Isolation of Lignocellulose-derived Sugars, Co-fermentation of Lactic Acid Bacteria Strains, and Evaluation of L-lactic Acid Productivity

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High-productivity lactic acid bacteria (LAB) strains were screened and their capability to ferment lignocellulose-derived sugars into lactic acid were evaluated. Fifteen LAB strains were successfully isolated from cow dung, haystack, and sheep manure, respectively. Four relatively good strains were selected based on Gram stain, colony morphology, and catalase activity tests. The four strains and commercial inoculants (*Lactobacillus pentosus* and *Enterococcus faecalis*) were used to ferment cellobiose/ glucose/xylose to produce high-purity L-lactic acid. One of the strains (N4) presented the highest production of L-lactic acid after fermentation for 12 h and showed a L-lactic acid production of 15.1 g/L, 18.5 g/L, and 2.8 g/L and a productivity of 1.01 $g \cdot L^{-1} \cdot h^{-1}$, 3.68 $g \cdot L^{-1} \cdot h^{-1}$, and 0.47 $g \cdot L^{-1} \cdot h^{-1}$ by metabolizing cellobiose, glucose, and xylose, respectively. Through a phylogenetic tree analysis, strain N4 was identified as *Enterococcus faecium* and named *Enterococcus faecium* N4. *Enterococcus faecium* N4 has a great potential to ferment lignocellulose-derived sugars into L-lactic acid.

Keywords: Lignocellulose-derived sugars; L-lactic acid; Lactic acid bacteria; Microbial isolation; Phylogenetic tree

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INTRODUCTION

Lignocellulosic biomass is an organic material derived from a biological origin; it is the most abundant global source of biomass but large amounts of it have been unutilized (Lin and Tanaka 2006; Sun *et al.* 2006). It is mainly composed of cellulose, hemicellulose, and lignin, which account for 90% of the dry matter, as well as small amounts of minerals, oils, and other components (Molina-Sabio and Rodríguez-Reinoso 2004; Yang *et al.* 2009; Balat 2011). Biomass includes forest and crop residues (Chen and Lee 1997; Melzoch *et al*. 1997), municipal solid waste (John *et al.* 2007), waste paper (McCaskey *et al*. 1994), and wood (Linko *et al*. 1984). Its ratio of effective utilization is relatively low, so it has a great potential for biomass energy materials. In China, more than 700 million tons of crop straw are produced annually, more than 33% of which is burned or discarded. The burning or discarding of crop straw results in environmental pollution and energy waste.

Lactic acid (LA) is one of the most important chemical commodities. Recently, it has been employed in new applications in the plastics industry, whereby polylactic acid (PLA) is utilized as a biodegradable and biocompatible plastic material, and the PLA production process requires chirally pure L-lactic acid to be synthesized (Tanaka *et al*. 2002; Zheng *et al.* 2017).

Chirally pure LA can be produced through microbial fermentation, which is an alternative to chemically derived plastics (Drumright *et al.* 2000; Xu *et al.* 2004). Such an approach can potentially reduce net emissions of carbon dioxide and the demand for petroleum (Neu *et al*. 2016). Furthermore, microbial production of lactic acid is economically important because of the prospect of using cheap and widely available feedstock materials, such as lignocelluloses (Akerberg and Zacchi 2000; Hofvendahl and Hahn–Hgerdal 2000). Research has shown that effective utilization of lignocellulose-derived sugars can reduce the production cost of biomaterials by as much as 25% (Mohamed *et al.* 2011).

Conventional processes of producing lactic acid from lignocellulosic biomass involve pretreatment, enzymatic hydrolysis, fermentation, separation, and purification (Abdel-Rahman *et al.* 2011). Pretreatment includes physical (milling and grinding), chemical (alkali, dilute acid, oxidizing agents, and organic solvents), physicochemical (steam explosion/autohydrolysis, hydrothermolysis, and wet oxidation), and biological methods (Sun *et al*. 1995; Chandel *et al*. 2007).

Enzymatic hydrolysis is the most promising means to yield fermentable sugars from pretreated lignocellulosic biomass, and such a process makes it possible to produce LA when utilizing polysaccharides as a carbon source. Cellulases and hemicellulases are two general categories of enzymes necessary to convert cellulose and hemicellulose into soluble sugars (Yu and Zhang 2004). The hydrolysate of a lignocellulosic biomass is a mixture of hexoses (*e.g*., glucose) and pentoses (*e.g*., xylose and arabinose). Lignin cannot be used for lactic acid fermentation.

