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ORIGINAL ARTICLE

D-Lactic acid fermentation performance and the enzyme activity of a novel bacterium *Terrilactibacillus laevilacticus* SK5–6

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Abstract

Purpose The aim of this study was to prove that *Terrilactibacillus laevilacticus* SK5-6, a novel D-lactate producer, exhibited a good fermentation performance comparing to the reference D-lactate producer *Sporolactobacillus* sp.

Methods Glucose bioconversion for D-lactate production and the activity of five key enzymes including phosphofructokinase (PFK), pyruvate kinase (PYK), D-lactate dehydrogenase (D-LDH), L-lactate dehydrogenase (L-LDH), and lactate isomerase (LI) were investigated in the cultivation of *T. laevilacticus* SK5–6 and *S. laevolacticus* 0361^T.

Results *T. laevilacticus* SK5–6 produced D-lactate at higher yield, productivity, and optical purity compared with *S. laevolacticus* 0361^T. *T. laevilacticus* SK5–6, the catalase-positive isolate, simultaneously grew and produced D-lactate without lag phase while delayed growth and D-lactate production were observed in the culture of *S. laevolacticus* 0361^T. The higher production of D-lactate in *T. laevilacticus* SK5–6 was due to the higher growth rate and the higher specific activities of the key enzymes observed at the early stage of the fermentation. The low isomerization activity was responsible for the high optical purity of D-lactate in the cultivation of *T. laevilacticus* SK5–6.

Conclusion The lowest specific activity of PFK following by PYK and D/L-LDHs, respectively, indicated that the conversion of fructose-6-phosphate was the rate limiting step. Under the well-optimized conditions, the activation of D/L-LDHs by fructose-1,6-phosphate and ATP regeneration by PYK drove glucose bioconversion toward D-lactate. The optical purity of D-lactate was controlled by D/L-LDHs and the activation of isomerases. High D-LDH with limited isomerase activity was preferable during the fermentation as it assured the high optical purity.

Keywords D-Lactic acid · *Terrilactibacillus laevilacticus* · Fermentation · Glycolytic enzymes · Lactate isomerization · Lactate dehydrogenase

The GenBank/EMBL/DBBJ accession number for the 16S rRNA of *T. laevilacticus* strain SK5-6 is LC222555

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Introduction

Lactic acid as a chiral compound provides essential roles in industrial applications in food industries and life sciences. Approximately 70% of lactic acid produced is utilized in the food industry (Es et al. 2018). During the last decade, attention was given to the commercially valuable products by microbial biotechnology due to the raised issues in global energy and environmental concerns. This provides the great potential for producing green, biodegradable, and biocompatible polylactic acid (PLA) for replacement of the plastic commodities from petrochemical based feedstocks. The optical purity of lactic acid (both D- and L-lactic acid) plays a crucial role in controlling the physical properties of PDLLA (Cingadi et al. 2015). Although microbial fermentation of L-lactic acid has long been commercially established, there are relatively few studies on D-lactic acid fermentation (Tsuge et al. 2014). Microbial

fermentation can produce three forms of lactic acid, e.g., L-lactate, D-lactate, and DL-lactate, depending on the microbes employed (John et al. 2009). A few wild-type strains such as *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Sporolactobacillus inulinus*, *Sporolactobacillus laevolacticus*, *Sporolactobacillus terrae*, *Lactobacillus coryniformis* subsp. *torquens*, and *L. delbrueckii* subsp. *lactis* QU41 and some metabolically engineered strains such as *Lactobacillus plantarum*, *Escherichia coli*, *Corynebacterium glutamicum*, *Enterobacter asburiae*, and *Saccharomyces cerevisiae* including a novel genus *Terrilactibacillus* used in this study have been reported for the production of optical pure D-lactic acid (Tashiro et al. 2011; Mimitsuka et al. 2012; Li et al. 2013; Zhao et al. 2014; Cingadi et al. 2015; Bai et al. 2016; Prasirtsak et al. 2016).

Terrilactibacillus genus is comprised of the catalase-positive bacteria that can be grown in the presence of oxygen (Prasirtsak et al. 2016). It produces the higher cell biomass and lactic acid than other previously reported D-lactate producers that usually lack catalase, and thus, required growth and lactic acid production under anaerobic conditions (Prasirtsak et al. 2017). Owing to the above-mentioned evidences, *Terrilactibacillus laevilacticus* SK5–6 was selected for further investigating the fermentation performance and understanding its bioconversion of glucose to lactic acid from the specific activities of the key enzymes in glycolytic pathway as well as lactate production and isomerization. It is believed that by understanding the regulation of the key enzymes involving in glucose bioconversion for D-lactate production, one can apply this knowledge in process optimization to achieve high fermentation performance.

Materials and methods

Microorganism and inoculum preparation

Terrilactibacillus laevilacticus SK5–6, a catalase-positive D-lactic acid producing bacterium, was used for D-lactic acid production and enzyme activity assay (Prasirtsak et al. 2017). The GenBank/EMBL/DBBJ accession number for the 16S rRNA of strain SK5–6 is LC222555. A catalase-negative D-lactic acid producing bacterium, *Sporolactobacillus laevolacticus* NRIC 0361^T, was obtained from NODAI Culture Collection Center (NRIC) for use as the reference D-lactic acid producer. Both strains were maintained on the GYP agar containing (per liter) 10 g glucose, 5 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, 20 g agar, 5 g $CaCO_3$, and 10 mL salt solution. Salt solution consisted of (per 10 mL) 400 mg $MgSO_4{\cdot}7H_2O$, 20 mg $MnSO_4{\cdot}5H_2O$, 20 mg $FeSO_4{\cdot}7H_2O$, and 20 mg NaCl. The pH of the GYP agar medium was adjusted to 6.80. Both isolates were subcultured onto new GYP agar slants and incubated at 37 °C for 24–48 h prior to preparing the bacterial suspension for inoculation.

