RESEARCH ARTICLE

Lactic acid production from lignocellulosic residues: A case study towards process integration

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Abstract: In this study, the production of lactic acid from municipal forestry and greening wastes processed by steam explosion (SE) and enzymatic hydrolysis was addressed. The liquid fraction (d-LFH) obtained after filtration of the steam-exploded slurry (previously subjected to detoxification) and the enzymatic hydrolysate resulting from the insoluble solid fraction (WDH) were studied as substrate for lactic acid fermentation using an integrated strategy. The fermentation process of WDH using *Bacillus coagulans* and *Lactobacillus rhamnosus* showed similar sugar conversion yields of about 0.9 g g^{-1} (based on consumed glucose). In contrast, the use of d-LFH completely inhibited *B. coagulans*, making necessary a previous dilution with the WDH to trigger fermentation when using this microorganism. The best integrated strategy using both d-LFH and WDH streams and *B. coagulans* as fermentative strain was using a 1:3 d-LFH:WDH ratio, yielding 0.93 g lactic acid/g of consumed sugars and a lactic acid concentration of about 50 g L^{-1}.

Introduction

Fossil-based resources have been playing a major part in modern societies, shaping the economy around their use. Petroleum (5.1 billion tons), natural gas (3 billion tons) and coal (7.6 billion tons) are used worldwide as raw materials for an ample variety of fuels, products, commodities and chemicals [1]. The production of fossil-derived chemicals and products, most notably plastics, make up for an estimated 7-10% of the total usage, while being responsible to consume 30% of the global energy and to produce 20% of the greenhouse gas emissions within this sector [2]. The non-renewable nature of these resources and the serious environmental effects caused by their extensive use and exploitation make imperative the search for more sustainable and green alternatives. Biomass has been recognized as the most suitable option to replace fossil resources. This raw material can be transformed into a wide array of bio-based fuels and products in the so-called biorefinery facilities [3,4]. Lactic acid (LA) is one of the industrially relevant bioproducts that can be obtained from biomass. This compound is a precursor of polylactic acid (PLA), which is an important component in bioplastic production with multiple applications in the food and pharmaceutical sectors [5].

LA is a chiral compound, presenting two different enantiomers in the form of D-(-)-LA and L-(+)-LA. Its conversion to PLA requires enantiomeric purity to enhance the desired properties of the biopolymer. LA can be produced either chemically or biochemically. However, biochemical production methods are preferred since chemical LA production results in racemic mixtures [6]. Biochemical production of LA is performed via sugar fermentation using lactic acid bacteria (LAB). The common sugar sources for LA production are mainly starch- and sucrose-based biomass feedstocks (e.g., corn, grain, sugarcane), which are primarily meant for food and feed purposes. As an alternative, lignocellulosic biomass represents an inexpensive and abundant raw material with huge potential for developing sustainable bio-based processes [7].

Lignocellulosic biomass has multiple origins, including agricultural and forestry residues, industrial wastes, and the organic fraction of municipal solid waste. Within an urban biorefinery context, the municipal forestry and greening waste (MFGW) is an abundant residue –especially in large cities– that requires convenient management strategies to reduce waste production. MFGW is generated continuously and localised, thus contributing to simplify its collection and transportation. In addition, the high sugar content of MFGW makes this residue an attractive feedstock for certain bioprocessing approaches [8,9]. The use of MFGW as feedstock to produce valuable fuels and products has been previously researched to obtain biofuels [10], biomethane [11], bioethanol [9] or the aforementioned LA [12] by applying different biomass conversion technologies.

For an effective biotechnological conversion of MFGW, this substrate must be first subjected to pretreatment in order to fractionate biomass into its three main components: cellulose, hemicellulose and lignin. Steam explosion (SE), a hydrothermal technique that alters biomass by combining chemical, thermal and physical effects, is noted as one of the most efficient and most widely used methods for biomass fractionation [13,14]. In a typical SE process, hemicellulose is solubilized in the liquid fraction (LFH) or prehydrolysate, while the water-insoluble solid fraction

(WIS) is formed by the remaining cellulose and lignin. Both fractions can be easily separated by vacuum filtration of the whole slurry obtained after SE pretreatment. Enzymatic digestibility of the WIS fraction is drastically improved after SE, as this technology opens up the structure enhancing the accessibility of enzymes to cellulose polymer [15]. Negro et al. [9] studied SE to pretreat MFGW biomass under several process conditions and using different catalysts. In that study, acid-catalyzed SE pretreatment (using H_2SO_4 as catalyst) showed the best performance in terms of maximizing cellulose recovery and reaching high saccharification yields (up to 70% of the total initial sugars were recovered after pretreatment and hydrolysis of MFGW). Similarly, Bondesson et al. [16] also observed that the addition of an acid catalyst can increase the effectiveness of SE pretreatment of corn stover by mainly enhancing the recovery of hemicellulosic sugars in the liquid fraction, as well as improving the enzymatic hydrolysis (EH) yields of the pretreated solids. However, the acid-catalyzed SE pretreatment usually generates higher amounts of biomass-related degradation compounds that are inhibitors of the subsequent hydrolysis and fermentation steps. Major inhibitory products include 5-hydroxymethylfurfural (5-HMF) and furfural from hexose and pentose sugars degradation, respectively; acetic acid from the autohydrolysis process of hemicellulose; and a wide range of phenolic compounds derived from partial lignin solubilization [17]. These compounds can inhibit the microbial growth of LAB at considerably low concentrations, negatively influencing the sugar conversion by these microorganisms [18,19]. Different detoxification processes have been tested to overcome and/or reduced such inhibitory effects during LA production, underlying the importance of adding this process step to trigger sugar fermentation [20–22].

