Running title: Optical purity L-lactate synthesis in *B. coagulans*

Major Role of NAD-Dependent Lactate Dehydrogenases in High Optically Pure L-Lactic Acid Production by Thermophilic *Bacillus coagulans*

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Abstract

*Bacillus coagulans* 2-6 is an excellent producer of optically pure L-lactic acid. However, little is known about the synthesis mechanism for high optical purity of L-lactic acid produced by this strain. Three enzymes responsible for lactic acid production, L-nLDH (encoded by *ldhL*), D-nLDH (encoded by *ldhD*), and glycolate oxidase (encoded by *go*), were systematically investigated to study the relationship between enzymes and the optical purity of lactic acid. *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081 (D-lactic acid producer) and *L. plantarum* ssp. *plantarum* DSM 20174 (DL-lactic acid producer) were also examined as comparative strains in this study, in addition to *B. coagulans*. The specific activities of key enzymes for lactic acid production in the three strains were characterized *in vivo* and *in vitro*, and the levels of *ldhL*, *ldhD*, and *go* transcription during fermentation were also analyzed. Catalytic activities of L-nLDH and D-nLDH were different in L-, D-, and DL-lactic acid producers. Only L-nLDH activity was detected in *B. coagulans* 2-6 under native conditions, and *ldhL* transcription in *B. coagulans* 2-6 was much higher than that of *ldhD* or *go* at all growth phases. However, for the two *Lactobacillus* strains used in this study, *ldhD* transcription levels were higher than those of *ldhL*. The high catalytic efficiency of L-nLDH toward pyruvate and high transcription ratio of *ldhL* to *ldhD* and *go* provide the key explanations for the high optical purity of L-lactic acid produced by *B. coagulans* 2-6.

**Keywords:** Lactate dehydrogenase, Optical purity, L-Lactate, *Bacillus coagulans*
Introduction

Lactic acid is among the top 30 building block chemicals of biomass, and it has versatile applications in the food, pharmaceutical, cosmetic, and chemical industries. The use of lactic acid in the synthesis of polylactic acid (PLA) has been consistently on the rise (1). PLA is a renewable and biodegradable material and is regarded as an environmentally friendly alternative to traditional plastics derived from fossil fuels (2). Since only the optically pure L- and D-lactic acid monomers can be used as precursors of PLA, the production of optically pure lactic acid is a prerequisite for polymer synthesis (1, 3, 4).

Microbial fermentation is an efficient approach for producing lactic acid with high optical purity. Many microorganisms, such as fungi, Lactobacillus species, Bacillus coagulans, and various genetically modified strains, can produce lactic acid (1, 2, 5, 6). The bacterial genera that form lactic acid are often called lactic acid bacteria (LAB). The fermentative metabolism of LAB is characterized by the glycolytic breakdown of carbohydrates, and the late step in this pathway is distinguished by the conversion of pyruvate into lactic acid, a reaction that oxidizes the NADH formed during glycolysis, thus maintaining cellular redox balance (7). The enzymes responsible for the conversion of pyruvate to lactic acid and back are lactate dehydrogenases (LDHs), which fall into two broad classes—NAD-dependent lactate dehydrogenases (nLDHs) and NAD-independent lactate dehydrogenases (iLDHs). nLDHs are cytoplasmic proteins that catalyze the conversion of pyruvate to lactic acid in a reversible or irreversible manner. iLDHs are membrane bound, and they catalyze
the oxidation of lactic acid to pyruvate (8). Members of these two classes exist in a stereospecific (L- or D-specific) form.

Almost all LAB have LDHs, but the optical purities of lactic acid produced by various LAB are markedly different (1). NAD-dependent L-lactate dehydrogenases (L-nLDHs; EC 1.1.1.27) are responsible for the synthesis of L-lactic acid, whereas D-lactic acid can be produced in two ways: NADH-dependent reduction of pyruvate by the NAD-dependent D-lactate dehydrogenases (D-nLDHs; EC 1.1.1.28) or racemization of L-lactic acid by an L-lactic-acid-inducible lactate racemase (9, 10).

L-nLDHs differ across genera and species and even among strains of the same species; the same is true for D-nLDHs (11). Sequence alignment has shown that L-nLDHs and D-nLDHs, encoded by ldhL and ldhD, belong to two distinct families, the NAD-dependent L- and D-2-hydroxyacid dehydrogenases, respectively (12). Lactate racemase (EC 5.1.2.1) can only be found in strains that form D,L-lactic acid, such as Staphylococcus urease, Lactobacillus curvatus, L. paracasei, L. plantarum WCFS1, L. sakei, and Clostridium butylicum (1, 11). Although L-nLDHs and D-nLDHs are the key synthetic enzymes for L-lactic acid and D-lactic acid, respectively, studies showed that a mutation defective for both genes encoding L- and D-nLDHs from L. plantarum, an organism producing a mixture of 50% L- and 50% D-lactic acid, does not result in a complete lack of lactic acid production (10). The same situations were also found in other strains, such as L. fermentum (13) and L. casei BL23 (14). The different activities of L- and D-nLDHs contribute to variations in the ratio of the two isomers (1, 15). Because the optical purities of lactic acid differ, and there are few reported
studies focusing on the relationship between the enzymes and the optical purity of lactic acid, a study of the crucial factors affecting lactic acid optical purity is necessary.

