



Development of a Novel D-Lactic Acid Production Platform Based on *Lactobacillus saerimneri* TBRC 5746

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Abstract

D-Lactic acid is a chiral, three-carbon organic acid, that bolsters the thermostability of polylactic acid. In this study, we developed a microbial production platform for the high-titer production of D-lactic acid. We screened 600 isolates of lactic acid bacteria (LAB) and identified twelve strains that exclusively produced D-lactic acid in high titers. Of these strains, *Lactobacillus saerimneri* TBRC 5746 was selected for further development because of its homofermentative metabolism. We investigated the effects of high temperature and the use of cheap, renewable carbon sources on lactic acid production and observed a titer of 99.4 g/L and a yield of 0.90 g/g glucose (90% of the theoretical yield). However, we also observed L-lactic acid production, which reduced the product's optical purity. We then used CRISPR/dCas9-assisted transcriptional repression to repress the two *Lldh* genes in the genome of *L. saerimneri* TBRC 5746, resulting in a 38% increase in D-lactic acid production and an improvement in optical purity. This is the first demonstration of CRISPR/dCas9-assisted transcriptional repression in this microbial host and represents progress toward efficient microbial production of D-lactic acid.

Keywords D-Lactic acid · Bioplastic · Lactic acid bacteria · CRISPR/dCas9-assisted transcriptional repression

Introduction

Producing chemicals and fuels from biological sources, rather than petroleum, offers a promising alternative that could help reduce global greenhouse gas emissions (Liao et al., 2016). Examples of bio-based production include creating alternatives to gasoline and organic acids, and developing monomers for bioplastics (Chen et al., 2018; Choi & Lee, 2013; Kruyer & Peralta-Yahya, 2017; Moon et al., 2009). Lactic acid, an organic compound with three carbon atoms, is utilized across various sectors like food and plastic production (Castro-Aguirre et al., 2016; Lahtinen et al., 2012). In the plastic industry, lactic acid is especially valuable for making biodegradable and eco-friendly polylactic acid (PLA) plastics, which can replace non-biodegradable petroleum-based plastics. The chirality of lactic acid used,

whether it is pure L-lactic or D-lactic acid, or a mixture of both, significantly impacts the properties of the resulting PLA (Bai et al., 2017). Stereocomplexation of poly-D-lactic acid with poly-L-lactic acid leads to plastics that are notably heat resistant. L-Lactic acid production through fermentation by microbes is well-known and widely practiced by several large biotech companies, while D-lactic acid production is less common, with few published reports (Abdel-Rahman & Sonomoto, 2016; Abdel-Rahman et al., 2013). This gap signals the need to develop a more effective system for producing pure D-lactic acid.

Several strains of bacteria, naturally occurring in the wild, are known for their prolific production of D-lactic acid (Lahtinen et al., 2012). These include members of the *Sporolactobacillus*, *Leuconostoc*, and *Lactobacillus* genera. Many research teams have used these microorganisms effectively to achieve high levels of D-lactic acid through fermentation. For instance, fermenting sugarcane juice with *Leuconostoc mesenteroides* B512 in a shake-flask led to a yield of 60.2 g/L of D-lactic acid, equivalent to 0.51 g/g of glucose (Coelho et al., 2011). Similarly, fermenting corncob residue hydrolysates with *Sporolactobacillus inulinus* YBS1-5 in a fed-batch process produced 107.2 g/L of D-lactic acid,

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equivalent to 0.85 g/g of glucose (Bai et al., 2016). Apart from these natural D-lactic acid producers, certain industrial hosts that originally could not produce D-lactic acid have been genetically modified to do so (Juturu & Wu, 2016; Sornlek et al., 2022; Watcharawipas et al., 2021).

In this study, we systematically screened 600 lactic acid bacterial isolates archived at the Thailand Bioresource Research Center (TBRC) and BIOTEC's culture collection (BCC) to identify strains that produce high levels of D-lactic acid. Our screening led us to *Lactobacillus saerimneri* (recently reclassified as *Ligilactobacillus saerimneri*) TBRC 5746, which stood out due to its conversion yield of 0.96–0.99 g/g of glucose. Fed-batch fermentation of *L. saerimneri* TBRC 5746 resulted in a lactic acid titer of 99.4 g/L. However, at this high production level, the strain began to also produce a noticeable amount of L-lactic acid. Based on genome and real-time PCR analysis of *L. saerimneri* TBRC 5746, we found that two putative *Lldh* genes were likely responsible for this L-lactic acid production. By using CRISPR/dCas9-assisted transcriptional repression to down-regulate these genes, we achieved a 38% improvement in D-lactic acid production. This research represents a step forward towards the efficient microbial production of D-lactic acid.

