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Enhancement of the catalytic activity of D-lactate dehydrogenase from *Sporolactobacillus laevolacticus* by site-directed mutagenesis

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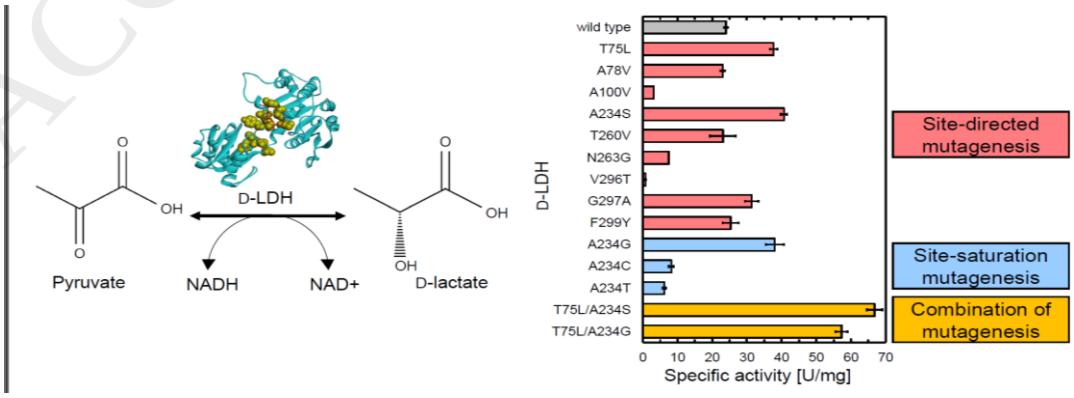
Graphical abstract



Highlights

- Finding of important amino acid residues that positively affect catalytic activity.
- Optimization of an amino acid residue to enhance the activity of D-LDH.
- Site-directed mutagenesis improved the activity of D-LDH from *S. laevolacticus.*
- Potential of the mutant D-LDH for microbial production of D-lactic acid.

ABSTRACT

Sporolactobacillus laevolacticus is a producer of D-lactic acid with high optical purity. The amino acid sequence of D-lactate dehydrogenase (D-LDH) from *S. laevolacticus* was compared with those of other lactate producers. To enhance the activity of D-LDH from *S. laevolacticus*, some amino acid residues close to the substrate binding site or the active center were replaced by site-directed mutagenesis. Ala234 of D-LDH from *S. laevolacticus* was found to be an important amino acid residue that positively affects catalytic activity. Site-saturation mutagenesis of the 234th residue was performed. The mutant D-LDH at the 234th residue from alanine to serine or glycine showed enhanced catalytic activity toward pyruvate. The kinetic analysis revealed that the k_{cat}/K_m of

D-LDH_A234S and _A234G on pyruvate increased 1.9- and 1.2-fold, respectively. Furthermore, the double mutant D-LDH_T75L/A234S improved k_{cat}/K_m by 6.8-fold compared to that of wild-type D-LDH. These results showed the potential of the mutant D-LDH for microbial production of D-lactic acid by heterologous expression.

Keywords: D-lactate dehydrogenase, D-lactic acid, polylactic acid, site-directed mutagenesis

1. Introduction

Lactic acid is a fermentation product required for the production of polylactic acid (PLA) [1]. PLA is a sustainable alternative to petrochemical-derived plastics because lactic acid can be produced on a large scale by the microbial fermentation of biomass [2]. Moreover, PLA is a biodegradable and biocompatible material that can be used in a wide variety of applications ranging from packaging to the production of fibers and foams [3]. PLA is regarded as an environment-friendly alternative to traditional plastics derived from fossil fuels [4].

PLA can exist in three stereochemical forms: poly (L-lactic acid) (PLLA), poly (D-lactic acid) (PDLA), and poly (DL-lactic acid) (PDLLA); these are polymers of L-lactic acid, D-lactic acid, and DL-lactic acid, respectively. The homopolymers, PLLA and PDLA, have a melting point of approximately 170°C, while PDLLA depresses the crystalline melting point to as low as 130°C [5, 6]. In addition, prior studies demonstrated that blending PLLA and PDLA could create a stereocomplex PLA with a melting point of 230°C, which is 50°C higher than those of the homopolymers [7, 8]. The fermentative production of L-lactic acid has advanced; however, the technology for D-lactic acid remains immature [9, 10] and there exists an increasing demand for

optically pure D-lactic acid production.

