New BIOTECHNOLOGY 72 (2022) 1–10

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt

Towards efficient production of highly optically pure D-lactic acid from lignocellulosic hydrolysates using newly isolated lactic acid bacteria

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ARTICLE INFO

Keywords:
d-lactic acid
Optical purity
Lignocellulosics
Leuconostoc sp.
Co-cultivation
Nanofiltration

ABSTRACT

This study presents the production of D-lactic acid with high enantiomeric purity using lignocellulosic hydrolysates from newly isolated lactic acid bacterial (LAB) strains. Six strains, 4 heterofermentative and 2 homofermentative, were investigated for their ability to grow and produce lactic acid on sugar beet pulp (SBP) hydrolysates, containing a mixture of hexose and pentose sugars. Among the strains tested, three were isolates designated as A250, A257 and A15, all of which belonged to the genus *Leuconostoc*. Only strain A250 could be reliably identified as *Leuconostoc pseudomesenteroides* based on cluster analysis of Maldi-ToF spectra. All strains produced D-lactic acid in the presence of SBP hydrolysates, but with varying optical purities. The homofermentative strains achieved higher D-lactic acid optical purities, but without assimilating the pentose sugars. Co-cultivation of the homofermentative strain *Lactobacillus coryniformis* subsp. *torquens* DSM 20005 together with the heterofermentative isolate A250 led to the production of 21.7 g/L D-lactic acid with 99.3 % optical purity. This strategy enabled the complete sugar utilization of the substrate. Nanofiltration of the SBP hydrolysate enhanced the enantiomeric purity of the D-lactic acid produced from the isolates A250 and A15 by about 5 %. The highest D-lactic acid concentration (40 g/L) was achieved in fed-batch cultures of A250 isolate with nanofiltered SBP, where optical purity was 99.4 %. The results of this study underline the feasibility of a novel isolate as an efficient D-lactic acid producer using lignocellulosic hydrolysates.

Introduction

The reduction in amount of petroleum-based plastics has been in the spotlight for the last four years, especially since December 2015, when an EU Action Plan for a circular economy was approved [1]. This action plan highlighted the need to develop an effective strategy for handling of plastics. In 2018, the EU declared an outlook for a more sustainable plastics industry foreseen until 2030 [2,3]. Apart from plastic recycling, the initiative also presented the advantages and disadvantages of (bio) degradable plastics [1]. Research on (bio)degradable plastics has been ongoing for many years [4], and bioplastics have already been employed in tissue engineering and medicine [5]. Poly(lactic acid) (PLA) is one of the most well-known biodegradable and biocompatible plastics, finding uses mainly as packaging material or in medical applications [6,7]. PLA covers 24 % of global biodegradable polymer production [8].

PLA is synthesized either from enantiomerically pure L-lactic acid resulting in PLLA – or from D-lactic acid – resulting in PDLA – whereas the racemic mixture of lactic acid results in an amorphous polymer [9]. The stereocomplex (scPLA) produced from enantiomerically pure PLLA and PDLA presents enhanced properties compared to its monomers in terms of thermal stability, mechanical performance and hydrolysis resistance [9]. It is estimated that by 2025, almost 50% of the lactic acid produced will be used for PLA production [10], the increasing demand for which has led to extensive research on sustainable lactic acid production. Optically pure D- or L-isomers can be produced biotechnologically, which is a considerable advantage in comparison to the production of lactic acid from petrochemicals.

Lactic acid can be produced from a wide range of substrates. L-lactic acid production in particular has been investigated using various renewable feedstocks as fermentation substrates, including lignocellulosic biomass [11]. The focus on L-lactic acid compared to the D- isomer is most probably related to the wide use of L-lactic acid in the food

Abbreviations: LAB, lactic acid bacteria; SBP, sugar beet pulp; PLA, Poly(lactic acid); scPLA, PLA stereocomplex; MRS, De Man, Rogosa and Sharpe; MALDI-ToF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MF, microfiltration; NF, nanofiltration.

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https://doi.org/10.1016/j.nbt.2022.08.003
Received 24 May 2022; Received in revised form 5 August 2022; Accepted 11 August 2022
Available online 15 August 2022

industry. Moreover, there are more wild-type strains able to produce L-lactic acid alone [9]. D-lactic acid attracted attention in the last ten years, especially due to its significance for a more stable PLA [9]. Final concentrations of more than 200 g/L D-lactic acid have been reported in the literature from pure glucose [12]. Obtaining high final concentrations with high optical purities using renewable feedstocks constitutes one of the main challenges in fermentative D-lactic acid production.

Lignocellulosic biomass has gained scientific attention, mainly due to its abundance and high content of structural polysaccharides. Lignocellulosic hydrolysates contain pentoses (xylose, arabinose) derived from hemicellulose degradation and glucose generated after cellulose hydrolysis. Lactic acid bacteria (LAB) are categorized as homofermentative and heterofermentative. In the former, LAB utilize the Embden-Meyerhof-Parnas (EMP) pathway to metabolize hexoses (e.g. glucose) and the pentose phosphate (PP) pathway to ferment pentoses, yielding lactic acid as the major end-product [9]. On the other hand, heterofermentative LAB metabolize both hexoses and pentoses via phosphoketolase (PK) pathway. In this case, acetic acid, ethanol or some polyols are formed as by-products [13]. Most D-lactic acid bacteria able to consume pentoses are heterofermentative, while the production of the racemic mixture is strain dependent. The identification of strains capable of growing on lignocellulosic hydrolysates and producing D-lactic acid with high optical purity is crucial for its industrial application. A metabolically engineered *Lactobacillus plantarum* able to utilize glucose and xylose was utilized for the simultaneous saccharification and fermentation of hardwood pulp [14]. The strain produced 102.3 g/L D-lactic acid with an optical purity of 99.2 %. A *Leuconostoc* sp. isolate was recently evaluated for the fermentation of rice husk hydrolysates, resulting in 27 g/L D-lactic acid and 97 % optical purity [15].

Sugar beet pulp (SBP) is a by-product of the sugar industry generated after sugar extraction from the beets. SBP is currently sold as animal feed, but different studies have demonstrated its potential as fermentation substrate [16–20]. Recently, the production of L-lactic acid from SBP hydrolysates in batch and continuous fermentations was presented [17].

