA production at high concentration, from hemp hurds was investigated. To this aim, the H_2SO_4 concentration for OS fractionation of hemp hurds and the enzyme and solid loadings for the enzymatic cellulose hydrolysis, were optimized toward the recovery of fermentable sugars (C5 and C6). Moreover, best B. coagulans strains XZL4 and DSM1 fermentation conditions were investigated and an efficient non-sterile process for LA production was developed, using the hemp hurds-derived sugars streams.

Methods

Raw materials Hemp hurds was provided by Assocanapa (Carmagnola, Italy) as chopped pieces of 5 cm in length or shorter. Biomass was manually separated from dust and short fiber, knife milled under 2 mm screen (MF-10, IKA, Germany) and extracted first with CH_2Cl_2 and then with acetone using a 1 L Soxhlet apparatus (12 h each, 3–4 cycles/h). The composition of hemp hurds was 44% cellulose, 23% lignin, 25% hemicellulose and 1.2% ash (Gandolfi et al., 2013). The enzymes blend CTec2 was generously provided by Novozymes (Bagsværd, Denmark). B. coagulans strain XZL4 was isolated by Prof. Xu (Shangai Jiao Tong University), while strain DSM1 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). Bacterial cells were stored in a 20% (v/v) glycerol stock solution at about 80 C. All chemicals were used as received and were of analytical grade.

Organosolv pretreatment

The pretreatment of hemp hurds was performed in a 300 mL high-pressure reactor system (Berghof, BR-300, Germany) with temperature control (Berghof, model BTC-3000) and continuous stirring, as previously described (Gandolfi et al., 2014) The solids loading used in all the experiments was 10% (w/v). Hemp hurds (10 g) were soaked with a 65% (v/v) of a methanolic solution and with a content of concentrated sulfuric acid 0–3% (w/w, based on hemp hurds dry weight) for 1 h, 150 rpm and at room temperature. After soaking, the reactor was pressurized to 5 bar with N_2 and heated at an average rate of 2.5 C/min. When the set point temperature of 165 C was reached, the reaction was left to proceed isothermally for 40 min, and then cooled down to 40 C in an ice bath. The reaction liquid was directly sampled from the reactor for quantification of sugars, degradation products and lignin. The solid residue was separated from the process liquor by vacuum filtration, and washed twice with 150 mL of warm methanolic 65% (v/v) solution adjusted to pH 1.5 with concentrated H_2SO_4. The washes and the process liquor were combined and methanol was removed by rotary evaporation, to precipitate the dissolved lignin (OSL). The resultant aqueous fraction was neutralized to pH 6 with $CaCO_3$, filtered and analyzed for quantification of sugars and degradation products. The aqueous fraction was concentrated by rotary evaporation and used as carbon source for LA fermentations (C5-fraction). The residual solids fraction was thus washed with water, until a neutral pH of the filtrate was obtained, dried and subjected to enzymatic hydrolysis (C6-fraction). The OS process severity was evaluated using the Combined Severity factor (CS) as described by Chum et al. (1990).

Enzymatic hydrolysis

The enzymatic activity (FPU) was determined according to NERL protocol (Adney and Baker, 1996). The hydrolysis of pretreated biomass was performed using the enzymes blend CTec2. The reaction was carried

out in a thermostated shaker for 72 h at 50 C and 150 rpm. A 0.01 M sodium citrate buffer pH 5.5 was used for all the experiments, while different enzyme and biomass loadings were assessed, 10–40 FPU/g glucan and 2.5–13% (w/v), respectively. At the end of the hydrolysis, unconverted solid biomass was allowed to settle and the recovered liquid was heated at 99 C for 5 min to denature the solubilized enzymes, which where then removed by centrifugation (10 min at 13,000 rpm; 15,000 g). The liquid was filtered through 0.45 μm nylon membranes and analyzed. The liquid fraction was then concentrated and used as carbon source for LA fermentations (C6-fraction).