Almost all lactic acid bacteria have both L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH). In lactic acid bacteria producing D-lactic acid, the specific activity of purified D-LDH is much higher than that of purified L-LDH [11]. *Sporolactobacillus laevolacticus* (a D-lactic acid producer) is known to produce D-lactic acid with relatively high optical purity [6, 12]. It was expected that as D-LDH of *S. laevolacticus* had high catalytic activity, *S. laevolacticus* could produce D-lactic acid with high optical purity and yield.

In this report, D-LDH from *S. laevolacticus* was overexpressed in *Escherichia coli*, purified, and characterized *in vitro*. Enhancement of the specific activity of D-LDH from *S. laevolacticus* was attempted by using site-directed mutagenesis. The mutation points were identified by multiple sequence alignment of three LDHs. Furthermore, site-saturation mutagenesis at the specific residue and combination of these mutations were performed to construct mutant D-LDH that has superior catalytic activity.

2. Materials and methods

2.1. Construction of the expression plasmid

The D-LDH gene was amplified by PCR from pBL2 containing the gene encoding D-LDH from *S. laevolacticus* JCM 2513 [13] using the forward primer SLLDHD-FP2 (recognition site of *Nde* I underlined, 5′-GGA GAA TTC ATA TGA AAT TCT TGA TGT ATG-3′) and the reverse primer SLLDHD-RP (recognition site of *Xho* I underlined, 5′-TTG TCT CGA GTT TTG CCG ATA GCG GTT GTG CTT GAA CAA G-3′). The PCR product was recovered by the High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN, USA), and its size was confirmed as a 1,007-bp band by 2% (w/v) agarose gel electrophoresis. The purified PCR product was digested with *Nde* I and *Xho* I and ligated with the *Nde* I-*Xho* I-digested pET-21b(+) vector by using Ligation high Ver. 2.0 (Toyobo, Osaka, Japan). The D-LDH gene was His-tagged [hexa-histidine (6×His)] at the C-terminus and cloned downstream of the T7 promoter in the pET-21b(+) expression vector. The recombinant plasmid DNA, pEDldh, was transformed into both *E. coli* JM109 and *E. coli* BL21(DE3) cells for protein expression. Mutant D-LDH genes were constructed using the QuikChange Site-Directed

Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) and the abovementioned recombinant plasmid vector, pEDldh, as a template. The recombinant vector and all mutant genes were confirmed by DNA sequencing.

2.2. Expression and purification of D-LDHs

E. coli BL21(DE3) transformed with pEDldh and its derivatives, which harbored the wild-type and mutant D-LDH genes, respectively, were cultured in Luria-Bertani medium (10 g tryptone, 5 g yeast extract, 5 g NaCl) containing 100 mg/L ampicillin sodium. A volume of 200 mL medium in a 500 mL baffled Erlenmeyer flask was inoculated with the transformed cells and incubated at 37°C for 2-3 h with rotary shaking at 150 rpm. When the optical density at 600 nm (OD_{600}) reached 0.6–0.8, the expression of D-LDHs was induced for 4 h at 25°C with shaking at 150 rpm by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM [14].

The cells were harvested by centrifugation at 7,150 × *g* for 5 min at 4°C and re-suspended in a 50 mM Tris-HCl buffer (pH 7.0) twice. The suspension was disrupted by intermittent ultrasonic disintegration at 80 W for 40 min in an ice bath. The supernatant and precipitate of the resulting suspension were separated by centrifugation

at 11,200 × *g* for 5 min at 4°C into soluble and insoluble fractions, respectively.