The effective utilization of hemicellulose-derived sugars, rather than relying on the cellulose-derived sugars alone, can reduce the production cost of biomaterials by as much as 25% (Okano *et al.* 2010). Fermentation technologies must be cost-competitive with chemical synthesis to validate the use of biotechnological processes on an industrial scale (Martinez *et al.* 2013; Zhang *et al.* 2015). In comparison with separate hydrolysis and fermentation (SHF) technology, the simultaneous saccharification and fermentation (SSF) technology offers many advantages, such as reduced reactor volume, rapid processing time, and an enhanced rate of hydrolysis (Temer *et al.* 2014). But the activity of β-glucosidase in SSF process is very low, so it causes significant accumulation of cellobiose that cannot be hydrolyzed to glucose (Xia and Shen 2004).

Studies have shown that cellobiose strongly inhibits endoglucanase and cellobiose hydrolysis, and this inhibition is much stronger than for glucose (Tomme *et al*.1995; Levine *et al.* 2010). Thus, highly active β-glucosidase is important in the process of L-lactic acid fermentation using lignocellulosic biomass (Chandel *et al*. 2007). The use of partial cellobiose as the carbon source metabolized to L-lactic acid directly can shorten the production pathway of L-lactic acid in SSF and significantly improve the efficiency of L-lactic acid (Temer *et al.* 2014).

This study identifies a strain of L-lactic acid bacteria that can metabolize lignocellulose-derived sugars with high efficiency to overcome the problem of cellobiose inhibition in the degradation process of lignocellulosic biomass and to improve fermentation efficiency. The L-lactic acid bacteria obtained can be used for lignocellulosic biomass utilization and high-optical-purity L-lactic acid production. The ideal strain was identified through a 16S rRNA gene sequence similarity analysis.

EXPERIMENTAL

Materials

Microorganism and culture media

Lactobacillus pentosus 22156 (LP) and *Enterococcus faecalis* 21057 (EF) were obtained from the China Center of Industrial Culture Collection (Beijing, China). Three samples (cow dung, haystack, and sheep manure) were collected from various sources in Hohhot City, China. The modified Man, Rogosa, and Sharpe (mMRS) medium was used for the cell growth, inoculum preparation, and fermentation as reported previously (Shibata *et al.* 2007), except that glucose, cellobiose, or xylose was used as the carbon source for the mMRS-glucose, mMRS-cellobiose, or mMRS-xylose medium with a sugar content of 2%(w/v), respectively. Complete medium (CM)-cellobiose was used for cell cultivation (Tokuhiro *et al.* 2008).

Methods

Isolation methods with cellobiose as the carbon source

The samples (5 g) were blended with 100 mL of sterilized distilled water. Serial dilutions were performed to isolate lactic acid bacteria (LAB) strains by using mMRS-cellobiose agar with calcium carbonate as the medium. The cultures were grown on mMRS-cellobiose agar at 30 °C for 12 h to 24 h. The large transparent circle of LAB stains was removed and transferred to the CM-cellobiose agar medium. Three samples were selected from 15 LAB strains, and each LAB colony was purified twice by streaking on mMRS agar and then preserved at -4 °C. The 15 strains (C1–C5, N1–N5, and Y1–Y5) were inoculated in mMRS-glucose and mMRS-xylose media for lactic acid fermentation. Fermentation was conducted at 30 °C with agitation at 200 rpm, and the initial pH was adjusted to 7.0 with calcium carbonate; the excess calcium carbonate was added to maintain a neutral pH during the process (Xu *et al.* 2007). Four strains (C3, N4, Y3, and Y5) were selected because of their high acidification activity. Gram stain, colony morphology, and catalase activity were determined according to the methods for LAB identification.

Glucose/xylose as carbon sources for lactic acid fermentation

To investigate the acid-producing capacity of the four strains in glucose/xylose substrates, each strain was cultivated overnight in 100 mL of mMRS-glucose and mMRS-xylose media at 37 °C, 200 rpm, and a pH of 7.0. Then, 10 mL of the culture medium was inoculated in 90 mL of mMRS-glucose and 90 mL of mMRS-xylose media for 24 h. Afterwards, 4 mL of the fermentation broth was sampled every 3 h. After centrifugation, the L- and D-lactic acids in the samples were detected through high-performance liquid chromatography (Alltech, Nicholasville, KY, USA) at 25 °C. The mobile phase was 60% methanol (v/v) at a flow rate of 0.2 mL/min. The instrument was equipped with a tunable UV detector at 210 nm. Two commercial LAB inoculants, LP and EF, were used to compare their effectiveness with that of the isolates.