Another important aspect towards cost-effectiveness in biorefining processes is maximizing biomass-to-product conversion yields. Glucose and xylose are the most abundant sugars in MFGW. They represent over 90% of the total sugars in this raw material, and therefore, efficient conversion of these two components is of utmost importance during bioprocessing. In this work, glucose and xylose fermentation into LA was studied by integrating the resulting SE-pretreated MFGW streams: the detoxified LFH and the WIS-derived hydrolysate (WDH), the latter obtained after enzymatic hydrolysis of the resulting pretreated WIS fraction. The bacteria *Bacillus coagulans* was chosen as the homofermentative LA producer due to its ability to convert both glucose and xylose into LA with an optical purity of L-(+)-LA (the preferred isomer for PLA production) above 99.5% [23]. This bacteria is also capable of converting and tolerating to some extend certain biomass inhibitory compounds, providing an extra *in situ* biodetoxification of the pretreated lignocellulosic hydrolysates and thus contributing to ease the fermentation of these complex media [24]. In addition, the non-xylose-fermenting bacteria *Lactobacillus rhamnosus* (another homofermentative strain for L-LA production) was used for comparison purposes to assess major benefits/drawbacks of the proposed integration strategy (*e.g.*, higher/lower product titers/yields, tolerance to inhibitors). During fermentation assays, product titers, yields and productivities, as well as sugar consumption rates and cell biomass concentration were monitored to evaluate process performance. Overall, the results presented herein, targeting at integrating the pretreated solids and liquid fractions during LA fermentation, will contribute to develop effective process strategies to convert MFGW biomass within a sustainable urban biorefinery perspective.

Results and Discussion

SE Preteatment of MFGW biomass

SE pretreatment (195ºC, 10 min and 60 mg H_2SO_4) of MFGW resulted in a slurry with 78.2% moisture and 21.8% total solids (14.0% of insoluble solids and 7.8% of soluble compounds), with a total solid recovery yield of about 96% from the initial biomass inlet. The slurry was then subjected to filtration to separate the WIS and LFH fractions. The WIS fraction showed the following composition in % (dry weight): cellulose, 41.4; hemicellulose, 2.7 (xylan, 1.9; arabinan, 0.5; galactan, 0.2; mannan, 0.1); lignin 43.8; and ash 3.1. When compared to the raw biomass, cellulose and lignin significantly increased from 35.4% and 25.4% to 41.8% and 43.8%, respectively, mainly due to the solubilization of hemicellulosic sugars (hemicellulose content decreased from 14% to 2.7%) and other biomass components such as the water soluble fraction of ash and extractives [25]. This is a well-known effect when subjecting lignocellulosic biomass to SE pretreatment [14,26] and contributes to improve the accessibility of enzymes to the corresponding carbohydrates during the EH step. In contrast, the harsh conditions of SE pretreatment also generates several biomass degradation compounds that can inhibit the saccharification and fermentation steps, including furfural, 5-HMF, low molecular weight organic acids and phenolic compounds [27]. Table 1 shows the composition of LFH, which mainly contains the hemicellulosic sugars and degradation compounds solubilized and/or generated during SE pretreatment. It is noteworthy to highlight that 90% of the sugars quantified in the LFH are in monomeric form (data not shown).

Table 1: Composition of LFH obtained after steam explosion pretreatment

Sugars		**Degradation products (g/L)**		**Phenolic compounds (g/L)**	
	Monomers (g/L)				
Glucose	16.2 ± 2.0	Formic Acid	2.5 ± 0. 1	Vanillin	0.61 ± 0.002
Xylose	17.5 ± 0.8	Acetic Acid	8.3 ± 1.7	Syringaldehyde	0.09 ± 0.04
Galactose	5. 9 ± 0.2	Furfural	2.9 ± 1.2	OH Benzoic acid	0.09 ± 0.04
Arabinose	3.4 ± 0.1	HMF	1.7 ± 0.3		
Mannose	2.5 ± 0.1				

Regarding inhibitory compounds, in addition to furfural, 5-HMF and organic acids, certain phenolic compounds including vanillin, syringaldehyde and 4-hydroxybenzaoic acid were also quantified at minor concentrations (10-60 mg L-1). It has been reported that inhibitory compounds directly affect LA production process [24,28]. In this context, the LFH was detoxified by using an ionic exchange resin to remove most of the inhibitory compounds prior to the fermentation process. The mass balance related to the pretreatment process of MFGW is shown in Figure 1. This figure underlines a good pretreatment performance of MFGW by SE, showing recovery yields of 91% and 75% for glucose and xylose, respectively, considering both the WIS and LFH. Overall, 69% of glucose and 67% of xylose of raw MFGW would be available for lactic acid production in the pretreated substrate. It is also interesting to note an increase in the acid insoluble (AI) lignin content from 25.5 g 100 g^{-1} of raw biomass to 28.2 g 100 g^{-1} of raw biomass after SE pretreatment (Figure 1). During SE, key intermediates derived from furfural and 5-HMF may also polymerize/condense with lignin leading to the formation of the so-called acid insoluble pseudo-lignin [29,30], which may be quantified as AI lignin in the composition analysis. This can result in an increase of this biomass component after the pretreatment process, as it is found in this study.

