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Overexpression and characterization of L-lactate dehydrogenases from *Lacticaseibacillus casei* HY2782 to control post-acidification in yogurt

Abstract

Post-acidification is a common phenomenon in fermented milk that can cause adverse effects, in which lactic acid bacteria used as starter cultures continue the lactic acid fermentation during storage. Lactate dehydrogenase (LDH) is a key enzyme for lactic acid fermentation. Therefore, the control of LDH could be one of solutions to prevent post-acidification. In the present study, four genes (*ldhL*1, *ldhL*2, *ldhL*3, and *ldhL*4) encoding L-LDH were identified by whole genome sequencing of the *Lacticaseibacillus casei* HY2782, cloned into expression vector, pET22b (+), transformed into *Escherichia coli* BL21 (DE3), and expressed by IPTG induction. Four recombinant L-LDHs were purified using Ni-NTA agarose column and characterized. The molecular weight of purified L-LDHs were 30~37 kDa on SDS-PAGE. Among the four L-LDHs, L-LDH3 exhibited highest enzyme activity. The L-LDH3 exhibited maximal activity at 43℃ and pH 4.0 with 8 mM fructose 1,6-diphosphate (FDP) by 302,343.16 U/mg. In acidic condition, L-LDH3 was activated by 2 mM of Mg^{2+}, Ca^{2+} and Mn^{2+} ions, inhibited by Zn^{2+} and Cu^{2+} ions. In addition, activity of L-LDH3 was gradually decreased as KH_2PO_4 concentration increased. Consequently, the L-LDH3 seems to be major enzyme among the L-LDHs of *L. casei* HY2782 and may play an important role in lactic acid fermentation of this strain. Therefore, the inhibition of L-LDH3 activity using Zn^{2+}, $Cu^{2+}$and KH_2PO_4 could be one of solutions to mitigate the post-acidification caused by *L. casei* HY2782.

Keywords: Post–acidification; *Lacticaseibacillus casei*; lactate dehydrogenase, gene cloning

Introduction

Yogurt is one of the most popular fermented food products, and its consumption is thought to be beneficial to human health (Shiby and Mishra, 2013). The yogurt is produced through lactic acid fermentation using lactic acid bacteria (LAB), traditionally, *Lactobacillus delbruekii* subsp. *bulgaricus* and *Streptococcus thermophilus* are used. Yogurt also often contains probiotics, such as *Lactobacillus acidophilus*, *Lacticaseibacillus casei* and *Bifidobacteria* spp. (Heller, 2001; Kulp and Rettger, 1924; Lourens-Hattingh and Viljoen, 2001). Dairy companies use different combinations of these LAB starters or probiotics to give their yogurt products specific flavors and textures. However, the LAB often continue their lactic acid fermentation during the product's shelf life, and this phenomenon is known as "post-acidification". The phenomenon causes several adverse effects on yogurt products, such as too much acidic taste, decrease of viable LAB count and higher syneresis, which ultimately reduces the shelf life of the product (Abu-Jdayil and Mohameed, 2002; Donkor *et al*., 2006; Lubbers *et al*., 2004; Vinderola *et al.*, 2000). Post-acidification increases the size of casein particles, hydrophobic and electrostatic interactions between proteins, and consequently causes restructuring of the protein network (re-curding), increasing viscosity of the products (Deshwal *et al.*, 2021). The point is that lactic acid fermentation continues even with cold chain and the situation is much worse in tropical regions or developing countries with poor refrigeration systems. Therefore, this phenomenon needs to be controlled to maintain good quality of yogurt during transportation and storage.

Several methods have been suggested for the control of post-acidification, including adding preservative such as benzoic or sorbic acids, nisin, vanillin, bacteriocin, changing the ratio of *Lactobacillus* spp. and *Streptococcus* spp., modifying milk composition, applying pulse electric field, and using weak post-acidification bacteria or genetically modified bacteria as starter (Chanos *et al.*, 2020; Dave and Shah, 1997; Han *et al.*, 2012; Zhang *et al.*, 2011). However, these

methods have not provided satisfiable solution for post-acidification. Even though genetically modified bacteria have obvious potential, it still has trouble to be used in food due to consumer's negative perception (Bawa and Anilakumar, 2013; Maghari and Ardekani, 2011). Lately, Vieira *et al.* (2021) showed positive correlation between accumulation of bioactive amines in cow's fermented milk and post-acidification which further emphasizes the importance of controlling post-acidification. Consequently, the post-acidification is still a problem to be solved.

Lactic acid fermentation can be simply described as a metabolic process that produces cellular energy and the lactic acid, from glucose utilization. A glucose in converted to two molecules of pyruvate through glycolysis then the pyruvates are converted to lactate by lactate dehydrogenase (LDH) in the presence of cofactor NADH. For the LAB, this reaction is important in the concept of regeneration of NAD^+, which is needed in early fermentation stage as the oxidizing agent. Therefore, LDH is considered as a key enzyme of lactic acid fermentation, and control of this enzyme could be one of solutions to prevent post-acidification.

Different species of LAB produce D- and L-lactic acid with different ratio (Kandler and Weiss, 1986; Schleifer, 1986). In the case of *Lacticaseibacillus casei*, L-LDHs are important for lactic acid fermentation because *L. casei* produces L-lactic acid predominantly (Vijayakumar *et al.*, 2008). The L-LDHs of *L. casei* need fructose 1,6-diphosphate (FDP) for their proper activities (Hensel *et al.*, 1983), and these allosteric L-LDHs are reported to possess much higher activities then the non-allosteric ones (Jiang *et al.*, 2014).

In this sense, the objectives of this study were to identify the L-LDHs produced by *Lacticaseibacillus casei* HY2782; to clone and overexpress these enzymes in *Escherichia coli* BL21 (DE3) strain; and characterize enzymatic properties on the L-LDHs.

Materials and Methods

Escherichia coli strain DH5α and BL21 (DE3) were purchased from BioFACT (Daejeon, Korea) and Invitrogen (Massachusetts, USA), respectively. The cloning vector pGEM-T Easy was from Promega (Wisconsin, USA) and the expression vector pET22b (+) was from Merch KGaA (Darmstadt, Germany). Whole-genome sequencing of *L. casei* HY2782 and annotation were conducted by the ChunLab Whole Genome Analysis Service (ChunLab, Seoul, Korea). DNA extraction and purification kit from Qiagen (Hilden, Germany), enzymes from Takara Bio (Shiga, Japan) and T4 DNA ligase from Roche (Basel, Switzerland) were used for DNA cloning. The Ni–NTA agarose column was purchased from Qiagen. All the medium for microbial growth were purchased from Difco (New Jersey, USA) and all chemicals were from Sigma-Aldrich (Missouri, USA).

Bacterial strains, plasmids and culture conditions

L. casei HY2782 was supplied by hy Co., Ltd. (Seoul, Korea). *L. casei* HY2782 was inoculated into MRS broth at 37℃ anaerobically. *E. coli* DH5α and BL21 (DE3) were inoculated into Luria-Bertani (LB) broth with shaking or LB agar (1.5% agar, w/v) at 37℃. These strains were preserved in 10% (w/v) skim milk supplemented with 25% (v/v) glycerol at -80℃. pGEM-T Easy and pET22b (+) were used to construct expression vector (Table 1).

Plasmid construction

Four $_{L}$-LDH genes (Fig. S2) amplified from genomic DNA (Fig. S1) of *L. casei* HY2782 were inserted into the pGEM-T Easy vector, a linearized vector with a single 3-terminal thymidine at both ends (TA cloning; Holton and Graham, 1991). The constructed pJE vectors (Table 1, Fig. 1) were transformed into *E. coli* DH5α. Ampicillin (Amp, 100 μg/mL) and 5-bromo-4-chloro-3-indolyl-β-$_{D}$-galactoside (X-gal, 40 μg/mL) were supplemented in the LB agar for blue/white

screening for *E. coli* DH5α harboring pJEs (Sambrook *et al.*, 1989a). The pJEs were extracted from the recombinant strains (white colonies) and inserted to pET22b (+) vector through *Nde*I/*Xho*I digestion and ligation. Each constructed pET22b-*ldhL* (Table 1, Fig. 1) was transformed into *E. coli* BL21 (DE3) respectively.