Species identification by 16S rRNA sequencing

To specifically identify the best bacterial strain, 16S rRNA sequencing was employed. The genomic DNA of bacterial strains was extracted through the method mentioned in (Sambrook and Russell 2008) and stored at -20 °C prior to use. Then, 1 μL of diluted DNA was used as a template for the PCR reactions. The PCR primers were 27F

(5′–AGA GTT TGA TCC TGG CTC AG–3′) and 1492R (5′–TAC GGC TAC CTT GTT ACG ACT–3′) (Cai *et al.* 1999). The PCR products were purified, cloned, and sequenced by the Beijing Center for Physical and Chemical Analysis. The 16S rDNA sequences were aligned with 16S rRNA sequences from GenBank to identify the organisms through a MEGA analysis. Finally, the 16S rRNA gene sequence of the isolate was compared with the sequences from the LAB-type strains contained in GenBank (Kimura and Ohta 1972).

RESULTS AND DISCUSSION

LAB Isolation with Cellobiose as the Carbon Source

The lignocellulose-derived sugars are mainly cellobiose, hexose (glucose), and pentose (xylose and arabinose) (Mohamed *et al.* 2011). Thus, the use of mixed sugar fermentation to improve the yield of lactic acid is critical (Gao *et al.* 2011; Ouyang *et al.* 2013; Pessione *et al.* 2014).

In the first stage, 15 bacterial isolates were obtained from three environmental samples according to the procedures described above. Four relatively good strains (C3, N4, Y3, and Y5) were selected according to the results of Gram stain, colony morphology, and catalase activity tests. The four strains and two commercial inoculants used cellobiose (sugar content of 2%(w/v)) as the substrate for lactic acid fermentation. The control group was without adding cellobiose. Results are shown in Fig. 1. The LA concentration was generally less than 1 g/L in the control group due to the absence of a carbon source. Therefore, the effect of the medium without a carbon source on the lactic acid production was ignored. This was because the amount of nitrogen in beef extract, peptone, and yeast powder was much larger than the amount of carbon. This imbalanced carbon–nitrogen ratio seriously affected the growth of LAB and the lactic acid yield. Strains C3, Y3, Y5, and N4 showed high lactic acid production, and they were used as the primary screening bacteria.

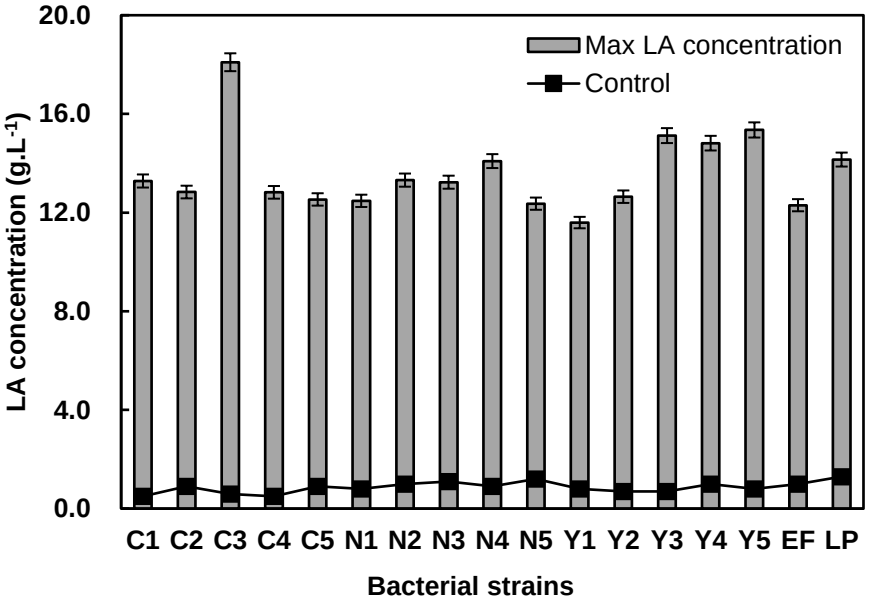

Fig. 1. Lactic acid production of bacteria from haystack (C1 through C5), cow dung (N1 through N5), sheep manure (Y1 through Y5), *lactobacillus* pentose (LP), *Enterococcus faecalis* (EF), and the control group experiments at 37 °C, 200 rpm, initial pH 7.0 for 12 h of fermentation (working volume 100 mL; sugar content, 2%); [a] Produced L-lactic acid (g/L) per glucose (g/L)