Microorganism strains, culture medium and fermentations

The microbial strains were cultured in a medium composed of [g/L]: sugar (glucose or xylose) 10, yeast extract 10, $(NH_4)_2HPO_4$ 2, $(NH_4)_2SO_4$ 3.5, BIS-TRIS 20. Pre-cultures were grown under aerobic conditions in 5 mL of medium in a shaker at 130 rpm and 40 C (strain DSM1) or 50 C (strain XZL4) starting from an overnight-growth culture. Cells used as inoculum for LA fermentation experiments were collected at mid-exponential phase (8 h growth cultures). Medium used for LA production and for parameters optimization (temperature, pH and initial sugar concentration) contained 50–220 g/L of sugar (glucose and/or xylose) and 10 g/L yeast extract. $CaCO_3$ was added as 60% (w/w) of sugar, as buffer agent to maintain the pH around 5–6. All the experiments were performed in 300 mL screw cap bottles containing 150 mL of non-sterile medium. The inoculum volume was 10% (v/v) and fermentations were carried out in a thermostated shaker for 144 h at 130 rpm. Samples were collected periodically to check pH, cell growth (OD600), LA and sugars concentrations. All experiments were performed in triplicate.

Analytical methods

Analyses of untreated, pretreated solid biomass and OS process liquor were performed following the NREL protocols (Sluiter et al., 2012; Sluiter et al., 2006). Quantification of monomeric sugars was carried out by HPLC using a Rezex RPM or a ROA column (Phenomenex) at 85 C and an evaporative light-scattering ELS-2041 detector (Jasco). The mobile phase was ultrapure H_2O with a flow rate of 0.6 mL/min. Total reducing sugars were spectrophotometrically determined by BCA method (Zhang and Lynd, 2005). Analysis of furfural, HMF, acetic acid, levulinic acid and LA were performed with a Rezex ROA column at 60 C and a diode array detector MD-910 (Jasco). The mobile phase was 0.005 M H_2SO_4 with a flow rate of 0.6 mL/min. Furfural and HMF were detected at a wavelength of 270 nm while acetic acid, levulinic acid and LA were detected at 205 nm. Identification and quantification of the compounds were done via calibration curve to corresponding standards. The enantiomeric excess (e.e.) of L-(+)-LA was determined by HPLC equipped with a Sumichiral OA-6100 chiral column (Sumika Chemical Analysis Service) and a UV-975 detector (Jasco) at wavelength of 254 nm. The mobile phase was 0.002 M $CuSO_4$ and acetonitrile (ratio 98:2) at flow rate of 0.6 mL/min. and LA retention time were 3.76 and 5.56 min, respectively.

Results and discussion

Effect of catalyst concentration OS pretreatment

OS approach to lignocellulosic materials typically results in a pretreated biomass readily hydrolyzable by cellulolytic enzymes, an aqueous fraction containing hemicellulosic-derived sugars, and an organic solvent fraction rich in fragmented lignin (Zhao et al., 2009). The present study focuses on the evaluation of catalyst (H_2SO_4) concentration (0–3% w/w) to maximize the recovery of fermentable sugars; other pretreatment parameters (e.g. temperature, reaction time and solvent concentration) were selected considering previously described results (Gandolfi et al., 2014). OS pretreatments were carried out using 10% (w/v) of solids loading and the process harshness, was evaluated by the CS value (Chum et al., 1990). The generated OS process streams, namely the residual solids and the process liquor (Fig. 1) were characterized and the results are summarized in Tables 1 and 2. As expected, dissolution of biomass increased as a function of CS, thus affecting the chemical composition of the residual solids. Xylan decreased from 25.5% to 9.6% and lignin was reduced from 23.3% to 5.9%. Conversely, the glucan content ranged from 48.8% to 73.0% and between CS -0.3 and 1.3 over 97% of glucan recovery in the residual solids was obtained. These results are consistent to those observed for other hardwood (Chum et al., 1990; Lai et al., 2014), and to those previously obtained at lower solids loading and particle size (Gandolfi et al., 2014), suggesting the scalability of the OS process. The use of high solids loading, as well as higher particle size, would decrease the capital costs of unit operations and water consumption, making the process more environmentally friendly and competitive. The change observed in the residual solids composition was reflected on that of the process