Overexpression of recombinant proteins

The recombinant *E. coli* BL21 strains were grown in baffled flask containing LB broth supplemented with Amp (100 μg/mL) at 37°C and 150 rpm for 3 h, the optical density at 600 nm (OD_{600}) reached about 0.6. To induce the expression of *ldhL* genes, isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added to a final concentration of 1 mM. After incubated under each condition (Table 2) for each strain, the cells were collected by centrifugation and washed two times using 1× PBS buffer containing NaCl (8 g/L), KCl (0.2 g/L), Na_2HPO_4 (1.44 g/L), and KH_2PO_4 (0.24 g/L). Cell pellets were resuspended in same buffer and disrupted by sonication (pulse on, 2 s; pulse off, 10 s; 58% amplitude; VCX 500, Sonics, Connecticut, USA) on ice for 6 min, four cycles. Disrupted cells were centrifugated and the supernatants were filtered using syringe with 0.45 μm filter.

Recombinant protein purification and SDS–PAGE

Each cell lysates were loaded on Ni-NTA agarose column, and loaded samples were washed two times using wash buffer (Table 3). Then the L-LDHs were eluted using elusion buffers containing different concentration of imidazole (Table 3). The fractions containing eluted proteins were identified using 12% (w/v) SDS-PAGE. To identify approximate molecular weight of the proteins, Precision Plus Protein™ Dual Color Standards (Bio-Rad, California, USA) was loaded together. Electrophoresis was conducted at 100 V for 1 h and 40 min. The gels were stained in staining buffer (Table 3) with shaking for 1 h. Then the stained gels were destained in

destaining buffer (Table 3) with shaking for 4 h and further destained in distilled water with overnight (Sambrook *et al.*, 1989b). The factions indicated single band on SDS-PAGE were pooled. The purified proteins were concentrated and buffer-changed by centrifugation using Amicon Ultra (30 kDa, Merck Millipore, Massachusetts, USA), two times with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM ethylenediamine-tetraacetic acid (TE buffer, Bioneer, Daejeon, Korea).

Enzyme assays

The L-LDH activity for conversion of pyruvate to lactate was analyzed by measuring the reduction rate of NADH at 340 nm using UV spectrophotometer (Cary 300 UV VIS, Agilent technologies, USA). The enzyme reaction mixture contained citrate-citric acid buffer (50 mM, pH 4.0), 10 mM sodium pyruvate, 0.2 mM NADH, and 8 mM fructose 1,6-diphosphate (FDP) in a total volume of 3 ml. The reactions were conducted at 43℃ and initiated by adding each enzyme (Jiang *et al.*, 2014).

One unit was defined as the amount of enzyme required to catalyze the oxidation of 1 μmol of NADH per min. Beer-Lambert law was used to convert absorbance to concentration. Specific activity was expressed as units per mg of protein (Powers *et al.*, 2007).

$$\text{Absorbance per time} = \text{Absorptivity} \times \text{Concentration per time} \times \text{path length (cm)}$$

$$\text{Concentration per time (U)} = \frac{Absorbance\ per\ time}{Absorptivity \times Path\ length\ (cm)}$$

$$\text{Specific activity (U/mg)} = \frac{Absorbance\ per\ time}{6.3 \times \frac{enzyme\ (mg)}{reaction\ mixture\ (ml)}}$$

Effect of pH and temperature on enzyme activity

The optimal pH for L-LDH3 activity was determined in following buffers for each pH condition: 50 mM citrate-citric acid buffer (pH 3.0~6.5; 0.5 pH unit intervals), 50 mM Tris-HCl buffer (pH 7.0~8.0; 0.5 pH unit intervals), and 50 mM carbonate-bicarbonate buffer (pH 9.0~11.0; 1.0 pH unit intervals). The enzyme reactions were conducted as described previously at 43℃ with 8 mM FDP. The effect of pH on the enzyme stability was investigated using the enzyme pre-incubated at 37℃ for 1 h with different pH buffer (pH 3.0~11.0; 1.0 pH unit intervals), same buffers with specific pH that used for optimal pH assay, and the reaction was conducted at 43℃ in citrate-citric acid buffer (50 mM, pH 4.0) with 8 mM FDP.

The optimal temperature for L-LDH3 activity was determined by conducted the enzyme reaction at following temperature with 8 mM FDP: 4, 10, 25, 30, 37, 43, 50, 55, 60, and 65℃. The effect of temperature on the L-LDH3 stability was examined using the enzyme pre-incubated at different temperatures (25, 37, 43, 50, 55, 60, and 65℃) for 10 min in citrate-citric acid buffer (50 mM, pH 6.0).

The relative activity was determined by the highest specific activity as 100%. Each experiment was conducted in triplicate.

Effect of metal ions and KH_2PO_4 on enzyme activity

To investigate the effect of metal ions on L-LDH3 activity, $MgSO_4$, $CaCl_2$, $MnSO_4$, $ZnSO_4$, and $CuSO_4$ was added to the reaction mixture at final concentration of 2 mM. The enzyme reaction was conducted at 43℃ in citrate-citric acid buffer (50 mM, pH 4.0) with 10 mM sodium pyruvate, 0.2 mM NADH, 8 mM FDP, and 2 mM metal ions.

KH_2PO_4 was added to the reaction mixture at different final concentration (2, 4, 8, and 16 mM) to examine their inhibitory effects on L-LDH3 activity. The enzyme reaction was

performed at 43℃ in citrate-citric acid buffer (50 mM, pH 4.0) with 10 mM sodium pyruvate, 0.2 mM NADH, 8 mM FDP and different concentration of KH_2PO_4.

The relative activity was determined by the control (without metal ions or KH_2PO_4) as 100%. All experiments were conducted in triplicate.

Statistical analysis

All experiments were conducted in triplicate, and the results were expressed as means and standard deviation. Statistical significance ($p<0.05$) was determined using one-way analysis of variance (ANOVA; IBM SPSS Statistics 30, IBM, Armonk, NY, USA) along with Ducan's multiple range test.

Results and Discussion

Expression and purification of recombinant L-LDHs

The recombinant strains of *E. coli* BL21 harboring pET22b-*ldhL*s were induced the expression of each L-LDH after IPTG induction at each condition (Table 2). Then the expressed proteins were purified using Ni-NTA agarose column. The expression and purification of the recombinant protein were identified by SDS-PAGE analysis (Fig. 2). The molecular weight of L-LDHs was approximately 30~37 kDa on SDS-PAGE.

About the L-LDH1, the fractions eluted by elusion buffers containing 150, 200, 250 mM imidazole (Fig. 2) were pooled and concentrated using Amicon Ultra. First, the enzyme reaction was conducted at 25℃ in Tris-HCl buffer (50 mM, pH 7.0) with 10 mM sodium pyruvate, 0.2 mM NADH and 5 mM FDP. Absorbance at 340 nm was recorded every 2 min, however, no reduction was observed until 30 min (Fig. 3).

The L-LDH2 fractions eluted by elusion buffers containing 150, 200 mM imidazole were pooled and concentrated (Fig. 2). The enzyme activity was examined at same condition for L-LDH1. The L-LDH2 also exhibited no activity (Fig. 3).

The fractions of purified L-LDH3 eluted by elusion buffers containing 150, 200 mM imidazole were pooled and concentrated by centrifugation with Amicon Ultra (Fig. 2). The enzyme reaction mixture was prepared as described above. The reduction of absorbance at 340 nm was recorded every 2 s (Fig. 4). The specific activity of purified L-LDH3 was 3,990.42 U/mg.

Purified fractions of L-LDH4 eluted by elusion buffers containing 200, 250 mM imidazole were pooled and concentrated (Fig. 2). The enzyme reaction was performed as described before. Absorbance at 340 nm of reaction mixture was recorded every 2 min (Fig. 4). Purified L-LDH4 exhibited 165.82 U/mg of specific activity.

