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Contributed Paper

Characteristics of D-lactate Dehydrogenase from the High Potential D-lactic Acid Producer *Leuconostoc pseudomesenteroides* TC49 Isolated from Thailand

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ABSTRACT

D-lactate dehydrogenase obtained from *Leuconostoc pseudomesenteroides* TC49, a D-lactic acid producing bacterium isolated from a *Tithonia diversifolia* flower in Thailand, was studied its properties for use in D-lactic acid production. Successful protocols of protein precipitation, dialysis, ultrafiltration and chromatography were used for D-LDH purification. The purified D-LDH with its N-terminal amino acid sequence as MKIFAYGIRE displayed the molecular weight of approximate 40.6 kDa with preferred pH of 8.5 and temperature of 30°C for its highest activity. The enzyme stability was decreased with increasing temperature and was completely vanished at 50°C. The k_{cat}, K_m and k_{cat}/K_m of the purified enzyme in the pyruvate reduction were 180 s^{-1}, 0.5 mM and 360 mM^{-1} s^{-1} while the values in the D-lactate oxidation were 117 s^{-1}, 69.6 mM and 1.681 $mM^{-1}s^{-1}$, respectively. $AgNO_3$ and $ZnCl_2$ slightly inhibited the purified enzyme.

Keywords: *Leuconostoc pseudomesenteroides*, D-lactate dehydrogenase, D-lactic acid, N-terminal amino acid sequence, pyruvic acid, native-PAGE

1. INTRODUCTION

Nowadays, global warming has been the hot issue in many aspects as it causes many negative impacts to the earth. Petroleum-based plastics are one of the problems for which large amounts of CO_2 are released during plastic manufacturing and their degradation takes a long period of time. Development of new material alternative to petroleum-based plastics is certainly needed in order to reduce and replace the traditional plastics. Bio-plastic development has been extensively studied mainly due to its bio-degradable and promising for large scale production. Polylactic acid (PLA) is a high potential and commercially attractive bio-plastic made of optically pure lactic acid monomers, both D- and L-isomeric forms [1, 2]. The low melting point of Poly L-lactic acid (PLLA), around 175°C, makes it unfit for some applications such as drug delivery systems and

alternative commercial polymers. The thermal stability of PLLA, up to 230°C, can be enhanced by the 1:1 ratio blending with poly D-lactic acid (PDLA). High efficient bio-plastics cannot solely be obtained from PLLA or PDLA. In addition, the degradation rate of PLLA is significantly slower than PDLA, due to the larger size of PLLA crystalline regions [3]. While the chemical synthesis of lactic acid provides racemic lactic acids, the microbial fermentation can simply produce high optically pure L(+)- or D(-)-lactic acid using the most suitable microorganisms harboring L- or D- lactate dehydrogenase [4, 5].

Lactic acid bacteria (LAB) are the well-known LDH proprietors as they are used as starter cultures for lactic acid production both in food and non-food industries [6, 7]. Although *Lactobacillus* spp. are mainly used, other LAB as well as some engineered *Escherichia coli* have been demonstrated to produce high quantity of lactic acid. In particular, some LAB species, i.e. *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 [4], *L. coryniformis* ATCC 25600 [7], *L. delbrueckii* subsp. *bulgaricus* [8], *L. rhamnosus* [9], *L. pentosus* [10], *L. fermentum* JN248 [11], *Pediococcus acidilactici* [12], *L. delbrueckii* [13], *Weisella* sp. S26 [14], *Sporolactobacillus inulinus* [15], *L. delbrueckii* subsp. *lactis* JCM 1107 [16] and *Terrilactibacillus laevolacticus* SK5-6 [17], have been reported to provide efficient D-LDH for D-lactic acid production. In fact, D-lactic acid naturally exists in lesser amount when compared with its enantiomeric L-lactic acid. Presently, many engineered lactic acid producing bacteria have been reported. However, the isolation of the new D-lactic acid producer cannot be excluded because the wild strains can provide more enzyme diversity involved in the lactic acid production which can be used for production enhancement in terms of specificity, sensitivity and stability or in future useful applications. To find out a new source of D-LDH for D-lactic acid production, biosensors and other applications involving the lactic acid existence in organisms such as the detection of D-lactic acid in humans as clinical indicators, LAB capable of producing D-lactate were screened from natural habitats in Thailand including soils, water, fermented foods and flowers. Among the isolates obtained, *Leu. pseudomesenteroides* TC49 isolated from *Tithonia diversifolia* flower displayed the highest D-lactate production with 99% of the optical purity [18]. The optical purity of D-lactic acid production has been reported by several research groups with lesser purity. For examples, D-lactic acid production by *L. delbruekii* subsp. *delbruekii* NBRC3202 gave 97.79% optically pure D-lactic acid [19] while 98.3% of optically pure D-lactic acid was produced by engineered *Escherichia coli* [20]. To elucidate the *Leu. pseudomesenteroides* TC49 enzymes involved in D-lactic acid production, this study aimed to purify and characterize D-LDH of the strain. The enzymatic information obtained will be beneficially for further D-lactic acid production process development for serving as a monomer in bio-plastic industries, for genetic diversity provided in recombinant technology and for biosensor development.