Thermophilic Bacillus species have several traits, such as the capacity to withstand relatively low pH, high temperature, and various other harsh conditions, which could be beneficial in industrial strains and improve the commercial competitiveness of lactic acid production (16). B. coagulans can metabolize an extensive range of sugars, such as pentoses and hexoses in lignocellulosic biomass (16, 17). Compared to the most frequently used lactic acid producers such as Lactococcus lactis and L. rhamnosus, B. coagulans can grow optimally at 50-55°C, which is expected to minimize contamination in industrial-scale fermentations under non-sterile fermentation conditions. Therefore, in recent years, there has been an interest in studies on optical purity of L-lactic acid produced by this species (17, 18, 19).

However, the mechanism of high optically pure L-lactic acid production by this species has never been demonstrated.

In this study, a high optical purity L-lactic acid producer (optical purity >99%), B. coagulans 2-6, was chosen as a representative strain. Three enzymes responsible for lactic acid production, L-nLDH, D-nLDH, and glycolate oxidase ([GOX]; EC 1.1.3.15, encoded by go), were annotated from its whole-genome sequence (21). To systematically investigate the relationship between enzymes and the optical purity of lactic acid, L. delbrueckii ssp. bulgaricus DSM 20081 (D-lactic acid producer) and L. plantarum ssp. plantarum DSM 20174 (D,L-lactic acid producer) were also selected as
comparative strains for study.

Materials and Methods

Bacteria and culture conditions. B. coagulans 2-6 was isolated by our laboratory and used in this study. It is a homofermentative producer of L-lactic acid, with an optical purity of 99% (20, 21). L. delbrueckii ssp. bulgaricus DSM 20081 (D-lactic acid optical purity of 98%) and L. plantarum ssp. plantarum DSM 20174 (1:1 ratio of L-lactic acid to D-lactic acid) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). These three strains were chosen, because they produce different types of lactic acid (l-, d-, and dl-, respectively). These strains were maintained on de Man, Rogosa, and Sharpe (MRS) agar slants. The pH was adjusted to 6.2-6.5. Unless otherwise stated, the incubation temperature for B. coagulans 2-6, L. delbrueckii ssp. bulgaricus DSM 20081, and L. plantarum ssp. plantarum DSM 20174 were 50°C, 37°C, and 30°C, respectively. Fully grown slants were stored at 4°C.

The seed culture was prepared as follows: a loop of cells from the fully grown slant was inoculated into 50 mL MRS broth in 100 mL Erlenmeyer flasks and incubated for 12 h at an appropriate temperature without agitation.

Cloning and overexpression of the enzymes responsible for lactic acid production. Both ldhL and ldhD genes were shown to be distributed in the three strains. According to the whole-genome sequence of B. coagulans 2-6 (GenBank accession number: CP002472), the go gene (coding GOX) was also present.
lactate racemase genes or homologs were found in any of the three strains (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Genes of interest were amplified by PCR using *Pfu* DNA polymerase (Thermo Fisher Scientific) from genomic DNA of the respective organisms. Seven DNA fragments were cloned into the expression vector (*pETDuet-1*, with T7 promoter), respectively. The resultant recombinant plasmids were as follows: *pETDuet-1*-2-*6ldhL*, *pETDuet-1*-2-*6ldhD*, *pETDuet-1*-2-*6go*, *pETDuet-1*-20081-*ldhL*, *pETDuet-1*-20081-*ldhD*, *pETDuet-1*-20174-*ldhL*, and *pETDuet-1*-20174-*ldhD*. The recombinant plasmids containing *ldhL*, *ldhD*, and *go* genes were transformed into competent *E. coli* BL21 (DE3) cells, respectively. For the expression of the recombinant proteins, transformed cells were grown to an optical density of $A_{600}=0.6$ at 37°C, then induced with 1 mM isopropyl-$\beta$-D-1-thiogalactopyranoside, and grown for an additional 5 h at 23°C.

**Purification of the enzymes.** The *E. coli* cultures were pelleted by centrifugation (10,540 × g, 30 min). Cells were resuspended in binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 10 mM imidazole [pH 7.0]) and then disrupted by sonication for 10 min to obtain crude enzyme solutions. After the cell debris was removed by centrifugation (13,000 × g, 30 min, 4°C), the supernatant was used to obtain purified nLDHs and GOX using FPLC system with nickel column chromatography. Proteins were resolved by SDS-PAGE using 4-16% Bis-tris gel (Invitrogen) and detected by Coomassie Blue staining. Protein content was determined with a Bradford Protein Assay Kit (Bio-Rad). Absorption at 595 nm was measured and compared to a standard curve prepared with bovine serum albumin.
This purification was performed at 4-10°C, and the buffer used was potassium phosphate (pH 7.0) unless otherwise stated.