To summarize, the L-LDH3 showed highest enzyme activity among four L-LDHs of *L. casei* HY2782 at 25℃ with 5 mM FDP. Although L-LDH4 also indicated activity at same condition, the specific activity was almost 4% of that of L-LDH3. Other two L-LDHs, L-LDH1 and L-LDH2, exhibited no activity at this condition. Consequently, L-LDH3 seems to be a major L-LDH of *L. casei* HY2782.

Effect of FDP on L-LDHs activities

To verify the L-LDHs are FDP-dependent allosteric enzyme or not, effect of FDP on L-LDH3 and L-LDH4 activities was examined (Fig. 5). Different concentration of FDP (0~10 mM at 1 mM unit intervals) was added to enzyme reaction mixture comprising Tris-HCl buffer (50 mM, pH 7.0) with 10 mM sodium pyruvate, 0.2 mM NADH. Without FDP in enzyme reaction mixture, L-LDH3 exhibited specific activity by 112.04 U/mg that was similar to the L-LDH4. The specific activity increased as concentration of FDP increased to 8 mM. With 8 mM FDP, L-LDH3 indicated highest specific activity by 30,736.99 U/mg. Increasing the concentration of

FDP over 8 mM did not increase the activity further. The results indicate that the L-LDH3 is an allosteric enzyme that needs FDP for their proper activities which are similar to previous studies (Arai *et al.*, 2002, 2010; Hensel *et al.*, 1983). In contrast, enzyme activity of L-LDH4 was unaffected by the concentration of FDP. The specific activity maintained a constant level from 56.58 to 94.30 U/mg. The result that L-LDH3 showed higher activities compare to non-allosteric L-LDH4 is consistent with Jiang *et al.* (2014) report, which allosteric L-LDH possess much higher activities then the non‑allosteric ones.

Effect of pH and temperature on L-LDH3 activity

Various pH values (pH 3.0~11.0) were tested to determine the optimal pH for L-LDH3 activity. The maximal activity of L-LDH3 was observed at pH 4.0 by 302,343.16 U/mg. The activity increased drastically at pH 4.5~4.0 and decreased at pH 3.5 (Fig. 6A). Although the optimal pH for L-LDH activity of *L. casei* HY2782 was much lower than that from previous studies (pH 5.0-5.5), it is similar in that it shows higher activity in acidic conditions (Holland and Pritchard, 1975; Mayr *et al.*, 1980). It was described that L-LDH of *L. casei* indicates higher sensitivity to FDP and exhibits marked activity even without FDP under acidic conditions (Arai *et al.*, 2001, 2002; Garvie, 1980; Hensel *et al.*, 1977, 1983; Holland and Pritchard, 1975; Mayr *et al.*, 1980). Arai *et al.* (2010) explained the high pH-dependence in the allosteric effects in relation to the structure of *L. casei* L-LDH, which is the unique intersubunit salt bridges in the active state. Therefore, the higher activity at below the pH 5.0 could be due to the higher FDP sensitivity. The optimal temperature was determined by comparison the activity at various temperature values (4~65℃). The L-LDH3 exhibited maximal activity at 43℃ (Fig. 6B).

The enzyme stability was investigated at different pH (pH 3.0~11.0) and temperature (25~65℃) values. L-LDH3 retained more than 80% of the enzyme activity after incubation at pH

5.0~7.0 and 45% was retained after incubation at pH 4.0 and 8.0 (Fig. 7A). Effect of temperature on the enzyme stability was assessed by pre-incubation at pH 6.0 and different temperature for 10 min. The enzyme activity rapidly decreased after incubation at 50℃ and over (Fig. 7B).

Effect of metal ions on L-LDH3 activity

The enzyme activity with various metal salts was examined by adding each metal ion to the reaction mixture at final concentration of 2 mM. The reaction was conducted at 43℃, pH 4.0 with 8 mM FDP. About L-LDHs of different bacteria, manganese (Mn^{2+}) exhibited positive effect on the enzyme (Crow and Pritchard, 1977; Hensel *et al.*, 1977). According to the Holland and Pritchard (1975), Co^{2+}, Cd^{2+}, Cu^{2+}, and Ni^{2+} ions activated the L-LDH of *L. rhamnosus* as effective as Mn^{2+} at pH 6.3, Fe^{2+} and Zn^{2+} were less effective but also activated the L-LDH, Mg^{2+} exhibited no activation effect. In this study, Mg^{2+}, Ca^{2+}, and Mn^{2+} indicated positive effect on L-LDH3 of *L. casei* HY2782, especially Ca^{2+} was more effective than Mn^{2+}, and Zn^{2+}, Cu^{2+} ions inhibited the activity of L-LDH3 (Fig. 8A). The results are consistent with several previous studies that the activation of *L. casei* L-LDH markedly improved in the presence of certain divalent metal ions such as Mn^{2+}, Mg^{2+} and Ca^{2+} at acidic condition (pH 5.0-5.5) (Furukawa *et al.*, 2014; Mayr *et al.*, 1980). Under pH 6.5, on the other hand, Zn^{2+}, Cu^{2+} showed positive effects like Mn^{2+}, but not Mg^{2+} for L-LDH of *L. casei* (Holland and Pritchard, 1975). The differences between the studies may come from the difference of enzyme origin or assay conditions, especially pH. However, the information about the effect of metal ions at acidic condition could be more useful in view of control of L-LDH activity in fermented milk.

Effect of KH_2PO_4 on L-LDH3 activity

According to the results of amino acid sequence analysis (Fig. S3) and effect of FDP concentration on enzyme activity, the L-LDH3 of *L. casei* HY2782 seems to be allosteric enzyme

that activated by FDP. Several studies (Arai *et al.*, 2002; Taguchi and Ohta, 1995) revealed that His-188 residue of allosteric L-DH interacts directly with phosphate group of FDP. Therefore, phosphate is generally considered as competitive inhibitor for FDP-dependent L-LDHs (Anders *et al.*, 1970; Crow and Pritchard, 1977; Garvie, 1978, 1980; Garvie and Bramley, 1979a, b; Neimark and Tung, 1973). However, the tendency of inhibition by phosphate is vary among L-LDHs of different bacteria. The sensitivity about FDP can affect the inhibitory effect of phosphate, higher FDP sensitivity lower inhibition by FDP (Garvie and Bramley, 1979b). Some LDH could even be stimulated by phosphate (e.g. LDH of *Streptococcus faecalis* at neutral pH; Garvie, 1980). To verify whether phosphate inhibits L-LDH3 of *L. casei* HY2782, effect of KH_2PO_4 on L-LDH3 activity was investigated by adding different concentration of KH_2PO_4 in enzyme reaction mixture. Considering the concentration of FDP (8mM), KH_2PO_4 concentrations for experiment were decided. The activity of L-LDH3 decreased to 19% of that of control (without KH_2PO_4) with 8 mM KH_2PO_4, same concentration to FDP. By adding 16 mM of KH_2PO_4, the enzyme activity was completely inhibited (Fig. 8B).

Conclusion

Post-acidification is main obstacle to extending shelf-life of yogurt, which is still unsolved problem in yogurt industry. In the present study, we overexpressed and characterized L-LDH s of *L. casei* HY2782 to control the post-acidification caused by this strain. Four genes encoding L-LDH were identified from *L. casei* HY2782 by whole genome sequencing. These genes were successfully cloned and expressed in *E. coli* BL21 (DE3). Among the four L-LDHs, L-LDH3 exhibited highest enzyme activity with maximal activity at 43℃ and pH 4.0 with 8 mM FDP by 302,343.16 U/mg. The enzyme activity was retained more than 80% after incubation at pH 5.0~7.0 and rapidly decreased after incubation at over 50℃. In acidic condition, Mg^{2+}, Ca^{2+}, and

Mn^{2+} ions stimulated L-LDH3 activity, and Zn^{2+} and Cu^{2+} ions had inhibitory effects on L-LDH3. In addition, L-LDH3 was inhibited by KH_2PO_4. The results indicated that L-LDH3 may play important role in lactic acid fermentation of *L. casei* HY2782 as major L-LDH of this strain. Therefore, control of L-LDH3 with potential inhibitors, Zn^{2+}, Cu^{2+}, and KH_2PO_4, could be one of solutions to mitigate the post-acidification caused by *L. casei* HY2782. However, further study is needed to verify whether these inhibitors actually reduce the post-acidification. Nevertheless, this study provides some basic information to better understand the lactic acid fermentation of *L. casei* strains and could be new approach to control the post acidification in fermented dairy products such as yogurt.