Fermentation cultivation of the catalase-positive *T. laevilacticus* SK5–6 for D-lactic acid

D-lactic acid production by the catalase-positive *T. laevilacticus* SK5–6 was previously optimized for the flask cultivation and reported in Prasirtsak et al. (2017). The fermentation consisted of two phases, (i) the preculture phase for cell growth and (ii) the production phase for producing D-lactic acid by cells obtained in the preculture phase. *T. laevilacticus* SK5–6 was subcultured onto the GYP agar slant and incubated at 37 °C for 24 h and suspended in the sterile NaCl solution (0.85% (w/v)). The optical density (OD) at 600 nm of the bacterial suspension was adjusted to approximately 30–40 using sterile NaCl solution. The diluted suspension was then used to inoculate into the sterile preculture medium at 1% (v/v) inoculum size. The preculture medium for the catalase-positive bacteria (pH 6.8) contained (in 1 L) 10 g glucose, 15 g yeast extract, 4 g NH_4Cl, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 5 g $CaCO_3$, and 20 mL salt solution. The culture was incubated at 37 °C, 200 rpm for 4 h in a flask plugged with a C-type silicone stopper. Then, 25 mL of this preculture broth was transferred into 25 mL of sterile fermentation medium for the catalase-positive isolate which contained (in liter) 240 g glucose and 160 g $CaCO_3$ and continued to incubate in a flask plugged with a T-type silicone stopper in a W-zip pouch containing AnaeroPack-Anaero gas pack (Mitsubishi Gas Chemical, Tokyo, Japan) at 37 °C, 150 rpm for 48 h. Samples were taken every 12 h for analyses of OD600 reading, the remaining glucose, D-lactate and byproduct formation, and the optical purity of D-lactate product. Cell biomass was also collected for enzymatic activity assays.

Fermentation cultivation of the catalase-negative *S. laevolacticus* 0361^T for D-lactic acid

S. laevolacticus 0361^T, the catalase-negative D-lactate producer, was also used for lactic acid production in this study for a comparison purpose to *T. laevilacticus* SK5–6. The fermentation of *S. laevolacticus* 0361^T consisted of two phases but slight differences in the optimized operating conditions previously reported elsewhere (Prasirtsak et al. 2017; Thitiprasert et al. 2017). An active 48 h GYP agar slant grown under anaerobic condition was used to prepare the bacterial suspension using the same procedure mentioned above. The bacterial suspension was inoculated into the sterile preculture medium at 1% (v/v). The preculture medium for the catalase-negative bacteria (pH 6.8) contained (in 1 L) 10 g glucose, 5 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, 5 g $CaCO_3$, and 10 mL salt solution. The culture was

incubated at 37 °C under anaerobic condition in the flask plugged with the T-type silicone stopper and placed into the W-zip pouch containing AnaeroPack-Anaero gas pack. After that, the preculture broth was inoculated into the fermentation medium for the catalase-negative isolate. Such medium contained (in 1 L) 120 g glucose, 10 g yeast extract, 5 g peptone, 0.25 g KH_2PO_4, 0.25 g K_2HPO_4, 80 g $CaCO_3$, and 10 mL salt solution. The culture was incubated at the same operating conditions as those during the preculture stage for another 72 h to complete glucose consumption. Samples were taken every 12 h for analyses of OD600 reading, the remaining glucose, D-lactate and byproduct formation, and the optical purity of D-lactate product. Cell biomass was also collected for enzymatic activity assays.

Cell biomass determination

A broth sample was centrifuged at 10,000 g for 5 min to separate the cell-free supernatant from the biomass. The supernatant was collected for further analyses of the remaining glucose, lactic acid, and byproducts as well as the optical purity of D-lactic acid. The solid cell biomass (pellet) was acidified with 1 M HCl to remove residual insoluble $CaCO_3$ and then centrifuged as above to pellet the cells. The cell pellets were resuspended in the same volume of deionized water for the OD reading at 600 nm using spectrophotometer.

Substrate and product analyses

Residual glucose and products formed during the fermentation were analyzed using a high-performance liquid chromatography (HPLC). The cell-free fermentation samples were filtered through a PTFE (hydrophilic) membrane and diluted with double-deionized water. For analyses of glucose, lactic acid (total acid including both D- and L-lactic acid), and acetic acid present in the sample, 15 μL diluted particle-free samples were automatically injected (Shimadzu-SIL-10A) into an organic acid analysis column (Biorad, Aminex HPX-87H in exclusion organic acid column; 300 mm × 7.8 mm) and maintained at 45 °C in a column oven (Shimadzu-CTO-10A), eluting with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min (Shimadzu-LC-10Avp). A refractive index detector (Shimadzu-RID-10A) was used to detect the organic compounds. Standards, containing 0–2 g/L of each component (glucose, lactate, and acetate), were injected as references to determine the sample concentration from the respective peak areas. To determine the optical purity of lactic acid, 5 μL diluted particle-free samples were automatically injected into a chiral column (Sumipack, Sumichiral OA5000) maintained at 40 °C and eluted with 1 mM $CuSO_4$ at a flow rate of 1.0 mL/min. A UV detector was used to detect the lactate isomers at 254 nm. Standards containing 0–2 g/L of D- and L-lactic acid were injected as references to determine the sample concentration from the peak areas.

