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Production of optically pure L-lactic acid from lignocellulosic hydrolysate by using a newly isolated and D-lactate dehydrogenase gene-deficient *Lactobacillus paracasei* strain

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HIGHLIGHTS

- *L. paracasei* 7B could produce lactic acid with excellent production efficiency.
- *L. paracasei* 7B could tolerate to inhibitors produced from pretreated lignocellulose.
- *ldhD* deficient strain 7BL could produce optically pure L-lactic acid.
- *L. paracasei* 7BL could produce L-lactic acid from lignocellulosic hydrolysate.
- *L. paracasei* 7BL represents a potential for large-scale industrial application.

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ABSTRACT

The use of lignocellulosic feedstock for lactic acid production with a difficulty is that the release of inhibitory compounds during the pretreatment process which inhibit the growth of microorganism. Thus we report a novel lactic acid bacterium, *Lactobacillus paracasei* 7BL, that has a high tolerance to inhibitors and produced optically pure L-lactic acid after the interruption of *ldhD* gene. The strain 7BL fermented glucose efficiently and showed high titer of L-lactic acid (215 g/l) by fed-batch strategy. In addition, 99 g/l of L-lactic acid with high yield (0.96 g/g) and productivity (2.25–3.23 g/l/h) was obtained by using non-detoxified wood hydrolysate. Rice straw hydrolysate without detoxification was also tested and yielded a productivity rate as high as 5.27 g/l/h. Therefore, *L. paracasei* 7BL represents a potential method of L-lactic acid production from lignocellulosic biomass and has attractive application for industries.

1. Introduction

Lactic acid is a valuable organic acid widely used in the food, pharmaceutical, cosmetic, and chemical industries. Recently, there has been growing interest in the use of lactic acid as a building block of poly-lactic acid (PLA), a biodegradable and environmentally friendly alternative to petrochemical-derived plastics. Lactic acid is chiral, and thus, has two optical isomers, L-(+)-lactic acid and D-(−)-lactic acid. The optical purity of L- and D-lactic acid is important for the industrial application of PLA because poly (DL-lactic acid) (PDLLA) is an amorphous polymer (Nair and Laurencin, 2007).

Presently, almost all lactic acid used globally is produced via microbial fermentation and accordingly, lactic acid bacteria (LAB) have been subjected to considerable investigation relative to other microorganisms (Abdel-Rahman et al., 2013). The genome of LAB encode for stereospecific L- or D-lactate dehydrogenase that can convert fermentable sugars, such as hexoses and pentoses, to lactic acid. However, the optical purity of the produced lactic acid depends on the type of LAB. For example, *Lactobacillus delbrueckii* subsp. *Lactobacillus coryniformis*, *Lactobacillus jensenii*, and *Lactobacillus vitulinus* produce D-lactic acid; *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* produce L-lactic acid; and *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus sake*, *and Lactobacillus acidophilus* produce DL-lactic acid (Mack, 2004).

The use of inexpensive non-food materials appears to be more attractive for biorefining because these materials are cheap, abundant, and do not compete with the food market. For example, rice straw is the most abundant agricultural waste worldwide and 90% of this biomass is found in Asia (Hsu et al., 2006; Kim and Dale,

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2004). Wood is another potential feedstock that contains higher proportions of glucose and lignin than gramineous feedstock. In addition, the establishment of co-localized biorefinery plants and plywood factories would be an advantageous step toward the production of biofuel or biochemical from waste wood.

Lignocelluloses comprise three main components: cellulose, hemicelluloses, and lignin. Different pretreatment methods and enzymatic hydrolytic procedures to release fermentable sugars prior to fermentation have been studied. However, these methods also produce some substances with inhibitory activity against microorganisms, such as furan derivatives, phenolic compounds, and weak organic acids. Many studies have been devoted to the development of different detoxification processes to remove or neutralize such inhibitors (Cavka and Jonsson, 2013; Huang et al., 2009; Jonsson et al., 2013; Koopman et al., 2010; Lin et al., 2012b; Soudham et al., 2014; Thomsen et al., 2009). However, the additional detoxification processes may complicate the fermentation process and increase the manufacturing costs of lactic acid. Therefore, the investigation of strains resistant to these inhibitors has received considerable attention (Boguta et al., 2014; Peng et al., 2013; Zhang et al., 2014).

The purpose of this study was aimed to isolate an ideally tolerant strain for lactic acid production from lignocellulosic biomass. The potential candidate strain was further engineered to produce absolutely pure and polymer-grade L-lactic acid. Furthermore, the experimental conditions of batch and fed-batch fermentation for pure L-lactic production were optimized using glucose or lignocellulosic hydrolysates as a carbon source. This work has attractive applications for large-scale production of lignocellulose-based L-lactic acid.