From about 55 g of potential sugars contained in raw MFGW, a total of 17 g were recovered in the LHF and 21.6 were obtained after EH of the WIS fraction. These figures correspond to a high overall sugar production yield of about 70% from the potential initial sugars in MFGW. The solid residue after EH contains about 8 g of carbohydrates (mainly cellulose), thus highlighting the presence of parts in the cellulose polymer that are more recalcitrant to fractionation and EH. This finding has been previously observed by Alcántara et al. [31] after pretreatment of corn stover and sugarcane bagasse with sulfuric-acid-catalyzed SE. These authors attributed the difficulty to reaching maximum sugar yield after EH due to the presence of a carbohydrate fraction still being recalcitrant to hydrolysis, even though when using a very high enzyme dose. The conditions used in this study for SE pretreatment were previously optimized by Negro et al. [9]. In that study, different conditions were tested according to the following aspects: *i*) SE pretreatment under different temperatures (185, 205, and 220 °C); *ii*) combining SE with the addition of $FeCl_3$ (0.1 M, 90 mg/g MFGW biomass); *iii*) combining SE with organosolv (ethanol 50% v/v); and *iv*) combining SE with dilute acid impregnation (60 mg/g MFGW biomass). All these pretreatment processes were evaluated according to sugars recovery, generation of degradation product, and enzymatic hydrolysis yield. The best pretreatment performance resulted with the acid-catalyzed (60 mg H_2SO_4/g MFGW) SE at 195 °C for 10 min, which allowed to recover up to 77% of the sugars contained in MFGW biomass.

Lactic acid fermentation in synthetic defined media

L. rhamnosus DSM 20711 and *B. coagulans* DSM 2314 were first grown in synthetic MRS media supplemented with glucose and/or a glucose/xylose mixture in order to assess their fermentation performance in the absence of inhibitory compounds. Figure 2 shows the fermentation profiles of *L. rhamnosus* and *B. coagulans* in glucose-containing media. Regardless of the strain, similar fermentation profiles were observed in the presence of glucose, reaching complete sugar depletion within 24 h. In total, 13.1 ± 0.3 g of lactic acid were produced from MRS glucose media (containing 13.9 ± 0.4 g of glucose with an initial concentration of 50-55 g L^{-1}), thus showing yields of about 0.95 g g^{-1} for these microorganisms. Although similar yields were observed, *L. rhamnosus* showed slightly higher glucose consumption rates when compared to *B. coagulans* (Figure 2), which can be attributed to a higher cell biomass formation during fermentation with *L. rhamnosus* (2.4 ± 0.1 g vs 0.8 ± 0.1 g). In this context, maximum growth rates (μ_{max}) for *L. rhamnosus* and *B. coagulans* were 0.232 and 0.098 h^{-1}, respectively.

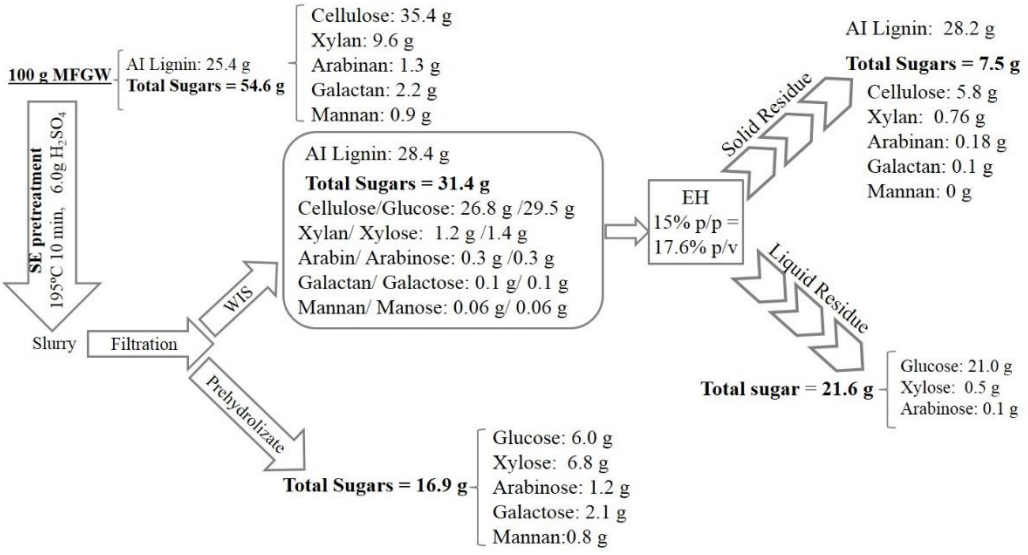

Figure 1. Mass balance resulting after steam explosion (SE) pretreatment and enzymatic hydrolysis (EH) of municipal forestry and greening waste (MFGW). AI Lignin: acid insoluble lignin; WIS: water insoluble solids.