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Tables and Figures

Table 1. Bacteria strains and plasmids used in this study.

Strains or plasmid	Characteristics	Source or reference
Strain		
L. casei HY2782	Wild–type strain	hy Co., Ltd.
E. coli DH5α	φ80 *lacZ*ΔM15 Δ(*lacZYA–argF*)U169 *recA*1 *endA*1 *hsdR*17 *supE*44λ–*thi*–1	BioFACT
E. coli BL21 (DE3)	F^- *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3)	Invitrogen
Plasmid		
pGEM–T Easy	Cloning vector, Amp^r	Promega
pET22b (+)	Expression vector (His–Tag), Amp^r	Merch KGaA
pJE1	pGEM-T Easy containing 951 bp PCR fragment corresponding to the *ldhL*1 gene	This study
pJE2	pGEM-T Easy containing 903 bp PCR fragment corresponding to the *ldhL*2 gene	This study
pJE3	pGEM-T Easy containing 993 bp PCR fragment corresponding to the *ldhL*3 gene	This study
pJE4	pGEM-T Easy containing 918 bp PCR fragment corresponding to the *ldhL*4 gene	This study
pET22b–*ldhL*1	pET22b (+) containing 939 bp *Nde*I–*Xho*I fragment derived from pJE1	This study
pET22b–*ldhL*2	pET22b (+) containing 891 bp *Nde*I–*Xho*I fragment derived from pJE2	This study
pET22b–*ldhL*3	pET22b (+) containing 981 bp *Nde*I–*Xho*I fragment derived from pJE3	This study
pET22b–*ldhL*4	pET22b (+) containing 906 bp *Nde*I–*Xho*I fragment derived from pJE4	This study

Table 2. IPTG induction conditions for each *E. coli* BL21 (pET22b–*ldhL*s).

Strain	Condition
E. coli BL21 (pET22b–*ldhL*1)	16℃, 170 rpm, 10 h
E. coli BL21 (pET22b–*ldhL*2)	37℃, 150 rpm, 4 h
E. coli BL21 (pET22b–*ldhL*3)	37℃, 150 rpm, 4 h
E. coli BL21 (pET22b–*ldhL*4)	37℃, 225 rpm, 4 h

Table 3. Composition of buffer solution for Ni–NTA purification and SDS–PAGE.

Buffer solution	Composition
Ni–NTA purification	
Wash buffer	6.9 g/L $NaH_2PO_4 \cdot H_2O$, 17.54 g/L NaCl, 20 mM imidazole (pH 8.0)
Elusion buffer	6.9 g/L $NaH_2PO_4 \cdot H_2O$, 17.54 g/L NaCl, different concentration of imidazole (50, 100, 150, 200, 250 mM)
SDS–PAGE	
Staining buffer	1 g/L Coomassie blue R–250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid
Destaining buffer	10% (v/v) methanol, 10% (v/v) glacial acetic acid

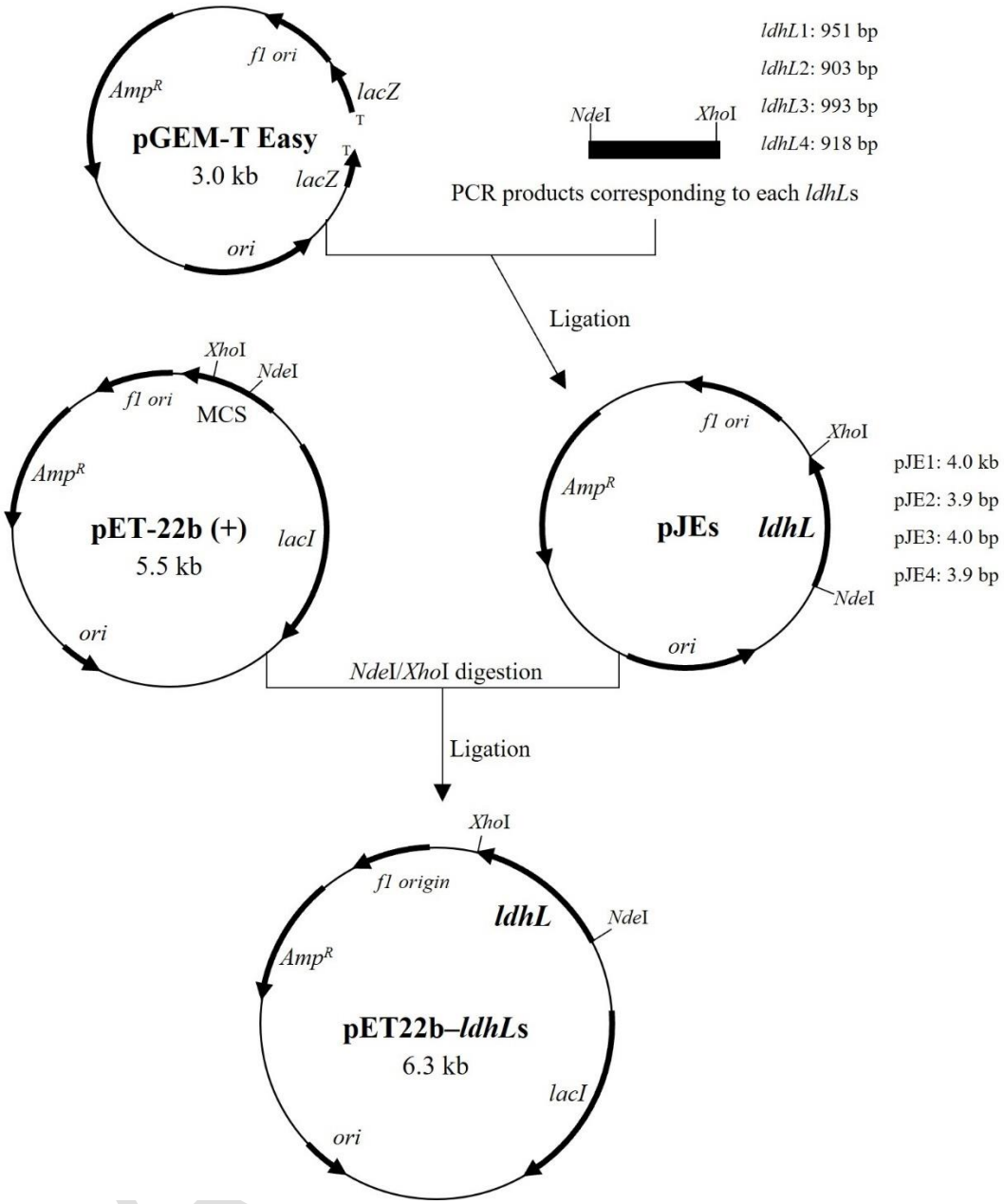


Figure 1. Plasmid construction of pET22b–*ldhL*s. Each amplified *ldhL* genes were cloned into the pGEM–T Easy vector and recloned into the pET22b (+) vector. *Amp*^{R}, ampicillin resistance gene; *ori*, origin of replication.