liquor, which increased in monomeric sugars (xylose and glucose) and lignin content, as a function of the OS process harshness (Table 2). The water-soluble fraction of the process liquor, was shown to be composed mainly of hemicellulosic and cellulosic sugars. Sugars-derived degradation products and organic acids were also detected, but in small quantities. Xylose was found to be the most abundant monomeric sugar. It should also be noted that between CS 0.5 and 1.7, xylose accounted for 98% of the hydrolyzed xylan, suggesting its almost complete conversion into monomeric form. This is of significance as monomers are readily fermentable, avoiding a subsequent hydrolysis step. On the other hand, in the CS range studied, cellulose was hardly hydrolyzed and low glucose concentrations were found in the process liquor. This was expected as acidic pretreatments typically result in a selective removal of the hemicellulosic component. Increasing the pretreatment harshness, generation of furans and acids, due to degradation of sugars, is often observed (Chen et al., 2007). Because of their well-known toxicity for fermenting microorganisms the presence of these compounds is undesirable. Concentration of those compounds is reported in Table 2, remarkably it was low between CS 0.5 and 1.3 and no detoxification step prior to fermentation was required.

Enzymatic hydrolysis
Effect of OS pretreatment
The enzymatic hydrolysis of OS pretreated hemp hurds was performed using the industrial cellulase blend CTec2. As given in Table 3, OS pretreatment increased enzymatic susceptibility of residual solids as a function of the CS. An almost eightfold increase in glucan to glucose conversion, compared to untreated hemp hurds, was achieved at CS 1.7. Although the highest glucan hydrolysis was observed for the CS 1.7, maximum overall glucose recovery (66.0%) was obtained from CS 1.3. This was due to the higher residual solids recovery obtained (Table 1). Interestingly, as seen in Fig. 2, enzymatic hydrolysis showed a linear increase when AIL was reduced from 25 to 13%, while a further delignification did not improve hydrolysis. Even though it is well known that lignin impedes enzyme access to glucan chains by unproductive binding and steric hindrance (Chang and Holtzapple, 2000), according to our results, only 50–60% of lignin should be removed from hemp hurds to obtain high levels of hydrolysis by CTec2. Although other authors stated the need of higher delignification degrees, to achieve satisfactory enzymatic hydrolysis levels (Choi et al., 2013; Siqueira et al., 2013), the extent of delignification required seems to be dependent on biomass source.

Effect of enzyme and solids loadings
Cellulolytic enzymes contribute significantly to the total cost of biorefinery and their dosage should be optimized as much as possible. Therefore, the effect of enzyme loading (10– 40 FPU/gglucan) and solid loading (2.5–13% w/v) on glucan to glucose conversion, of residual solids from CS 1.3, was investigated and results are shown in Fig. 3. As shown, hydrolysis increased by 40% raising loading from 10 to 20 FPU/gglucan, while a further doubling of enzyme activity provided only an additional 2% of hydrolysis (Fig 3a). The effect of solid loading is shown in Fig 3b. Between 2.5 and 10% of loading, hydrolysis yield was stable around 65%, while a sharp decrease to 55% was observed when loading was increased to 13%. Since there is a clear positive relationship between biomass loading and glucose yield, as well as a negative relationship between biomass loading and glucan hydrolysis, a compromise was found at 10% (w/v) of solid loading. Under optimized hydrolysis conditions (i.e., 20 FPU/gbiomass and 10% of solid loading), glucose concentration was 31.4 g/L.

Lactic acid fermentation
OS pretreatment performed at CS 1.3 followed by enzymatic hydrolysis, was found the most appropriate for high C5 and C6 sugar recoveries, 0.17 and 0.29 g/g (raw material), respectively. These conditions were thus selected to obtain sugar-rich streams for LA fermentation. The compositions of concentrated cellulosic and hemicellulosic hydrolysate (expressed in g/L) were as follows: xylose 417.4, glucose 54.1, furfural 3.8 and HMF 0.48 for C5-fraction or glucose 355.6 and xylose 27.3 for C6-fraction. The reducing sugars concentrations, as determined by BCA method, were 486.4 ± 9.7 g/L for C5 and 384.7 ± 8.1 g/L for C6. Due to its homolactic metabolism, ability to grown on pentose sugars and low nutritional requirement, B. coagulans has become an attractive organism for the industrial production of enantiomerically pure LA

from lignocellulosic material (Abdel-Rahman et al., 2013; De Clerck et al., 2004; Su and Xu, 2014). The capacity of B. coagulans strains XZL4 and DSM1, to produce LA from hemp hurds-derived carbohydrates was therefore evaluated. Strain XZL4, was the most efficient in converting glucose and xylose to LA. On the other hand, strain DSM1 showed growth only on glucose as a substrate. This feature can be genetically explained by the presence of an incomplete xyl operon in the genome of strain DSM1, while the full form is present in strain XZL4 (Su and Xu, 2014).