2. MATERIALS AND METHODS

2.1 Bacteria, Growth Conditions and Packed Cell Preparation

Leuconostoc pseudomesenteroides TC49 (accession number MG493259) isolated from *Tithonia diversifolia* flower using de Man, Rogosa and Sharpe (MRS) Medium (Merck™, Germany) with 0.02% (w/v) bromocresol green, pH 5.7 ± 0.2 and kept as a stock culture at -20°C in the Microbiology Laboratory 2711 of the Department of Biology, Faculty of Science, Chiang Mai University, Thailand [18] was used throughout the study. The bacterial isolate was grown at pH 6.5 ± 0.2, 37°C for 72 hours in the modified MRS medium composed of 2% (w/v) glucose, 1% (w/v) proteose peptone, 0.8% (w/v) beef extract, 0.4% (w/v) yeast extract, 0.5% (w/v) sodium acetate trihydrate, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 0.05% (w/v) $MnSO_4 \cdot 7H_2O$ and 0.2% (w/v) K_2HPO_4. Cells were cultivated to late exponential phase and harvested at 4°C using centrifugation, washed with 20 mM potassium

phosphate buffer (KPB), pH 7.0, and kept at -20°C until used.

2.2 Preparation of the Crude Enzyme Extract

Six grams of packed cells were dispersed in the KBP buffer, 30 ml. Cell disruption was performed by an ultrasonication method using a Sonicator (TOMY, Ultrasonic disruptor, UD-201, Tokyo, Japan; set at 50 W and 60 MHz) for 1 minute at 30 cycles [21]. The cell free supernatant harboring crude enzyme was obtained by centrifugation at 17,860 × g, 4°C for 10 minutes to remove the cell debris. A volume of 30 ml of crude enzyme was kept at 4°C for further enzyme purification and native-PAGE evaluation.

2.3 Determination of *Leu. pseudomesenteroides* TC49 D-LDH Activity

The enzyme activity was examined spectrophotometrically by a Hitachi U-2910 spectrophotometer (Tokyo) fitted with a thermostat. The D-LDH activity was demonstrated in terms of pyruvate reduction and D-lactate oxidation. Substrate solution containing 0.1 mM NADH, 10 mM pyruvate and 100 mM Tris/HCl buffer, pH 8.0 was used for pyruvate reduction while the solution containing 5 mM NAD^+, 10 mM D-lactate and 100 mM Tris/HCl buffer, pH 8.5 was applied for D-lactate oxidation. For both reactions, the enzyme, 20 µl, was mixed with the appropriate substrate in a 1 cm width cuvette, a total volume of 1,000 µl, and measured the absorbance by the spectrophotometer at 340 nm, 30°C for 5 minutes. One unit of enzyme was characterized as the oxidation of 1 µmol NADH per minute or the reduction of 1 µmol NAD^+ per minute. The protein was quantitated according to Lowry's method [22].

2.4 Native-polyacrylamide Gel Electrophoresis (PAGE) and Activity Stain of the D-LDH

The native-PAGE was performed by the modified method of Davis, 1964 [23] in capillary tubes (4.0 mm ID × 80 mm L) with separating gel, 9.5% and stacking gel, 2.25%. The crude enzyme, 500 µl, was mixed with 500 µl of loading dye composed of 0.12 M Tris-HCl buffer pH 6.8, 10% sucrose and 0.004% bromophenol blue. The mixture was loaded into each capillary tube with a total volume of 200 µl and run at 25 mA for 60 minutes using the Maxi Vertical Electrophoresis (20 × 20 cm; Topac Inc., USA). Afterwards, the gels were extruded from the capillary tubes, washed with distilled water and evaluated the enzyme activity by soaking in each test substrate solution. The activity of the purified enzyme was tested against the following substrates: 10 mM D-lactate; 10 mM D-lactate and 5 mM NAD^+; 10 mM D-lactate and 5 mM $NADP^+$; no substrate, no cofactor (negative control for D-LDH); 0.025% Coomassie brilliant blue G-250 in 50% methanol and 10% acetate (control for potential protein); 10 mM L-lactate; 10 mM L-lactate and 5 mM NAD^+; 10 mM L-lactate and 5 mM $NADP^+$; no substrate, no cofactor (negative control for L-LDH). All substrate solution contained 0.1 mM *p*-iodonitrotetrazolium violet (INT), 0.1 mM phenazine methosulfate (PMS), and 100 mM KPB (pH 9.0). The reaction mixtures were incubated for 30 minutes at 30°C before observing a red band of positive enzyme activity. Meanwhile, Coomassie brilliant blue G-250 was used to stain the protein for 20 minutes, as a control. All stained gels were subsequently placed on a plastic sheet for photography.

2.5 Purification of *Leu. pseudomesenteroides* TC49 D-LDH

Each purification step was conducted in triplicate at 4°C unless otherwise stated.

Step 1 Ammonium sulfate fractionation

The crude enzyme extract was precipitated at 4°C using $(NH_4)_2SO_4$ at 10%, 20%, 30%, 40% and 50% saturation under agitation by a magnetic stirrer (Magnetic stirring plate 115 VA, 60 Hz, STERLITECH Corporation., USA). Afterwards, the sample was kept at 4°C for 10 minutes. The crude enzyme was separated by centrifugation at

13,250 × g for 10 minutes at 4°C to obtain pellets and supernatant. Crude enzyme supernatant was kept at 4°C for further enzyme assay.