**In vitro enzymatic activity assays.** Enzyme activity assays were performed in transparent 96-well plates. Briefly, the reaction mixture (total volume, 200 μL) containing 100 mM sodium phosphate (pH 6.5), 200 μM NADH, and 0.1 mg/mL enzyme was pre-incubated at 45°C for 10 min. To start the reaction, sodium pyruvate was added to a final concentration of 20 mM, and NADH oxidation was monitored at 340 nm in a 96-well plate reader. Assay conditions for the nLDHs originating from *L. delbrueckii* ssp. *bulgaricus* DSM 20081 and *L. plantarum* ssp. *plantarum* DSM 20174 were similar to those described for *B. coagulans* 2-6, except that the reaction temperature for strain DSM 20081 was 35°C and 30°C for strain DSM 20174.

To measure the reverse enzymatic activities of nLDHs from lactic acid to pyruvate, the reaction mixture (total volume, 200 μL) contained 100 mM sodium phosphate (pH 6.5), 15 mM NAD, and 0.1 mg/mL enzyme. Different concentrations of L- or D- lactic acid were added to the reaction mixture to start the reaction and the NAD reduction was monitored at 340 nm under the respective optimal temperature.

Conditions for the assay of the GOX enzyme from *B. coagulans* 2-6 were as described previously (22). The reaction mixture (total volume, 200 μL) contained 200 mM Tris-HCl (pH7.5), 100 μM dichlorophenolindophenol, and 0.1 mg/mL enzyme. The reaction was initiated with the addition of 20 mM sodium lactate, and the change in absorbance at 578 nm was determined in a 96-well plate reader at 30°C.

Kinetic parameters of purified enzymes were determined by varying the
concentration of one substrate over a wide range while maintaining the other at near-saturating levels. The reaction mixture (total volume, 200 μL) contained 100 mM sodium phosphate (pH 6.5), 200 μM NADH, and 0.1 mg/mL enzyme. To start the reaction, different concentrations of pyruvate were added to the reaction mixture, and NADH oxidation was monitored at 340 nm in a 96-well plate reader.

One unit (1 U) of nLDH activity was defined as the amount of enzyme required to reduce 1 μmol NAD per minute. One unit (1 U) of GOX activity was defined as the amount of enzyme that caused an initial change in absorbance of 0.001/min at 578 nm (22).

**In vivo enzymatic activity assays.** To assay the enzymatic activities in vivo, whole-cell extracts were used as described previously (20), with some modifications. Fermentations were performed in 500 mL Erlenmeyer flasks containing 200 mL of MRS broth. The cultures were inoculated for 24 h at an appropriate temperature without agitation. Exponentially growing cells were harvested by centrifugation (10,540 × g, 30 min, 4°C) and washed with 0.85% (w/v) physiological saline. Cell pellets were subsequently suspended in 100 mM potassium phosphate buffer (pH 7.0) and disrupted by sonication in an ice bath. After centrifugation at 12,000 ×g for 10 min, the supernatants were used as the crude cell extracts. The reaction mixture, containing 100 mM sodium phosphate (pH 6.5), 200 μM NADH, 20 mM pyruvate, 0.1 mg/mL crude cell extract, was pre-incubated at 37°C for 10 min. After incubation, the concentrations of L- and D-lactic acid were analyzed by high performance liquid chromatography (HPLC).
In vivo enzymatic activities were also confirmed by active staining of nLDHs, after native-PAGE, according to a previous report (20) with some modifications. Crude enzymes of the three representative strains were concentrated by ultrafiltration and separated by native-PAGE on gradient 4-20% native polyacrylamide gels. After electrophoresis, gels were cut into sections for active staining. The gel section with the molecular weight marker was soaked in Coomassie Brilliant Blue R-250. The other parts were soaked with 100 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM phenazinemethosulfate; 0.1 mM nitrotetrazolium blue chloride; 2 mM NAD; and 100 mM D-lactate, L-lactate, or DL-lactate, depending on the enzyme substrate preference. The staining mixture and gel sections were shaken gently until clear blue bands appeared.

**Quantitative real-time (RT)-PCR.** Quantitative RT-PCR was employed to determine the transcription levels of the genes encoding key enzymes at different fermentation periods. Initially, *B. coagulans* 2-6, *L. delbrueckii* ssp. *bulgaricus* DSM 20081, and *L. plantarum* ssp. *plantarum* DSM 20174 were inoculated in MRS broth at 50°C, 37°C, and 30°C, respectively, to measure the growth curve and time courses of optical purity during fermentation for the determination of sample points. Cells of the three representative strains were harvested by centrifugation (5,000 × g for 10 min, 4°C) for RNA isolation by using an E.Z.N.A.™ Bacterial RNA Kit (Omega). Total RNA concentration was determined from the absorbance at 260 nm (NanoVue, GE). Using appropriate gene-specific primers (Table 1), cDNA copies were synthesized with a FastQuant RT Kit (with gDNase) (Tiangen, China) and amplified with SYBR
Premix Ex Taq (TaKaRa, China). The threshold cycle for each PCR with different concentrations of cDNA was determined and compared against a standard DNA (16S rRNA gene) that was also analyzed at the same time (23, 24). From these results, a ratio of the concentration of gene-specific mRNA in the sample was calculated. Reported results are the average of at least three experiments with variation less than 15%.