Effect of temperature, pH and initial sugar concentration on LA production

Optimum fermentation conditions (e.g., pH, temperature and initial sugars concentration) were studied by batch fermentations of authentic sugars samples. Effects of temperature and pH on sugar conversion to LA are shown in Fig. 4. The two strains had the following optima: 50 C and pH 5.5 for XZL4, 40 C and pH 6.5 for DSM1. The thermophilic character of the selected strains enables non-sterile processes to be carried out, thus all fermentations in the present study were run openly, and no media sterilization was used. To evaluate the effect of initial sugar concentration, fermentations were carried out using different concentrations of sugars (Table 4). When glucose concentration was below 150 g/L, LA yields were over 0.93 g/g for strain XZL4 and over 0.91 g/g for strain DSM1. When strain XZL4 was cultured in a medium containing 120 g/L of xylose LA yield was over 0.92 g/g. Mixtures of monomeric carbohydrates are usually obtained from lignocellulosic pretreatment processes therefore; use of Carbon Catabolite Repression (CCR)-positive strain is highly desirable in biorefinery. As shown in Fig. 5, B. coagulans XZL4 was able to utilize glucose and xylose simultaneously, although glucose was utilized faster than xylose.

OS sugars fermentation

Batch fermentations of OS sugars stream (C5 and C6 fractions) were accomplished in 150 mL of fresh medium containing yeast extract, OS sugars and CaCO3. The fermentation profile of OS-derived sugar fractions are shown in Fig. 6, and results were compared in terms of LA volumetric productivity, LA yield (based on consumed sugar) and L-LA e.e. When strain DSM1 was cultured in a medium containing 120 g/L of C6-glucose LA productivity was 1.19 g/L h and yield 0.99 g/g (Fig. 6a). Similarly, when strain XZL4 was cultivated on 150 g/L of C6-glucose LA productivity was 1.79 g/L h and yield 0.99 g/g (Fig. 6b). These values are in the range of LA productivity reported for B. coagulans 36D1 (2.5 g/L h) fermenting glucose (Ou et al., 2011). Moreover, strain XZL4 on 120 g/L of C5-xylose gave a LA productivity of 1.08 g/L h, and yield 0.95 g/g (Fig. 6c). The latter value is close to the theoretical conversion (1.0 g/g) if xylose is used trough the Pentose Phosphate Pathway (PPP), instead of the less efficient phosphoketolase pathway (PKP), which can generate less than 0.6 g/g (Patel et al., 2006). For strain XZL4, the LA productivity using biomass-derived xylose, was comparable to the value reported by Ouyang et al. (2012) for B. coagulans NL01 (1.04 g/L h). Interestingly, the presence of low concentrations of furanic compounds in C5-fraction seems not to affect the fermentation performance of B. coagulans XZL4. It is well known that the optical purity of LA affects its applicability. Particularly, if it has to be used for the synthesis of PLA, e.e. >98% are required (Ye et al., 2013). The optical purity of the produced LA was determined by chiral HPLC. Remarkably, strain XZL4 gave e.e.P99.3% for the L-LA using both C5 and C6 sugar fractions. Conversely, strain DSM1 showed lower e.e. and values around 96% for the L-form were achieved for fermentation of C6 fraction.

Conclusions

In the present study L-LA production was used as an example for bulk chemical production from hemp hurds, a widely available and cheap by-product from fiber production. OS pretreatment and enzymatic hydrolysis were optimized to maximize the recovery of fermentable sugars. High LA yield, titer and e.e. were achieved by non-sterile fermentations of C5 and C6 sugar streams. The excellent performances of B. coagulans XZL4 makes it a promising strain for industrial L-LA production from lignocellulose.