The aim of this study lies in the production of highly optically pure D-lactic from SBP hydrolysates. Different bacterial strains were initially evaluated for their ability to consume the sugars present in the hydrolysate and produce D-lactic acid with high optical purity. Among those tested, four were heterofermentative and two homofermentative lactic acid producers. The heterofermentative strains were evaluated in order to fully exploit the monosaccharides of the SBP hydrolysate. Apart from pure culture fermentations, co-cultivation was also tested as an alternative strategy, targeting both pentose utilization and high D-lactic acid optical purity. Finally, a fed-batch fermentation was employed for testing both the selected strain's capacity and also aiming for by-product reduction.

Materials and methods

Substrate

Sugar beet pulp (SBP) was kindly provided by Pfeifer & Langen GmbH & Co. KG (Cologne, Germany) in dried form. Its composition is presented in [16].

Hydrolysis of SBP

Hemicellulose and cellulose hydrolysis to fermentable sugars was carried out according to the optimized conditions determined in [16]. Initially, SBP (10 % solid loading) was subjected to dilute acid pretreatment using 0.5 % (v/v) H_2SO_4, at 121 °C for 30 min in an autoclave. Enzymatic hydrolysis was then carried out in a 72 L bioreactor (B-Braun Biotech, Hessen, Germany), after adjustment to pH 5 with 20 % (w/w) NaOH using the commercial enzyme preparation Accellerase 1500 (Genencor). Enzyme loading was set to 0.5 mL per g cellulose, and hydrolysis was carried out at 50 °C for 24 h, at 400 rpm. The composition of the resulting hydrolysate was (in g/L): glucose, 19.2; xylose, 0.9; galactose, 3.7; arabinose, 15.8; fructose, 4; acetic acid, 4.1 [16]. At the end of the process, the hydrolysate was subjected to coarse filtration using filter bags (Schwegmann Filtrations-Technik GmbH, Grafschaft-Ringen, Germany) with 400 μm pore size for solids removal.

Micro- and nanofiltration of SBP-derived hydrolysate

The permeate obtained after coarse filtration was then subjected to microfiltration (MF) using a UFI-TEC cross-flow system (UFI-TEC GmbH, Oranienburg, Germany) with ceramic membranes with a pore size of 0.2 μm. The micro-filtered permeate was further treated by a UFI-TEC cross-flow nanofiltration (NF) system equipped with OS 15 membranes (INSIDE CéRAM 0.2 μm TAMI industries, Nyons, France) with a cut-off of 150–300 Da. Operation conditions for both unit operations have been previously described in [21]. Nanofiltered hydrolysate was also concentrated to a sugar concentration of 125 g/L via vacuum evaporation to be used as feeding solution for the fed-batch fermentation experiments.

Microorganisms and inoculum preparation

Six strains, three isolated at ATB with internal codes A250 (isolated from ensiled white cabbage), A257 (isolated from rye) and A15 (isolated from carrot juice) and three obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany), namely *Lactobacillus coryniformis* subs. *torquens* DSM 20004 and 20005 and *Leuconostoc mesenteroides* subsp. *mesenteroides* DSM 20241, were evaluated for their ability to ferment SBP hydrolysates to D-lactic acid. Inoculum preparation was the same for all strains and involved growth in De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany), for 20 h, at 100 rpm and at a temperature of 30 °C. Each inoculum was prepared in 250 mL flasks, containing 60 mL MRS broth.

Fermentations

Batch and fed-batch fermentations were carried out in 2 L BIOSTAT bioreactors (Sartorius AG, Goettingen, Germany), with 1 L working volume at 30 °C, using SBP hydrolysate as carbon source and 10 g/L yeast extract. Stirring was set at 200 rpm, and the pH was controlled to 6.0 with 20% (w/w) NaOH. The growth of *Lb. coryniformis* subs. *torquens* DSM 20004 and 20005 was also supported with the following mineral salt solution: $MgSO_4 \bullet 7H_2O$ (0.2 g/L), K_2HPO_4 (2 g/L) and $MnSO_4$ (0.05 g/L). Inoculum was 6 % (v/v) for all fermentations. For the fed-batch fermentations, feeding solution was concentrated nanofiltered SBP hydrolysate with approximately 125 g/L total sugars.

Strain identification

Selected isolates were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) based on ribosomal MS fingerprints. To obtain best possible identification, two different preparation procedures were conducted. For the direct-smear technique, material from the grown colonies was directly transferred to the target, overlaid with malic acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and α-cyano-4-hydroxy cinnamic acid (CHCA) matrix (RIPAC-LABOR GmbH, Potsdam, Germany) and after a short drying step measured in the MALDI-ToF MS (Axima Confidence, Shimadzu Deutschland GmbH, Duisburg, Germany). For the second sample preparation, cell material from the grown colonies was suspended in purified water and 96 % ethanol (Carl Roth + Co. KG) was added and incubated for 5 min. Thereafter, the samples were centrifuged at 12,400 × *g* for 3 min and the supernatant was discarded. After a second centrifugation step the remaining supernatant was removed and the pelleted material re-suspended in malic acid (70 %) and acetonitrile