Because the expressed D-LDHs were fused with a 6×His affinity purification motif at their C-termini, affinity chromatography using the His-Trap^{FF} column (GE Healthcare, Little Chalfont, UK) was performed to purify the recombinant D-LDHs. Supernatants containing the soluble recombinant D-LDHs were applied to a His-Trap^{FF} column, which was pre-equilibrated with an equilibration buffer (pH 7.0) containing 50 mM sodium phosphate and 10 mM imidazole at a flow rate of 1 mL/min. After the His-Trap^{FF} column was washed with 5 mL of washing buffer (pH 7.0) containing 50 mM sodium phosphate and 20 mM imidazole, the recombinant D-LDHs were finally eluted with 2 mL of elution buffer (pH 7.0) containing 50 mM sodium phosphate and 200 mM imidazole. The 2 mL of eluate was packed into a cellulose tube and dialyzed against 300 mL of 50 mM Tris-HCl buffer (pH 7.0) at 4°C overnight.

The protein concentrations were determined according to the Bradford method using Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan).

The expression and purity of the recombinant D-LDHs were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed by using stacking and separating gels containing 4% and 14.8% (w/v) acrylamide, respectively. The SDS treatment was performed by mixing 10

μL of samples and 10 μL of SDS sample buffer (pH 6.8) containing 62.5 mM Tris-HCl, 2% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, and 0.1% (w/v) bromophenol blue and incubating at 95°C for 5 min.

2.3. D-LDH activity assay

The activity of D-LDHs was measured using sodium pyruvate and NADH. A volume of 3 mL of the reaction mixture containing 10 mM sodium pyruvate and 0.2 mM NADH in 50 mM Tris-HCl buffer (pH 7.0) was pre-incubated at 27°C for 5 min. To start the reaction, 2 μL of D-LDH solution was added. The NADH concentration was continuously recorded by monitoring absorbance at 340 nm for 5 min during redox reactions catalyzed by D-LDH. One unit of activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol NADH per minute at 27°C and pH 7.0.

2.4. Kinetic parameters of D-LDHs

A kinetic study of D-LDHs was performed using various concentrations of sodium pyruvate. A volume of 3 mL of the reaction mixture containing 0.2, 0.5, 1, 5, 10,

20, 30, 60, 80 mM sodium pyruvate and 0.5 mM NADH in 50 mM Tris-HCl buffer (pH 7.0) was pre-incubated at 27°C for 5 min. To start the reaction, 5 μg of D-LDH was added. The kinetic parameters (k_{cat}/K_m) were determined by Lineweaver–Burk plots. All experimental data in this study were the average values of triplicate measurements.

3. Results and discussion

3.1. Multiple sequence alignment of D-LDHs

Lactic acid bacteria have both L-LDH and D-LDH. The specific activity of D-LDH from a D-lactic acid producer is much higher than that of D-LDH from an L-lactic acid producer [11]. To identify the target sites for site-directed mutagenesis, amino acid sequence alignment of D-LDH from *S. laevolacticus* JCM 2513 (a D-lactic acid producer) [13], *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (a D-lactic acid producer), and *Lactobacillus casei* ATCC 334 (an L-lactic acid producer) was used as shown in Figure 1. Amino acid sequences of D-LDHs from *L. bulgaricus* (accession number: **CAI96942**) and *L. casei* (accession number: **YP_805451**) were retrieved from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov). Amino acid residues adjacent to the substrate binding site or the active center, but not the active center, were selected as candidates. Ala234, Thr260, Asn263, and Val296 of D-LDH from *S. laevolacticus* were different from those from *L. bulgaricus* and conserved in those from *L. casei* compared with corresponding residues in amino acid type. Especially, Thr260 of D-LDH from *S. laevolacticus*