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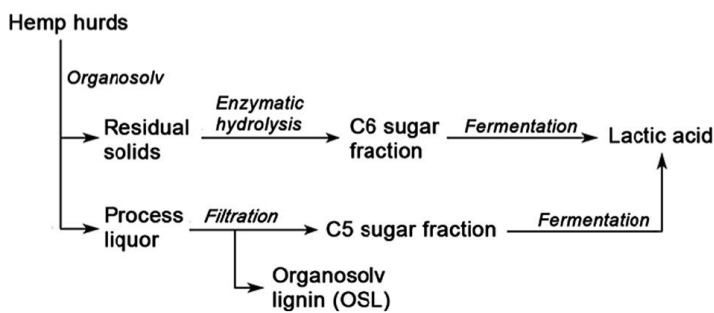Figure. 1. Schematic overview of the experimental methodology.

Figure. 2. Enzymatic hydrolysis of hemp hurds. Effect of AIL content in the solids residue on the enzymatic glucan hydrolysis. Standard deviation of three replicates was below 1%.
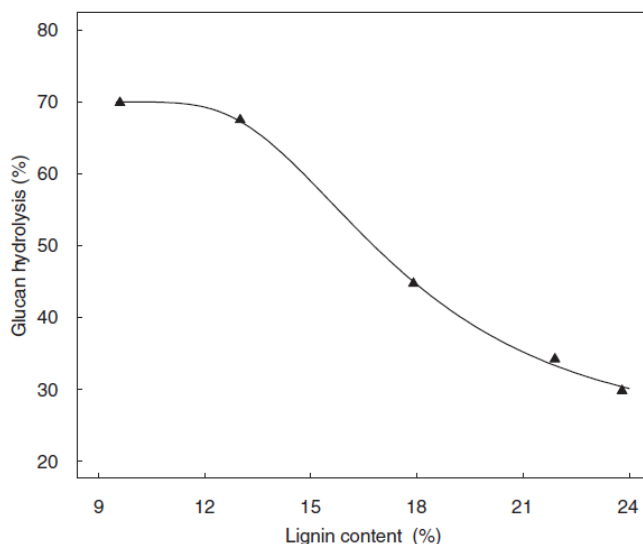