The crude enzyme supernatant was loaded into the tubing prior to dialysis against 1 L of 20 mM KPB (pH 7.0) for 1 hour at 4°C. Buffer was changed and repeated the process twice before leaving the dialysis tubing in 3 L of the same buffer for 12 hours. D-LDH activity was determined as mentioned earlier in the section 2.3.

Step 2 Anion exchange chromatography

The enzyme obtained from Step 1 was added into the TOYOPEARL GigaCap DEAE-650M column (2.0 × 6.0 cm, Tosoh™), which was equilibrated with 20 mM KPB, pH 7.0. Elution of the column was conducted with a linear gradient of KPB containing 0 to 1 M NaCl at a flow rate of 0.2 ml/min. Fractions of 3 ml were collected and subjected to D-LDH activity analysis. The fractions exhibiting D-LDH activity were pooled and dialyzed at 4°C for 12 hours against 3 L of the 20 mM KPB, pH 7.0.

Step 3 Hydrophobic interaction chromatography

The sample was loaded into TOYOPEARL Butyl-650M column (2.0 × 3.2 cm; Tosoh™) after equilibrated with 20 mM KPB containing 35% $(NH_4)_2SO_4$. Elution of the column was performed with a linear gradient of 35 to 0% $(NH_4)_2SO_4$ in 20 mM KPB. Fractions were collected and subjected to D-LDH activity analysis. The fractions exhibiting D-LDH activity were pooled and dialyzed at 4°C for 24 hours against 3 L of KPB.

Subsequently, the partial purified enzyme was concentrated by an ultrafiltration using centrifugal filter devices (Amicon® Ultra-15 10K, Merck™) at 8,000 × g, 4°C for 10 minutes.

Step 4 Gel filtration chromatography

The concentrated enzyme obtained was loaded into 20 mM KPB pre-equilibrated Superdex 200 10/300 GL (10 × 300-310 mm, GE Healthcare Bio-science™). The enzyme was eluted at a flow rate of 0.2 ml/min at 4°C for 2 hours by 20 mM KPB containing 0.1 M NaCl. The fractions showing D-LDH activity were gathered and stored at -20°C until used.

2.6 Molecular Weight Evaluation of the Purified D-LDH

The sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme was performed according to the method described by Laemmli, 1970 [24] using 12.5% separating gel and 2.25% stacking gel running by the AE-6530 mPAGE Mini-slab size electrophoresis system (ATTO Corporation, Japan) at 25 mA, 300 V for 60 minutes. The Protein Molecular Weight Marker (Broad) for SDS-PAGE (Takara Bio USA, Inc.) containing standard proteins with molecular mass of 6.5 - 200 kDa was used. The gel was stained with 0.025% Coomassie brilliant blue R-250 in 7.5% ethanol and 7.5% acetic acid for 20 minutes.

2.7 N-terminal Amino Acid Sequence Analysis of the Purified D-LDH

The SDS-PAGE separated proteins were subsequently transferred to a polyvinylidene fluoride (PVDF) membrane and stained with the Ponceau S solution (Sigma-Aldrich™ Co., USA). The N-terminal amino acid of purified D-LDH was performed by the Edman degradation technique [25] prior to sequencing analysis by the Hokkaido System Science Co. Ltd., Japan.

2.8 Phylogenetic Analysis of the Purified *Leu. pseudomesenteroides* TC49 D-LDH

Sequence was compared with other D-LDH amino acid sequence in the database of NCBI (National Center for Biotechnology Information) and KEGG (Kyoto Encyclopedia of Genes and Genome). Multiple alignments of the amino acid sequences were determined with the CLUSTAL X program. Phylogenetic tree analysis was conducted by Maximum Likelihood method based on the JTT matrix-based model using the MEGA 7 program.

2.9 pH and Temperature Effects on the Purified D-LDH Activity and Stability

To investigate the optimal pH of the D-LDH activity and thermal stability, the D-lactate oxidation was employed. The buffers used were citrate buffer (pH 4.0-6.0), potassium phosphate buffer (pH 6.0-7.5), Tris-HCl buffer (pH 7.0-9.0), N-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer (pH 9.0-10.0), sodium carbonate buffer (pH 10-10.5) and N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (pH 10.5-11.0). For the optimal pH of the enzyme activity, the purified protein was measured its activity by the method described in the section 2.3 against different pH buffers. To investigate the pH stability, the enzyme was incubated in each pH buffer at 30 °C for 30 minutes prior to D-LDH activity determination as mentioned above.

Temperature ranges of 20-60 °C and 25-60 °C were evaluated on the enzyme activity and thermal stability, respectively. The purified enzyme was mixed with the reaction mixture prior to D-LDH activity determination by the spectrophotometer at 340 nm for 5 minutes at each temperature. To monitor the thermal stability, the enzyme was incubated at each temperature for 30 minutes and cooled on ice for 10 minutes prior to D-LDH activity determination as mentioned above [4, 5].

2.10 Effect of Metal Ions and Chemicals on the Activity of D-LDH

Each metal ion or chemical, 1 mM, including $Ca(NO_3)_2$, $CuSO_4$, $AgNO_3$, NaCl, $CoCl_2$, $MgCl_2$, NH_4Cl, $ZnCl_2$, $MnCl_2$, $FeCl_2$, DTT and EDTA was incubated with the purified enzyme at 30 °C for 30 minutes prior to D-LDH activity determination.