Product yield, volumetric productivity, and optical purity of lactic acid

Product yield ($Y_{p/s}$) was determined from the ratio of the product formed to carbon substrate consumed during the fermentation stage. Volumetric productivity was defined as the total amount of product formed per unit volume per time. The optical purity of D-lactate, reported in the percentage of enantiomer excess (%ee), was defined from the respective peak areas of the chromatogram:

$$Optical\ purity = \frac{(D{-}lactate{-}L{-}lactate)}{D{-}lactate + L{-}lactate} \times 100$$

Cell extraction, partial enzyme purification, and protein determination

The fermentation sample (5 mL) was centrifuged at 4 °C, 10,000×*g* for 10 min. The supernatant was discarded while the cell pellets were washed twice with 0.05 M ice-cold phosphate buffer (pH 7.4). The cell pellets were resuspended in 5 mL phosphate buffer and then homogenized in an ultrasonic disruptor with glass beads (425–600 μm) for 5 min (30 s sonication interval with 45 s break to cool down) to obtain the cell lysate. The glass beads were removed by centrifugation as above, and the supernatant was used for protein determination by the Lowry method using bovine serum albumin as the standard and enzyme activity assays.

Enzyme activity assays

The crude extract was assayed for the activities of phosphofructokinase (PFK), pyruvate kinase (PYK), lactate dehydrogenases (LDH) (both D- and L-LDH), and lactate isomerase (LI) to investigate the changes of their activities during D-lactate fermentation by *T. laevilacticus* SK5–6 and *S. laevolacticus* 0361^T.

PFK activity was determined using a commercial PFK colorimetric assay kit (Cat. No. MAK093, Sigma) according to the manufacturer's instructions. The assay uses a coupled enzymatic reaction, in which PFK (crude extract) catalyzed the conversion of fructose-6-phosphate (F-6-P) and ATP to fructose-1,6-phosphate (F-1,6-P) and ADP and a subsequent conversion of ADP to NADH and AMP by an enzyme mix (provided in the assay kit), where the NADH was formed and reduced the colorless probe to a colorimetric product at 450 nm proportionally to the PFK activity present in the reaction mixture. The reaction mixture contained 42 μL PFK

assay buffer, 2 μL PFK enzyme mix, 2 μL PFK developer, 2 μL ATP, and 2 μL PFK substrate. To start the reaction, 10 μL crude extract was added into the reaction mixture. The resulting NADH reduced the colorless indicator resulting in a colorimetric product at 450 nm proportionally to the PFK activity. The absorbance at 450 nm of the reaction mixture was recorded every 50 min for 5 min. The average rate of the increasing absorbance at 450 nm per min was calculated. The activity of PFK was subsequently determined using the calibration plot of absorbance at 450 nm versus NADH concentration in micromoles. One unit of PFK was determined by the amount of enzyme that generated 1 μmol NADH per 1 min at 37 °C, pH 7.4 (Songserm et al. 2015).

The activity of PYK was assayed by monitoring the changes in the absorbance of NADH at 340 nm (A340) using L-LDH as a coupling enzyme. The reaction mixture contained 50 mM imidazole-HCl (pH 7.5), 5 mM phosphoenol pyruvate (PEP), 2 mM ADP, 100 mM KCl, 10 mM $MgCl_2$, 0.6 mM NADH, 2 U L-LDH from rabbit muscle, and 2 mL cell extract in a total volume of 3.15 mL, and the absorbance at 340 nm was monitored for 5 min at 37 °C. One unit of PYK activity was defined as the activity that converted 1 μmol of PEP to pyruvate per min (Zheng et al. 2014).

The activity of LDH was assayed by following the oxidation of NADH at 340 nm. A reaction mixture of 3.15 mL contained 0.25 mL sodium pyruvate (0.10 M), 0.10 mL NADH (0.01 M), 2.5 mL phosphate buffer (0.10 M) at pH 6.5 (for L-LDH assay) or pH 7.3 (for D-LDH assay), and 0.2 mL cell extract. The reaction was started after adding cell extract into the reaction mixture at 37 °C. The absorbance at 340 nm was monitored for 5 min. The amount of NADH oxidized in micromoles was determined from the calibration plot of the absorbance at 340 nm versus NADH (in micromoles present in the reaction mixture) as previously described in Thitiprasert et al. 2011. The unit activity was defined as the amount of enzyme that converted 1 μmol NADH per min at 37 °C.

Lactate isomerization was determined using a commercial assay kit (Cat. No.11112821035, Boehringer Mannheim, R-biopharm) according to the manufacturer's instructions. D-LDH catalyzed the conversion of lithium D-lactate and NAD^+ to pyruvate and NADH while L-LDH catalyzed the conversion of L-lactate and NAD^+ to pyruvate and NADH. Pyruvate and L-glutamate were subsequently converted by glutamate-pyruvate transaminase to L-alanine and 2-oxoglutarate. The reaction mixture of 2.22 mL contained 1 mL glycylglycine assay buffer, 0.2 mL NAD^+, 0.02 mL glutamate-pyruvate transaminase, 0.1 mL crude extract, and 0.9 mL distilled water. Lithium L-lactic acid (0.02 mL) was added into the reaction mixture, and the reaction mixture was incubated at 37 °C for 5 min before reading the absorbance at 340 nm (A1). Later, lithium D-lactate (0.02 mL) was added into the reaction mixture. The reaction mixture was incubated at 37 °C for 30 min before reading the absorbance at 340 nm (A2). The amount of NADH produced in the reaction mixture) was determined from the calibration plat of the absorbance at 340 nm versus NADH (in micromoles). The amount of NADH produced in the reaction mixture was stoichiometrically equal to the amount of L-lactic acid and D-lactic acid reacted. The unit activity of LI is defined as the amount of the enzyme required to transform 1 μmol of L-lactic acid or D-lactic acid to the other isomer in 1 min.