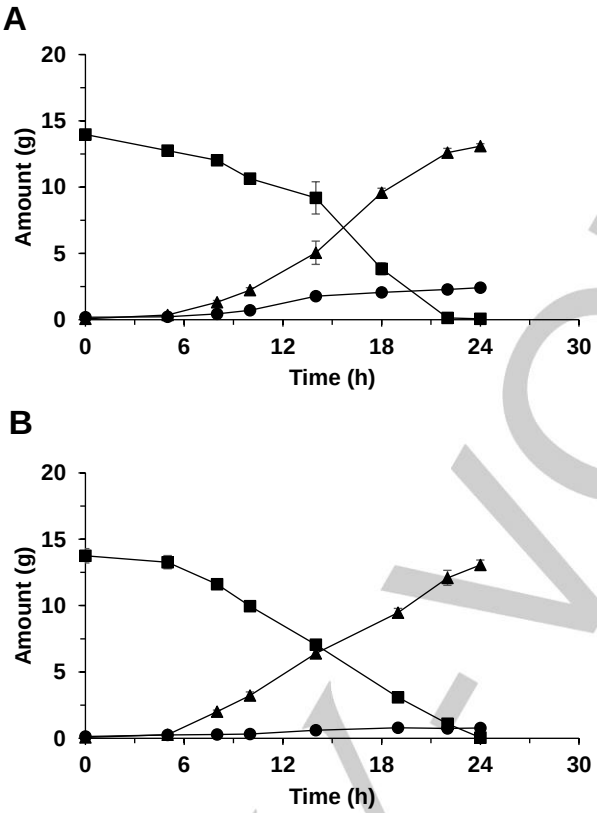

Figure 2. Time-course fermentation for lactic acid production using (A) *L. rhamnosus* and (B) *B. coagulans* in MRS media supplemented with glucose. Glucose (square), lactic acid (triangle), biomass (circle).

Despite the lower growth rates and the differences in cell dry weight (CDW), higher specific productivity values of lactic acid were observed for *B. coagulans*. A specific productivity of 0.71 g_{LA} $(g_{CDW}\ h)^{-1}$ was estimated for *B. coagulans* after 24 h, compared to 0.23 g_{LA} $(g_{CDW}\ h)^{-1}$ of *L. rhamnosus*. Furthermore, maximum specific productivity values of about 1.90 g_{LA} $(g_{CDW}\ h)^{-1}$ and 0.70 g_{LA} $(g_{CDW}\ h)^{-1}$ were estimated respectively for these microorganisms between 5-10 h. This result clearly offers an advantage of *B. coagulans* against *L. rhamnosus* for the production of lactic acid. Considering the presence of xylose in the LFH (18 g L^{-1}), another advantage of *B. coagulans* in biorefining processes is its ability for converting xylose into lactic acid. In the absence of inhibitors, the fermentation with *B. coagulans* on a synthetic medium with a glucose/xylose concentration similar to that of the LFH (i.e., about 20 g L^{-1} each) showed complete depletion of both glucose and xylose within 43 h (Figure 3). As a control test, fermentation assays of the same glucose/xylose media was performed using *L. rhamnosus*, confirming the inability of this microorganism to assimilate and convert xylose (Supplementary Figure S1).

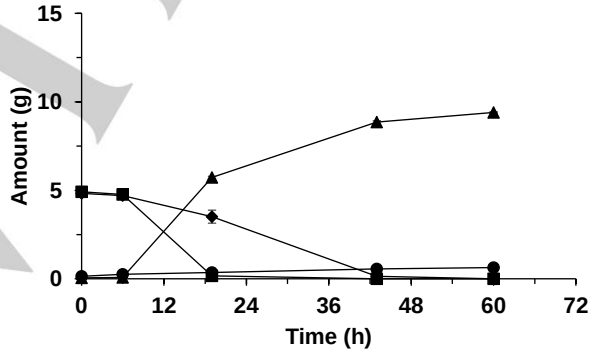

Figure 3. Time-course fermentation for lactic acid production using *B. coagulans* in MRS media supplemented with a glucose/xylose mixture. Glucose (square), xylose (diamonds), lactic acid (triangle), biomass (circle).

Fermentation of *B. coagulans* in a glucose/xylose mixture (containing 4.9 ± 0.1 g of glucose and xylose, respectively) resulted in a total production of lactic acid of 9.4 ± 0.1 g. These values correspond to a sugar conversion yield of 0.96 ± 0.02 g g^{-1}, similar to that obtained in media with glucose only. In contrast, lower sugar consumption rates were observed for this media, with growth rates of 0.021 h^{-1} (6-43 h) and yielding 0.7 ± 0.0 g of cell biomass. This also resulted in a lower specific productivity 0.25 g_{LA} (g_{CDW} h)$^{-1}$ within 60 h, with a maximum value of 1.2 g_{LA} (g_{CDW} h)$^{-1}$ at the fermentation range of 6-19 h.

Production of lactic acid from WIS-derived hydrolysate and detoxified liquid fraction

After evaluating the fermentation performance of *L. rhamnosus* and *B. coagulans* in the absence of inhibitory compounds, these microbial strains were used to evaluate lactic acid production from the WDH and the detoxified LFH (d-LFH).

In contrast to d-LFH, the WIS fraction resulting after pretreatment must be first hydrolysed to obtain the monomeric sugars from the corresponding polymers, being cellulose the carbohydrate with a higher content (41.4% w/w of the WDH). EH of WIS fraction was performed at 15% (w/w) substrate concentration for 72 h, resulting in a liquid fraction with a glucose concentration of 64.0 ± 2.2 g L^{-1}. This value represents an overall EH yield of 70% (after subtracting glucose from enzyme preparation). Similar EH yields have been previously reported by Negro et al. [9], who studied the effects of catalyst addition, temperature, and residence time of SE pretreatment in MFGW towards improving overall sugar recovery yield. In the present work, after EH of MFGW, a solid residue with the following composition in dry weight: 76% lignin, 16% glucan and 3% of other carbohydrates was yielded. Within a biorefinery approach, this solid residue could be also valorized into alternative bioproducts such as lubricants [32], thus contributing to the overall process economy and the zero-waste target of our current society.