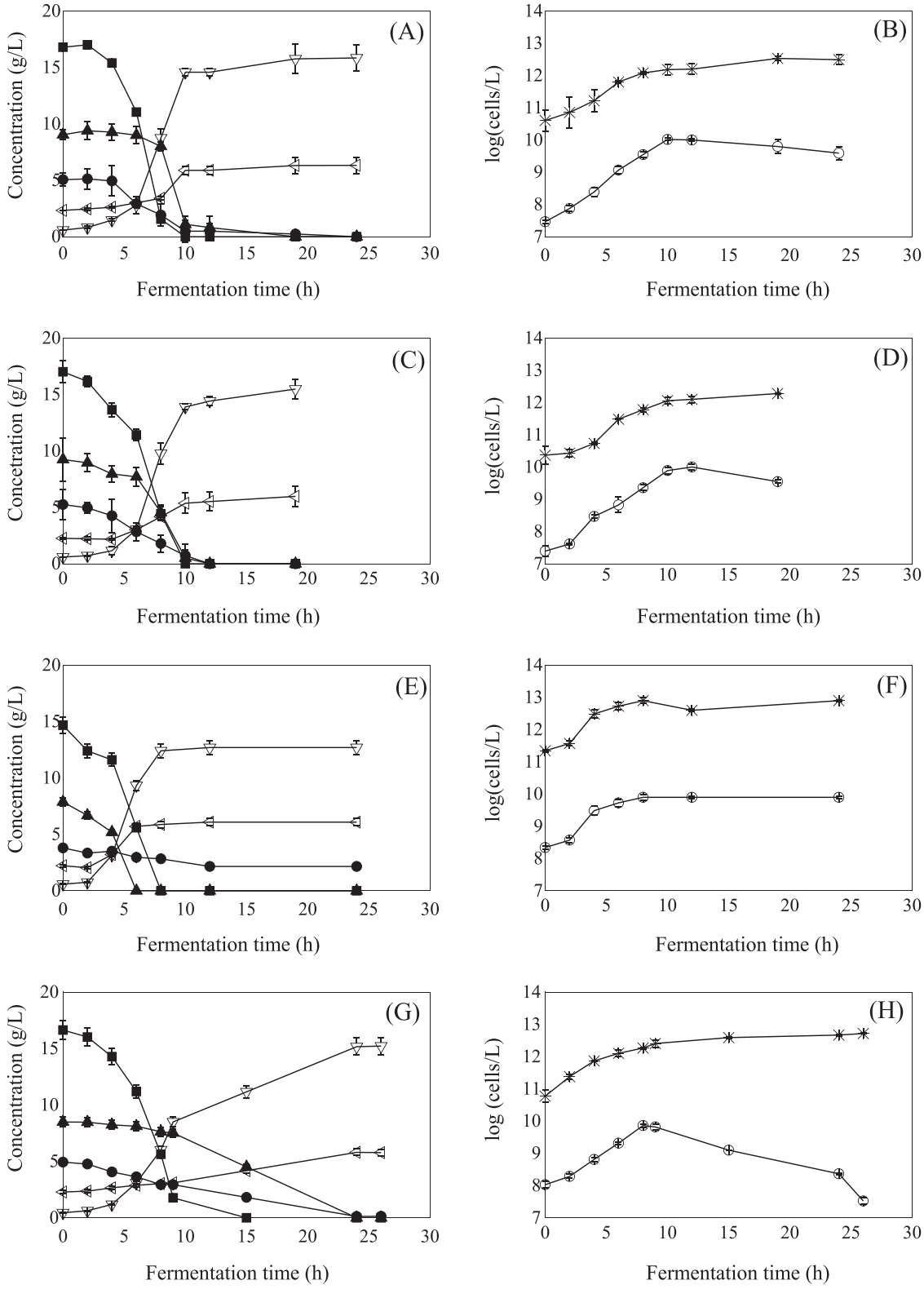

Fig. 1. Lactic acid fermentations with SBP hydrolysate using heterofermentative strains. (A), (B) isolate A250 (*most likely L. preudomesenteroides*); (C), (D) isolate A15; (E), (F) A257, (G), (H) *L. mesenteroides* subsp.*mesenteroides* DSM 20241. Glucose (■), fructose (●), arabinose (▲), lactic acid (▽), acetic acid (◁), total cells (✳), living cells (⊙).

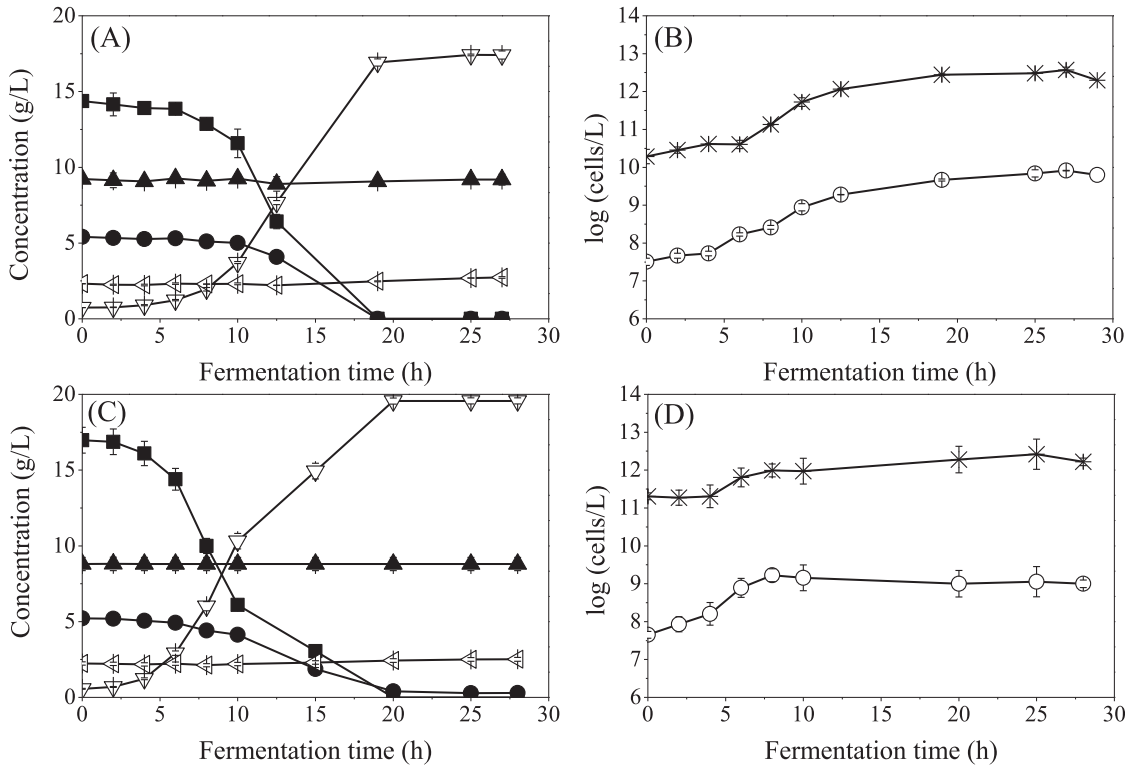

Fig. 2. Lactic acid fermentations with SBP hydrolysate using homofermentative strains. (A), (B) *Lb. coryniformis* subsp. *torquens* DSM 20005; (C), (D) *Lb. coryniformis* subsp. *torquens* DSM 20004. Glucose (■), fructose (●), arabinose (▲), lactic acid (▽), acetic acid (◁), total cells (✳), living cells (○).