Materials and Methods

Strain, Media, and Transformation

The bacterial strains used in this study were obtained from Thailand Bioresource Research Center (TBRC) and BIOTEC Culture Collection (BCC). The primers used in this study are listed in Table S1. The bacterial strains were stored in 25% glycerol at a temperature of -80°C . Lactic acid bacteria were grown in de Man, Rogosa and Sharpe (MRS) medium, with the addition of 5 $\mu\text{g/ml}$ chloramphenicol as needed. *Escherichia coli* was grown in Luria–Bertani (LB) medium, with the addition of 100 $\mu\text{g/ml}$ ampicillin and 5 $\mu\text{g/ml}$ chloramphenicol as necessary. *Lactobacillus saerimneri* TBRC 5746 cells were transformed via electroporation using a modified protocol (Huang et al., 2019). The transformed bacteria were selected on MRS medium supplemented with 5 $\mu\text{g/ml}$ chloramphenicol. The CRISPR–Cas9 plasmids used for gene repression were generated from pHSP02 and pLH01 (Huang et al., 2019).

Screening of Lactic Acid Bacteria from TBRC

Lactic acid bacteria strains from TBRC and BCC were cultured in 5-ml aliquots in MRS medium in 15-ml Falcon tubes. The cultures were grown at either 30°C or 37°C in an incubator without shaking. Samples were taken at 24 h

to determine optical density at 600 nm (OD_{600}) and D-lactic acid production. The amounts of D-lactic acid and L-lactic acid were determined using high-performance liquid chromatography (HPLC) as previously described with some modifications (Watcharawipas et al., 2021). Briefly, 1 ml of the bacterial culture was filtered through a 0.2-micron nylon syringe filter (Filtrex), and the purified sample was then analyzed on an Agilent 1100 series HPLC with a Shodex ORpak CRX-853 chiral column at 50°C . The eluent used was 0.5 mM CuSO_4 at a flow rate of 1.0 ml/min. D-lactic acid and L-lactic acid were detected using a UV detector set at 230 nm.

Plasmid and Strain Construction

To construct the CRISPR/Cas9 plasmids for specific gene repression, the specific crRNA for each gene (*Lldh_Scaffold9* and *Lldh_Scaffold15*) was designed using the CRISPR RGEN tools (Bae et al., 2014; Park et al., 2015).

Plasmid pHSP02-dCas9: The gene encoding SpCas9 was mutated to encode the nuclease-deficient mutant (*SpCas9*_{D10A,H840A} or *SpdCas9*) using overlap-extension PCR (OE-PCR). Three DNA fragments were amplified from pHSP02 using primers pHSP02dCas9-Fr1-F and pHSP02dCas9-Fr1-R, primers pHSP02dCas9-F2-F and pHSP02dCas9-Fr2-R, and primers pHSP02dCas9-Fr3-F and pHSP02dCas9-Fr3-R. The three fragments were then assembled by OE-PCR and ligated to the *PstI/BglIII* sites of pHSP02 to create pHSP02-dCas9.

Plasmid pHSP02-dCas9-Lldh-Scaffold9-Cm: The crRNA for *Lldh_Scaffold9* was amplified from pHSP02 using primers Sc9pHSP-F3 and Sc9pHSP-XbaI-R3. The 1.0-kb upstream and downstream fragments of *Lldh_Scaffold9* were amplified from the genomic DNA of *L. saerimneri* TBRC 5746 using primers Sc9pHSP-SOE-F and Sc9pHSP-R1, and primers Sc9pHSP-F2 and Sc9pHSP-R2, respectively. The three DNA fragments were assembled by OE-PCR and ligated to the *ApaI/XbaI* sites of pHSP02-dCas9 to create pHSP02-dCas9-Lldh-Scaffold9. The chloramphenicol selectable marker (Cm) was amplified from pLH01 using primers Cm-ApaI-F and Cm-ApaI-R and ligated to the *ApaI* site of pHSP02-dCas9-Lldh-Scaffold9 to create pHSP02-dCas9-Lldh-Scaffold9-Cm.