Figure 4 shows the fermentation performance of both *L. rhamnosus* and *B. coagulans* in WDH media. Different fermentation profiles could be observed for these microorganisms. Complete glucose depletion was observed during fermentation of WDH with *L. rhamnosus* within 32 h, while 15% of the initial glucose content remained after 48 h of fermentation with *B. coagulans*. In total, *L. rhamnosus* produced 13.9 ± 0.1 g of lactic acid, while fermentation of WDH with *B. coagulans* resulted in 12.6 ± 0.1 g of lactic acid. These values correspond to sugar conversion yields of 0.86 ± 0.01 and 0.92 ± 0.03 g g^{-1} (based on consumed glucose), respectively. In addition, a total cell biomass of 3.1 ± 0.2 and 0.9 ± 0.1 g (in dry weight) were also obtained for *L. rhamnosus* and *B. coagulans*, respectively.

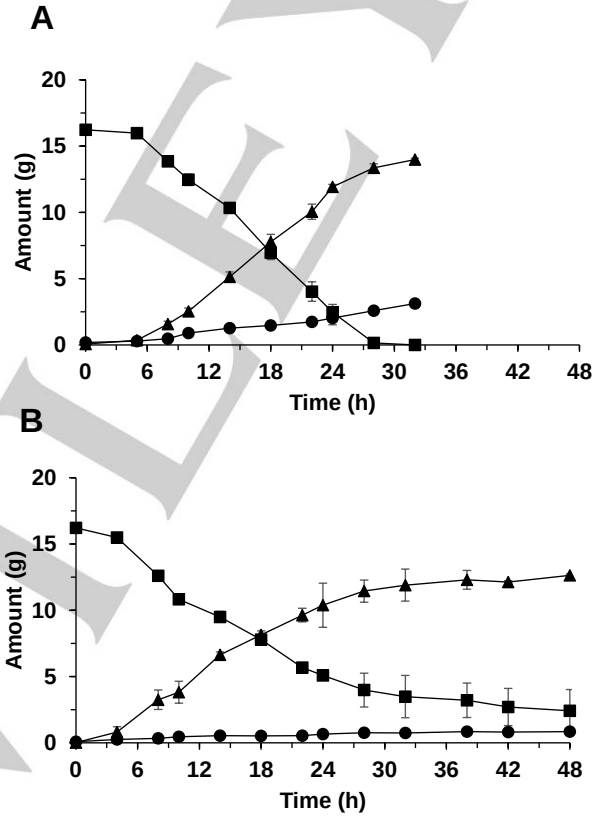

Figure 4. Time-course fermentation for lactic acid production using (A) *L. rhamnosus* and (B) *B. coagulans* in WDH media. Glucose (square), lactic acid (triangle), biomass (circle).

Even though *B. coagulans* showed an incomplete fermentation profile, it is interesting to note that this microbial strain exhibited a higher conversion yield and a higher specific productivity rate when compared to *L. rhamnosus* (Table 2). At the end of the fermentation, a specific productivity rate of 0.31 g_{LA} (g_{CDW} h)$^{-1}$ was observed for *B. coagulans* (at 48 h), while *L. rhamnosus* showed 2.2 times lower values for this kinetic parameter 0.14 g_{LA} (g_{CDW} h)$^{-1}$ within 32 h. Furthermore, after 32 h of fermentation, when *L. rhamnosus* showed complete glucose depletion and fermentation with *B. coagulans* started to slow down, the specific productivity of the latter was 3.5 times higher 0.49 g_{LA} (g_{CDW} ·h)$^{-1}$. Nonetheless, fermentation of WDH showed, in general, lower yields and specific productivity values to those obtained in absence of inhibitory products (MRS media) (Table 2).

In addition of using the WDH, the integration of LFH in the conversion process would benefit its overall performance by potentially converting all the sugar components contained in the raw material. LFH is mainly composed of the solubilized hemicellulosic sugars (e.g., xylose) and the glucose released from the partial cellulose solubilization. Usually, xylose is the sugar with the highest concentration in the LFH. The use of xylose-fermenting microbial strains such as *B. coagulans* would be therefore of interest to potentially increase the production of lactic acid using this pretreated fraction. The use of the LFH is however very challenging due to the presence of several biomass degradation compounds generated during pretreatment.

Fermentation of the LFH obtained after steam explosion pretreatment of MFGW completely inhibited the growth of *B. coagulans*. Major inhibitory compounds contained in the LFH were 5-HMF, furfural, formic acid, and acetic acid (Table 1). In order to overcome cell inhibition and trigger the conversion of sugars into lactic acid, the LFH was detoxified through a resin column, yielding 73.5% of the initial liquid with the same sugar concentration as the non-detoxified LFH. Detoxification completely removed formic acid and acetic acid, as well as traces of vanillin, syringaldehyde, and benzoic acid. The concentration of 5-HMF and furfural were also reduced by 65% and 73%, showing final concentrations of 0.6 g L^{-1} and 0.8 g L^{-1}, respectively. Nevertheless, fermentation by *B. coagulans* of the resulting d-LFH lead to no sugar consumption nor lactic acid production, highlighting the necessity of searching an alternative fermentation strategy to valorize this sugar-rich stream. As a control assay to evaluate the inhibitory potential of the d-LFH, this fraction was also subjected to fermentation with *L. rhamnosus*, showing complete glucose depletion (but no xylose consumption) within 30 h (Supplementary S2). This result underlines the need of increasing cell robustness on *B. coagulans*, in order to be capable of tolerating higher concentrations of lignocellulosic-derived inhibitors.