Table 1
Summary of the final lactic acid and acetic acid concentrations, as well as lactic acid yield and productivity for all the different strains used in batch fermentation mode with SBP hydrolysate as substrate. Heterofermentative strains: A257, A250, A15, L. *mesenteroides* subsp. *mesenteroides* DSM 20241. Homofermentative strains: *Lb. coryniformis* subsp. *torquens* DSM 20005 and 20004.

Strain	Final LA (g/L)	Final AA (g/L)	Y_{LA} (g/g)	P_{LA} (g/L/h)	LA optical purity (%)
A257	12.1	3.9	0.49	1.01	89.1
A250	14.0	3.6	0.54	1.17	93.9
A15	13.8	3.3	0.46	1.15	94.5
***L. mesenteroides* subsp. *mesenteroides* DSM 20241**	14.7	3.5	0.52	0.61	88.8
***Lb. coryniformis* subsp. *torquens* DSM 20005**	16.2	0.2	0.61	0.85	99.5
***Lb. coryniformis* subsp. *torquens* DSM 20004**	19.3	0.2	0.68	0.96	95.5

(1:2) (Carl Roth). One μL of this suspension was transferred to the target and dried briefly, overlaid with CHCA and after another short drying step measured in the MALDI-ToF MS and analyzed using BioNumerics software (version 7.6; Applied Maths NV, Sint-Martens-Latem, Belgium) as described in [22].

Analytical methods

Sugar and organic acid determinations were carried out by HPLC (Dionex, Sunnyvale, CA, USA), coupled with a refractive index detector (RI-71, Shodex, Yokohama, Japan). Sample was injected (volume 10 μL) in a Eurokat H column (300 mm × 8 mm × 10 μm, Knauer, Berlin, Germany) and 5 mL H_2SO_4 was used as mobile phase, at a flow rate of 0.8 mL/min.

Enantiomeric purity of D-lactic acid was determined via HPLC (Dionex), coupled with a UV detector. Separation was achieved with a Phenomenex Chirex 3126 (150 × 4.6 mm ID, Phenomenex, Torrance, USA) column, using 1 mM Cu_2SO_4 at a flow rate of 1 mL/min, at 30 °C.

Analysis of specific ions present in the SBP hydrolysates was performed by ion chromatography (Dionex). An IonPac CS 16 column (250 mm × 4 μm, Dionex) eluted with 30 mM CH_3SO_3H (1 mL/min, 40 °C) was utilized for cation analysis, while an IonPac AS9-HC column (250 mm × 4 μm, Dionex) was used for ion analysis with 9 mM Na_2CO_3 (1.2 mL/min, room temperature).

Living cells, expressed as colony forming units (cfu), were used for growth monitoring. One mL of fermentation sample was diluted sequentially in sterile tubes containing Ringer solution [23]. One hundred μL of the dilutions were plated on a Petri dish with MRS Agar (Merck) and incubated at 30 °C for 48 h. Total cells were calculated using a THOMA cell chamber (Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim, Germany) after adequate dilution in Ringer solution.

Results and discussion

Strain selection

Six strains, four heterofermentative (A257, A250, A15, and *L. mesenteroides* subsp. *mesenteroides* DSM 20241) and two homofermentative (*Lb. coryniformis* subsp. *torquens* DSM 20005 and 20004), were evaluated for their ability to produce high purity D-lactic acid using SBP hydrolysate as the sole carbon source. All the tested heterofermentative strains belonged to the genus *Leuconostoc*. Isolate A250 was reliably identified as *Leuconostoc* sp. using the SARAMIS™ database, and further analysis showed that it clustered with other mass spectra

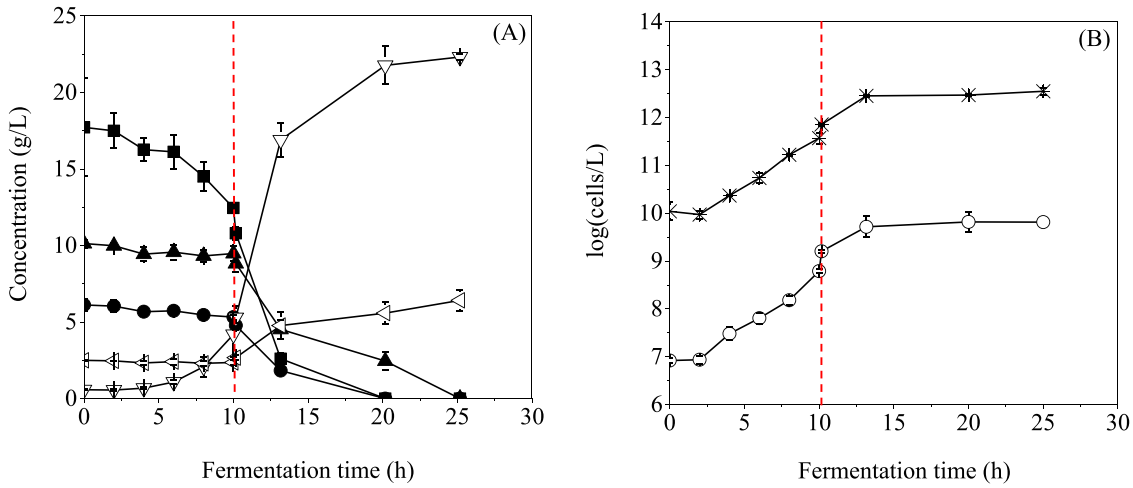

Fig. 3. Co-cultivation of *Lb. coryniformis* subsp. *torquens* DSM 20005 and isolate A250 (most likely *L. preudomesenteroides*) in SBP hydrolysate. (A) sugar consumption and organic acid production, (B) cell growth. Glucose (■), fructose (●), arabinose (▲), lactic acid (▽), acetic acid (◁), total cells (✳), living cells (○). The red dashed line indicates the time point when isolate A250 was added.

obtained from other *L. pseudomesenteroides* implying that isolate A250 also belongs to the species *L. pseudomesenteroides*. Isolate A15 could not be reliably identified using the AnagnosTec SARAMIS™ database (Spectral Archive and Microbial Identification System, bioMérieux Deutschland GmbH, Germany). However, cluster analysis revealed that this isolate most probably belonged to the family *Leuconostocaceae*. Isolate A257 could not be identified using the SARAMIS™ database, and neither could the cluster analysis help identify this isolate.