**Analytical methods.** The optical purity of L- and D-lactic acid was analyzed using HPLC (Agilent 1260 Series, Hewlett-Packard, USA) equipped with a chiral column (MCI GEL CRS10W, Japan) (1). The mobile phase was 2 mM CuSO$_4$ at a flow rate of 0.5 mL/min (25°C), with UV detection at 254 nm. The optical purity of L-lactic acid was defined as $\frac{(L-\text{lactic acid})}{(L-\text{lactic acid})+(D-\text{lactic acid})} \times 100\%$. The optical purity of D-lactic acid was defined as $\frac{(D-\text{lactic acid})}{(L-\text{lactic acid})+(D-\text{lactic acid})} \times 100\%$. Glucose and L-lactic acid concentrations were measured by an SBA-40C Biosensor Analyzer (Institute of Biology, Shandong Academy of Sciences, China). The growth curves were measured at $A_{600}$ by a 7200 Visible Spectrophotometer (UNICO, Shanghai, China).

**Results**

Characterization of specific activities of L- and D-nLDHs and GOX *in vivo* and *in vitro*. On the basis of obtaining the purified heterologously expressed His-tagged L- and D-nLDHs and GOX, the kinetic parameters of purified enzymes were studied (Table 2). For *B. coagulans* 2-6, both L-nLDH and D-nLDH were detected to have
catalytic activities. The catalytic efficiencies of recombinant nLDHs were calculated by $k_{\text{cat}}/K_m$. The catalytic efficiency toward pyruvate of L-nLDH ($5.8 \times 10^3 \, \text{M}^{-1} \, \text{s}^{-1}$) was higher than that of D-nLDH ($1.9 \times 10^3 \, \text{M}^{-1} \, \text{s}^{-1}$). Furthermore, the $K_m$ value of L-nLDH (3.8 mM) was lower than that of D-nLDH (5.9 mM), which indicated that L-nLDH had a higher affinity for pyruvate than D-nLDH. Although the go genes (coding GOX) were found in the B. coagulans 2-6 genome, heterologously expressed GOX exhibited no detectable activity. Furthermore, our study showed that nLDHs of B. coagulans 2-6 could convert pyruvate to lactic acid in a reversible manner. It could convert lactic acid to pyruvate when strains were grown in the media containing L- or D-lactic acid (Table 3). For L. delbrueckii ssp. bulgaricus DSM 20081 (D-lactic acid producer), L-nLDH activity was not detected. Furthermore, D-nLDH of L. delbrueckii ssp. bulgaricus DSM 20081 exhibited the highest catalytic efficiency among the three D-nLDHs. For L. plantarum ssp. plantarum DSM 20174 (DL-lactic acid producer), the activities of both L-nLDH and D-nLDH were detected, and D- and L-nLDH showed similar catalytic efficiencies with pyruvate as the substrate.

To further address whether nLDHs showed different catalytic activities in vivo, enzymatic activities of L-nLDHs and D-nLDHs in the three strains were determined using whole-cell extracts of wild strains. Exponentially growing cells were collected, and the reduced products, L- and D-lactic acid, were measured to evaluate enzymatic activity. As shown in Table 4, contrary to the results obtained in vitro, D-nLDH of B. coagulans 2-6 showed no enzymatic activity. High activity was observed for L-nLDH of B. coagulans 2-6. In L. delbrueckii ssp. bulgaricus DSM 20081 (D-lactic acid
producer), the specific activity of D-nLDH (8.30 μmol min⁻¹ mg⁻¹) was much higher than that of L-nLDH (0.17 μmol min⁻¹ mg⁻¹). In *L. plantarum* ssp. *plantarum* DSM 20174 (DL-lactic acid producer), the specific activities of L- and D-nLDHs were similar.

The *in vivo* enzymatic activities were also confirmed by active staining of nLDHs after native-PAGE (Figure 1). D-nLDH activities were detected in *L. plantarum* ssp. *plantarum* DSM 20174 (DL-lactic acid producer) and *L. delbrueckii* ssp. bulgaricus DSM 20081 (D-lactic acid producer) but not in *B. coagulans* 2-6. Both D- and L-nLDH activities were detected in *L. plantarum* ssp. *plantarum* DSM 20174 (DL-lactic acid producer) and only D-nLDH activities were detected in *L. delbrueckii* ssp. bulgaricus DSM 20081 (D-lactic acid producer). Furthermore, GOX was reported to catalyze the oxidation of lactic acid to pyruvate. For *B. coagulans* 2-6, only one fragment was observed when gels were soaked with L-lactate and DL-lactate, which mean that only L-nLDH activities were detected in *B. coagulans* 2-6. GOX activities were undetectable using active staining by native-PAGE analysis.