The goal of this work was to screen for high-optical-purity LAB. According to the conventional definition, LAB with an optical purity more than 80% is considered homo-fermentative LAB (Arriola *et al*. 2011). Figure 2 shows that the optical purity of Y3, Y5, N4, and *Lactobacillus pentosus* exceeded 95%; it follows that these strains could be potentially applied to produce L-lactic acid. Strain Y5 had the highest lactic acid production rate. After 21 h of fermentation, Y5 accumulated an L-lactic acid yield of 0.82 g/g cellobiose consumed. This phenomenon indicated that the strains selected from animal feces had better metabolic cellobiose capacity than natural plant strains. This might be the due to the presence of some LAB strains in the intestines of herbivores. The intestinal acidic environment was equivalent to a natural culture medium, so high-optical-purity LAB was obtained by suitable long-term natural selection in the intestinal tract. Strain N4 had an L-lactic acid yield of 0.78 g/g after 12 h of fermentation.

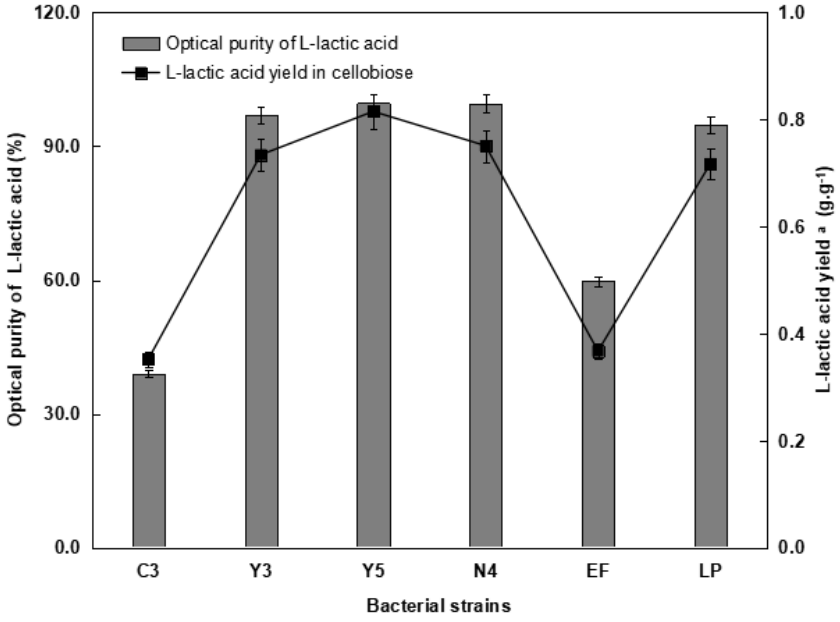

Fig. 2. L-lactic acid yield and optical purity of L-lactic acid from cellobiose at 37 °C, 200 rpm, initial pH 7.0 for 12 h fermentation (working volume 100 mL; sugar content, 2%); [a]: Produced L-lactic acid (g/L) per glucose (g/L)

Analysis of L-lactic Acid Production with Glucose as the Carbon Source

According to the above conclusions, the strains (Y3, Y5, N4, and LP) metabolized cellobiose into high-optical-purity L-lactic acid; therefore, further investigation to metabolize glucose was necessary.

In this part, glucose as the carbon source for lactic acid fermentation was investigated; the concentration was 2%(w/v). Figure 3 shows the results. The four strains had a stronger capability to metabolize glucose to lactic acid than cellobiose, and strain N4 had the highest L-lactic acid concentration and productivity. After fermentation for 6 h, lactic acid in strain N4 was achieved with a production of 18.5 g/L and a L-lactic acid yield of 0.93 g/g glucose consumed. The reason might be that in the metabolism of homo-fermentative LAB, glucose was metabolized to lactic acid *via* the Embden–Meyerhof-Parnas pathway (EMP) (Sun and Cheng 2002; John *et al*. 2006). However, cellobiose was hydrolyzed into glucose to enter the EMP pathway. Therefore, the LAB strains easily metabolized the monosaccharides.