2.11 Substrate Specificity of D-LDH

Pyruvate, D-lactate, 2-oxoglutaric acid, oxaloacetic acid, 2-ketobutyric acid and malic acid were used to evaluate substrate specificity of the purified enzyme. The D-LDH activity was conducted as mentioned above with the activity toward pyruvate and D-lactate was defined as 100%.

2.12 D-LDH Enzyme Kinetics

For the pyruvate reduction of the D-LDH, each pyruvate concentration (20, 10, 3.33, 1.11, 0.37, 0.18, 0.12, 0.06 mM) was investigated with 0.15 mM NADH was used as a cofactor. Consequently, each NADH concentration (0.18, 0.15, 0.1, 0.066, 0.05, 0.033, 0.025, 0.02 mM) was evaluated with 20 mM pyruvate was used. Likewise, the lactate oxidation of the D-LDH was performed using D-lactate (50, 40, 30, 15, 10, 7.5, 5, 3.75 mM) against 15 mM NAD^+ and using NAD^+ (15, 10, 5, 1.66, 1, 0.55, 0.4, 0.33, 0.25 mM) against 100 mM D-lactate. All tests were conducted in triplicate. The K_m and V_{max} were calculated using Lineweaver-Burk plots.

3. RESULTS AND DISCUSSION

3.1 Activity Stain of the Crude Enzyme Supernatant

Leu. pseudomesenteroides TC49 was grown at 37 °C for 72 hours in the modified MRS medium, 100 ml working volume, to obtain 2 g of the cell mass. Cells were subsequently disrupted by the ultrasonication method prior to the native-PAGE determination and the activity stain against substrates and cofactors including D-lactate, L-lactate, NAD^+ and $NADP^+$. The results showed that D-LDH activity was clearly illustrated the red band of positive activity when D-lactate and NAD^+ existed in the reaction mixture (Figure 1b) while the other reaction mixtures with different pair of substrate and cofactor displayed no positive band indicating the existence of the enzyme and its highly specific activity toward D-lactate and NAD^+. The activity stains were consistent with the potential protein band presented after incubated with Coomassie brilliant blue G-250 for 20 minutes (Figure 1e). The crude enzyme was further purified and evaluated its properties.

3.2 D-LDH Purification

The cell-free crude enzyme obtained was purified stepwise. Fold purification was demonstrated (Table 1) with the highest of 91 folds achieved.

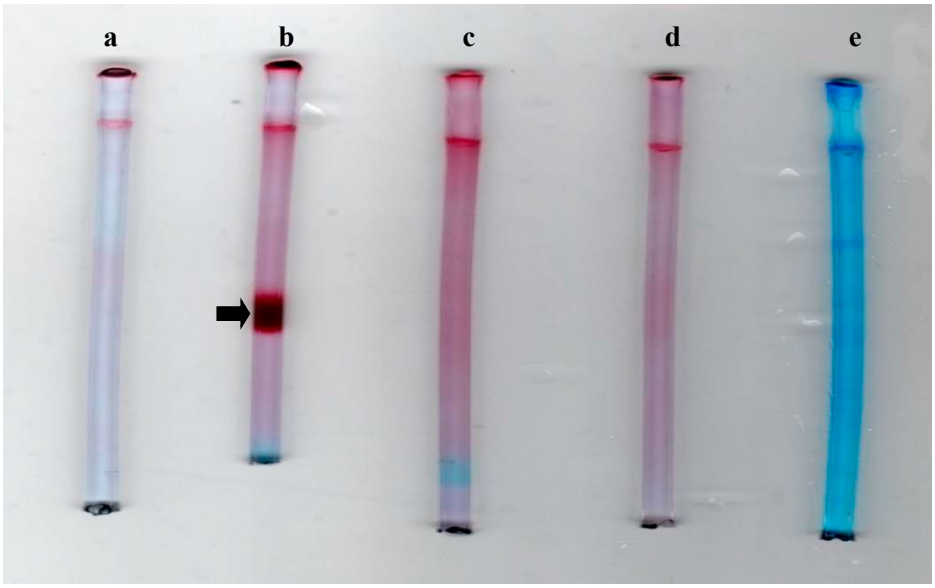

Figure 1. Native-PAGE of the crude enzyme was performed with 9.5% separating gel and 2.25% stacking gel in 4 × 80 mm capillary tubes, and run at 25 mA for 60 minutes using the Maxi Vertical Electrophoresis. Subsequently, each gel was pushed out of the tubes for enzyme activity stain with different reaction mixture, which were the followings: (a) D-lactate; (b) D-lactate and NAD^+; (c) D-lactate and $NADP^+$; (d) no substrate and no cofactor; (e) Coomassie brilliant blue G-250. Each gel was immersed in each reaction mixture and incubated at 30°C for 30 minutes prior to observation the red band of positive activity (pointed arrow). Afterwards, each stained gel was rinsed with distilled water, wiped out the water with napkin and placed onto a plastic sheet arranged in the order of activity stain substrate reaction for photography.

Table 1. Purification of D-LDH from *Leu. pseudomesenteroides* TC49.

Purification step	Total protein (mg)	D-LDH			
		Total activity (Unit)	Specific activity (Unit/mg)	Yield (%)	Fold purification
Cell-free crude enzyme	105.24	14.88	0.14	100	1
Ammonium sulfate precipitation	39.08	12.64	0.32	85	2
Anion exchange chromatography	6.405	10.19	1.60	69	11
Hydrophobic interaction chromatography	0.416	3.91	9.4	26	67
Gel filtration chromatography	0.167	2.13	12.75	14	91

One unit of enzyme activity characterized as the oxidation of 1 μmol NADH per minute. Total amount of enzyme before purification was defined as 100%.