corresponds to Val261 of D-LDH of *L. bulgaricus*, which is located near the catalytic active center [15]. The 263th residue of D-LDH from *S. laevolacticus* is amino acid with a hydrophilic side chain, Asn and Tyr in D-LDHs from *S. laevolacticus* and *L. casei*, a L-lactic acid producer, respectively, while Gly in D-LDH from *L. bulgaricus*, D-lactic acid producer. The 296th residue is amino acid with a hydrophobic side chain, Val and Ile in D-LDHs from *S. laevolacticus* and *L. casei*, respectively, while Thr with a hydrophilic side chain in D-LDH from *L. bulgaricus*. Therefore, it was assumed that the replacements of Asn263 and Val296 of D-LDH from *S. laevolacticus* by Gly and Thr would affect the catalytic activity. Thr75, Thr77, Ala78, Ala100, Gly297, and Phe299 of D-LDH from *S. laevolacticus* were different from those from both *L. bulgaricus* and *L. casei*. Amino acid residues of D-LDH from *S. laevolacticus*, which were same or similar to those from *L. casei* and different from those from *L. bulgaricus*, were replaced to match those found in D-LDH from *L. bulgaricus*. Variants of D-LDH_A234S, _T260V, _N263G, _V296T, _T75L, _T77N, _A78V, _A100V, _G297A, and _F299Y of *S. laevolacticus* were therefore constructed by site-directed mutagenesis. In Fig. 2, the replaced amino acid residues were simply mapped onto the three-dimensional structure of D-LDH from *S. inulinus* (PDB ID 4XKJ) that is 49% identical to that from *S. laevolacticus*.

3.2. Heterologous expression of wild-type D-LDH of S. laevolacticus in E. coli

The wild-type D-LDH gene of *S. laevolacticus* was cloned into the expression vector pET-21b(+), and 6×His-tagged wild-type D-LDH was overexpressed in *E. coli* BL21(DE3) under the control of the T7 promoter. Protein expression was induced with 1.0 mM IPTG, and the expressed proteins were analyzed by SDS-PAGE (Fig. 3). Most of the expressed 6×His-tagged wild-type D-LDH was found in a soluble fraction. After purification by affinity chromatography with the His-Trap^{FF} column and dialysis, a single band of approximately 36 kDa appeared, confirming the calculated molecular mass of 38 kDa derived from the amino acid sequence of a single subunit. The yield of 6×His-tagged wild-type D-LDH purified from the cell disruption supernatant was 39.6%. The specific activity of purified 6×His-tagged wild-type D-LDH was calculated to be 24.0 U/mg in the reduction of sodium pyruvate with NADH as a cofactor at 27°C.

Remaining activities of the wild-type D-LDH after 15 min-incubation at various temperatures are showed in Fig. 4. The wild-type D-LDH was stable under 30ºC.

3.3. Characterization of specific activities of mutant D-LDHs

Most 6×His-tagged mutant D-LDHs (D-LDH_T75L, _A78V, _A100V, _A234S, _T260V, _N263G, _V296T, _G297A, and _F299Y) were expressed in the soluble fraction, while D-LDH_T77N was expressed in the insoluble fraction. After the purification and measurement of activity, the expressed 6×His-tagged mutant D-LDHs except for D-LDH_T77N were found to have catalytic activity. Figure 5 shows the specific activities of mutant D-LDHs, except for those of D-LDH_T77N.

The specific activities of D-LDH_A78V, _T260V, and _F299Y were 23.0 ± 0.6, 23.1 ± 3.7, and 25.3 ± 2.3 U/mg, respectively, which are similar to that of the wild-type D-LDH. The D-LDH_T75L and _A234S showed higher catalytic efficiency than the wild-type D-LDH. The specific activities of D-LDH_T75L and _A234S were 37.7 ± 1.1 and 40.7 ± 0.9 U/mg, respectively.

3.4. Site-saturation mutagenesis of residue 234 of D-LDH

In D-LDH from *S. laevolacticus*, Ala234 neighbors the active center Arg235. The D-LDH_A234S showed the highest specific activity among the single mutant