Integration strategy for lactic acid production from MFGW

Due to the presence of several inhibitory compounds, the LFH obtained after pretreatment of lignocellulosic biomass requires detoxification and/or dilution to trigger fermentation [33]. With the aim of lowering the inhibitory potential of the d-LFH obtained in the present study, different dilution approaches with the WDH were tested to allow a better process integration of the pretreated fractions. Fermentation of d-LFH/WDH mixtures with different ratios were tested as following (d-LFH:WDH): 3:1, 1:1 and 1:3. Similar to non-diluted LFH, dilutions at 3:1 and 1:1 d-LFH:WDH rates were completely inhibitory for *B. coagulans*, showing no sugar consumption or lactic acid production. In contrast, as depicted in Figure 5, dilution at 1:3 d-LFH:WDH ratio allowed the cells to overcome inhibition and triggered fermentation. This integration strategy resulted in a production of 12.6 ± 1.1 g of lactic acid with 0.93 ± 0.04 g g^{-1} sugars conversion yields (based on consumed sugars).

Table 2. Fermentation kinetic parameters during lactic acid production in synthetic MRS media, WDH and d-LFH

Microorganism	Medium	Yield (g g^{-1}) [a]	μ_{max} (h^{-1})	Specific Productivity g_{LA} (g_{CDW} h)$^{-1}$ [b]
L. rhamnosus	MRS glucose	0.94 ± 0.01	0.232	0.23 (24 h)
	WDH	0.86 ± 0.01	0.147	0.14 (32 h)
B. coagulans	MRS glucose	0.95 ± 0.02	0.098	0.71 (24 h)
	MRS glucose/xylose	0.96 ± 0.02	0.021	0.25 (60 h)
	WDH	0.92 ± 0.03	0.079	0.31 (48 h)
	d-LFH	-	-	-
	1:3 LFH:WDH	0.94 ± 0.01	0.059	0.29 (30 h)

MRS, synthetic medium; WDH, WIS-derived hydrolysate; d-LFH, detoxified liquid fraction hydrolysate

[a]Yields have been estimated considering the maximum concentration of lactic acid observed and the consumed sugars (i.e., glucose when using *L. rhamnosus* and glucose and xylose when using *B. coagulans*)

[b]The corresponding time points at which specific productivities were estimated are indicated in brackets

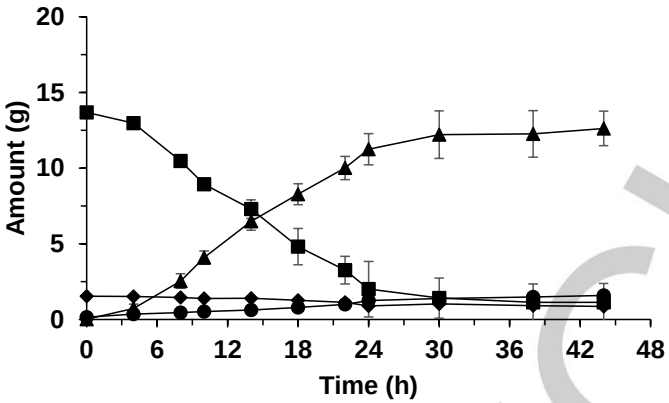

Figure 5. Time-course fermentation for lactic acid production using *B. coagulans* in 1:3 d-LFH:WDH media. Glucose (square), xylose (diamonds), lactic acid (triangle), biomass (circle).

Similar high conversion efficiencies (around 90%) of sugars to lactic acid were reported by van der Pol et al. [34] during the production of lactic acid from acid-catalyzed SE sugarcane bagasse via simultaneous saccharification and fermentation process using the *Bacillus coagulans* DSM 2314 strain. The authors tested the addition of sterile water or a simulated liquid fraction (with a similar composition to the real one obtained in the solid/liquid separation process after SE) to the pretreated solid, in order to obtain the fermentation media. Similar to the results obtained in the present work, the presence of inhibitory by-products in the liquid fraction influenced the fermentation performance of the corresponding *Bacillus* strain, extending the lag phase (40 *vs* 32 h) and a reducing conversion values (80% *vs* 92%) when compared to using the washed substrate.

Despite the high conversion yields observed in this work using the integrated strategy with WDH and d-LFH, *B. coagulans* was unable to assimilate all the sugars and about 10% and 50% of the initial glucose and xylose concentrations, respectively remained in the fermentation media after 44 h. This result is in agreement with the results obtained during the fermentation of WDH, where 15% of the initial glucose content remained in the media. A specific productivity value of 0.29 g_{LA} (g_{CDW} h)$^{-1}$ was observed after 30 h of fermentation, where the maximum lactic acid concentration was achieved (ca. 50 g L^{-1}). This specific productivity value is lower to the one obtained when using the WDH alone, being indicative of the high inhibitory potential of this media on *B. coagulans.*