Plasmid pHSP02-dCas9-Lldh-Scaffold15-Cm: The crRNA for *Lldh_Scaffold15* was amplified from pHSP02 using primers Sc15pHSP-F3 and Sc15pHSP-XbaI-R3. The 1.0-kb upstream and downstream fragments of *Lldh_Scaffold15* were amplified from the genomic DNA of *L. saerimneri* TBRC 5746 using primers Sc15pHSP-SOE-F and Sc15pHSP-R1, and primers Sc15pHSP-F2 and Sc15pHSP-R2, respectively. The three DNA fragments were assembled by OE-PCR and ligated to the *ApaI/XbaI* sites of pHSP02-dCas9 to create pHSP02-dCas9-Lldh-Scaffold15. The

chloramphenicol selectable marker (Cm) was amplified from pLH01 using primers Cm-ApaI-F and Cm-ApaI-R and ligated to the *ApaI* site of pHSP02-dCas9-Lldh-Scaffold15 to create pHSP02-dCas9-Lldh-Scaffold15-Cm.

Isolation and Sequencing of Genomic DNA from *L. saerimneri* TBRC 5746

Lactobacillus saerimneri TBRC 5746's genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). The purified DNA sample was sent to Novogene for whole genome sequencing. The 150 nt pair-end reads were checked for quality using FASTP, and the clean sequences were compared to the genome sequence of *L. saerimneri* 30a (Romano et al., 2013). The genome sequence was uploaded to GenBank with the Accession Number JAPXFN000000000.

Quantification of D-Lactic Acid Production and Other Fermentation Metabolites in Lactic Acid Bacteria Strains

For small-scale fermentation, the engineered strains were first grown in 5 ml of MRS medium overnight, then used to inoculate 5 ml of fresh MRS medium in 50-ml Falcon tubes to reach an initial OD₆₀₀ of 0.05. The cultures were incubated at 30 °C without shaking. At various time points, samples were taken to measure OD₆₀₀, biomass, extracellular metabolites, and the production of D-lactic acid. D-Lactic acid and other extracellular metabolites were analyzed using HPLC. Specifically, 1 ml of the culture was filtered through a 0.2-micron nylon syringe filter (Filtrex), and the purified sample was then analyzed on an Agilent 1100 series HPLC with an Aminex HPX-87H ion exchange column (Bio-Rad). The LC program was performed using 5 mM H₂SO₄ as the solvent at a flow rate of 0.68 ml/min for 30 min. The column was maintained at 60 °C. All metabolites were detected with Agilent 1200 series DAD and RID detectors.

Batch and Fed-Batch Bioreactor Fermentations

Fed-batch fermentation of *L. saerimneri* TBRC 5746 was performed in a 5-L stirred-tank bioreactor (Biostat B; Sartorius) with an initial volume of 2 L semi-defined medium with the following composition: 10 g/L peptone No.3, 10 g/L meat extract, 5 g/L yeast extract, 2 g/L K₂HPO₄, 2 g/L C₆H₈O₇·3NH₃, 5 g/L CH₃COO-Na, 1 g/L Tween 80, 0.2 g/L MgSO₄·7H₂O, 0.05 g/L MnSO₄·H₂O, and 0.1 ml/L antifoam. The pH was maintained at pH 6 by adding NH₄OH solution (25% solution in water). Glucose was prepared separately and added at a concentration of 40 g/L during batch fermentation. Fed-batch fermentation using intermittent feeding started after 10 h by adding 60 ml of 800 g/L

glucose for 20 min at 0, 4, 8, 12, and 18 h time points to maintain approximately 15 g/L glucose per feeding cycle.

RNA Isolation and Transcript Quantification

Fed-batch fermentation of *Lactobacillus saerimneri* TBRC 5746 was performed in a 5-L stirred-tank bioreactor as described above. At 2, 4, 6, and 8 h time points, 1 ml aliquots of each culture were collected and centrifuged for 2 min at 9000 × *g*. Total RNA was extracted from the samples using Qiagen's RNeasy Kit and the protocol provided by the manufacturer. The RNA was treated with DNaseI enzyme to remove any genomic DNA contaminants. The quantity of the RNA was measured using a NanoDrop ND-1000 spectrophotometer, and the samples were stored at – 80 °C until they were used for RT-PCR analysis. cDNA was produced from the RNA using the RevertAid Reverse Transcriptase kit and protocol provided by Thermo Fisher Scientific. The relative expression levels of the genes *Dldh*, *Lldh_Scaffold 9*, and *Lldh_Scaffold 15* were then quantified using the iQ SYBR Green Kit (Bio-Rad) on a CFX96 Touch Real-time PCR Detection System (Bio-Rad). Real-time PCR was performed in triplicates, and the 16S ribosomal RNA (*rRNA*) gene was used to normalize the amount of the total mRNA in all samples. Primers for real-time PCR are listed in Table S1.