D-LDHs. This suggested that 234th amino acid residue played an important role in catalytic reaction. Site-saturation mutagenesis of residue 234 was subsequently performed. Although D-LDH_A234P was expressed in the insoluble fraction, mutant D-LDHs, except for D-LDH_A234P, were expressed in the soluble fraction. The site-saturation mutagenesis at position 234 revealed that only D-LDH_A234S, _A234G, _A234C, and _A234T retained catalytic activity. Figure 5 shows the results of these mutant D-LDH activity assays. D-LDH mutants, in which the 234th residue was changed from Ala to Cys or Thr, showed lower catalytic activity than the wild-type D-LDH. In contrast, D-LDH_A234S and D-LDH_A234G respectively demonstrated 1.7-fold and 1.6-fold improvement in specific activity over the wild-type D-LDH (40.7 ± 0.9 and 38.0 ± 2.6 U/mg, respectively). The capacity of the substrate binding pocket depends greatly on the size of the side chain [16]. Because the size of Ser is similar to that of Ala and Gly is the smallest residue, the replacement of Ala234 by a smaller amino acid significantly increased the specific activity. Thus, the catalytic activity was lost because of mutation at position 234 of D-LDH to amino acids larger than Thr because of the steric constraint, and the catalytic activity increased with decreasing sizes of the amino acid residues. According to the D-LDH from *S. inulinus* (PDB ID 4XKJ), Ser233 (Ala234 in that from *S. laevolacticus*) forms hydrogen bonds to the conserved Asn231

(Asn232 in that from *S. laevolacticus*). The possible hypothesis is that mutation from Ala to Ser at position 234 of D-LDH from *S. laevolacticus* may have affected conformation of substrate binding site.

Since two mutations with improved activity were identified, the double mutant D-LDH_T75L/A234S and _T75L/A234G were created by site-directed mutagenesis. The specific activity of the double mutant D-LDH_T75L/A234S and _T75L/A234G were improved to 66.8 ± 2.2 and 57.3 ± 1.7 U/mg, respectively, which are much higher than that of wild-type D-LDH. In the D-LDH from *L. bulgaricus*, Leu76 and Ser235 are located on adjacent structural elements but not close enough to interact with each other (15.6 Å) [17]. Therefore, this suggested that the mutations at position 75th and 234th independently enhanced the activity of D-LDH. Double mutation at 75th and 234th synergistically enhanced the activity of D-LDH.

3.5. Kinetic study of D-LDH

The kinetic parameters of D-LDHs were determined using sodium pyruvate as a substrate. The kinetic parameters K_m and k_{cat} of D-LDHs are summarized in Table 1. D-LDH_A100V, _T260V, _N263G, and _F299Y had higher K_m than the wild-type

D-LDH, leading to decreased k_{cat}/K_m values. D-LDH_A234S had lower K_m and higher k_{cat}, resulting in the 1.9-fold increased k_{cat}/K_m compared with that of the wild-type D-LDH. The k_{cat}/K_m value of D-LDH_A234G increased by 1.2-fold because of the increased k_{cat}. From this result, the mutation at position 234 would affect rather the catalytic efficiency than the affinity between D-LDH and pyruvate. The sequence similarity of D-LDH from *S. laevolacticus* and *L. bulgaricus* is 80%. The residue 234 of D-LDH from *S. laevolacticus* may have significantly affected the catalytic activity because Arg236 of D-LDH, which is the active center, from *L. bulgaricus* corresponds to Arg235 of D-LDH from *S. laevolacticus*. Most strikingly, D-LDH_T75L/A234G and _T75L/A234S had lower K_m and much higher k_{cat}. Therefore, the k_{cat}/K_m value of D-LDH_T75L/A234G and _T75L/A234S increased by 5.0 and 6.8-fold, respectively. These results suggest that the replacement of Tyr75 and Ala234 synergistically improves catalytic efficiency.

4. Conclusions

Ala234 of D-LDH played an important role in catalytic reaction. Site-saturation mutagenesis indicated that the catalytic efficiency depends greatly on the size of the side chain at position 234. Furthermore, double mutation at 75th and 234th amino acid residues enhanced the catalytic activity. The catalytic efficiency (k_{cat}/K_m) of D-LDH_T75L/A234S was 6.8-fold higher than that of wild-type D-LDH. In previous researches, various characterizations of D-LDHs have been reported [18, 19]. The previous research showed that the improvement of enzymatic efficiency of the mutated D-LDH increased the microbial production of D-lactic acid [20]. In this study, enhancement of the catalytic activity of D-LDH from *S. laevolacticus* was achieved. This is fist report on enhancement of the catalytic activity of D-LDH. Mutated D-LDH with high catalytic activity would contribute to the improvement of D-lactic acid productivity in recombinant microorganism. Thus, the mutant D-LDH could be applied for the production of D-lactic acid.