SBP hydrolysate consists of a mixture of sugars with glucose and arabinose being the predominant ones. According to the recent study [16], SBP hydrolysate contained 43.6 ± 0.5 g/L total sugars, of which 44 % was glucose, 36 % arabinose, 9.2 % fructose, 8.5 % galactose and 2.1 % xylose. Due to analytical restrictions, it was not possible to separate fructose, galactose and xylose with the HPLC method employed. For this reason, the consumption of these three sugars (fructose, xylose, and galactose) is expressed as fructose, being the major one.

Fig. 1 and Fig. 2 present the sugar consumption as well as the organic acid production from the selected hetero- and homofermentative strains, respectively. Among the heterofermentative strains, only isolate A257 was unable to assimilate all sugars present in the hydrolysate (Fig. 1E) leading to the lowest lactic acid concentration (12.1 g/L). Isolates A250 and A15 presented the highest productivities, with values of 1.17 and 1.15 g/L/h, respectively. All heterofermentative strains co-produced acetic acid with an average concentration of 3.6 g/L (Table 1). Regarding the optical purity of the D-lactic acid produced, the lowest value was observed from *L. mesenteroides* subsp. *mesenteroides* DSM 20241 (88.8 %), and the highest from isolate A15 (94.5 %).

For all strains studied, lactic acid production coincided with the exponential growth phase, as shown in Fig. 1(B, D, F, H). The fermentation kinetics of the *Leuconostoc* strains were quite different from each other, as shown in Fig. 1. Isolate A257 started consuming fructose and arabinose only when glucose concentration reached about 10 g/L (4 h of fermentation). After this point, the strain consumed all arabinose, and a peak in acetic acid production was also observed (Fig. 1E). The connection between pentose metabolism and acetic acid production was very evident for this strain. When pentoses were completely consumed, acetic acid production ceased, and only lactic acid was still being produced (Fig. 1E). Isolate A15 had a similar behavior to A257 (Fig. 1C, E), even though a slight decrease in arabinose concentration was observed from the first 4 h of fermentation. Arabinose consumption by strain A250 started only when glucose was almost completely depleted (Fig. 1A). Isolate A15 was previously employed on rice husk hydrolysates [15] resulting in the production of 27 g/L D-lactic acid, with an optical purity of 97 %. On this substrate, acetic acid concentration reached 14.4 g/L.

L. mesenteroides DSM 20241 started to consume arabinose only when glucose- and most probably all the hexoses- was fully consumed (Fig. 1G). There was also an apparent decrease in living cells of DSM 20241, from 7.3×10^9 cfu/L (8 h) to 3.33×10^7 cfu/L at the end of the fermentation (Fig. 1H).

The homofermentative strains could not assimilate pentoses (Fig. 2), resulting in residual arabinose in the medium. *Lb. coryniformis* subsp. *torquens* DSM 20004 had a lag phase of about 4 h (Fig. 2C, D). There was an evident preference towards glucose, which was consumed within 20 h of fermentation. Similar observations have been described in [24], where the homofermentative strain *Lb. delbrueckii* spp. *delbrueckii* consumed glucose at a much faster rate in comparison to fructose and galactose, when grown in sugar mixtures. This phenomenon can be attributed to sugar competition for the membrane transporters [25]. Fructose is progressively phosphorylated prior to being divided into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) [26]. On the other hand, galactose is phosphorylated after its isomerization to glucose and then catabolized according to Leloir or tagatose pathways [26].

The DSM 20005 strain had a better performance than DSM 20004. Glucose consumption started rapidly and was consumed within 19 h of fermentation (Fig. 2A). When glucose concentration started to be limited (approximately 12.5 h of fermentation), the strain began consuming the other hexoses (mainly fructose), which were also completely depleted after 19 h. Final D-lactic acid concentration was 16.2 g/L, with a yield and productivity of 0.61 g/g and 0.85 g/L/h, respectively. This strain produced D-lactic with the highest optical purity (99.5 %), compared to all the other strains tested.

Lb. coryniformis subsp. *torquens* DSM 20004, even though a well-known D-lactic acid producer, has not been well-studied in the literature. Although considered a homofermentative strain [27], its ability to co-produce acetate has been reported by various researchers [28–30]. A previous study [31] claimed that phosphoketolase activity was not detected in this strain. When the strain is found under glucose limitation, acetic acid production occurs using alternative pyruvate catabolic pathways [32]. Small amounts of acetic acid were observed in the study of [31], when the strain was cultivated in MRS medium, as well as in the work of [32], when cultivated in dried distiller's grains hydrolysates.

Leuconostoc strains have been reported as efficient D-lactic acid producers, while *L. mesenteroides* has been tested for lactic acid production, especially in fermented foods [15,33]. It has been shown that, when cultivated under anaerobic conditions, *L. mesenteroides*

Table 2
Compositional analysis of SBP hydrolysate after micro- and nanofiltration.