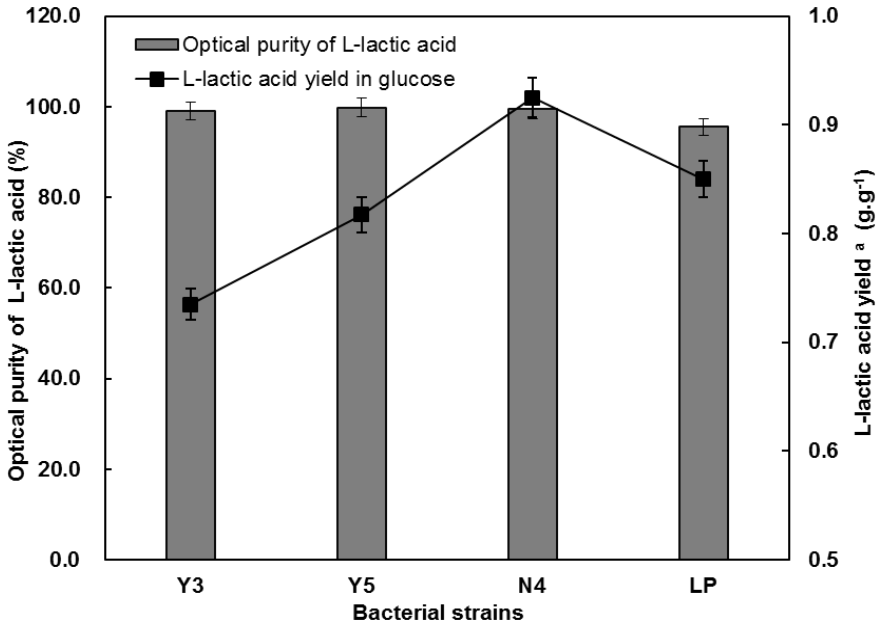

Fig. 3. L-lactic acid yield and optical purity of lactic acid from glucose at 37 °C, 200 rpm, initial pH 7.0 for 12 h fermentation (working volume 100 mL; sugar content, 2%); [a]: Produced L-lactic acid (g/L) per glucose (g/L)

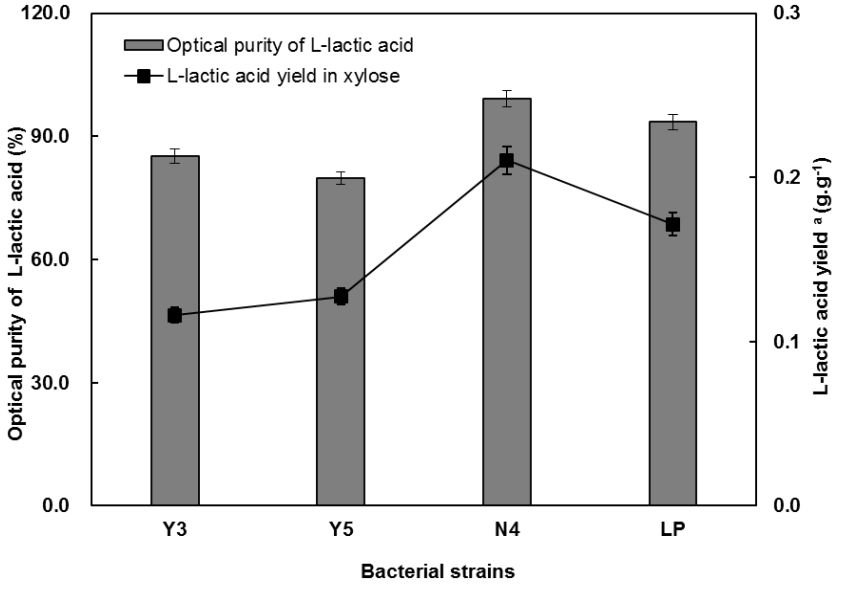

Fig. 4. L-lactic acid yield and optical purity of L-lactic acid from xylose at 37 °C, 200 rpm, initial pH 7.0 for 12 h fermentation (working volume 100 mL; sugar content, 2%); [a]: Produced L-lactic acid (g/L) per glucose (g/L)

Analysis of L-lactic Acid Production with Xylose as the Carbon Source

Based on the results shown in Fig. 3, the next experiments were conducted with Y3, Y5, N4, and *Lactobacillus pentosus* to study the lactic acid production capacity of xylose. The L-lactic acid yield and optical purity of lactic acid from xylose are shown in Fig. 4. The L-lactic acid production of the four strains is generally low because of the minimal

lactic acid fermentation from xylose by LAB (Patel *et al.* 2006). Only several reported species of LAB, such as *Lc. lactis* IO-1, *Streptococcus sp.*, and *Lb. thermophilus* T1, can metabolize xylose as a substrate to lactic acid (Oshiro *et al.* 2009). Figure 4 shows that the strain N4 produced more L-lactic acid than *Lactobacillus pentosus*, and the optical purity of lactic acid exceeded 90%. Thus, it has the potential to demonstrate high productivity through genetic engineering.