The results presented in this study allowed the integration of the resulting cellulosic and hemicellulosic streams obtained after steam explosion pretreatment of MFGW for the production of lactic acid. When considering consumed sugars, the xylose-fermenting bacteria *B. coagulans* showed to be capable of producing lactic acid with yields above 90% of the theoretical. This high yield offers potential to increase the lactic acid that can be obtained from MFGW by 26% in comparison to the use of a non-xylose-fermenting microorganism, when considering both glucose and xylose content in the raw material (Figure 1). The selected pretreatment methods determines, among other variables, the concentration of the inhibitory products, enzymatic hydrolysis rates and yields, the enzyme dose required, and the final product concentration. In addition to the acid-catalyzed steam explosion pretreatment used in this work, other pretreatment technologies have been also tested for LA production with different impacts in the downstream steps. Hu et al. [35] tested the pretreatment of various agro stovers such as corn stover, corncob and wheat stover with 5% NaOH at 75 °C for 3 h and 20% (w/w) substrate loading to produce LA from the solid fraction separated from the slurry and using the *B. coagulans* LA204 strain. These authors highlighted the presence of inhibitors in the pretreated biomass as an important limiting factor that clearly affect LA production, leading to inhibition of sugar consumption and acetic acid accumulation in the fermentation media. In this context, a washing step of the solid fraction overcomes these effects leading to increased LA yield, product titer, and the average volumetric productivity during the conversion processes. Schroedter et al. [36] studied a novel LX-pretreatment process to promote rye straw dissolution using 75–80% phosphoric acid at 50-75 °C and atmospheric pressure at a ratio of about 1:3 (w/w) for 15–45 min. After biomass dissolution, carbohydrates were precipitated and subjected to fermentation with *B. coagulans* 14-300 strain for LA production. These authors observed high LA titers (39.3 g L^{-1} of L-LA after 48 h of fermentation process) with a high optical purity (99%). In the present work, the integrated strategy still represents challenging conditions for *B. coagulans*, which requires further optimization of this process. In addition to reduce the inhibitory concentration by better balancing pretreatment process, optimization of the detoxification step might be also necessary. Major detoxification methods are classified into adsorption (e.g., resins, activated carbon), chemical treatment (e.g., overliming, surfactant addition) and biological treatment (e.g., enzyme and microbial treatment) processes [37]. Adsorption detoxification methods have been considered effective methods to reduce the concentration of inhibitory compounds in general. Therefore, the Microionex MB200 resin was selected in this study as detoxification agent. Combination of different detoxification methods can be also an interesting strategy to overcome microbial inhibition. Combination of adsorption methods with *in situ* biological detoxification approaches would be preferred to avoid increasing operational costs during the subsequent scaling up. In this context, metabolic and/or evolutionary engineering strategies to genetically modify the fermentative microorganisms

are key to improve cell robustness. Tran and Zhao [38] have recently reviewed some of the successful strategies that have been applied to increase cell tolerance to low pH, high concentrations of organic acids and lignocellulosic inhibitors. The implementation of these strategies are considered crucial towards achieving an economic and efficient fermentation process. *B. coagulans* have proven to be capable of assimilating both glucose and xylose from MFGW with high conversion yields (above 90% of the theoretical), thus representing an attractive candidate for further improvements and efficiently utilize MFGW sugars within a biorefinery perspective.

The strategy followed in this works to integrate both the cellulosic and hemicellulosic streams obtained after preatreatment of MFGW in a single fermentation process is an innovative approach towards maximizing the utilization of the carbohydrates contained in lignocellulosic biomass. The results presented herein represent a step forward to process integration in the biological transformation of MFGW into industrially relevant bio-based products such as lactic acid and offer a more efficient utilization of carbohydrates by supporting very high conversion yields. Still, the fermentation step is highly challenged by the presence of biomass degradation compounds, therefore representing an important bottleneck that requires further attention.

Conclusion

The integration of the solid and liquid fractions resulting after SE pretreatment of MFGW may represent an interesting strategy towards maximizing LA concentrations and yields. The use of *B. coagulans* as fermentative strain for LA production exhibits some advantages in comparison to other non-xylose-fermenting microorganisms such as *L. rhamnosus*, based on its ability to assimilate both glucose and xylose with yields above 90% of the theoretical. However, further research is needed to overcome the lower tolerance of *B.coagulans* to the inhibitory compounds present in the corresponding LFH in order to fully exploit the potential of this bacteria.

Experimental Section

Raw material and pretreatment

MFGW - collected from the public gardens in the Community of Madrid was used as raw material in this study. MFGW was first chopped and shredded at the Migas-Calientes composting plant (Madrid, Spain), and then transported to CIEMAT facilities for further use. Chopped biomass was characterized according to the methodology described by Sluiter et al. [39], showing the following composition (% in dry weight basis): extractives, 8.3 ± 0.6; cellulose, 35.4 ± 1.4; hemicelluloses, 14.0 ± 0.1 (xylan, 9.6 ± 0.1; galactan, 2.2 ± 0.9; arabinan, 1.3 ± 0.1; mannan, 0.9 ± 0.1), acetyl groups, 4.7 ± 0.1; total lignin, 29.1 ± 1.3, and ash, 3.7 ± 0.1.

After characterization, MFGW was subjected to SE pretreatment under previously optimized conditions [7]. In brief, biomass was impregnated on a 1:1 (w/w) basis with 0.6 M H_2SO_4 during 18 h, resulting in a final proportion of 60 mg H_2SO_4 g dry $MFGW^{-1}$. Impregnated biomass was subjected to SE at 195 ºC for 10 min in a 2-L reactor. Pretreated solid and LFH fractions were then separated from the resulting collected slurry by vacuum filtration. Solids were thoroughly washed with water to collect the WIS fraction and subsequently analysed in terms of macromolecular composition, following the same procedure described for the raw material.

The LFH was also analysed to determine the concentration of sugars and inhibitory compounds (see the Analytical methods section below). Due to its high inhibitory potential, the LFH was subjected to detoxification according to the method described by Álvarez et al. [40] and Negro et al. [41]. For that, the ion-exchange resin Microionex MB200 (Rohm Haas, Denmark) was used as detoxification agent in a solid:liquid ratio of 1:5.3. The resulting detoxified LFH (d-LFH) was then supplemented with peptone (10 g L^{-1}), yeast extract (4 g L^{-1}), K_2HPO_4 (2 g L^{-1}), and $MgSO_4 \cdot 7H_2O$ (0.2 g L^{-1}), filter-sterilized through 0.22 µm (NalgeneTM Rapid-FlowTM; ThermoFisher Scientific, Waltham, MA, USA) and stored at 4 °C until further use.