Sample	Glucose (g/L)	Fru + Xyl + Gal (g/L)	Arab. (g/L)	Acetic acid (g/L)	N_{kjel} (mg/L)	PO_4^{3-}-P (mg/L)	Cl^- (mg/L)	SO_4^{2-} (mg/L)	Na^+ (mg/L)	K^+ (mg/L)	Mg^{2+} (mg/L)	Ca^{2+} (mg/L)	NH_4^+-N (mg/L)
Coarse filtration	17.8 ± 1.4	5.1 ± 0.6	14.10 ± 0.71	2.7 ± 0.6	1231.0 ± 61.6	29.9 ± 1.5	16.2 ± 0.8	6395.0 ± 322.6	2589.0 ± 125.3	373.1 ± 18.8	184.1 ± 9.2	430.9 ± 21.5	24.4 ± 1.2
MF permeate	17.0 ± 1.4	5.2 ± 0.6	8.72 ± 0.35	2.8 ± 0.1	685.8 ± 34.3	20.7 ± 1.3	14.9 ± 0.8	5801.0 ± 292.1	2207.0 ± 111.4	316.4 ± 15.8	154.4 ± 7.7	333.9 ± 16.7	24.7 ± 1.2
MF retentate	18.3 ± 1.4	5.6 ± 0.5	9.49 ± 0.47	3.1 ± 0.2	3226.0 ± 161.3	29.1 ± 1.5	12.6 ± 0.7	6978.0 ± 352.9	3414.0 ± 170.5	484.7 ± 24.2	267.5 ± 13.5	582.6 ± 29.1	37.1 ± 1.9
NF permeate	0.250 ± 0.001	0.050 ± 0.003	n.d.	1.7 ± 0.1	29.3 ± 1.5	n.d.	3.8 ± 0.22	81.7 ± 4.2	109.7 ± 6.5	20.2 ± 1.2	0.94 ± 0.05	3.2 ± 0.2	3.5 ± 0.2
NF retentate	38.3 ± 1.5	11.7 ± 0.7	20.45 ± 1.20	2.8 ± 0.1	1002.0 ± 52.3	n.d.	20.4 ± 1.1	11,682.5 ± 585.1	4646.0 ± 235.3	652.1 ± 32.7	341.0 ± 17.1	720.7 ± 37.7	18.1 ± 0.9

metabolizes glucose into D-lactic acid, ethanol and CO_2 through the phosphoketolase pathway [34]. Recently, the production of D-lactic acid (99 % optical purity) from broken rice hydrolysates (starch-based hydrolysis) with an isolate identified as *L. pseudomesenteroides* was reported [35]. It can be deduced that *L. pseudomesenteroides* presents a high potential, though yet unexplored.

Co-cultivation strategy

As already stated, optical purity is crucial for the efficient polymerization of lactic acid. Strain *Lb. coryniformis* subsp. *torquens* DSM 20005 produced high optical purity D-lactic acid (99.5%), but was unable to consume arabinose, resulting in about 9 g/L of unconsumed sugars in the medium. Co-cultivation (or co-fermentation) has been proposed in the literature as an alternative fermentation strategy, especially when lignocellulosic hydrolysates are used as substrate [15,36]. Depending on the desired end-product, different types of co-cultivation strategies have been developed [36].

In this study, the homofermentative strain *Lb. coryniformis* subsp. *torquens* DSM 20005 was initially employed to convert the majority of hexoses into high pure D-lactic acid. The heterofermentative isolate A250 (identified most probably as *L. mesenteroides* subsp. *mesenteroides*) was then added to the broth to consume the remaining pentoses. Fig. 3 presents the fermentation kinetics of the co-cultivation strategy. The performance of *Lb. coryniformis* subsp. *torquens* was similar to that presented in Fig. 2(A, B). The strain started consuming glucose rapidly and, after 10 h of fermentation, half of glucose concentration was consumed (Fig. 3A). At this point isolate A250 was added to the medium. The fermentation lasted 25 h with a final lactic acid concentration of 21.7 g/L and optical purity of 99.3 %. The co-cultivation strategy resulted not only in higher final lactic acid concentration (25.4 % and 35.5 % higher when compared to *Lb. coryniformis* subsp. *torquens* and A250 isolate pure cultures, respectively), but also to a higher optical purity when compared to a pure culture of a heterofermentative strain. Yield and productivity were 0.78 g/g and 0.89 g/L/h, respectively, in the co-cultivation system. Lactic acid yield was also higher when compared to pure cultures of both selected strains (0.54 g/g for A250 isolate and 0.61 g/L/h for *Lb. coryniformis* subsp. *torquens*), indicating that co-cultivation is an interesting approach for the fermentative production of D-lactic acid. The final acetic acid concentration was 3.9 g/L, which is similar to the amount produced in pure cultures of the A250 isolate (3.6 g/L). However, it should be noted that the ratio of acetic acid: lactic acid was lower in the co-cultivation strategy (1:0.18 compared to 1:0.25). The presence of acetic acid is still problematic, since it constitutes one of the main impurities in the lactic acid purification process [17].

It appears that the D-lactic acid optical purity achieved here (99.3 %) is the highest in the literature when a co-cultivation strategy is employed for the fermentation of a lignocellulosic hydrolysate. In another study [37], the fermentability of a mixture of corn stover and poplar hydrolysates was enhanced via sequential cultivation of *Lactobacillus brevis* and *Lactobacillus plantarum*. In this case, the addition of *Lb. brevis* was carried out after 20 h of fermentation at the time that poplar hydrolysate was also added in the initial medium. Lactic acid yield was 13 % higher, but its optical purity was only 50 %. An increased lactic acid yield of 18.6 % and 29.6 % when compared to pure cultures of *Lb. rhamnosus* and *Lb. brevis*, was achieved when the two strains were co-cultivated in NaOH-treated corn stover hydrolysates [38]. Lactic acid was 0.70 g/g, but the optical purity was not determined.

Fermentations with nanofiltered SBP hydrolysate

Nanofiltration (NF) was tested not only in order to remove particles and phenolic compounds that could possible inhibit bacterial performance, but also to increase the total sugar concentration of the hydrolysate. As shown in Table 2, sugar concentration increased from about