Analysis of L-lactic Acid Productivity

The L-lactic acid production of the four strains from different carbon sources is shown in Figs. 5 and 6.

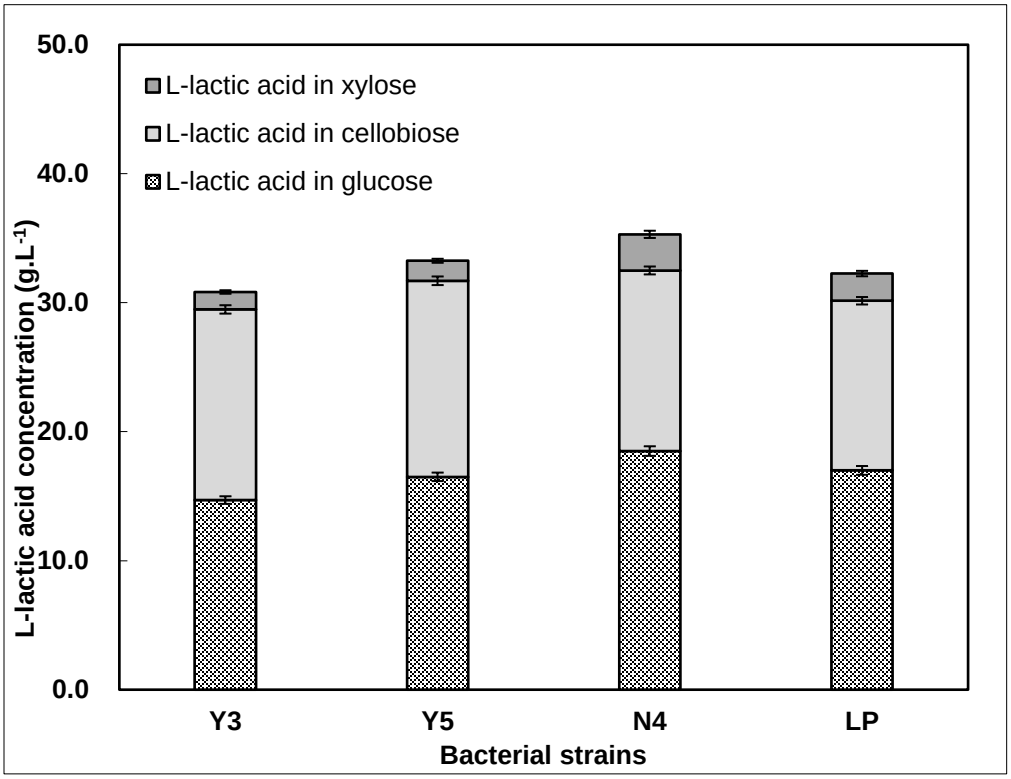

Fig. 5. Comparison of L-lactic acid production from different carbon sources at 37 °C, 200 rpm, initial pH 7.0 for 12 h fermentation (working volume 100 mL; sugar content, 2%)

Strain N4 metabolized cellobiose, glucose, and xylose to produce lactic acid and obtained an L-lactic acid production of 15.1 g/L, 18.5 g/L, and 2.8 g/L carbohydrates and a productivity of 1.01 $g \cdot L^{-1} \cdot h^{-1}$, 3.68 $g \cdot L^{-1} \cdot h^{-1}$, and 0.47 $g \cdot L^{-1} \cdot h^{-1}$, respectively, which were higher than the values for the three other strains. The lactic acid and L-lactic acid production of strain N4 was 1.64 and 1.59 times that of *Lactobacillus pentosus* when cellobiose was metabolized, 1.95 and 1.40 times when glucose was metabolized, and 2.33 and 1.56 times with xylose as the substrate. This results indicated that strain N4 has great potential to produce high-purity L-lactic acid from lignocellulose-derived sugars.