Results and Discussion

Screening of Lactic Acid Bacteria to Identify High D-Lactic Acid-producing Strains

In this study, 600 lactic acid bacteria (LAB) strains previously isolated from various sources, such as fermented meat, fermented fish, and vegetables, were tested for their ability to produce D-lactic acid. These strains had been deposited at the Thailand Bioresource Research Center (TBRC) and the BIOTEC Culture Collection (BCC). Each LAB isolate was cultured in de Man, Rogosa and Sharpe (MRS) broth at 37 °C for 24 h. The cell-free supernatant was then used to analyze D-lactic acid production using an HPLC equipped with a chiral column. The screening results showed that among 600 isolates from 8 genera of LAB including *Lactobacillus*, *Weissella*, *Tetragenococcus*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, *Trichococcus*, and *Fructobacillus*, only 15 isolates from the genera *Weissella* and *Leuconostoc* showed potential as D-lactic producers (Supplementary file 1). Of these 15 isolates, 12 produced exclusively D-lactic acid (Table 1).

Interestingly, during the screening, we found that *L. saerimneri* TBRC 5746 had the highest production of D-lactic acid without any other metabolic by-products such as ethanol or acetic acid. This is likely due to *L. saerimneri*

Table 1 The potential D-lactic acid producer strains obtained from screening

| BCC/TBRC code | Genus | D-Lactic acid Titer (g/L) | L-Lactic acid Titer (g/L) | D:L-lactic acid ratio | Source |
|---------------|--|---------------------------|---------------------------|-----------------------|-------------------------|
| TBRC 5746 | <i>Lactobacillus saerimneri</i> | 27.08 | 0.00 | 100:0 | Pickled cabbage |
| BCC 38033 | <i>Weissella paramesenteroides</i> | 14.18 | 0.00 | 100:0 | Fermented pork (Nham) |
| BCC 38023 | <i>Leuconostoc pseudomesenteroides</i> | 12.36 | 0.00 | 100:0 | Nham |
| BCC 27654 | <i>Weissella bombi</i> | 11.91 | 0.00 | 100:0 | Nham |
| BCC 75402 | <i>Weissella paramesenteroides</i> | 11.68 | 0.00 | 100:0 | Feces |
| BCC 42492 | <i>Weissella paramesenteroides</i> | 11.67 | 0.00 | 100:0 | Fermented bean paste |
| BCC 38189 | <i>Weissella paramesenteroides</i> | 10.56 | 0.00 | 100:0 | Fermented fish |
| BCC 38096 | <i>Weissella thailandensis</i> | 8.88 | 0.00 | 100:0 | Fermented fish (Pla Ra) |
| BCC 3702 | <i>Weissella cibaria</i> | 6.95 | 5.42 | 56:44 | Nham |
| BCC 7365 | <i>Weissella cibaria</i> | 6.05 | 5.08 | 54:46 | Nham |
| BCC 7485 | <i>Weissella thailandensis</i> | 5.79 | 0.00 | 100:0 | Pla Ra |
| BCC 62792 | <i>Leuconostoc lactis</i> | 5.14 | 0.00 | 100:0 | Fermented fish |
| BCC 42499 | <i>Weissella thailandensis</i> | 3.55 | 0.00 | 100:0 | Pla Jaw |
| BCC 42400 | <i>Weissella thailandensis</i> | 3.14 | 0.00 | 100:0 | Nham |
| BCC 7312 | <i>Weissella confusa</i> | 1.44 | 7.75 | 16:84 | Nham |
| BCC 7435 | <i>Weissella confusa</i> | 1.36 | 8.28 | 14:86 | Nham |

TBRC 5746 belonging to the group of homofermentative LAB, which produce only lactic acid as the primary by-product in glucose fermentation. Homofermentative LAB produce two lactic acid molecules as a major end-product per molecule of consumed glucose, with a theoretical yield of 1.0 g/g. In contrast, the isolates from the genera *Weissella* and *Leuconostoc* belong to the group of heterofermentative LAB, which produce lactic acid simultaneously with several organic acids such as succinic acid and acetic acid, resulting in lower lactic acid production. Because of this potential, we further investigated *L. saerimneri* TBRC 5746.