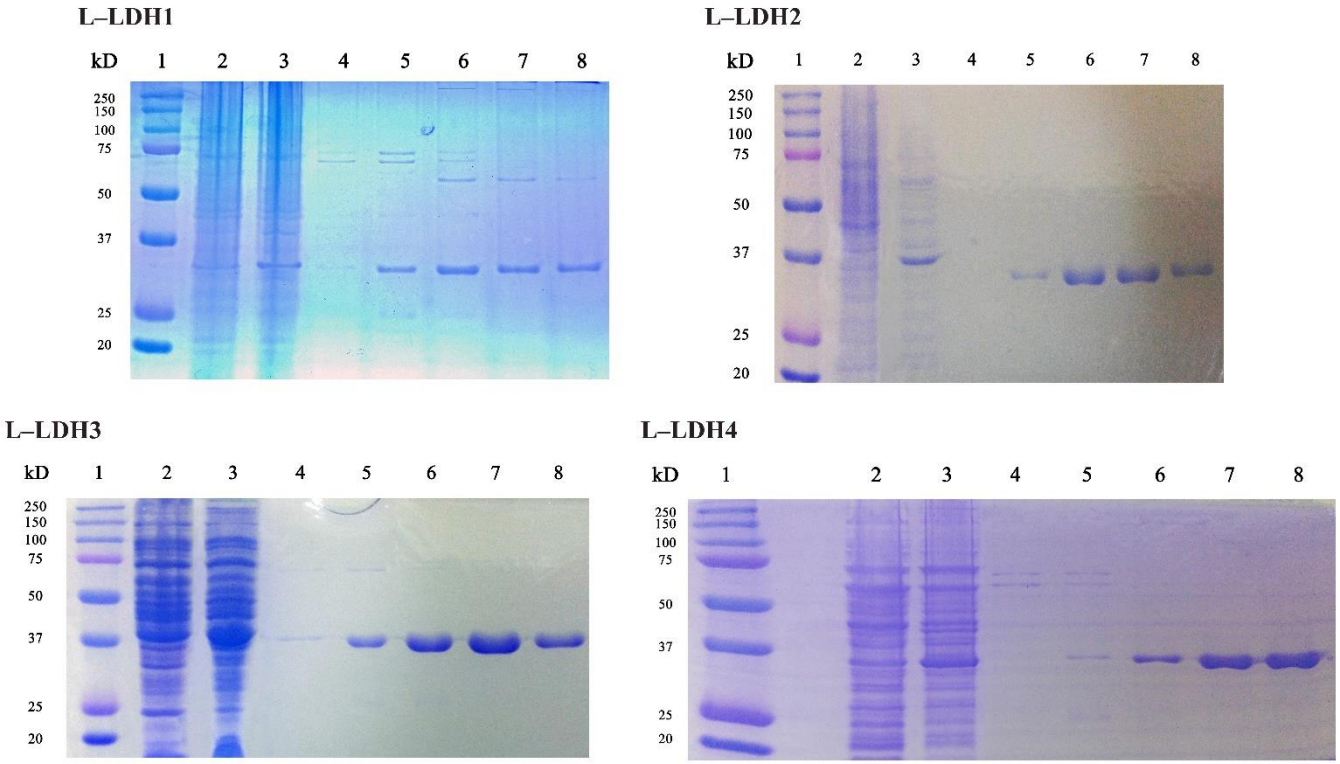
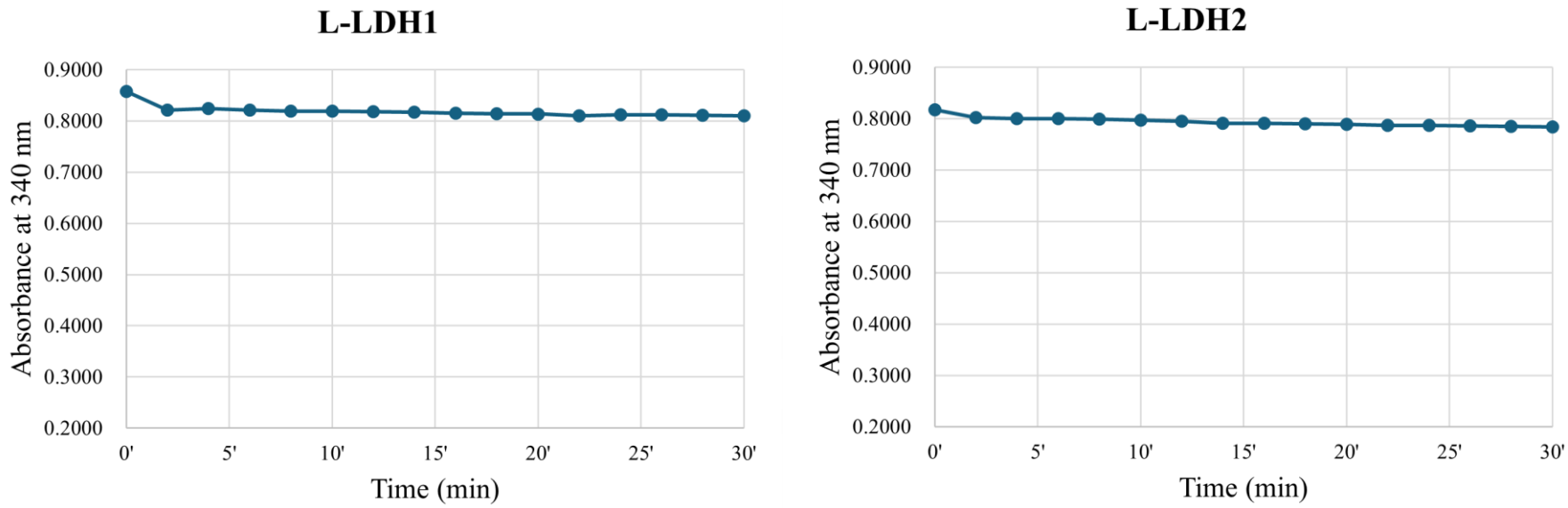


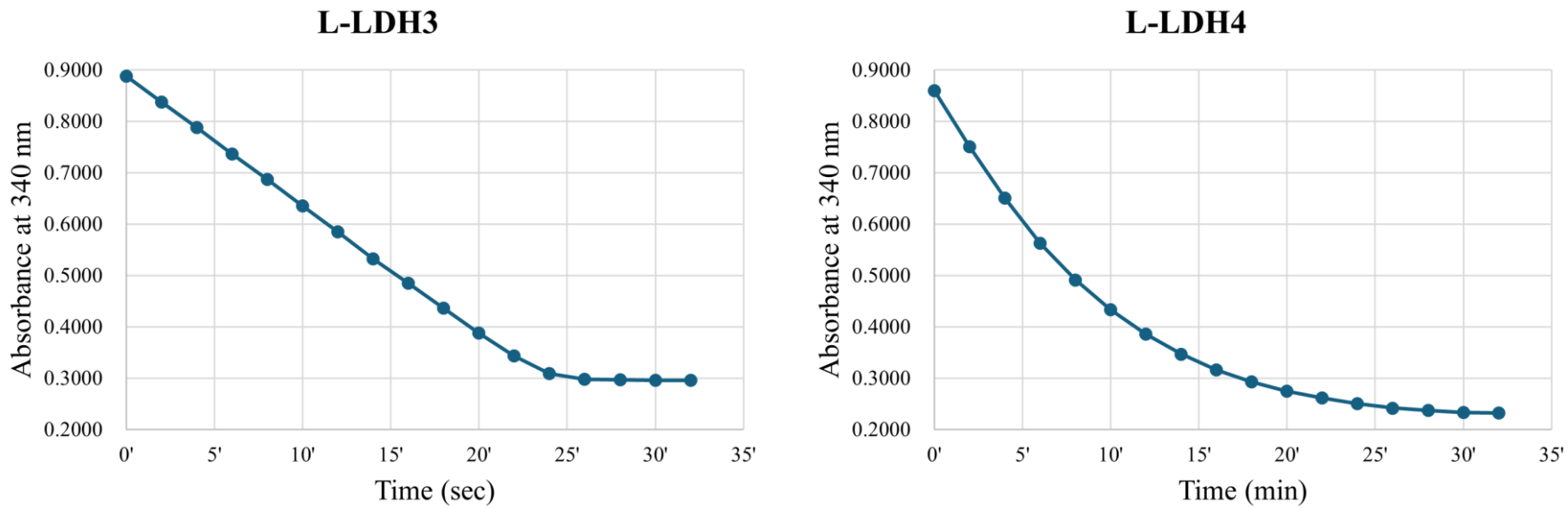
Figure 2. SDS–PAGE analysis of L–LDHs. Lane 1, molecular weight standards; Lane 2, the cell lysate of *E. coli* BL21 harboring pET22b–*ldhL*s without IPTG induction; Lane 3, cell lysate of *E. coli* BL21 harboring pET22b–*ldhL*s with IPTG induction; Lane 4, eluted proteins by 50 mM imidazole; Lane 5, eluted proteins by 100 mM imidazole; Lane 6, eluted proteins by 150 mM imidazole; Lane 7, eluted proteins by 200 mM imidazole; Lane 8, eluted proteins by 250 mM imidazole.