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Figure captions

Fig. 1. Multiple sequence alignment of D-LDH from *S. laevolacticus*, *L. bulgaricu*s, and *L. casei*. Amino acid sequences of D-LDHs were aligned using multiple alignment program of the Genetyx software (Genetyx, Tokyo, Japan). The red characters show amino acid residues with hydrophobic side chains. The triangle and asterisk indicate the substrate binding site and active center of *L. bulgaricus*, respectively. The circle represents the mutated residue.

Fig. 2 The position of the replaced amino acid residues in D-LDH from *S. laevolacticus*. Ten replaced amino acid residues and active center (Arg235, Glu264, and His295) are represented by yellow CPK models and red stick models in the solid ribbon model of D-LDH from *L. bulgaricus* (PDB ID 1J49), respectively.

Fig. 3. SDS-PAGE analysis of the expressed and purified wild-type D-LDH. Lane M: protein markers, Lane 1: soluble fraction in cells from 50 μl-culture, Lane 2: insoluble fraction in cells from 50 μl-culture, Lane 3: purified wild-type D-LDH.

Fig. 4. Thermal stability of the wild-type D-LDH.

Fig. 5. The specific activities of the wild-type and various mutant D-LDHs of *S. laevolacticus*. Error bars represent the standard deviations of the means for three independent experiments.

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S. laevolacticus   1 -MKFLMYGVQDHERATIENWANQH-QVEIATTSDFLSEDTVAQSKGFDGI 48
L. bulgaricus      1 MTKIFAYAIREDEKPFLKEWEDAHKDVEVEYTDKLLTPETVALAKGADGV 50
L. casei           1 -MKIIAYGARVDEIQYFKQWAKDT-GNTLEYHTEFLDENTVEWAKGFDGI 48

S. laevolacticus  49 CIQQPIALGGPNLYTQLKNNGIKQIATRTAGYDMIDLNEAEKNSLLVTNV 98
L. bulgaricus     51 VVYQQLDYTA-ETLQALADNGITKMSLRNVGVDNIDMAKAKELGFQITNV 99
L. casei          49 NSLQITPYAA-GVFEKMHAYGIKFLTIRNVGTDNIDMTAMKQYGIRLSNV 97

S. laevolacticus  99 PAYSPYAVAELAVTQAMQLVRHIPEFNKRVAGKDFRWSG-LISREIRSLT 147
L. bulgaricus    100 PVYSPNAIAEHAAIQAARILRQDKAMDEKVARHDLRWAP-TIGREVRDQV 148
L. casei          98 PAYSPAAIAEFALTDTLYLLRNMGKVQAQLQAGDYEKAGTFIGKELGQQT 147

S. laevolacticus 148 VGIVGTGRIGATAAQLFKGLGAKIIGFDQYPNDRLNGILDYRPSLEDVLK 197
L. bulgaricus    149 VGVIGTGHIGQVFMQIMEGFGAKVIAYDIFRNPELEKKGYYVDSLDDLYK 198
L. casei         148 VGVMGTGHIGQVAIKLFKGFGAKVIAYDPYPMKGDHPDFDY-VSLEDLFK 196

S. laevolacticus 198 EADIISLHTPLFDSTRHMINKSTLKLMKNSAYLINVARGGLIKTEDLIEA 247
L. bulgaricus    199 QADVISLHVPDVPANVHMINDESIAKMKQDVVIVNVSRGPLVDTDAVIRG 248
L. casei         197 QSDIIDLHVPGIEQNTHIINEAAFNLMKPGAIVINTARPNLIDTQAMLSN 246

S. laevolacticus 248 LENGEIAGAALDTFENELMINKDLSKQ-PLNDPLLSKLLDMEQVLLTPHV 296
L. bulgaricus    249 LDSGKIFGYAMDVYEGEVGIFNEDWEGKEFPDARLADLIARPNVLVTPHT 298
L. casei         247 LKSGKLAGVGIDTYEYETEDLLNLAKHGSFKDPLWDELLGMPNVVLSPHI 296