Results and discussion

Fermentation kinetics of *T. laevilacticus* SK5–6 and *S. laevolacticus* 0361^T

Table 1 shows the fermentation kinetics data of *T. laevilacticus* SK5–6 and *S. laevilacticus* 0361^T. *T. laevilacticus* SK5–6 completely consumed glucose within 48 h for D-lactate and cell biomass. On the other hand, incomplete glucose consumption was observed in the fermentation by *S. laevilacticus* 0361^T at 72 h. The residual glucose concentration of 17.75 g/L was observed at the end of fermentation. D-lactic acid was only observed in the chromatograms of the fermentation samples collected from the cultures of these two lactate producers (data not shown). This referred that the two isolates in this study produced D-lactic acid via the homofermentative pathway. The final concentration of lactic acid obtained from the fermentation by *T. laevilacticus* SK5–6 was slightly higher than that obtained by *S. laevolacticus* 0361^T; however, the fermentation time was shorter. This led to the higher volumetric productivity. Almost twice higher final cell biomass concentration was obtained in the fermentation by *T. laevilacticus* SK5–6 compared to that obtained in the culture of *S. laevolacticus* 0361^T. The high cell biomass concentration during the fermentation by *T. laevilacticus* SK5–6 was responsible for the rapid bioconversion of glucose to D-lactate as a growth-associated product (Prasirtsak et al. 2017; Thitiprasert et al. 2017). With the higher cell biomass concentration, this led to the lower lactate yield and the lower ratio of lactate to cell biomass in *T. laevilacticus* SK5–6 compared to those obtained from the culture of *S. laevilacticus* 0361^T. To clearly explain the obtained experimental data, the high inoculum approach typically developed for the catalase-positive lactate producing isolate was applied in D-lactate production by *T. laevilacticus* SK5–6 (Prasirtsak et al. 2017; Thitiprasert et al. 2017). The preculture was carried out under an aerobic condition. This gave the rapidly increasing OD of the active biomass within a short preculture time. Together with the inoculum size at 50%, this resulted in the higher initial OD during the fermentation stage which eventually led to the higher D-lactate productivity with the lower yields of lactate to glucose and lactate to cell. It is suggested that

Table 1 Comparison of lactic acid production performance by the catalase-positive *T. laevilacticus* SK5–6 and the catalase-negative *S. laevilacticus* 0361^T

Strain	Fermentation time (h)	Cell biomass (OD600)	Lactic acid production performance					Remaining glucose (g/L)	pH
			Lactate conc. (g/L)	Lactate yield ($Y_{p/s}$)[c]	Lactate to cell ratio ($Y_{p/x}$)[d]	Productivity (g/L h)[e]	Optical purity (%ee)		
[a]*T. laevilacticus* SK5–6	0	1.481 ± 0.004	0.00 ± 0.00	0	0	0	100.00	122.15 ± 2.48	6.66 ± 0.01
	12	13.325 ± 0.009	45.40 ± 0.85	0.93	3.48	3.78	100.00	73.25 ± 3.61	5.71 ± 0.01
	24	15.938 ± 0.014	79.25 ± 2.48	0.94	5.07	3.30	100.00	37.65 ± 1.06	5.58 ± 0.03
	36	17.675 ± 0.003	93.05 ± 2.05	0.89	5.35	2.58	99.67	17.20 ± 2.40	5.58 ± 0.03
	48	17.500 ± 0.003	102.50 ± 1.70	0.84	5.96	2.14	99.74	0.00 ± 0.00	5.73 ± 0.04
[b]*S. laevolacticus* 0361^T	0	0.032 ± 0.002	0.00 ± 0.00	0	0	0	100.00	120.65 ± 0.50	6.91 ± 0.01
	12	0.567 ± 0.011	0.00 ± 0.00	0	0	0	DL[f]	118.30 ± 0.42	6.50 ± 0.01
	24	1.790 ± 0.005	4.75 ± 0.21	0.78	2.70	0.20	81.10	114.55 ± 2.33	5.82 ± 0.04
	36	8.080 ± 0.007	36.95 ± 4.60	0.74	4.59	1.03	97.73	78.40 ± 0.42	5.44 ± 0.02
	48	9.770 ± 0.006	64.80 ± 1.84	0.95	6.65	1.35	99.24	52.50 ± 2.40	5.39 ± 0.01
	60	9.385 ± 0.008	79.70 ± 0.99	0.90	8.52	1.33	98.79	31.75 ± 2.05	5.33 ± 0.04
	72	9.225 ± 0.013	97.70 ± 0.99	0.95	10.63	1.36	99.09	17.75 ± 0.21	5.32 ± 0.03

[a] *T. Laevilacticus* SK5–6 was precultured under the aerobic condition in the medium containing (per liter) 10 g glucose, 15 g yeast extract, 4 g NH_4Cl, and 5 g $CaCO_3$ supplemented with minerals and salt solution before transferring into the fermentation medium containing (per liter) 120 g glucose and 80 g $CaCO_3$ at 50% inoculum size. The fermentation was conducted under the anaerobic condition