Bioprocess Development for High-titer Production of D-Lactic Acid Using *L. saerimneri* TBRC 5746

Effects of temperature on lactic acid production: To investigate the effect of cultivation temperature on *L. saerimneri* TBRC 5746 growth and lactic acid production, a shake flask fermentation was performed at 37 °C, 42 °C, and 45 °C, using glucose, sucrose, and fructose as carbon sources. The results showed that the maximum growth and lactic acid production was obtained at 42 °C for all three carbon sources (Fig. S1). However, achieving higher titers of lactic acid

was difficult in shake flask experiments due to pH control issues. As the pH dropped to around 4.5, the growth of the microorganism was inhibited. Therefore, the effects of temperature were re-examined in a 5-L fermenter using batch fermentation with pH control at 6. Ammonium hydroxide was used to control the pH as it can also serve as an additional nitrogen source. The results showed that the maximum growth and lactic acid production of *L. saerimneri* TBRC 5746 was obtained after 11 h with a titer of 42.1 g/L, without ethanol production (Figs. S2–S4). This demonstrated the strain's ability to convert glucose to lactic acid with a conversion yield of 0.96–0.99 g/g glucose, close to the theoretical yield of homofermentative lactic acid production (1.0 g/g glucose).

To further improve lactic acid production, a fed-batch fermentation was performed using glucose as the carbon source (Fig. 1A and B). The initial glucose concentration was 40 g/L, with an additional 70 g/L added during the fed-batch fermentation. The fermentation was performed at 40 °C (Fig. 1A) and 42 °C (Fig. 1B) as these temperatures were deemed suitable for industrial application because of the potential for reduced operational costs for temperature control. The results showed that lactic acid production at

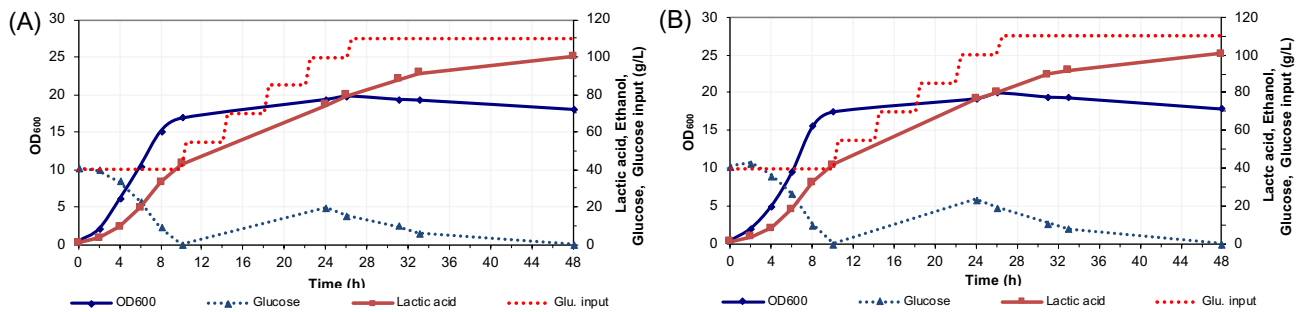


Fig. 1 Fed-batch fermentation profiles of *L. saerimneri* TBRC 5746 in a 5-L fermenter at 40 °C (A) and 42 °C (B). Fed-batch fermentation was performed with an initial volume of 2 L semi-defined medium. Fed-batch fermentation using intermittent feeding of 280 ml glucose stock solution (500 g/L) started after 10 h at 0, 4, 8, 12, and

18 h time points to maintain approximately 15 g/L glucose per feeding cycle. Total lactic acid and other metabolites were determined using HPLC equipped with an Aminex HPX-87H ion exchange column

40 °C and 42 °C were similar, with titer values of 98.7 g/L and 99.4 g/L, respectively. These values correspond to lactic acid productivity of 2.08 g/L/h and 2.09 g/L/h at 40 °C and 42 °C, respectively. No ethanol was detected during fermentation, confirming the homofermentative characteristic of the strain. This is an important characteristic to have, as ethanol production would decrease the yield and purity of the final lactic acid product. The ability to produce lactic acid at high temperatures allows for flexibility and potential scalability in industrial applications.