**Variation of optical purities during fermentation.** The optical purities of lactic acid produced by three representative strains differed during fermentation. For *B. coagulans* 2-6, the optical purity of L-lactic acid was 83.7% initially. The optical purity dramatically increased to 98.1% within 6 h and then reached 99.8% at 48 h (Figure 2a). For *L. delbrueckii* ssp. bulgaricus DSM 20081 (D-lactic acid producer), the upward trend in optical purity of D-lactic acid was similar to that of L-lactic acid produced by *B. coagulans* 2-6. The optical purity of D-lactic acid increased from 89.9%
14 at 0 h to 98.9% at 48 h (Figure 2b). For *L. plantarum* ssp. *plantarum* DSM 20174 (DL-lactic acid producer), the optical purity of L-lactic acid increased from 29.0% at 6 h to 51.8% at 48 h, and the optical purity of D-lactic acid decreased from 71.6% at 6 h to 48.2% at 48 h (Figure 2c and 2d). The ratio of L-lactic acid to D-lactic acid optical purities was approximately 1:1 at the end of the experiment.

**Changes in transcription levels of ldhL, ldhD, and go during fermentation.** To examine the transcription levels of the genes encoding nLDHs and GOX, RT-PCR assays were performed. According to the growth curves of the representative strains (data not shown) and the time courses of optical purity values, cells of the three representative strains at the different phases (exponential phase, stationary phase, and decline phase) were collected to measure the transcription levels.

The $2^{\Delta \Delta CT}$ relative quantification method was used to determine the mRNA levels, and 16S rRNA was used as the internal reference. For *B. coagulans* 2-6, *ldhL* transcription levels were higher than those of *ldhD* and *go* at different growth phases (Figure 3a and 3b). The transcription ratio of *ldhL* to *ldhD* increased from 130-fold at the exponential phase to 216-fold at the decline phase. Although *go* genes were found in the genome of *B. coagulans* 2-6, the GOX enzymatic activity was not detectable *in vivo* or *in vitro*. Thus, as expected, *go* transcription levels were also rather low, and the transcription ratio of *ldhL* to *go* was 1323-fold at the exponential phase. For *L. delbrueckii* ssp. *bulgaricus* DSM 20081 (D-lactic acid producer), *ldhD* transcription levels were higher than those of *ldhL*, from 13-fold to 22-fold at different growth phases (Figure 3c). For *L. plantarum* ssp. *plantarum* DSM 20174...
(DL-lactic acid producer), \(ldhD\) transcription levels were slightly higher than those of \(ldhL\), from 3-fold to 12-fold at different growth phases (Figure 3d). The transcription levels of key enzymes encoding genes in three representative strains were high at the exponential phase, and decreased gradually from the exponential phase to the stationary phase, then reached their minimum in the decline phase (Figure S1 in Supporting Information File).

**Discussion**

*Lactobacillus* species are representative lactic acid producers, and they are known to produce high yields of lactic acid (25). However, a limitation of lactic acid production is that the optical purity does not satisfy the demands for polymer-grade PLA (20). Efforts have been made to inactivate the nLDHs responsible for \(L\)- or \(D\)-lactic acid formation in *Lactobacillus* species (9, 10). Studies showed that in the absence of one active nLDH, pyruvate can be metabolized by several alternative pathways. Thus, this method does not dramatically increase the optical purity of lactic acid (7). *B. coagulans* 2-6 has merits of high fermentation temperature, high productivity, high yield, and the product with high optical purity (20). The 99.8% optical purity of L-lactic acid produced by *B. coagulans* 2-6 signifies that it is a good producer for polymer-grade L-lactic acid. In addition, *B. coagulans* 2-6 grows optimally at 50-55°C, which is also expected to minimize contamination caused by mesophilic DL-lactic acid producers in the industrial scale fermentation. Non-sterilized batch fermentations for L-lactic acid production were performed using *B. coagulans*...
2-6, and studies showed that the optical purity of L-lactic acid was higher than 99%, without contamination during the open operations (20). In recent years, there has been interest in lactic acid production by B. coagulans. Although optically pure lactates are synthesized from pyruvate by the catalysis of chiral-specific D- or L-LDHs (26), the mechanism to produce high optically pure L-lactic acid by B. coagulans has not been demonstrated.

L-nLDH (encoded by ldhL), D-nLDH (encoded by ldhD), and GOX (encoded by go) were annotated from the whole-genome sequence of B. coagulans 2-6 (21). In fact, both ldhL and ldhD are widely distributed in almost all sequenced or reported LAB (1). As a predominant redox product of catabolism, NADH plays an important role in over 700 biochemical reactions, a number of which are synthetically practical enzymatic reactions (27). The physiological role of nLDHs in bacteria is to regenerate NAD⁺ in a balanced way during fermentation. During this process, NADH, which is generated during the conversion of hexoses to pyruvate, is used as a cofactor. This reaction is an important step in the metabolism and energy conversion of living cells, because it allows re-oxidation of NADH, which is necessary for glycolysis. nLDHs play a complex role in B. coagulans. They catalyze pyruvate transformation to lactic acid and NADH oxidation. Furthermore, glucose consumption and growth rates were impaired in a B. subtilis strain defective for nLDHs (16).