Figure. 3. Enzymatic hydrolysis of solid residues from OS pretreatment CS 1.3. (a) Effect of enzyme loading. (b) Effect of biomass loading. Black triangles glucan conversion, gray squares glucose concentration. Error bars indicate standard deviation of three replicates.
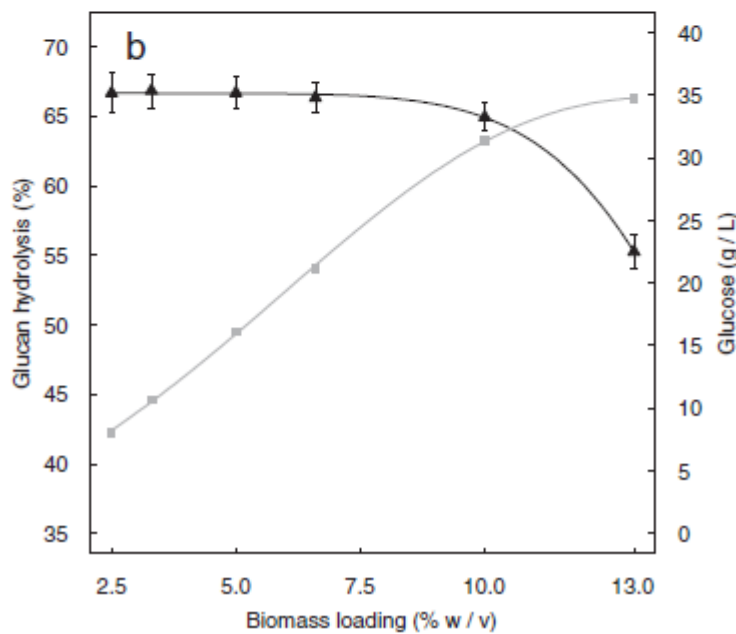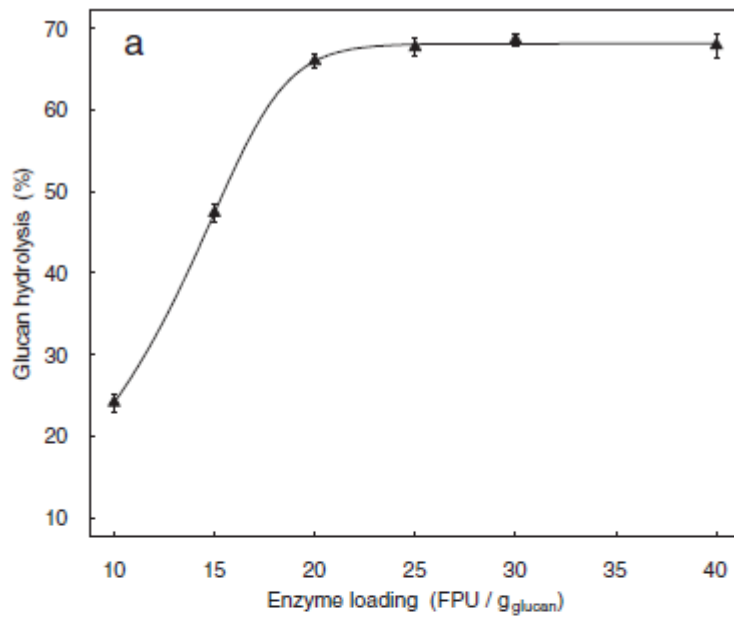
Figure. 4. Sugars conversion to LA. (a) Effect of temperature. (b) Effect of pH. Fermentations were carried out at 50 g/L of initial sugar concentration. Black symbols B. coagulans XZL4, gray symbols B. coagulans DSM1, circles glucose and triangles xylose. Error bars indicate standard deviation of three replicates.
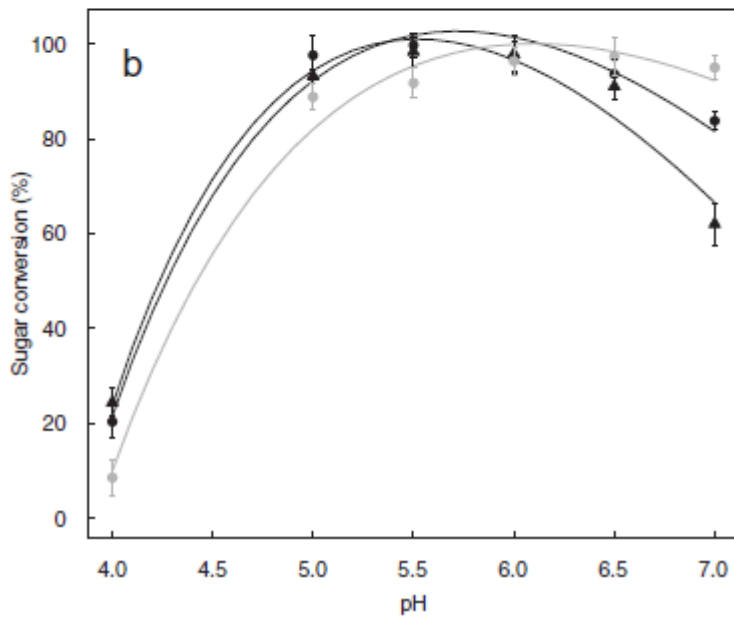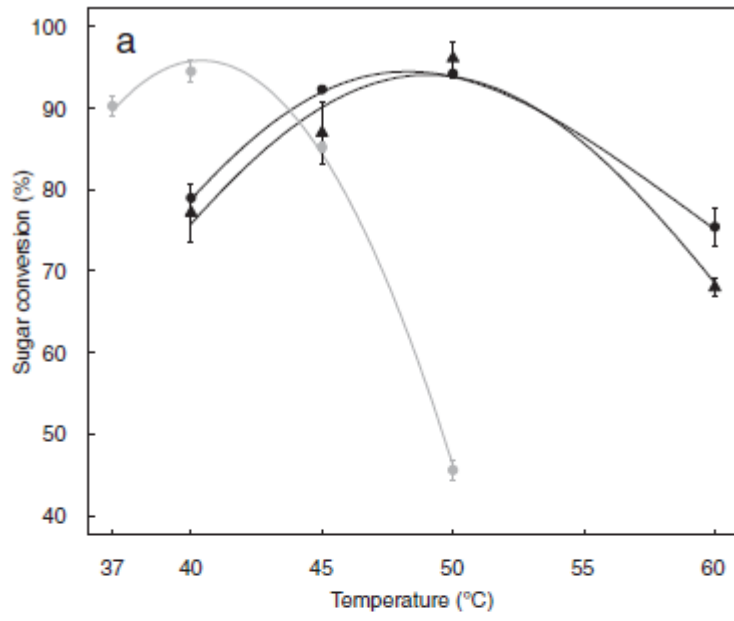
Figure. 5. Fermentation profile of B. coagulans XZL4. Circles LA, triangles xylose and squares glucose. Error bars indicate standard deviation of three replicates.
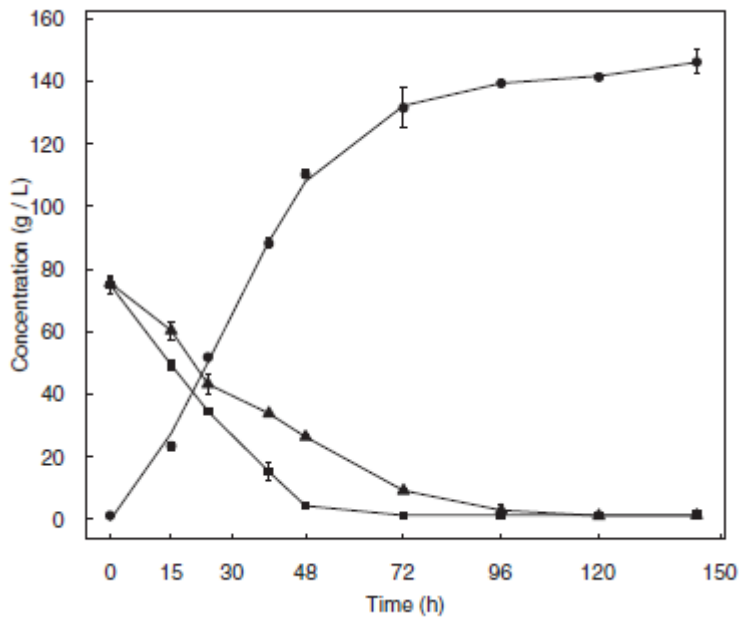