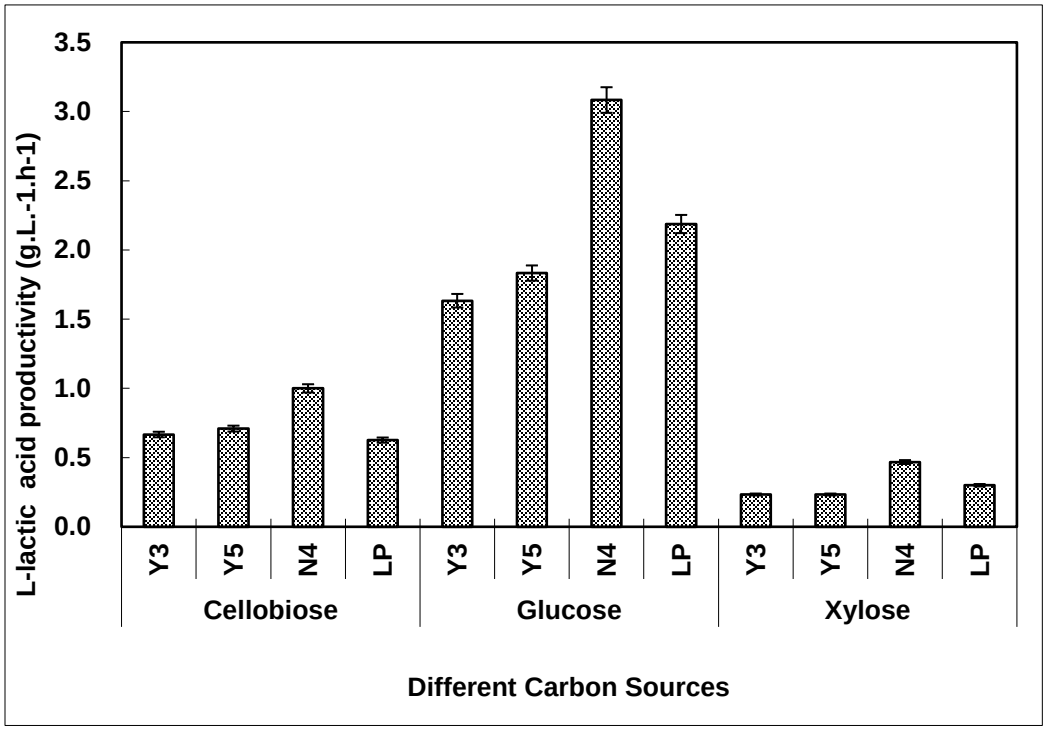

Fig. 6. L-lactic acid productivity from different carbon sources at 37 °C, 200 rpm, initial pH 7.0 for 12 h fermentation (working volume 100 mL; sugar content, 2%)

According to previous studies, hemicellulose forms a substantial fraction of the lignocellulosic biomass as well as cellulose that yields pentose sugars, such as xylose, by saccharification. The majority of LAB strains can convert cellulose-derived glucose to lactic acid, but not hemicellulose-derived sugars. Among the LAB strains reported so far, only the wild-type *E. mundtii* QU 25 (Abdel-Rahman *et al.* 2010) and the genetically modified *Lb. plantarum* ΔldhL1 (Okano *et al.* 2009) can perform homo-lactate fermentation of pentose sugars (Table 1). The strain *Lc. lactis* IO-1 (Tanaka *et al.* 2002) has been used to convert xylose to lactic acid and obtained an L-lactic acid yield of 0.62 g/g xylose. The strain *Lb. delbrueckii* mutant Uc-3 (Adsul *et al.* 2007) and *Lb. lactis* mutant RM2-24 (Singhvi *et al.* 2010) metabolize cellobiose to lactic acid and obtained an L-lactic acid productivity of 2.05 $g \cdot L^{-1} \cdot h^{-1}$ and $g \cdot L^{-1} \cdot h^{-1}$, respectively.

In this study, the strain N4 converted cellobiose to lactic acid and obtained an L-lactic acid yield of 0.78 g/g and a productivity of 1.05 $g \cdot L^{-1} \cdot h^{-1}$. When glucose was metabolized to lactic acid, it obtained an L-lactic acid yield of 0.93 g/g and a productivity of 3.68 $g \cdot L^{-1} \cdot h^{-1}$. When xylose was metabolized to lactic acid, it showed an L-lactic acid yield of 0.21 g/g and a productivity of 0.47 $g \cdot L^{-1} \cdot h^{-1}$. The lactic acid concentration by mixture of glucose, xylose and cellobiose with N4 is 92.7g/L, obtained the L-lactic acid yield of 0.68 g/g and L-lactic acid productivity of 2.03 $g \cdot L^{-1} \cdot h^{-1}$ in batch fermentation. Thus, the isolation of strain N4 is expected to generate substantial levels of lactic acid production from lignocellulosic biomass. Lactic acid fermentation from lignocellulose-derived sugars by LAB strains was achieved with different fermentation modes, as summarized in Table 1.