In this study, the catalytic efficiencies of nLDHs from B. coagulans 2-6 were investigated. In vitro, both L-nLDH and D-nLDH activities were detected, and L-nLDH catalytic efficiency toward pyruvate was 3-fold higher than that of D-nLDH.
The 3.8 mM $K_m$ of $L$-nLDH for pyruvate in comparison to the 5.9 mM $K_m$ of $D$-nLDH suggests that $D$-nLDH has a lower affinity for pyruvate in *B. coagulans* 2-6 (Table 2). Although both $L$-nLDH and $D$-nLDH catalytic activities were detected *in vitro, in vivo* analysis and active staining studies showed that $D$-nLDH activity was not detectable in *B. coagulans* 2-6. The low or undetectable $D$-nLDH activity under native conditions may explain the high optical purity of $L$-lactic acid produced by *B. coagulans* 2-6.

Furthermore, the catalytic activities of $L$-nLDH and $D$-nLDH from *L. delbrueckii* ssp. *bulgaricus* DSM 20081 and *L. plantarum* ssp. *plantarum* DSM 20174 were comparatively studied *in vivo and in vitro*. For *L. delbrueckii* ssp. *bulgaricus* DSM 20081 ($D$-lactic acid producer), $D$-nLDH catalytic activity ($k_{cat}/K_m$) was the highest among the three strains, which is also higher than that of $D$-nLDH reported previously (1). Moreover, only $D$-nLDH activity was detected by active staining. For *L. plantarum* ssp. *plantarum* DSM 20174 ($DL$-lactic acid producer), both $L$-nLDH and $D$-nLDH activities were detected *in vivo and in vitro*, with a ratio of approximately 1:1. Our study confirmed that $L$-nLDH and $D$-nLDH are mainly responsible for primary $L$- and $D$-lactic acid production in LAB.

According to previous reports of other *L. plantarum* strains, catalytic activity of nLDH varies during the exponential phase (28). For *B. coagulans* 2-6, the optical purity of $L$-lactic acid increased gradually during the exponential phase until it reached its maximum at the stationary phase (Figure 2). For the other two representative strains (*L. delbrueckii* ssp. *bulgaricus* DSM 20081 and *L. plantarum* ssp. *plantarum* DSM 20174), the optical purity of lactic acid also varied at different
growth phases. Transcription of the genes encoding key enzymes was analyzed to determine the relationship between optical purity variations and gene transcription levels. *B. coagulans* 2-6 *ldhL*, *ldhD*, and *go* transcription levels decreased gradually from the exponential phase to the stationary phase, and levels reached their minimum in the decline phase (*Figure S1* in Supporting Information File). The gene transcription was high at the exponential phase, which correlates with the marked change in optical purity of L-lactic acid when the cells were in the exponential phase of growth. Subsequently, gene transcription decreased gradually and accordingly the optical purities were constant. However, the relative transcription levels of *ldhL* in *B. coagulans* 2-6 were much higher than those of *ldhD* and *go* at different growth phases, and the transcription ratio of *ldhL* to *ldhD* increased from the logarithmic phase to decline phase (*Figure 3*). Previous reports of various Lactobacillus strains showed that the transcription levels of *ldhD* were higher than those of *ldhL* in representative LAB, and there are no other obvious distinctions between *ldhL* and *ldhD* transcript levels among various Lactobacillus strains (1). In this study, we comparatively investigated the transcription of *ldhL* and *ldhD* in *L. delbrueckii* ssp. *bulgaricus* DSM 20081 (D-lactic acid producer) and *L. plantarum* ssp. *plantarum* DSM 20174 (D,L-lactic acid producer). *ldhD* transcription levels were higher than those of *ldhL* in both strains (*Figure 3*), which was similar to the previous report (1). D-lactic acid is important for the biosynthesis of the *L. plantarum* cell wall and D-lactic acid is incorporated at the last position of the peptidoglycan precursor instead of the usual D-alanine. This feature has also been observed in other Lactobacillus strains, such as *L.
Therefore, D-lactic acid is required for the growth of Lactobacillus strains. Even for the L-lactic acid producers (L. casei with 92% enantiomeric excess of L-lactic acid), ldhD transcription levels are higher than those of ldhL (1). Unlike Lactobacillus strains, ldhL transcription in B. coagulans 2-6 was much higher than that of ldhD at all growth phases, which may explain the high optical purity of L-lactic acid produced. Although D-nLDH activities were not detected under the native condition, the transcriptions of D-nLDH-encoding genes were detected by RT-PCR analysis. Western blot analyses were also conducted to detect the expression of D-nLDH using whole cell extracts of Bacillus coagulans 2-6. Although D-nLDH could not be detected by native-PAGE, it was confirmed that D-nLDH’s expression could be detected by western blotting (Figure S2 in Supporting Information File). Since western blotting, which could detect less than 1 pg of protein per band, have higher sensitivity than native-PAGE (about 0.5 µg of protein per band), it was supposed that D-nLDH activity of B. coagulans 2-6 was too low to be detected by native-PAGE, and its contribution to the optical purity of lactic acid appeared to be minimal in vivo. Meanwhile, the presence of very low level of transcription of ldhD also explains why B. coagulans 2-6 produced very small amounts of D-lactic acid.