[b] *S. laevolacticus* 0361^T was precultured under the anaerobic condition in the medium containing (per liter) 10 g glucose, 10 g yeast extract, 5 g peptone, and 5 g $CaCO_3$ supplemented with minerals and salt solution before transferring into the fermentation medium containing (per liter) 120 g glucose and 80 g $CaCO_3$ supplemented with minerals and salt solution at 10% inoculum size. The fermentation was conducted under the anaerobic condition

[c] Lactate yield represented the cumulative value of the ratio of the amount of lactate produced to the amount of glucose consumed at a time

[d] Lactate to cell ratio referred to the specific production of lactate per unit of cell biomass at a time

[e] Lactate productivity was calculated from the volumetric production of lactate at a time

[f] DL referred to the presence of racemate of lactate at that time

further optimization to drive more glucose flux to lactate production instead of building up the biomass can be done by medium optimization in both the preculture and the fermentation stages. With the balanced carbon to nitrogen ratio in the medium, it is believed that the sufficiently high productivity and yields of D-lactate can be obtained (Thitiprasert et al. 2017).

Key enzymes in D-lactate fermentation

The evidence of a higher lactate productivity in *T. laevilacticus* SK5–6 compared with that in *S. laevilacticus* 0361^T was explained by the activities of the key enzymes involving sugar uptake and lactate conversion. Both *T. laevilacticus* SK5–6 and *S. laevolacticus* 0361^T use the glycolytic pathway to convert glucose to D-lactate. It was reported that lactic acid production was mainly regulated via the two irreversible steps that were catalyzed by the allosteric enzymes, e.g., PFK and PYK (Zheng et al. 2017). Glucose consumption and D-lactate production were corresponded with the specific activities of PFK, PYK, and LDH that controlled and regulated both cell growth and lactate production (Figs. 1 and 2; Table 1) (Sawai et al. 2011).

PFK and PYK involved in the bioconversion of glucose to the key intermediate pyruvate. Increasing activities of PFK and PYK were observed with the increasing cell growth in the cultures of both *T. laevilacticus* SK5–6 and *S. laevolacticus* 0361^T as the fermentation time preceded (Fig. 1; Table 1). From Fig. 1, it was found that the specific activity of PFK was approximately 10 times lower than that of PYK in both bacterial cultures. This evidence inferred that an ATP regeneration process occurred during the fermentation stage when the bacteria used the anaerobic respiration system (Songserm et al. 2015). Particularly, the phosphorylation of F-6-P to F-1,6-P by PFK required ATP while the conversion of PEP to pyruvate by PYK regenerated ATP (Fig. 3) (Zheng et al. 2017). Therefore, PYK was responsible for controlling the glycolytic intermediates (PEP and pyruvate) and regenerating ATP (Zheng et al. 2014). In homolactic acid pathway, the complete consumption of 1 mol of glucose for cell biomass and lactate yielded 2 mol of ATP. In some bacteria, PFK was activated by Mg-ADP which promoted the binding affinity of F-6-P at the catalytic site while this enzyme was inhibited by PEP (Zheng et al. 2017). The rapid consumption of glucose for cell biomass and lactate in *T. laevilacticus* SK5–6 in 24 h could be explained by the rapidly

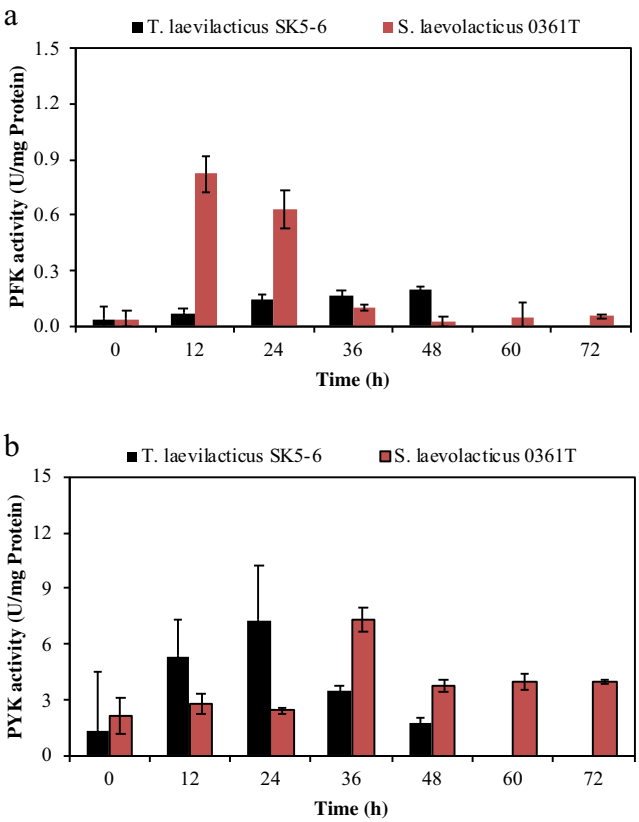

Fig. 1 The activities of PFK and PYK during the fermentation of **a** *T. laevilacticus* SK5–6 and **b** *S. laevolacticus* 0361^T. The samples used for the enzyme assays were collected from the fermentations reported in Table 1