Table 1. L-lactic Acid Production from Lignocellulose-derived Sugars by Lactic Acid Bacteria

Microorganism	Substrate	Fermentation Process	$Y_{L\text{-}LA}$[a] (g/g)	$P_{L\text{-}LA}$[b] ($g \cdot L^{-1} \cdot h^{-1}$)
E. mundtii QU 25	Cellobiose	Batch	0.75	1.01
	Xylose	Batch	0.76	0.81
	Glucose/cellobiose	Batch	0.81	1.65
	Glucose/xylose	Batch	0.78	1.25
	Glucose/xylose/ cellobiose	Batch	0.73	2.01
E. casseliflavus and Lb. casei	Glucose/xylose	Batch	0.68	1.13
Lb. plantarum ΔldhL1	Xylose	Batch	0.76	1.44
Lb. delbrueckii mutant Uc-3	Cellobiose	Batch	0.81	2.05
Lb. lactis RM 2-24	Cellobiose	Batch	0.72	1.51
	_α-Cellulose	SSF	0.67	1.43
Lc. lactis IO-1	Xylose	Batch	0.62	--
Lb. delbreuckii	Alfalfa fibers	SSF	0.35	0.75
Lb. rhamnosus and Lb. brevis	Corn stover	SSF	0.70	0.58
Lb. bifermentans DSM 20003	Wheat bran hydrolysate	Batch with cell immobilization	0.83	1.17
Lb. coryniformis ATCC 25600	Cellulose	SSF	0.89	0.50
Strain N4	Cellobiose	Batch	0.78	1.05
	Xylose	Batch	0.21	0.47
	Glucose	Batch	0.93	3.68
	Glucose/xylose/ cellobiose	Batch	0.68	2.03

* [a]: Yield of L-lactic acid produced (g) to substrate consumed (g); [b]: L-lactic acid productivity

The neighbor-joining method was used to construct the phylogenetic tree of N4, and the bootstrap was 1000. As shown in Fig. 7, Strain N4 was most closely related to *Enterococcus faecium*, with 98% similarity in their 16SrDNA gene sequences.

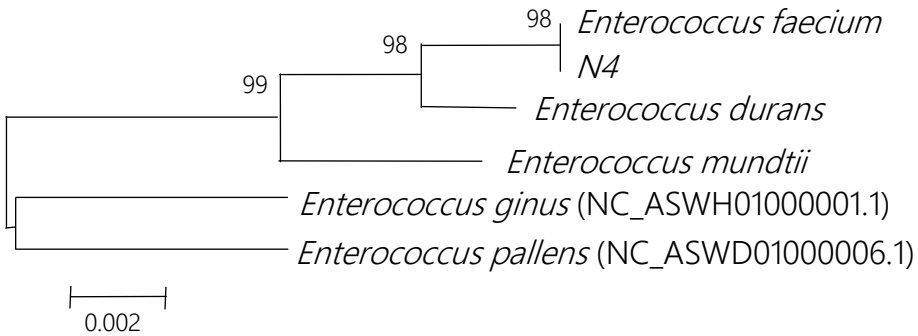

Fig. 7. Phylogenetic tree of partial 16S rDNA sequences of the N4 strain and sequences of identified bacteria in the nucleotide database of GenBank

Based on the above results, the authors designated strain N4 as *Enterococcus faecium* N4. The strain metabolized cellobiose, glucose, and xylose into high-purity L-lactic acid well, and has potential for metabolizing a glucose/xylose/cellobiose mixture to L-lactic acid.

CONCLUSIONS

1. Fifteen lactic acid bacteria strains were isolated from cow dung, haystack, and sheep manure. Four strains were evaluated through Gram stain, colony morphology, and catalase activity tests.
2. Strain N4 converted cellobiose, glucose, and xylose to lactic acid and obtained a high L-lactic acid yield and the optical purity exceeded 95%.
3. Through a phylogenetic tree analysis, strain N4 was most closely related to *Enterococcus faecium*, and the authors designated it as *Enterococcus faecium* N4.

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