Figure. 6. Fermentation profiles of biomass-derived sugars (black) and authentic sugars substrates as control (gray). (a) B. coagulans DSM1, circles LA and triangles glucose. (b) B.coagulans XZL4, circles LA and triangles glucose. (c) B. coagulans strain XZL4, circles LA and triangles xylose. Error bars indicate standard deviation of three replicates.
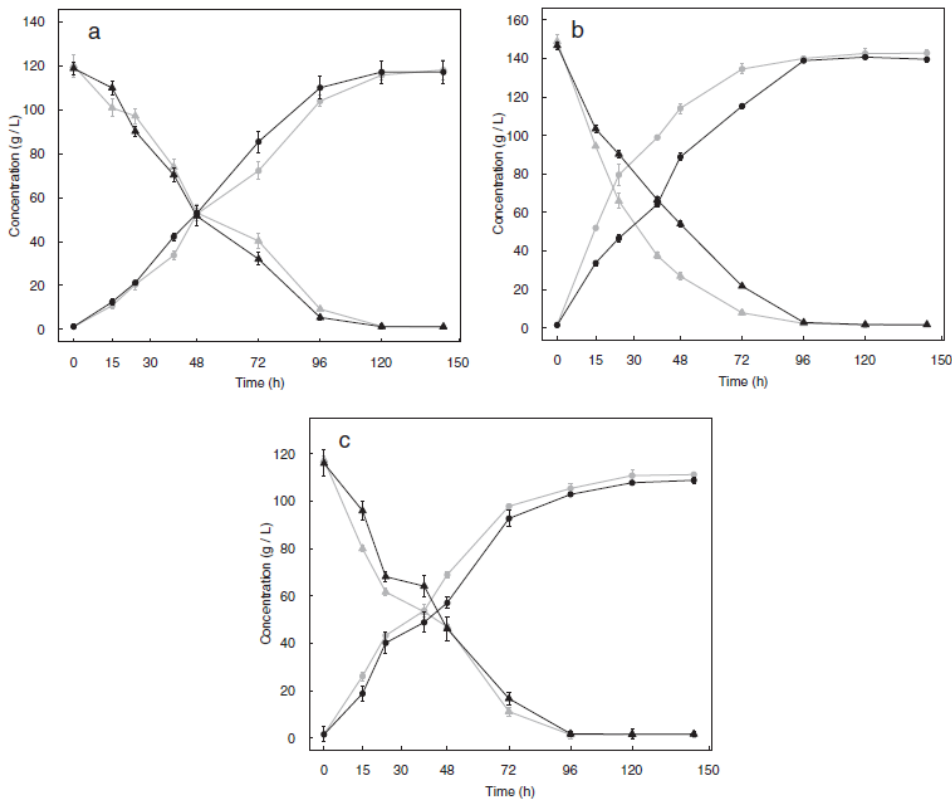
Table 1
Compositional analysis of untreated and pretreated hemp hurds.