19

20 **Figure 3.** Enzyme activities of L–LDH1 and L–LDH2 at 25℃ with 5 mM FDP. Absorbance at 340 nm of enzyme reaction mixtures with L–

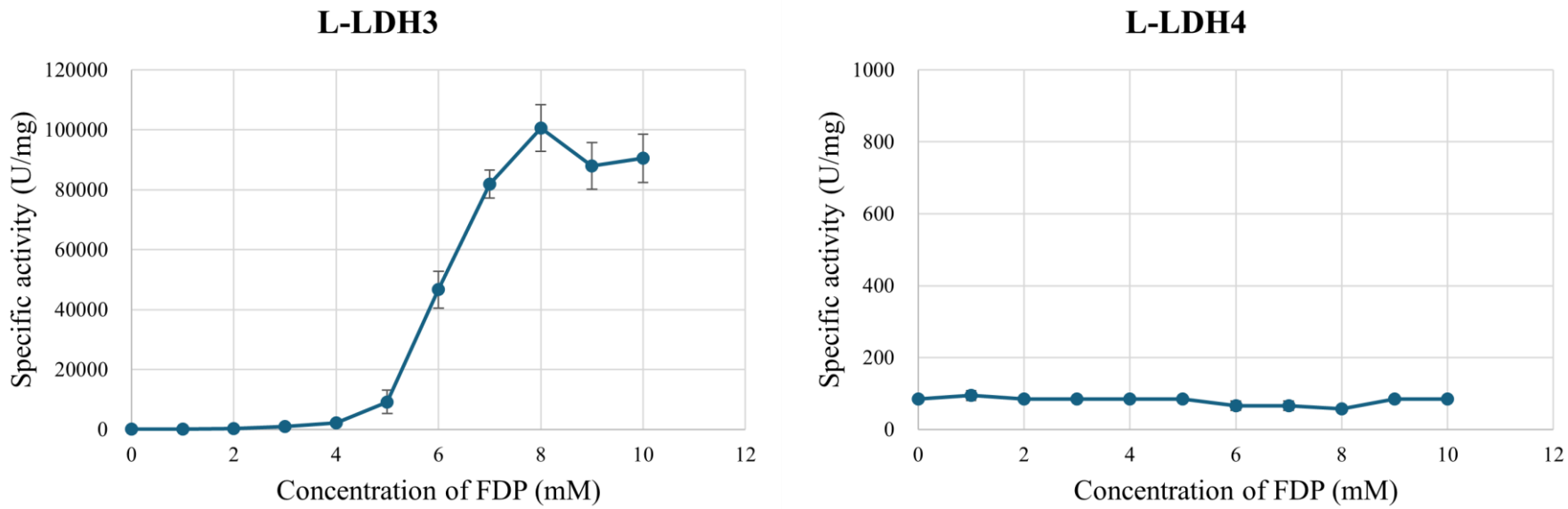
21 LDH1 and L–LDH2 were recorded every 2 minutes.



22

23 **Figure 4.** Enzyme activities of L–LDH3 and L–LDH4 at 25℃ with 5 mM FDP. Absorbance at 340 nm of enzyme reaction mixtures with L–

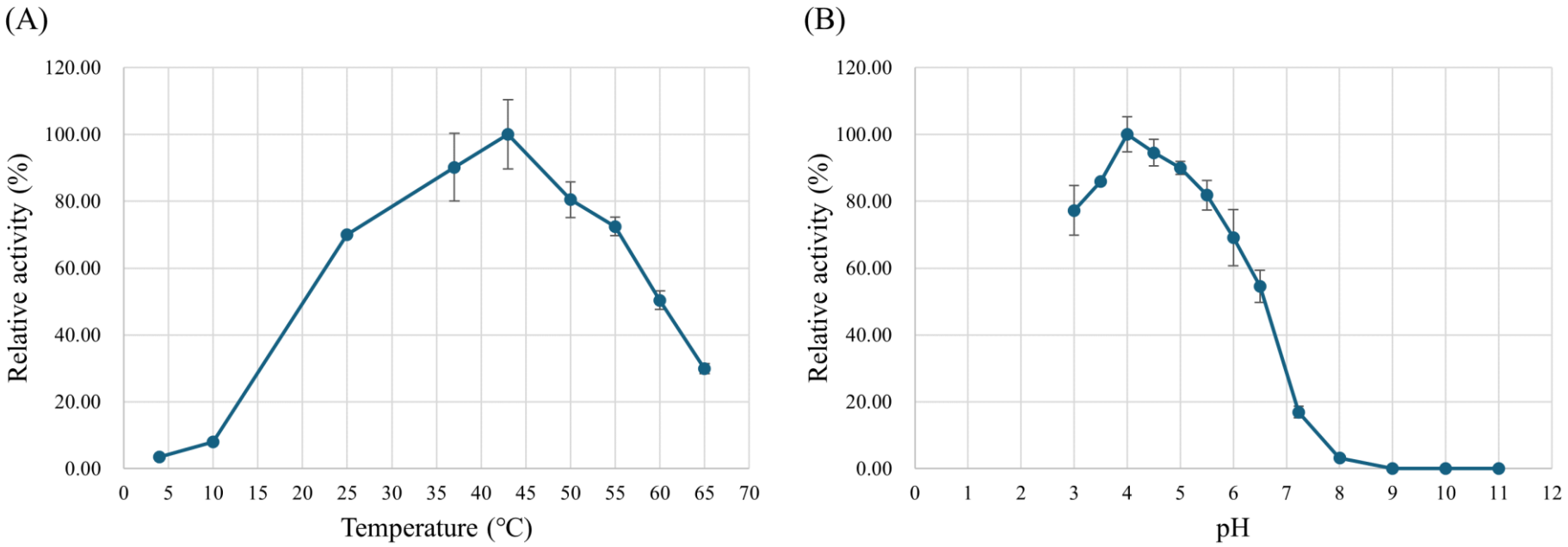
24 LDH3 and L–LDH4 were recorded every 2 seconds and 2 minutes, respectively.



25

26 **Figure 5.** Effect of FDP on L-LDH3 and L-LDH4 activities. The specific activity of L-LDH3 and L-LDH4 were assessed with 0–10 mM of FDP

27 in an interval of 1 mM. Values are expressed as means ± SDs (bars) of the results of three separate experiments.



28

29 **Figure 6.** Effect of pH and temperature on L–LDH3 activity. (A) Optimal pH for L–LDH3 activity. (B) Optimal temperature for L–LDH3

30 activity. The relative activity was determined by the highest specific activity as 100%. Values are expressed as means ± SDs (bars) of the results

31 of three separate experiments.