Enzymatic hydrolysis of pretreated WIS fraction

After pretreatment, the resulting WIS fraction was subjected to enzymatic hydrolysis to obtain the corresponding fermentable sugars from structural carbohydrates in a 15 L drum bioreactor (Infors Terrafors-IS, Infors Ag, Switzerland) with a working volume of 15 L. This step was performed according to Negro et al. [9], using 15% (w/w) substrate loading, 50 °C, 150 rpm and pH 5.0 (0.05 M sodium citrate buffer) for 72 h. For this purpose, the cellulolytic enzymatic preparation Cellic® CTeC2 from Novozymes (Bagsværd, Denmark) was use at an enzyme dose of 45 FPU/g glucan. After the saccharification stage, the corresponding WDH was supplemented with peptone (10 g L^{-1}), yeast extract (4 g L^{-1}), K_2HPO_4 (2 g L^{-1}), and $MgSO_4 \cdot 7H_2O$ (0.2 g L^{-1}), filter-sterilized through 0.22 µm (NalgeneTM Rapid-FlowTM; ThermoFisher Scientific, Waltham, MA, USA) and stored at 4 °C prior to fermentation assays.

Microorganisms and cell propagation

B. coagulans DSM 2314 from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunscheweig, Germany) was chosen as LA-producing microorganism. In addition, the LAB *L. rhamnosus* DSM 20711 from the same culture collection was used for comparison purposes. Regardless of the strain, cells were grown under anaerobic conditions in 0.5 L Biostat Q Sartorius fermenters (Sartorius AG, Goettingen, Germany) with magnetic stirring at pH 5.5 (pH control was done using 4 M KOH) and 50 °C or 37 °C for *B. coagulans* or *L. rhamnosus*, respectively. MRS broth (Merck, Darmstadt, Germany) containing glucose at a final concentration of 20 g/L was used as growth media. MRS was sterilized by autoclaving (121 °C for 15 min) and supplemented with 2 mL L^{-1} of a sterile antifoam solution (Merck, Darmstadt, Germany). Cells growing in exponential phase (OD600nm ≈ 2) were harvested by centrifugation (3000 g, 10 min), resuspended in 5-times lower

volume of MRS media containing 20% (v/v) glycerol, aliquoted in 5 mL vials and stored at -80 °C prior to inoculation.

Fermentation assays of pretreated MFGW fractions

Both d-LFH and WDH were subjected to fermentation assays, using either *B. coagulans* or *L. rhamnosus*. These two pretreated fractions were also mixed at 3:1, 1:1 and 1:3 d-LFH:WDH ratios for integrated glucose/xylose fermentation with *B. coagulans*. All fermentations were carried out under anaerobic conditions in 0.25-L Biostat Q Sartorius fermenters (Sartorius AG, Goettingen, Germany) with magnetic stirring and a working volume of 250 mL. Regardless of the microorganism used, pH was maintained at 5.5 using 4 M KOH, while temperature was set at 37 ºC for *L. rhamnosus* and 50 °C for *B. coagulans*. Cells were inoculated using 5-mL of the 5-times concentrated glycerol stocks. Inoculum concentration is similar to 10% (v/v) of cell propagation media. However, concentrated cells were used to avoid sugars dilution. After inoculation, fermentation assays were monitored periodically in terms of sugars, cell biomass and LA concentration following the procedures described in the Analytical methods section. For that, collected samples were first centrifuged at 3000 g for 10 min to separate and collect both cells and supernatant.

Analytical methods

Monomeric sugars (glucose, xylose, galactose, mannose and arabinose), lactic acid and inhibitors (furfural, 5-HMF, acetic acid, formic acid, vanillin, syringaldehyde, and benzoic acid) were analysed by High-Performance Liquid Chromatography (HPLC). Glucose, xylose, galactose, mannose and arabinose were determined in a 2695 HPLC Waters system (Milford, MA, USA) equipped with a 2414 Waters Refractive Index detector and a CARBOSep CHO-782 LEAD column (Transgenomic, USA), operating at 70 °C with Milli-Q water as the mobile-phase with a flow rate of 0.5 mL min^{-1}. Carboxylic acids (acetic acid, formic acid, and lactic acid) were identified and quantified using an Aminex HPX-87H column (Bio-Rad Labs, Hercules, CA, USA) operating at 65 °C with 0.6 mL min^{-1} of 0.5 mM H_2SO_4 as eluent. On the other hand, 5-HMF, furfural, vanillin, syringaldehyde, and benzoic acid were analysed using a 1200 HPLC Agilent series (Agilent, Waldbronn, Germany), equipped with a 1100 Agilent Diode-Array detector series (Agilent Technologies, CA, USA) and an IC Sep ICE-COREGel 87H3 analytical column (Transgenomic, San José, CA, USA) coupled to an ICSep-ICE-COREGEL 87H3 guard column. The column was maintained at 65 °C and 89% 0.005 M H_2SO_4 with 11% acetonitrile was used as eluent at a flow rate of 0.6 mL min^{-1}.

Cell biomass concentration was estimated by measuring dry cell weight after vacuum-filtering 2 mL of samples through 0.22 µm cellulose nitrate filters (Whatman, Merck, Darmstadt, Germany) and oven drying at 50 °C until constant weight.

Calculations

Maximum growth rate during the exponential phase (µmax) is calculated as the slope of the logarithm of biomass concentration versus time, noting the exponential phase as the period in which both parameters are linearly correlated. Product (YP S^{-1}) and biomass (YX S^{-1}) yields are calculated as the ratio between the lactic acid produced and the biomass generated over the amount of sugar consumed. LA productivity was calculated by considering the LA produced within the interval of time required to reach the highest lactic acid concentration.

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