S. laevolacticus 297 GFFTETAIQNIVEGALDSVVEVLKTGTSKNLVQAQPLSAK           336
L. bulgaricus    299 AFYTTHAVRNMVVKAFDNNLELVEGKEAETPVKVG-----           333
L. casei         297 AYYTETAVHNMVYFSLQHLVDFLTNGETSTEVTGPAK---           333
```

Fig. 1

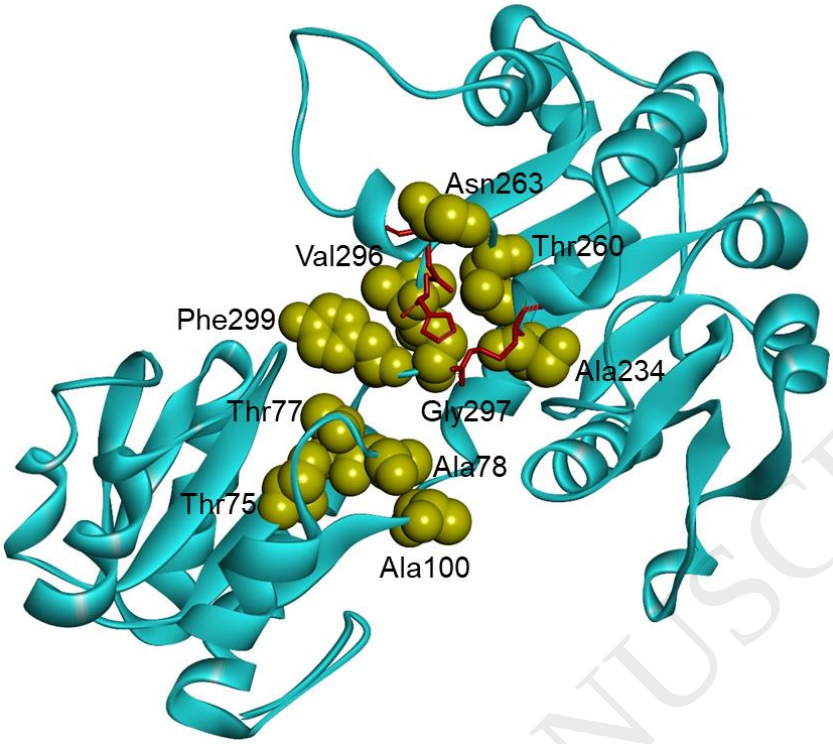

Fig. 2

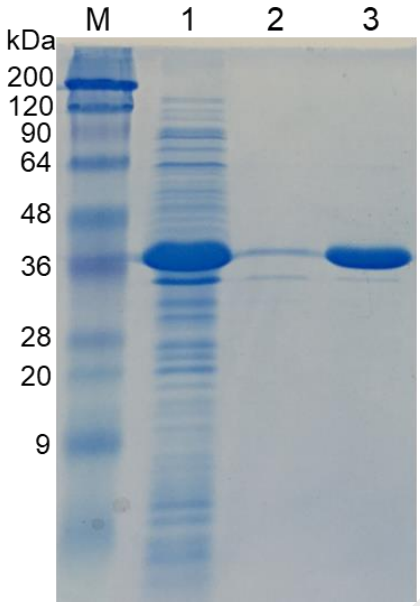

Fig. 3

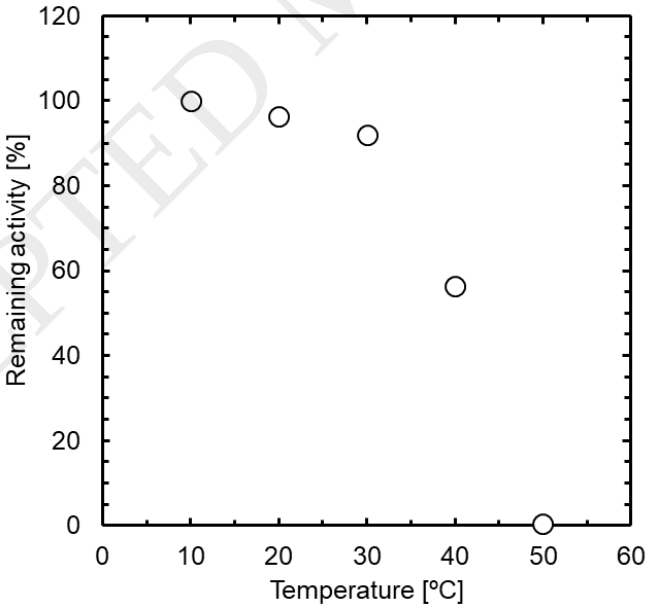

Fig. 4

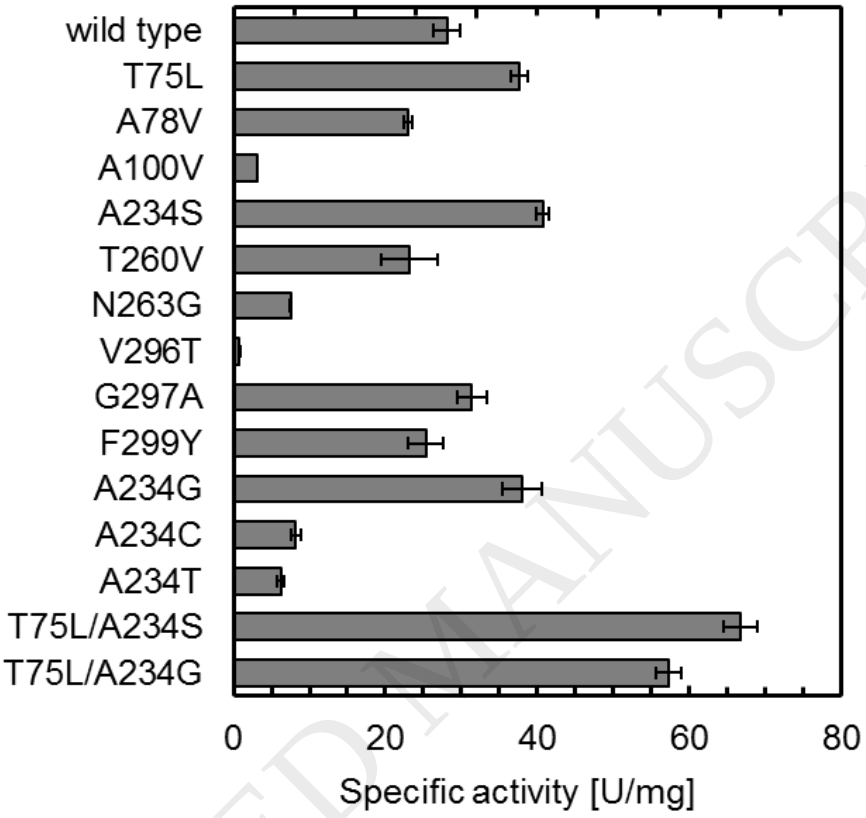

Fig. 5

Tables

Table 1: Kinetics parameters of the purified His-tagged D-LDHs.

D-LDH	$k_{cat}\times10^{-4}(s^{-1})$	K_m (mM)	$k_{cat}/K_m\times10^{-6}$ ($M^{-1}s^{-1}$)
D-LDH (wild-type)	4.62	14.8	3.12
D-LDH_T75L	7.77	14.1	5.51
D-LDH_A78V	3.11	9.20	3.38
D-LDH_A100V	1.59	67.3	0.236
D-LDH_A234S	5.68	9.49	5.99
D-LDH_T260V	5.66	21.4	2.65
D-LDH_N263G	7.21	136	0.530
D-LDH_G297A	1.90	6.10	3.11
D-LDH_F299Y	4.37	20.8	2.10
D-LDH_A234G	6.85	18.5	3.70
D-LDH_A234C	0.755	5.98	1.26
D-LDH_A234T	0.880	15.3	0.575
D-LDH_T75L/A234S	22.5	10.6	21.1
D-LDH_T75L/A234G	18.1	11.5	15.7