For the ammonium sulfate precipitation, the suitable condition was 50% (w/v) saturation, which yielded the highest enzyme activity, 0.32 U/mg. Meanwhile, 1 M of NaCl and 35% (w/v) of $(NH_4)_2SO_4$ provided the highest activities of the enzyme for the purification steps of anion exchange (1.60 U/mg) and hydrophobic interaction chromatography (9.4 U/mg), respectively. When the partial purified protein obtained from the hydrophobic interaction chromatography was subjected to further purification by the gel filtration chromatography, a specific enzyme activity of 12.75 U/mg and a total protein of 0.167 mg were accomplished with the maximum fold purification. Notably, the purified protein showed only the D-LDH activity while the L-LDH activity was not found. The purified enzyme displayed a molecular weight of 40.6 kDa conducted by the SDS-PAGE (Figure 2). During the purification process, D-LDH was soluble after cell disruption indicating the readily releasing membrane bound of the enzyme. This enzyme character was similar to D-LDH of *Sulfolobus tokodaii* strain 7 [26]. Furthermore, the set up protocol was highly successful for D-LDH purification because it provided the high purity of D-LDH and very promising for the upscale enzyme production in the future with some modifications will be applied such as the lower cost of materials used in chromatography.

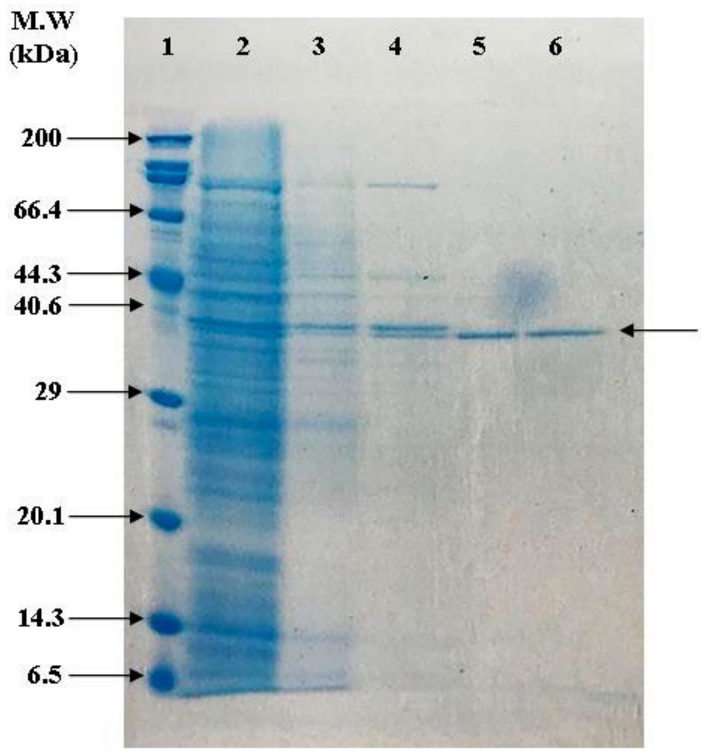

Figure 2. SDS-PAGE of D-LDH from each purification step: lane 1, markers; lane 2, crude enzyme; lane 3, enzyme after ammonium sulfate precipitation; lane 4, enzyme after anion exchange chromatography; lane 5, enzyme after hydrophobic interaction chromatography; lane 6, enzyme after gel filtration chromatography. Protein Molecular Weight Marker (Broad) for SDS-PAGE was used. All samples were performed simultaneously using AE-6530 mPAGE Mini-slab size electrophoresis system at 25 mA, 300 V for 60 minutes.

3.3 D-LDH N-terminal Amino Acid Sequence Investigation

The N-terminal amino acid sequence of the purified D-LDH was **MKIFAYGIRE**. The sequence was compared with other protein sequences in database of NCBI (National Center for Biotechnology Information) and KEGG (Kyoto Encyclopedia of Genes and Genome), and the phylogenetic tree was constructed (Figure 3). The N-terminal amino acid sequence of D-LDH from *Leu. pseudomesenteroides* TC49 was similar to that of other species within the genus by 10-90% while *Weissella paramesenteroides* ATCC 33313 (EER75533) and *Lactobacillus futsaii* JCM 17355 (KRK95745) expressed the maximum of 100% similarity. The N-terminal amino acid sequence of *Leu. pseudomesenteroides* TC49 D-LDH was clearly separated from that of *Leu. mesenteroides* subsp. *mesenteroides* J18: MI1_07960 (AET31034) and *Leu. mesenteroides* subsp. *mesenteroides* ATCC 8293 (ABJ62843). Although, the strain TC49 shared 100% similarity of N-terminal amino acid sequence with *W. paramesenteroides* ATCC 33313 and *L. futsaii* JCM 17355, however, the previous disclosure of D-LDH full-length amino acid sequence of *Leu. pseudomesenteroides* LMGCF08 (ORI97229) presented only 30 and 32% identity with *W. paramesenteroides* ATCC 33313 and *L. futsaii* JCM 17355, respectively. Meanwhile, the full-length D-LDH amino acid sequence of *Leu. pseudomesenteroides* LMGCF08 (ORI97229) had 35 and 27% sequence similarity with *Leu. mesenteroides* subsp. *mesenteroides* J18 and ATCC 8293, respectively. Possibly, the partial D-LDH gene sequences of *Leu. pseudomesenteroides* TC49 was horizontally inherited from *W. paramesenteroides*, *Lactobacillus* sp. or other strains rather than by the vertical gene transfer. Hence, the D-LDH gene transmission of *Leu. pseudomesenteroides* TC49 would be worth investigating in the future.