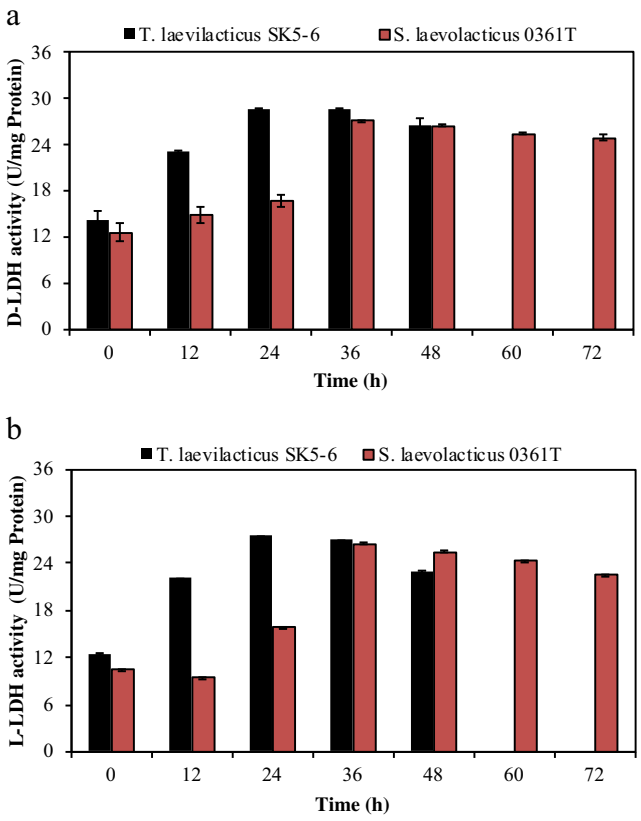

Fig. 2 The activities of lactate dehydrogenases (D-LDH and L-LDH) during the fermentation of **a** *T. laevilacticus* SK5–6 and **b** *S. laevolacticus* 0361^T. The samples used for the enzyme assays were collected from the fermentations reported in Table 1

increasing activities of PYK and D/L-LDHs (Figs. 1b and 2; Table 1). The slowly increasing activity of PFK from 0 to 48 h was claimed to be responsible for F-1,6-P synthesis that was required in simultaneous activation of LDH (Fig. 1a). The high growth rate as observed from the abruptly increasing cell biomass of *S. laevolacticus* 0361^T from 12 to 24 h (Table 1) was correlated with the high specific activity of PFK (Fig. 1a). After 24 h, the increase in the specific activities of PYK and D/L-LDHs was observed while the specific activity of PFK became lower. This evidence supported the findings of delayed D-lactate production in *S. laevolacticus* 0361^T during the first 24 h and incomplete glucose consumption at 72 h (Table 1).

Figure 2 shows the specific activities of D-LDH and L-LDH during the fermentation stage of both isolates. The activity of D-LDH was slightly higher than that of L-LDH, especially at earlier time points. The specific activities of both enzymes in *T. laevilacticus* SK5–6 was enhanced along with the increasing cell biomass and lactate production (Fig. 2; Table 1). The specific activities of both D- and L-LDHs remained highest from 24 to 48 h. The specific activity of D-LDH in *S. laevolacticus* 0361^T gradually increased from 0 to 24 h then dramatically increased from 24 to 36 h and remained highest until 72 h (Fig. 2a). This was associated with the rapidly increasing cell biomass and lactate production at that period (Table 1). It was found that the increasing activities of D/L-LDH was associated with the increase in the activities of PFK and PYK (Figs. 1 and 2). Garvie (1980) reported that LDH was stimulated through the consumption of pyruvate, the regeneration of NAD^+, and the formation of F-1,6-P by PFK. In addition, the activities of D/L-LDH were susceptible to the change of pH due to protonation of His imidazole and Arg guanidine groups at their catalytic sites (Iwata and Ohta 1993; Tsuge et al. 2015). Arai et al. (2002) studied the allosteric regulation of L-LDH in *Lactobacillus casei*. The allosteric mechanism of L-LDH absolutely required F-1,6-P for its catalytic activity under neutral pH but exhibited a remarkable catalytic activity in the absence of F-1,6-P under the acidic pH through the activation of its substrate, pyruvate. Therefore, as the fermentation preceded, the acidity increased as a consequence of lactic acid production. By this, LDH activity further enhanced which resulted in high production rate of lactic acid (Fig. 2).

Low optical purity of D-lactate was observed at the initial period of fermentation (from 12 to 36 h) by *S. laevolacticus* 0361^T (Table 1). Previous work reported the similar phenomenon during the batch cultivation of D-lactate by *S. inulinus* JCM6014 and *S. laevolacticus* JCM2513T. Both isolates yielded the low optical purity of D-lactate during the early