H2SO4 (%)[a]	CS	Solids[b]	(%) Glucan	(%) Xylan	(%) AIL (%)	ASL (%)
un.[c]	n.a.[d]	n.a.	44.5	23.3	25.5	2.0
0	-1.0	90.6	48.8	21.2	23.8	1.8
1	-0.3	82.3	52.9	17.9	21.9	2.2
2	0.5	68.4	63.3	10.6	17.9	1.5
2.5	1.3	59.1	73.7	9.4	13.0	0.9
3	1.7	43.9	73.0	5.9	9.6	1.1

a Based on biomass dry weight.
b Residual solids recovery.
c Untreated hemp hurds.
d Not applicable.

Table 2
Compositional analysis of OS process liquor.

CS	OSL (g)	Glu[a] (g/L)	Xyl[b] (g/L)	HMF (g/L)	Fur[c] (g/L)	LvA[d] (g/L)	AcA[e] (g/L)
-1.0	0.14	0.07	1.05	0.003	0.001	n.d.[f]	0.009
-0.3	0.19	0.30	6.82	0.008	0.003	0.009	0.014
0.5	0.62	1.94	14.48	0.017	0.137	0.037	0.169
1.3	1.26	2.73	16.14	0.019	0.141	0.058	0.163
1.7	1.48	3.63	19.63	0.154	0.163	0.281	0.357

a Glucose.
b Xylose.
c Furfural.
d Levulinic acid.
e Acetic acid.
f Not detected.

Table 3
Enzymatic hydrolysis of OS residual solids.

CS	% of glucan hydrolyzed	% of glucose recovery
un.[a]	7.9 (0.2)	7.9
-1.0	29.8 (0.3)	29.6
-0.3	34.2 (0.2)	33.4
0.5	44.7 (0.4)	43.5
1.3	67.5 (2.8	66.0
1.7	69.9 (0.8)	50.3

Values in parenthesis represent the standard deviation of three replicates.
a Untreated hemp hurds.

Table 4

Initial sugar concentration for B. coagulans fermentation.

Sugar (g/L)	XZL4 (Glucose)		DSM1 (Glucose)		XZL4 (Xylose)	
	LA (g/L)	Yield(g/g)	LA (g/L)	Yield(g/g)	LA (g/L)	Yield(g/g)
50					49.7 (1.6)	0.98
80	78.4 (2.3)	0.98	77.6 (1.9)	0.97	78.4 (2.0)	0.98
100					96.3 (2.3)	0.96
120	117.6 (1.7)	0.98	116.4 (1.2)	0.97	110.4 (1.5)	0.92
150	139.5 (3.3)	0.93	136.5 (2.3)	0.91	126.1 (3.7)	0.84
180	153.3 (2.4)	0.85	151.2 (2.4)	0.84	77.4 (6.2)	0.43
200	144.1 (3.2)	0.72	124.7 (4.7)	0.62		
220	90.2 (7.2)	0.41				

Values in parenthesis represent the standard deviation of three replicates.