GOX belongs to the flavoprotein oxidase class of enzymes, which is a large family of enzymes includes dehydrogenases and reductases that utilize nucleotide cofactors for oxidation-reduction reactions (29). GOX and iLDHs are members of the α-hydroxyacid-oxidizing flavoprotein group, and their main function is to oxidize lactate to pyruvate by a flavin-dependent mechanism (28). All iLDHs convert lactic
acid to pyruvate, and no evidence of a reverse reaction has been found (11). Although the genes encoding GOX were annotated in the *B. coagulans* 2-6 genome, the heterologously expressed enzyme showed no detectable activity *in vitro* (Table 2). Furthermore, active staining studies also showed that only L-nLDH activities were detected in *B. coagulans* 2-6 (Figure 1), which mean that GOX activities were too low to be detected *in vivo* using native-PAGE. However, the western-blot analysis showed that GOX’s expression could be detected (Figure S3 in Supporting Information File), which mean that the observed transcripts of the gene encoding GOX were not silenced. Notably, *ldhL* transcription levels were 209-fold to 1,323-fold higher than those of *go* *in vivo* (Figure 3). Lactic acid is a weak acid (*pK_a* = 3.86) that is uncharged and small enough to permeate the lipid membrane of cells (30). *B. coagulans* 2-6 cells likely encounter this acidic condition, especially in the decline growth phase. Under these conditions, GOX could catalyze the oxidation of L-lactic acid to pyruvate *in vivo* and allow strains to grow well in the medium containing L-lactic acid (31). Indeed, the transcription ratios of *ldhL* to *go* decreased from 1,323-fold at the exponential phase to 209-fold at the decline phase, indicating GOX might play growing role in *B. coagulans* 2-6. However, the contribution of GOX to the optical purity of lactic acid appeared to be minimal, if any, in *B. coagulans* 2-6.

In conclusion, L-nLDH and D-nLDH catalytic activities differed in L-, D-, and DL-lactic acid producers. Although L-nLDH (encoded by *ldhL*), D-nLDH (encoded by *ldhD*), and GOX (encoded by *go*) were annotated from the complete genome...
sequence of B. coagulans 2-6, only L-nLDH activity was detected in vivo. D-nLDH and GOX contributed weakly to L-lactic acid production in B. coagulans 2-6. L-nLDH’s high catalytic efficiency toward pyruvate and the high transcript ratio of ldhL to ldhD and go explains the mechanism of high optically pure L-lactic acid produced by B. coagulans 2-6. Studies revealing the synthesis mechanism of high optically pure L-lactic acid provided the direction for further strain improvements.

**Acknowledgments**

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**References**


Table 1. The specific primers for quantitative real-time PCR analyses.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Target gene</th>
<th>Sequence 5′→ 3′</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. coagulans 2-6</strong></td>
<td><em>ldhL</em></td>
<td>GTGTTTGGAACGGGTGCAGTTGGTAC&lt;br&gt;GTCTCCGAACTCTTTAAGCGCCAC</td>
</tr>
<tr>
<td></td>
<td><em>ldhD</em></td>
<td>CCTGAAACGGCAATGAAAGC&lt;br&gt;CCGCTCTCCATAAGGATACAAAG</td>
</tr>
<tr>
<td></td>
<td><em>go</em></td>
<td>AAAATCTGACGGTGACGGTTGAGC&lt;br&gt;CAACCGCTCCATTTCCGTCC</td>
</tr>
<tr>
<td></td>
<td>16S</td>
<td>GCAATGGAGAGGAAAGGAA&lt;br&gt;TAACACTCTGTGTTGCCGG</td>
</tr>
<tr>
<td><strong>L. delbrueckii ssp.</strong></td>
<td><em>ldhL</em></td>
<td>TGACAGAAGCAGCCTTAGATG&lt;br&gt;GCCACGCGATAGTAGGGTT</td>
</tr>
<tr>
<td><em>bulgaricus DSM 20081</em></td>
<td><em>ldhD</em></td>
<td>AAGATGAGCCTGCGTAAC&lt;br&gt;TTCGTCCATAGCCTTGTC</td>
</tr>
<tr>
<td></td>
<td>16S</td>
<td>TCAGTGCCCGAGCAACCG</td>
</tr>
<tr>
<td><strong>L. plantarum ssp.</strong></td>
<td><em>ldhL</em></td>
<td>ATCCTCGTTCCGTGTTGATG&lt;br&gt;AAGTTGATGATGCTGTAAGC</td>
</tr>
<tr>
<td><em>plantarum DSM 20174</em></td>
<td><em>ldhD</em></td>
<td>TGGTGTTATCGGTACTGGTC&lt;br&gt;TGTGGTAGTCTATCCTTAATGC</td>
</tr>
<tr>
<td></td>
<td>16S</td>
<td>CAGCCTACAACTCCGAACTGAGAA&lt;br&gt;TCGTTCGTGAGATGTTGGTT</td>
</tr>
</tbody>
</table>
Table 2. Kinetic parameters of purified His-tagged L-/D-nLDHs and GOX from the three representative strains.