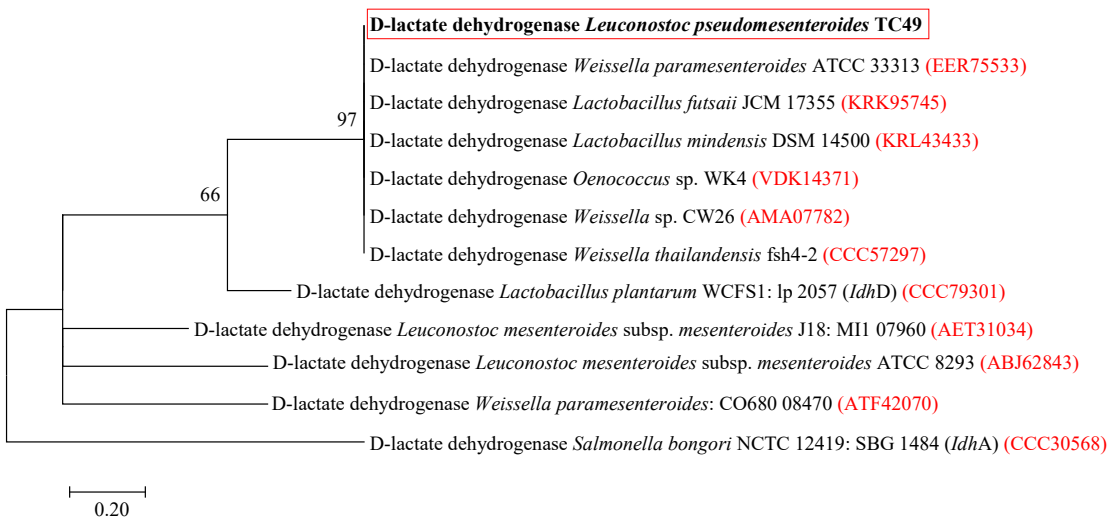

Figure 3. Phylogenetic tree analysis of D-lactate dehydrogenase amino acid sequences from *Leu. pseudomesenteroides* TC49 (bold) and some species generated D-lactate dehydrogenase by Maximum Likelihood method based on the JTT matrix-based model. Bootstrap values (expressed as percentages of 1,000 replications). 50% are shown at the branch points. Bar, 0.20 substitutions per nucleotide position. The D-lactate dehydrogenase amino acid sequences of *Salmonella bongori* NCTC 12419: SBG 1484 (*Idh*A) is presented as an outgroup sequence. Sequence alignment and phylogenetic tree were done using MEGA 7.0 software.

3.4 Effect of pH and Temperature on D-LDH Activity and Stability

The purified D-LDH was examined its activity and stability in various pH buffers (pH 4.0-11.0). The enzyme activity was measured at 30°C for 5 minutes while the pH stability was conducted by incubating the enzyme in the corresponding buffer for 30 minutes prior to enzyme activity measurement at 30°C for 5 minutes. The results showed that the maximum enzyme activity was observed in alkali condition with the pH value of 8.5 (Figure 4a). This result indicated that D-LDH of *Leu. pseudomesenteroides* TC49 is strongly resistant to alkali condition. This finding is not different from the previous reports on *E. coli* (D-LDH gene from *Leu. mesenteroides* ssp. *mesenteroides* J18), *L. jensenii* [5], *Leu. mesenteroides* subsp. *mesenteroides* ATCC 8293 [4] and *S. inulinus* [15] that their D-LDHs activity for lactate oxidation was found at pH 7.5-12.0 [27]. However, long time exposure of the enzyme with the buffer pH above 7.5 led to the enzyme disruption. In the study, the enzyme was highly stable in the pH ranging between 4.5 and 7.5 and was completely disrupted after 30 minutes incubation at pH above 9.5 (Figure 4b). The enzyme activity determination was conducted within 5 minutes but the enzyme stability determination was performed for 30 minutes. Therefore, the enzyme was challenged in a longer period of time in the stability test. Possibly, the alkaline buffer pH might disturb the active site of the enzyme and eventually abolished its function. Moreover, the data obtained were consistent with the character of the strain which had the optimal initial media pH and temperature for growth at pH 6.5 and 37°C, respectively [18].

To evaluate the effect of temperature on enzyme activity, it was found that the purified D-LDH displayed the highest activity at 30°C (Figure 5a). For the thermal stability, the enzyme was gradually inactivated when the temperature was lower or higher than the optimal temperature (30°C) and was completely disrupted at 50°C and the above (Figure 5b).

The optimal pH and temperature for enzyme activity of *Leu. pseudomesenteroides* D-LDH was similar to the report on D-LDH enzyme activity of *Leu. mesenteroides* subsp. *mesenteroides* ATCC 8293 (*Idh*D) [4]. The enzyme remained stable at acidic to neutral pH values (pH 4.5-7.5) and at temperatures between 25 and 40°C which was adjacent to the optimal growth conditions of *Leuconostoc* sp., optimal pH and temperature of *L. reuteri, E. coli* BL21 (DE3) D-LDH activities [27, 28].