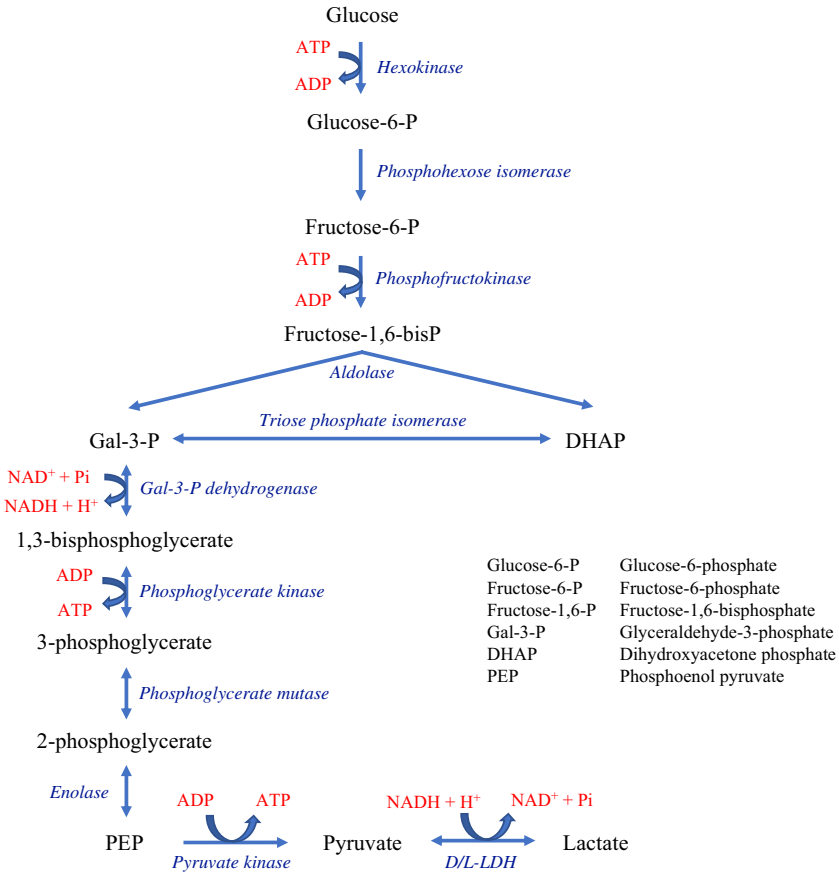

Fig. 3 Homolactic acid fermentation pathway in lactic acid producing bacteria and the key enzymes

phase, but later, the optical purity was increased (Sawai et al. 2011). On the other hand, the optical purity of D-lactate from the culture of S. terrae gradually increased during the cultivation period (Sawai et al. 2011).

Lactate isomerization was observed during the fermentation. Figure 4 shows the specific activities of lactate isomerases for both D-lactate and L-lactate. As the fermentation preceded, the transformation of L-lactate to D-lactate was observed in both isolates (Fig. 4a; Table 1). As a result, the highest optical purity of D-lactate was observed at the end of fermentation when growth reached the late log phase in batch cultivation. The low specific activity of isomerase for L-lactate was observed in both isolates (Fig. 4b). This finding confirmed that both isolates used in this study preferably produced D-lactate. The specific isomerization activities for both D-lactate and L-lactate observed in *S. laevolacticus* 0361^T were higher than those found in *T. laevilacticus* SK5–6. This rather indicated the higher degree of isomerization resulting in the fluctuation in the optical purity of lactic acid product during fermentation. This implies that the activities of D/L-LDH and isomerase depended on the bacterial strains besides the stage of bacterial growth. Previous studies claimed that both LDH and isomerase played key roles in controlling the optical purity of lactic acid product (Garvie 1980). Sakai et al. (2006) reported the DL-lactate racemate production by *Lactobacillus plantarum* although the low L-LDH activity was observed. They identified later the cause of low optical purity of lactic acid product was due to the high activity of D-LDH and isomerase. Some species of *Lactobacillus* and *Leuconostoc* were reported as the D-lactate producers that lacked L-LDH activity (Garvie 1980) while the D-lactate producers such as *S. inulinus*, *S. laevolacticus*, and *S. terrae* possessed L-LDH activity (Sawai et al. 2011).

Overall, the higher lactate productivity observed in *T. laevilacticus* SK5–6 compared with that in *S. laevolacticus* 0361^T can be explained by the well-optimized process conditions that led to the proper regulation of the key enzymes, e.g., PFK, PYK, and LDH since the initial stage of fermentation (right after inoculation). It was claimed that PFK was activated by both GDP and ADP and inhibited by PEP (Songserm et al. 2015). With approximately 10 times higher specific activity of PYK compared to that of PFK, this confirmed the rapid conversion of PEP to pyruvate; thus, no excess PEP pool remained causing the inhibition to PFK (Zheng et al. 2017). By the enzymatic conversion of PEP to pyruvate by PYK, this resulted in ATP regeneration for activation of PFK while at the same time providing F-1,6-P from the conversion of F-6-P by PFK for activating LDH and pyruvate as the substrate for LDH (Liu et al. 2012). With the highest activity of LDH compared with the other two key

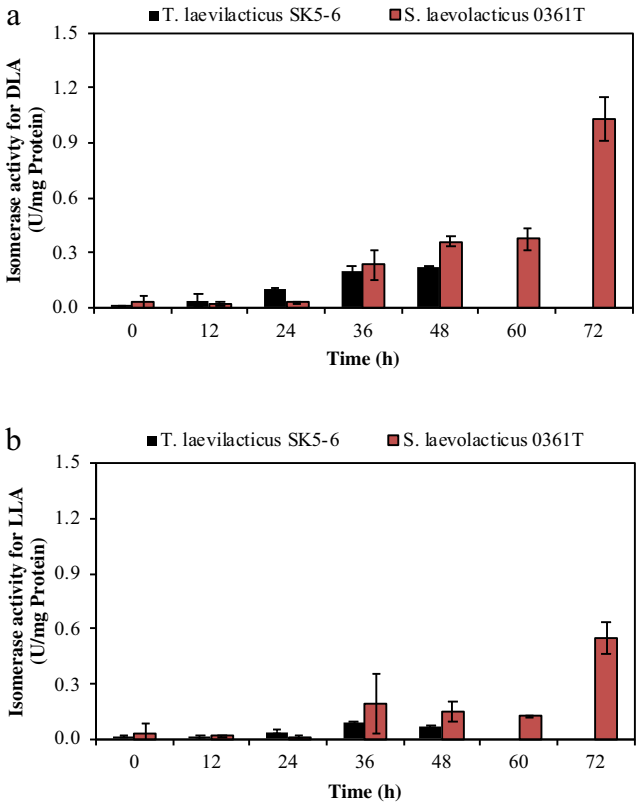

Fig. 4 The activities of isomerase for D-lactate and L-lactate during the fermentation of **a** *T. laevilacticus* SK5–6 and **b** *S. laevolacticus* 0361^T. The samples used for the enzyme assays were collected from the fermentations reported in Table 1