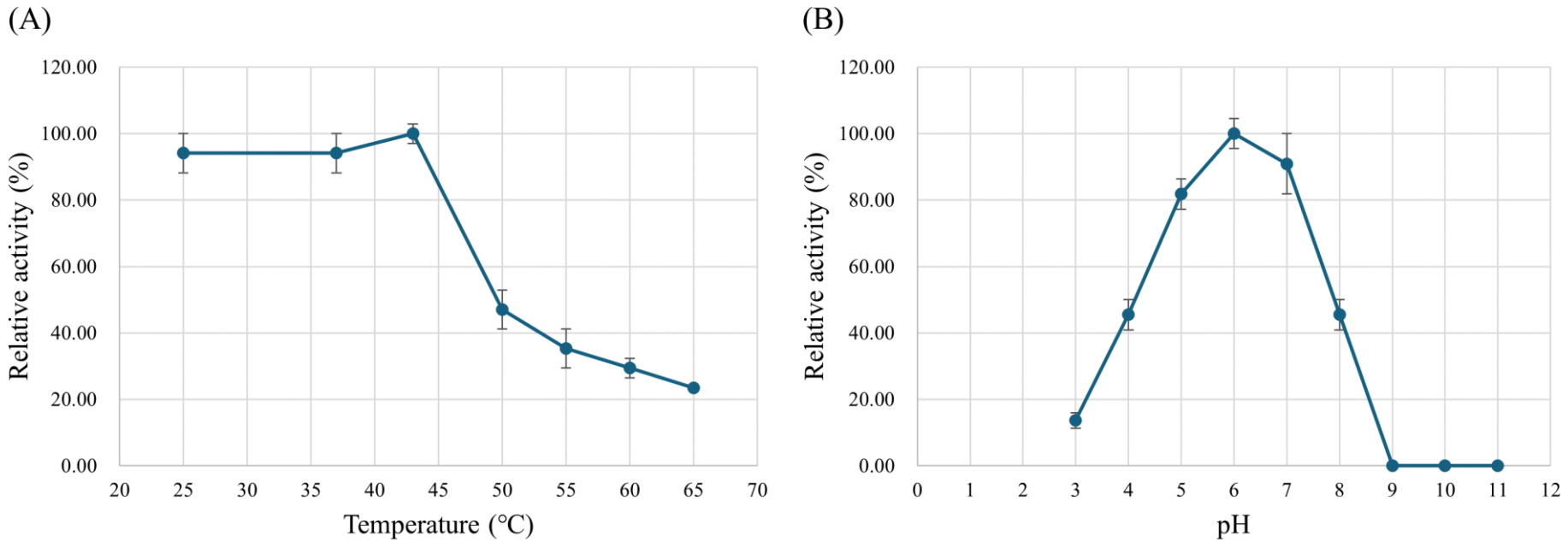


Figure 7. Effect of pH and temperature on L–LDH3 stability. (A) Effect of pH on the enzyme stability. (B) Effect of temperature on the enzyme stability. The relative activity was determined by the highest specific activity as 100%. Values are expressed as means ± SDs (bars) of the results of three separate experiments.

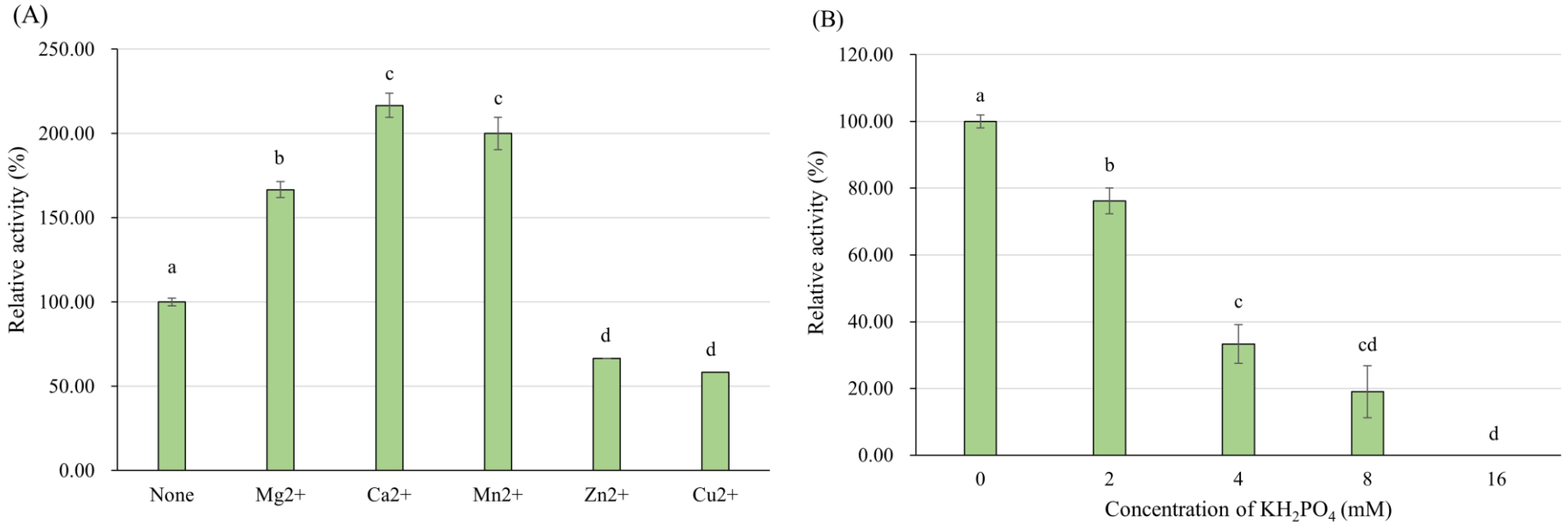


Figure 8. Effect of metal ions and KH_2PO_4 on $_L$–LDH3 activity. (A) Effect of metal ions on the enzyme activity. (B) Effect of KH_2PO_4 on the enzyme activity. The relative activity was determined by the control (without metal ions or KH_2PO_4) as 100%. Values are expressed as means ± SDs (bars) of the results of three separate experiments. Statistically significant differences ($p<0.05$) among groups are indicated by different letters.

Supplementary Figures

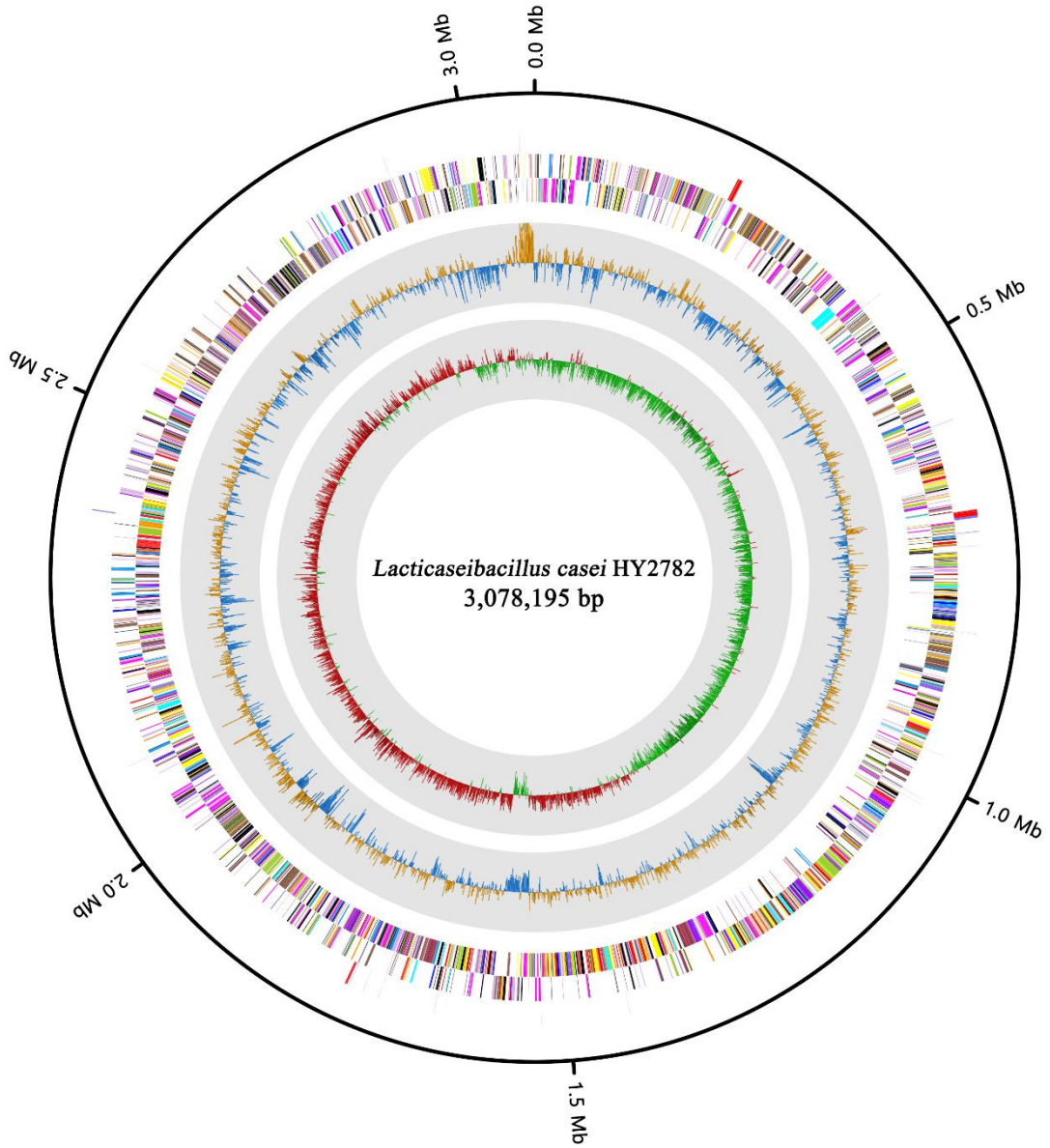


Figure S1. Circular genome map of *L. casei* HY2782. The circles of genome map represent following from outside to inside: rRNA/tRNA; the annotated reference genes (CDSs) in the reverse strand and forward strand; GC ratio; and GC skew. The genome size was 3,078,195 bp with 2,971 CDSs, 21 rRNAs, and 54 tRNAs.