Interestingly, while the optimal pH and temperature for enzyme activity of *Leu. pseudomesenteroides* TC49 D-LDH were similar

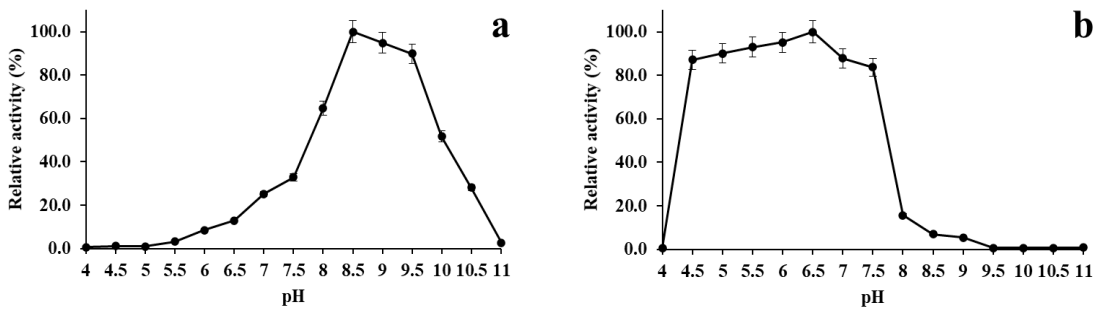

Figure 4. Effect of pH on D-LDH from *Leu. pseudomesenteroides* TC49: (a) enzyme activity, 5 minutes incubation time (b) enzyme stability, 30 minutes incubation time. The reactions were conducted at 30°C.

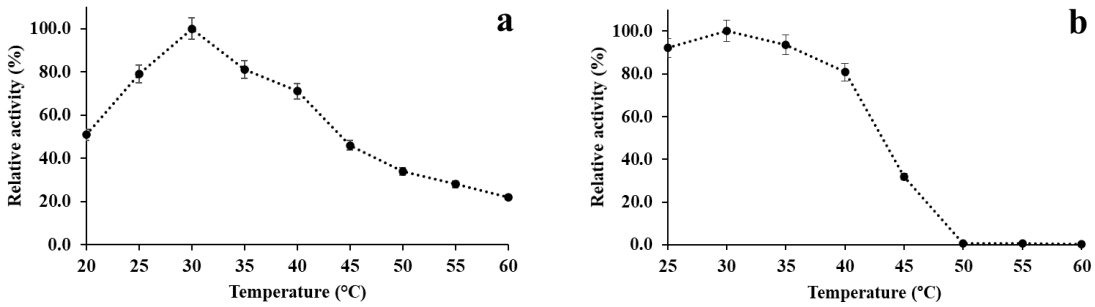

Figure 5. Effect of temperature on enzyme activity of D-LDH from *Leu. pseudomesenteroides* TC49: (a) optimal temperature of enzyme activity, 5 minutes incubation time; (b) range of temperature for enzyme stability, 30 minutes incubation time.

to that of *Leu. mesenteroides*, their N-terminal amino acid sequences were distinct. Rather, its N-terminal amino acid sequence is similar to that of *W. paramesenteroides* ATCC 33313 and *L. futsaii* JCM 17355. This phenomenon suggested that the N-terminal amino acid sequence of D-LDH may play no role on effect of pH and temperature of the enzyme activity. However, this hypothesis needs proof by elucidating the structure of the purified enzyme. Noteworthy, members of the genus *Weissella* were once designated in the genera *Leuconostoc* and *Lactobacillus*, hence, some phenotypic properties are difficult to separate among these bacterial groups while their genotypic characteristics are diverse [29].

3.5 Effects of Metal Ions and Chemicals on D-LDH Activity

Most test ions and chemicals slightly suppressed the enzyme activity except for $AgNO_3$ and $ZnCl_2$ which were able to inhibit the D-LDH activity by 49% and 81%, respectively (Figure 6). Furthermore, all test ions and chemicals could not enhance the enzyme activity. Likely, the heavy ions may inhibit disulfide bond formation in the protein by binding to an SH group [30]. Similarly, D-LDH of *Leu. mesenteroides* subsp. *mesenteroides* ATCC 8293 was inhibited by 1 mM Zn^{2+} and 1 mM SDS [3] by 72% and 100%, respectively. In addition, Zn^{2+} could inhibit the activity of *L. reuteri* D-LDH [28] while Ag^{+} was able to inhibit the activity of the α-1,3-glucanase from *Streptomyces thermodiasticus* HF 3-3 by 80% [28].

3.6 Substrate Specificity of D-LDH

When pyruvate was replaced by other substrates including 2-oxoglutaric acid, oxaloacetic acid and 2-ketobutyric acid, and lactate was replaced by malic acid, D-LDH activity was disappeared. The results suggested that the longer chain of carbon, the lower affinity of the enzyme. This purified *Leu. pseudomesenteroides* TC49 D-LDH was highly specific to pyruvate and D-lactate which was similar to other reports studying in *Leu. mesenteroides* subsp. *mesenteroides* ATCC 8293 and *E. coli* (*Idh*D) [4, 5].