enzymes in the glycolytic pathway (PFK and PYK), this simultaneously drove the glucose flux toward lactate production. A high level of ATP was reported to be the inhibitor of lactate production through the decreased affinity of PYK for substrate in *S. inulinus* (Zheng et al. 2014). Previous study also claimed that the glycolytic capacity of *Lactobacillus lactis* grown under microaerobic condition was significantly increased in engineered strains with increased PFK activity (Papagianni and Avramidis 2011). From the findings and literatures mentioned above, this is to confirm that PFK, PYK, and LDH together play key roles in converting glucose via glycolytic pathway for biosynthesis of lactate under the optimized conditions.

Comparing fermentation performance of *T. laevilacticus* SK5–6 with the commonly available D-lactate producers

The experimental data exhibiting the shorter lag phase with the higher final lactate concentration and productivity with the high and stable optical purity over fermentation of *T. laevilacticus* SK5–6 reported in Table 1 suggested that this novel D-lactate producer gave the promising advantages over *S. laevolacticus* 0361^T, the reference of the commonly available D-lactate producers. Table 2 compares the fermentation performance of D-lactate producers previously published with the fermentation results of *T. laevilacticus* SK5–6. The

Table 2 Previous literatures reporting D-lactic acid fermentation from various nitrogen sources compared with the results in this study

Microorganisms	Fermentation medium/Operation	D-lactic acid production performance				References
		Final lactate conc. (g/L)	Lactate yield (Yp/s)	Productivity (g/L h)	Optical purity (%ee)	
Sporolactobacillus sp. CASD	Glucose, peanut meal/fed batch	207.0	0.93	3.80	99.3	Wang et al. (2011)
Lactobacillus delbrueckii subsp. *lactis* QU 41	Glucose, yeast extract, peptone, meat extract/batch	20.1	0.90	1.67	> 99.9	Tashiro et al. (2011)
Sporolactobacillus laevolacticus DSM442	Glucose, cotton seed hydrolysate	144.4	0.96	4.13	99.3	Li et al. (2013)
Escherichia coli HBUT-D	Glucose, yeast extract	127.0	0.93	6.35	99.5	Liu et al. (2014)
Sporolactobacillus inulinus Y2–8	Corn flour hydrolysate, yeast extract	145.8	0.97	1.62	> 99.0	Zhao et al. (2014)
Sporolactobacillus spp. Y2–8	Glucose, yeast extract, corn steep liquor, wheat bran	127.0	–	1.72	99.0	Sun et al. (2015)
S. inulinus NBRC13595	Palm sugar, whey protein hydrolysate	189.0	0.94	5.25	> 98.0	Tadi et al. (2017)
S. inulinus YBS1–5	Corncob hydrolysate, cottonseed meal	107.2	0.85	1.19	99.2	Bai et al. (2016)
T. laevilacticus SK5–6	Glucose, yeast extract, NH_4Cl	102.5	0.84	2.14	99.74	This study
T. laevilacticus SK5–6	Glucose, yeast extract, $(NH_4)_2HPO_4$	92.0	0.98	1.92	99.09	This study

medium composition and the operating conditions were optimized for those isolates. As previously mentioned, *T. laevilacticus* SK5–6 is the catalase-positive isolate; thus, preculture stage can be conducted under the aerobic condition which results in a high cell concentration to be inoculated into the fermentation stage. By this, it provided a preferable outcome during the fermentation stage that was considered as the stage with limited energy source where the growth yield coefficient was only 10 mg dry weight produced per 1 mmol ATP regeneration (Forrest 1965). As a result, when comparing the kinetic rates with the type strain of *S. laevolacticus* growing in the similar medium compositions, it turned out that the fermentation performance of *T. laevilacticus* SK5–6 was better with the higher initial cell numbers. Therefore, with further scrutinized process optimization, *T. laevilacticus* SK5–6 can acquire strong evidences that support the usage of this novel bacterium in the industrial fermentation.

Conclusions

This study evaluated the fermentation performance and glucose bioconversion for D-lactate production in terms of the specific activities of the key enzymes in *T. laevilacticus* SK5–6, the potent D-lactate producer. Compared with *S. laevolacticus* 0361^T as the reference of the commercial D-lactate producer, *T. laevilacticus* SK5–6, the catalase-positive isolate, yielded a better fermentation performance in term of shorter lag phase during fermentation cultivation, complete glucose consumption within a shorter time, higher D-lactate productivity, yield, and optical purity. The key enzymes including PFK, PYK, and D/L-LDH were shown to be responsible for the rapid bioconversion of glucose to D-lactate in *T. laevilacticus* SK5–6. The results suggested that among the key enzymes studied, the specific activity of PFK was the lowest indicating the conversion of F-6-P to F-1,6-P was the rate limiting step. Under the well-optimized conditions, the activation of D/L-LDH by F-1,6-P and pyruvate and ADP/ATP regeneration by PYK could drive glucose bioconversion toward D-lactate. The optical purity of D-lactate was not only controlled by the specific activity of D/L-LDH, but it was reported that the activation of isomerases also played roles in isomerization of lactate. High D-LDH with limited isomerase activity was preferable during the fermentation as it assured the high optical purity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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