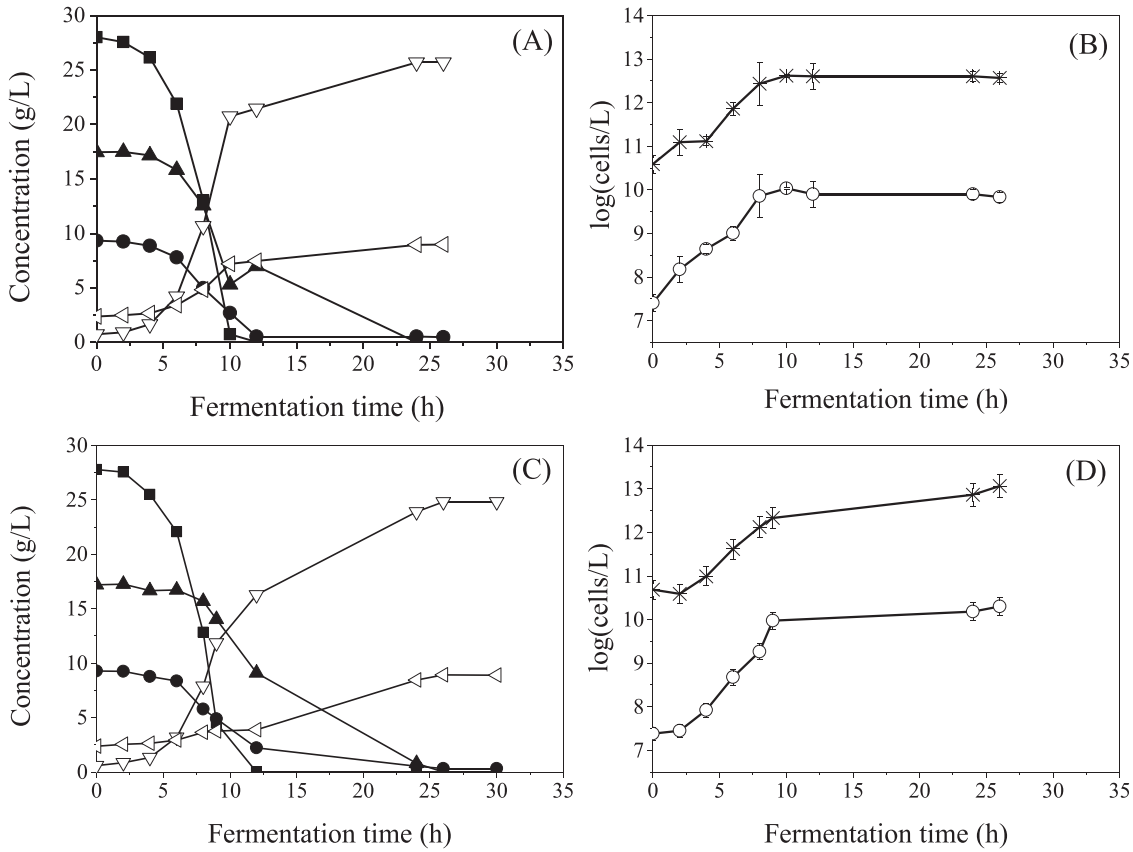

Fig. 4. Batch fermentations with nanofiltered SBP hydrolysate. (A), (B) isolate A15 and (C), (D) isolate A250 (most likely *L. preudomesenteroides*). Glucose (■), fructose (●), arabinose (▲), lactic acid (▽), acetic acid (◁), total cells (✳), living cells (○).

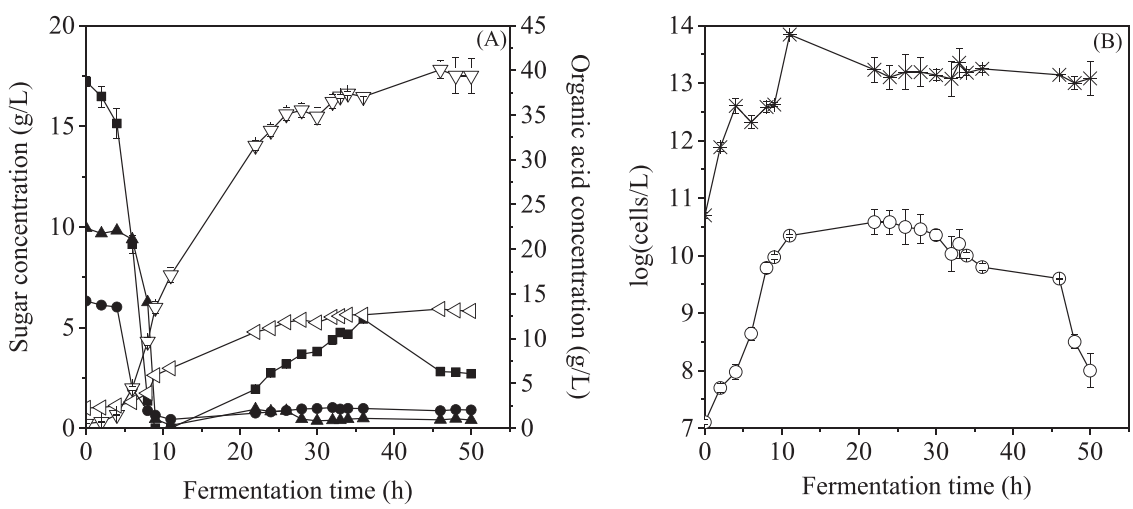

Fig. 5. Fed-batch fermentation with the isolate A250 (most likely *L. preudomesenteroides*) in SBP hydrolysate. (A) sugar consumption and organic acid production, (B) cell growth. Glucose (■), fructose (●), arabinose (▲), lactic acid (▽), acetic acid (◁), total cells (✳), living cells (○).

37 g/L in the hydrolysate obtained after coarse filtration to 70.5 g/L in the NF retentate. During nanofiltration all the hydrolysate components become concentrated, resulting in higher amounts of various ions such as Na^{+}, K^{+}, Mg^{2+} and Ca^{2+} (Table 2).

Batch fermentations with nanofiltered SBP hydrolysate

The concentrated SBP hydrolysate after nanofiltration was subsequently employed as fermentation substrate using the isolates A250 and A15, aiming to achieve higher final D-lactic acid concentrations. Initial sugars were about 54 g/L in both experiments. Sugar consumption profile was similar to that observed when coarse filtration was employed on SBP hydrolysate (Fig. 4). Lactic acid production followed the exponential phase for both strains as shown in Fig. 4(A, B) for A15 isolate and (C,D) for A250 isolate. Isolate A15 consumed all the available glucose in the first 10 h of fermentation, where the total living cells

Table 3
Current advances in D-lactic acid production from lignocellulosic hydrolysates.