3.7 Kinetic Characteristics of *Leu. pseudomesenteroides* TC49 D-LDH

D-LDH was analyzed against various concentrations of pyruvate or D-lactate, and NADH or NAD^{+} in accordance with pyruvate reduction or D-lactate oxidation determination. Lineweaver-Burk plots were performed to determine maximum velocity (V_{max}), Michaelis-Menten constant (K_m) and turnover number (k_{cat})

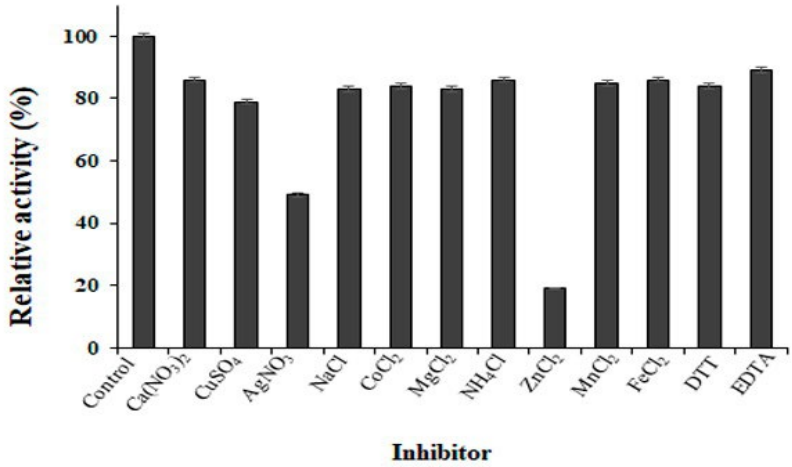

Figure 6. Influence of metal ions and chemicals on D-LDH activity. Each test ion or chemical, 1 mM, was added into the reaction mixture and incubated with D-LDH at 30°C for 30 minutes prior to determination of the enzyme activity. Control was referred to the reaction without test ions or chemicals added.

Table 2. Kinetic properties of *Leu. pseudomesenteroides* TC49 D-LDH.

Substrate	V_{max} (μmol/min/mg)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Pyruvate	270 ± 0.6	0.5 ± 0.02	180 ± 0.16	360.0
NADH	400 ± 0.57	0.08 ± 0.52	267 ± 0.28	3,337.5
D-Lactate	175 ± 0.22	69.6 ± 0.41	117 ± 0.15	1.681
NAD^+	125 ± 0.58	2.35 ± 0.10	83 ± 0.37	35.319

of pyruvate, D-lactate, NADH and NAD^+. K_m values for pyruvate, D-lactate, NADH and NAD^+ were 0.5, 69.6, 0.08 and 2.35 mM, respectively. In addition, k_{cat} values for pyruvate and D-lactate were 180 s^{-1} and 117 s^{-1}, respectively (Table 2). The K_m values obtained implied that the enzyme tended for pyruvate rather than D-lactate.

When compared the K_m for pyruvate of D-LDH in this study to the previous reports (Table 3), it was found that the *Leu. pseudomesenteroides* TC49 D-LDH (K_m = 0.50 mM) had lower K_m than that of *E. coli* BL21 (*Idh*D) from *Leu. mesenteroides* ATCC8293 (K_m = 0.58 mM) [4] < *P. cerevisiae* (K_m = 0.67 mM) [31] < *E. coli* BL21 (*Idh*D2) from *S. inulinus* YBS1-5 (K_m = 1.4 mM) [15] < *E. coli* BL21 (*Idh*D) from *Leu. mesenteroides* ssp. *mesenteroides* J18 (K_m = 2.66 mM) [27]. Potentially, the *Leu. pseudomesenteroides* TC49 D-LDH had higher affinity than D-LDH from other reports.

Table 3. Characteristics of *Leu. pseudomesenteroides* TC49 D-LDH compared with related species. T, temperature.

Strain	Optimal condition		K_m (mM)				k_{cat} (s^{-1})		k_{cat}/K_m ($mM^{-1}s^{-1}$)		Reference
	pH	T (°C)	Pyruvate	D-lactate	NADH	NAD^+	Pyruvate	D-lactate	Pyruvate	D-lactate	
Leu. pseudomesenteroides TC49	8.5	30	0.5 ± 0.02	69.6 ± 0.41	0.08 ± 0.52	2.35 ± 0.10	180 ± 0.16	117 ± 0.15	360.0	1.681	This study
P. cerevisiae	7.4	30	0.67	30	0.01	1.05	-	-	-	-	31
E. coli BL21 (*Idh*D) from *Leu. mesenteroides* ATCC8293	8.0	30	0.58	260	0.50	1.96	2900	2280	5,000	8.769	4
E. coli BL21 (*Idh*D) from *Leu. mesenteroides* ssp. *mesenteroides* J18	9.0	30	2.66	87.9	0.32	0.12	2,750	910	1,033.83	10.353	27
E. coli BL21 (*Idh*D2) from *S. inulinus* YBS1-5	7.5	30	1.4 ± 0.09	-	-	-	44.3 ± 0.03	-	-	-	15

4. Conclusions

Leuconostoc pseudomesenteroides TC49 D-LDH was successfully purified using the combination procedures of precipitation, dialysis, ultrafiltration and column chromatography. The high purified D-LDH would benefit in many applications from basic to advance ones such as a detection probe for product measurement and a tool in genetic engineering for bioconversion of high value products which will be elucidated in the future.

Conflict of interest

The authors have declared no conflict of interest.

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