2. Methods

2.1. Isolation of lactic acid bacteria

LAB were isolated from soil and putrid fruits according to the following protocol: 1 g of each sample was mixed with 3 ml phosphate-buffered saline (PBS). The mixed solution was diluted to 10^3–10^6 in PBS and the dilutions were spread directly on Man Rogosa Sharpe (MRS) agar plates containing 0.04% chlorophenol red as an acid indicator. The samples were incubated under anaerobic conditions (AnaeroPack, Thermo scientific, USA) at 37 °C for 1–3 days. After incubation, acid-producing bacteria were isolated and stored in MRS broth with 20% (v/v) glycerol at −80 °C. Subsequently, 0.1 ml of each stock bacterium was inoculated in 10 ml MRS broth in a 50-ml Erlenmeyer flask and cultured for 24 h without agitation. The lactic acid concentration and optical purity of the culture supernatants were subsequently analyzed.

2.2. Preparation of lignocellulosic hydrolysate

Waste wood chips were obtained from a plywood plant and rice straw was collected from a local farm near Taoyuan, Taiwan. A separate hydrolysis and fermentation (SHF) procedure was used to produce L-lactic acid. SHF media were obtained from the ton-scale pilot plant at the Institute of Nuclear and Energy Research (Taoyuan, Taiwan) via acid-catalyzed steam explosion processes and enzymatic hydrolysis procedures (Chen et al., 2013; Hsu et al., 2006). The compositions of the different SHF media used in this study are shown in Table 1. L1 and L2 SHF media were produced from wood chips but incorporated different solid contents in the enzymatic hydrolysis process, which resulted in differences in sugar and inhibitor compositions. L3 SHF medium was produced from rice straw.

Table 1
SHF mediums produced from lignocellulosic feedstocks.

No.	L1	L2	L3
Feedstock	Wood chip	Wood chip	Rice straw
Solid content[a] (%)	20	30	20
Glucose (g/l)	85.66 ± 0.01	120.22 ± 0.01	83.50 ± 0.01
Xylose (g/l)	15.56 ± 0.01	56.12 ± 0.01	26.11 ± 0.01
Acetic acid (g/l)	8.78 ± 0.01	15.91 ± 0.01	4.05 ± 0.01
HMF (g/l)	0.71 ± 0.01	1.53 ± 0.01	0.21 ± 0.01
Furfural (g/l)	0.85 ± 0.01	2.86 ± 0.01	0.25 ± 0.01

HMF: 5-hydroxymethylfurfura.
[a] The proportion of dry solid residue in enzymatic hydrolysis.

2.3. Screening of lactic acid bacteria using an enzymatic lignocellulosic hydrolysate

Among the isolated bacteria, 11 potential strains, including *Lactobacillus*, *Bacillus*, and Weissella spp., and another five strains obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan), were used to test the ability to utilize SHF media as well as resistance towards a furan inhibitor from pretreated lignocellulose.

Experiments were conducted in a 1-l bioreactor containing 600 ml L1 SHF medium in which 5 g/l yeast extract was added as a nitrogen source. The seed culture was prepared by adding 0.1 ml stock culture to a test tube containing 5 ml MRS medium, followed by incubation at 37 °C for 16 h without agitation. This culture was added to 150 ml MRS medium in a 250-ml Erlenmeyer flask and incubated at 37 °C on a shaking incubator until the optical density (OD) at 600 nm was approximately 5.0. Following incubation, the seed culture was inoculated into the bioreactor and cultured at 37 °C, pH 6.5, 200 rpm. Samples were taken at 48 h for further analysis.

2.4. Constructing ldhD-deficient L. paracasei 7B

In *L. paracasei* 7B, *ldhD* gene was disrupted using a non-replicative pBTEC-dD plasmid with a double-crossover homologous integration-based method described by Okano et al. (2009). The primers and plasmids used in this study are listed in Table 2. A region upstream of *ldhD* was amplified from *L. paracasei* 7B genomic DNA using the dDN-F/R primer pair and digested with *BamH*I and *EcoR*I. The resulting fragment was cloned into the pBTE plasmid at the corresponding site. Similarly, a downstream fragment of *ldhD* was amplified using the dDC-F/R set and cloned into the *EcoR*I and *Cla*I site to yield the pBTE-dD plasmid. For a selection marker, a chloramphenicol resistance gene was amplified from pC194 using the Cm-F/R primer pair, digested with *EcoR*I, and cloned into pBTE-dD at the *EcoR*I site to yield the recombination plasmid pBTEC-dD.

pBTEC-dD was introduced into *L. paracasei* 7B via electroporation as described previously (Lin et al., 2012a). After electroporation, pBTEC-dD-transfected *L. paracasei* 7B were cultivated at 37 °C under antibiotic-selective conditions to obtain *ldhD*-deficient *L. paracasei* 7B (Δ*ldhD*) mutants that had undergone gene excision through a double-crossover homologous event. The gene deletion was confirmed via polymerase chain reaction (PCR) with the DldhA-F/R primers, which are the forward and reverse primers that anneal to the upstream and downstream regions of *ldhD*, respectively. The resulting mutant strain was designated as 7BL.

2.5. Optimization of 7BL fermentation conditions

All experiments were performed in a 5-l bioreactor at an agitation speed of 200 rpm to ensure complete broth mixing. Samples