Substrate	Strain	Fermentation type	Final LA concentration	LA yield (g/g)	LA productivity (g/L/h)	LA optical purity (%)	Ref.
Rockrose hydrolysate	Metabolic engineered *E.coli* JU15	Batch	30.42	1.14*	2.17	–	[42]
Cassava fibrous waste enzyme hydrolysate	*S. inulinus* NBRC 13595	Batch, static conditions	19.16	0.96	0.48	99.57	[43]
Organosolv pretreated beechwood & pine	*L. delbrueckii* subsp. *bulgaricus*	Simultaneous saccharification & fermentation in batch cultures (hexose conversion)	62 (beechwood) 36.4 (pine)	0.69 (beechwood) 0.40 (pine)	0.86 (beechwood) 0.51 (pine)	–	[40]
Bio-detoxidied corn stover hydrolysate	Engineered *P. acidilactici*	Simultaneous saccharification & fermentation in batch cultures	115	0.61	1.6	–	[44]
Avocado seed hydrolysates	Metabolically engineered *E.coli* JU15	Batch fermentation	37.8	0.31	0.52	–	[45]
Orange peel waste hydrolysate	*L. delbrueckii* subsp. *bulgaricus* CECT 286	Separate hydrolysis and batch fermentation	45	0.86	0.63	99.5	[46]
	L. delbrueckii subsp. *bulgaricus* CECT 5037		39	0.84	0.55	99.3	
Rice straw hydrolysate supplemented with B vitamins	*L. delbrueckii* subsp. *delbrueckii*	Membrane-integrated continuous fermentation	46.6	0.92	18.56	99.5	[41]
Orange peel waste hydrolysates & CSL	*L. delbrueckii* spp. *delbrueckii*	Batch fermentation	49.6	0.83	1.71	98.1	[47]
Orange peel waste hydrolysates	*L. delbrueckii* spp. *delbrueckii*	Resting cells previously grown on fructose/batch fermentation	45	0.88	6.72	98	[48]
Dried distiller's grains with solubles	*L. coryniformis* subsp. *torquens*	Simultaneous saccharification & fermentation in batch cultures (hexose conversion)	27.9	0.85	1.5	99.9	[32]
Pretreated and detoxified corn stover	*P. acidilactici* ZY15	Simultaneous saccharification and co-fermentation	124.8	0.81	1.73	–	[49]
Sugar beet pulp hydrolysate	*L. coryniformis* subsp. *torquens* DSM 20005 & isolate A250 (most likely *L. preudomesenteroides*)	Co-cultivation in batch mode	21.7	0.78	0.89	99.3	This study
	isolate A250 (most likely *L. preudomesenteroides*)	Pure culture in fed-batch mode	40	0.45	0.86	99.4	

* The authors attributed the higher than 1 D-lactic acid yield on the consumption of other sugars beside glucose, which were not quantified by HPLC.

also peaked (1.8×10^{10} cfu/L). Final lactic acid concentration was 25 g/L, while 6.6 g/L of acetic acid was also co-produced. Yield and productivity presented values of 0.51 g/g and 1.04 g/L/h, respectively, which are very similar to those previously obtained (Table 1). Even though product yields were not significantly enhanced, D-lactic acid optical purity increased to 99.4 % in comparison to coarse filtered medium where optical purity was 94.5 % (Table 1). The ratio of D- and L-enantiomeric forms is affected by various parameters such as substrate composition, pH, temperature and incubation time [31]. The variations in nanofiltered SBP, especially in terms of the cations present in the medium (Table 2), could possibly affect the expression of the enzymes involved in the racemic mixture production. Others [39] isolated the gene responsible for D-lactate synthesis in *L. mesenteroides* subsp. *mesenteroides* ATCC 8593 and their findings indicated that the encoded enzyme was not inhibited by Ca^{2+} and Mg^{2+}, which are also present in SBP hydrolysates. It can be deduced that nanofiltered hydrolysate is preferred for achieving higher final D-lactic acid optical purities.

Fed-batch fermentation with A250 isolate and nanofiltered SBP hydrolysate

Given the fact that *Leuconostoc* sp. strains have not been extensively studied in the literature for D-lactic acid production, a fed-batch fermentation with isolate A250 was also investigated using nanofiltered SBP. Feeding was carried out with concentrated SBP hydrolysate. As shown in Fig. 5A, feeding started after 9 h of fermentation, when the remaining sugars were 1.1 g/L. During the process, total sugars never exceeded the threshold of 6 g/L. Arabinose values were kept below 1 g/L throughout the process, indicating that the strain did not present carbon catabolite repression. Final lactic acid concentration reached 40 g/L, with yield and productivity of 0.45 g/g and 0.86 g/L/h, while enantiomeric purity was 99.4 %. Acetic acid production rate decreased significantly when feeding started, and the final concentration was 13.3 g/L.

Table 3 summarizes the recent studies regarding D-lactic acid production from lignocellulosic hydrolysates. Even though high titers and productivities have been reported, most of the studies only focused on hexose sugars conversion, leaving pentoses unexploited. High D-lactic acid final concentrations were achieved using organosolv treated beechwood and pine in simultaneous saccharification and fermentation broth, using the homofermentative strain *Lb. delbrueckii* subsp. *bulgaricus* [40]. The strain was able to consume only the hexoses present in the hydrolysates, reaching final lactic acid concentrations of 62 g/L and 36.4 g/L, for each hydrolysate respectively. However, the degree of product optical purity was not presented. Enhanced D-lactic acid productivity (18.56 g/L/h) and optical purity (99.5 %) were also reported using an integrated membrane system and rice straw hydrolysate as nutrient source [41]. The authors highlighted the supplementation of the medium with B vitamins as vital for both lactate production and yeast extract reduction. It should be noted that the focus was only on glucose bioconversion. Comparing the findings of the present study with the literature, isolate A250 is a promising strain for sustainable D-lactic acid production from lignocellulosic hydrolysates. Further studies on sequencing the isolate for full characterization of the strain together with the nutritional requirements would explain the preferred approach to achieving higher productivities in substrates containing sugar mixtures.

Conclusions

This study indicated the production of high enantiomeric purity D-lactic acid (> 99 %) using novel LAB isolates, which are able to utilize both the hexoses and pentoses present in SBP hydrolysates. Isolates A250 and A15, both belonging to the genus *Leuconostoc*, were found to be promising D-lactic acid candidates. Co-cultivation and nanofiltration of SBP were evaluated as strategies to enhance D-lactic acid optical

purity and sugar consumption efficiency. Nanofiltration was critical for optical purity when isolates A250 and A15 were employed, implying possible influence of metal ions on the racemic mixture production. Further studies on these novel strains would further elucidate their potential as efficient D-lactic acid producers from lignocellulosic hydrolysates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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