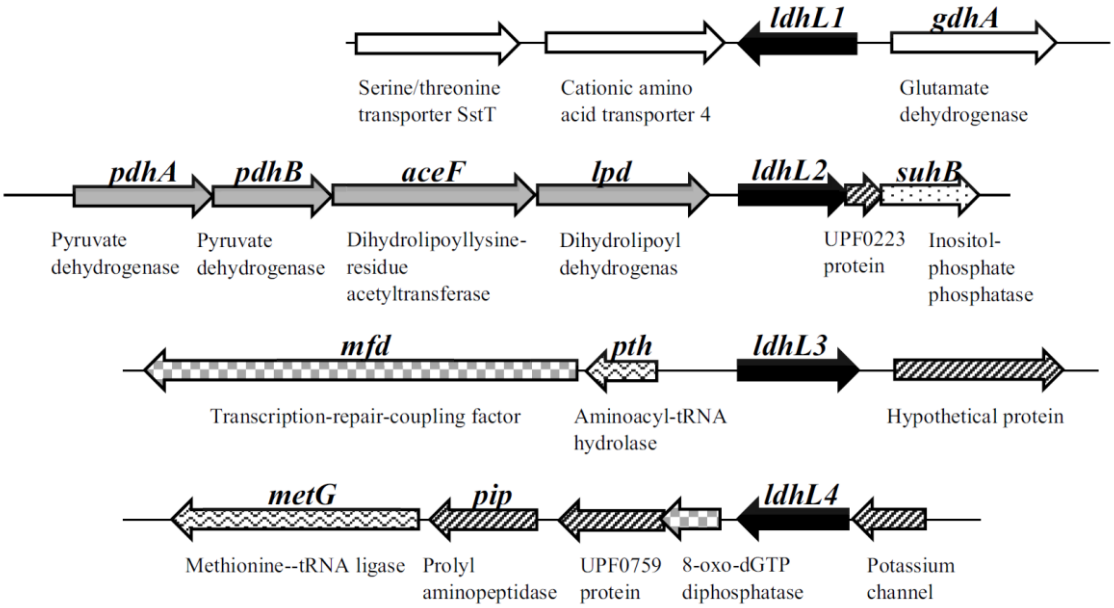


Figure S2. Gene arrow diagram indicating the location of *ldhL* genes of *L. casei* HY2782. Patterns of the arrows representing the clusters of orthologous groups (COGs) categories and *ldhL*s: *black arrows*, L–LDH genes (*ldhL*s); *white arrows*, amino acid transport and metabolism (initial E); *grey arrows*, energy production and conversion (initial C); *cross-hatched arrows*, function unknown (initial S); *dotted arrows*, carbohydrate transport and metabolism (initial G); *checkerboard arrows*, replication, recombination and repair (initial L); *zigzagged arrows*, translation, ribosomal structure and biogenesis (initial J). *L. casei* HY2782 possesses four L–LDH genes.

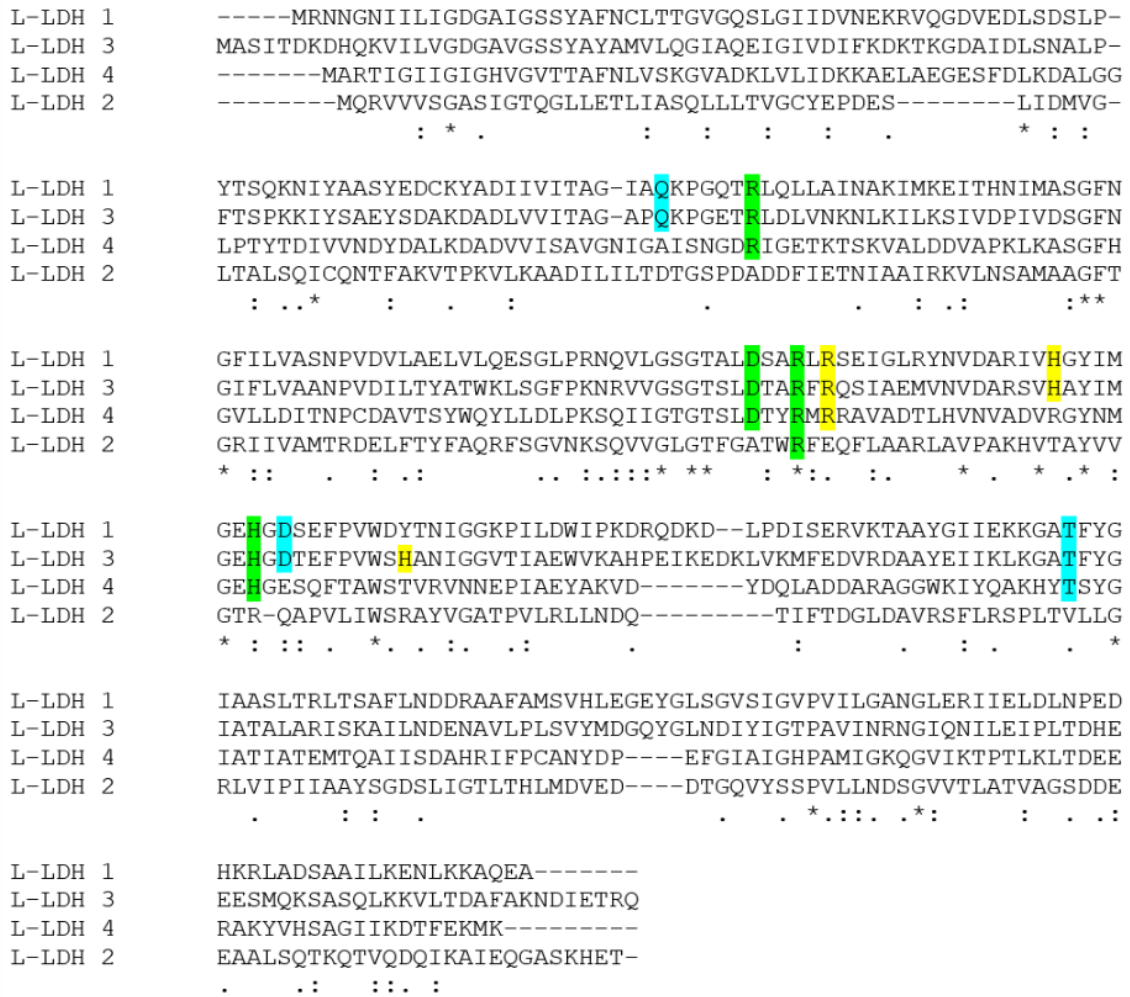


Figure S3. Multiple alignment of the amino acid sequences of four L–LDHs. The colors indicate following residues: *blue*, substrate recognition residues (Gln 103, Asp 197, and Thr 246); *green*, catalytic residues (Arg 109, Asp 168, Arg 171, and His 195); *yellow*, FDP-activated residues (Arg 173, His 188, and His 205).