<table>
<thead>
<tr>
<th></th>
<th>2-6</th>
<th>2-6</th>
<th>2-6</th>
<th>DSM 20081</th>
<th>DSM 20081</th>
<th>DSM 20174</th>
<th>DSM 20174</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-nLDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Km (mM)</td>
<td>5.9±0.2</td>
<td>3.8±0.3</td>
<td>ND</td>
<td>0.3±0.1</td>
<td>ND</td>
<td>2.7±0.3</td>
<td>1.24±0.1</td>
</tr>
<tr>
<td>Vmax (U/mg)</td>
<td>18.7±2.8</td>
<td>38.5±1.0</td>
<td>ND</td>
<td>384.6±2.6</td>
<td>ND</td>
<td>149.6±1.5</td>
<td>32.3±0.7</td>
</tr>
<tr>
<td>kcat (s⁻¹)</td>
<td>11.3±1.7</td>
<td>22.1±0.6</td>
<td>ND</td>
<td>235.5±1.6</td>
<td>ND</td>
<td>91.3±0.9</td>
<td>19.0±0.4</td>
</tr>
<tr>
<td>kcat/Km (M⁻¹s⁻¹)</td>
<td>(1.9±0.2) ×10⁻³</td>
<td>(5.8±0.2) ×10⁻³</td>
<td>ND</td>
<td>(7.9±0.5) ×10⁻⁶</td>
<td>ND</td>
<td>(3.4±0.0) ×10⁻⁵</td>
<td>(1.5±0.0) ×10⁻⁴</td>
</tr>
</tbody>
</table>

L-nLDH      |     |     |     |           |           |           |           |
| Km (mM)     |     |     |     |           |           |           |           |
| Vmax (U/mg) |     |     |     |           |           |           |           |
| kcat (s⁻¹)  |     |     |     |           |           |           |           |
| kcat/Km (M⁻¹s⁻¹) |     |     |     |           |           |           |           |

GOX        |     |     |     |           |           |           |           |
| Km (mM)     |     |     |     |           |           |           |           |
| Vmax (U/mg) |     |     |     |           |           |           |           |
| kcat (s⁻¹)  |     |     |     |           |           |           |           |
| kcat/Km (M⁻¹s⁻¹) |     |     |     |           |           |           |           |

ND, not detected

aD-nLDH, NAD-dependent D-lactate dehydrogenase

bL-nLDH, NAD-dependent L-lactate dehydrogenase

cGOX, glycolate oxidase
Table 3. Kinetic parameters of purified heterologously expressed His-tagged L- and D-nLDHs of *B. coagulans* 2-6 from lactic acid to pyruvate

<table>
<thead>
<tr>
<th></th>
<th>L-nLDH</th>
<th>D-nLDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mM)</td>
<td>4.96±2.37</td>
<td>9.54±1.12</td>
</tr>
<tr>
<td>$V_{max}$ (U/mg)</td>
<td>0.10±0.15</td>
<td>0.12±0.38</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>0.06±0.08</td>
<td>0.07±0.09</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</td>
<td>0.012±0.003</td>
<td>0.008±0.080</td>
</tr>
</tbody>
</table>
Table 4. The specific activities of L- and D-nLDHs from the three strains in exponential phase.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific activity (μmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-nLDH</td>
</tr>
<tr>
<td>2-6</td>
<td>11.35±1.17</td>
</tr>
<tr>
<td>DSM 20081</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>DSM 20174</td>
<td>4.72±0.38</td>
</tr>
</tbody>
</table>

*ND, not detected.*
Figure Captions:

Figure 1. Active staining of nLDHs after native-PAGE.

Cell extracts of *B. coagulans* 2-6 (lane 1, 4 and 7), *L. plantarum* ssp. *plantarum* DSM 20174 (lane 2, 5 and 8), and *L. delbrueckii* ssp. *bulgaricus* DSM 20081 (lane 3, 6 and 9) were used for the native-PAGE. DL-Lactate (lanes 1, 2, and 3), D-lactate (lanes 4, 5, and 6), and L-lactate (lanes 7, 8, and 9) were used as substrates for active staining. M, molecular weight marker.

Figure 2. Variation of lactic acid optical purities during the fermentation process.

a) Time course of optical purity of L-lactic acid produced by *B. coagulans* 2-6. b) Time course of optical purity of D-lactic acid produced by *L. delbrueckii* ssp. *bulgaricus* DSM 20081. c) Time course of optical purity of D-lactic acid by *L. plantarum* ssp. *plantarum* DSM 20174, and d) Time course of optical purity of L-lactic acid produced by *L. plantarum* ssp. *plantarum* DSM 20174.

Figure 3. Determination of the relative transcription levels of *ldhD*, *ldhL*, and *go* by RT-PCR analyses.

Transcript ratio of (a) *ldhL* to *ldhD* at different growth phases in *B. coagulans* 2-6, (b) *ldhL* to *go* at different growth phases in *B. coagulans* 2-6, (c) *ldhD* to *ldhL* at different growth phases in *L. delbrueckii* ssp. *bulgaricus* DSM 20081, and (d) *ldhD* to *ldhL* at
different growth phases in *L. plantarum* ssp. *plantarum* DSM 20174. Error bars represent the standard deviations of the means of three independent experiments.