Although accelerated growth and sugar consumption rates were obtained when the strain was cultivated at elevated temperatures, a reduced enantiopurity of D-lactic acid was observed as shown in Fig. 2. At 30 °C, a trace amount of L-lactic acid was detected after 24 h of fermentation. In contrast, at higher fermentation temperatures (37 °C, 40 °C, and 42 °C), L-lactic acid was detected from the early stages and persisted throughout the fermentation, culminating at 24 h. This resulted in decreased optical purity of D-lactic acid at higher temperatures. This phenomenon aligns with numerous reports indicating lower thermostability of D-lactate

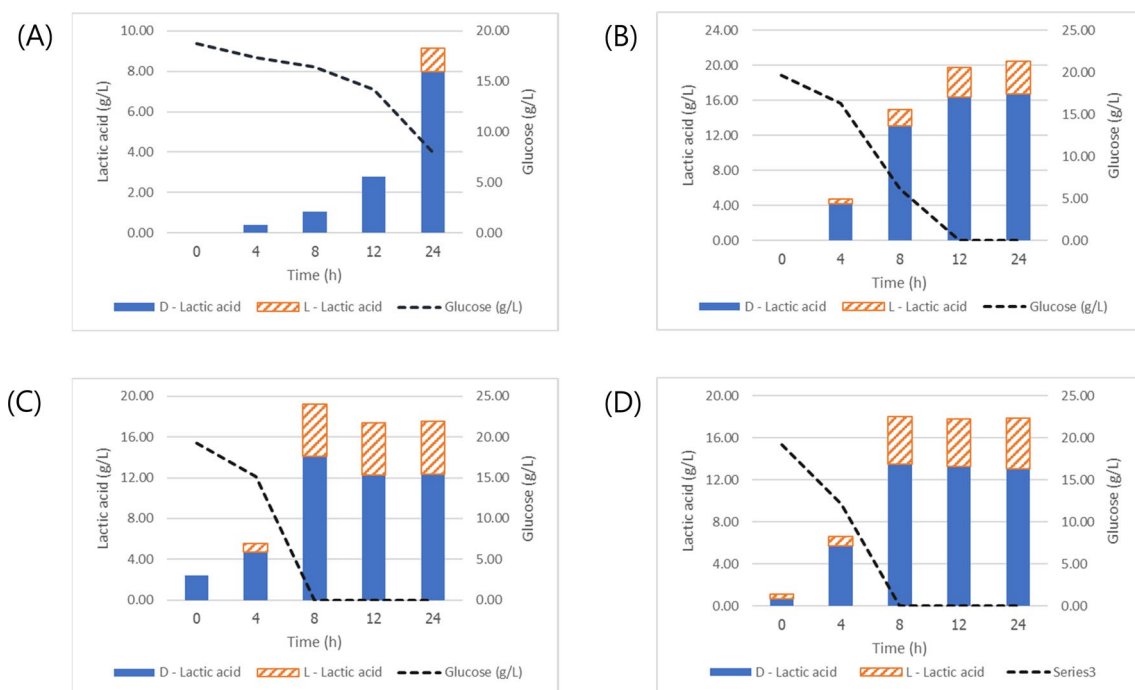


Fig. 2 Enantiopurity evaluation of D-lactic acid production at different fermentation temperature in *L. saerimneri* TBRC 5746 in a 5-L fermenter using MRS. (A) 30 °C, (B) 37 °C, (C) 40 °C, (D) 42 °C

dehydrogenase compared to L-lactate dehydrogenase, which directly impacts the efficiency of commercial microbial production of D-lactic acid (Gu et al., 2014; Jun et al., 2013; Kim et al., 2014).

Effects of carbon source (glucose and sucrose) on lactic acid production: Reducing operational costs is crucial for developing a bioprocess that is economically feasible and can be scaled up for industrial production (Alves de Oliveira et al., 2018). In bacteria, a disaccharide such as sucrose is transported into the bacterial cell via a phosphotransferase system (PTS)-dependent sucrose system or a non-PTS permeases transport system. For sucrose catabolism in lactobacilli, the major mechanism of sucrose uptake is mediated by phosphotransferase system (PTS) coupled to a phosphoenolpyruvate (PEP) dependent phosphorylation leading to sucrose-6-phosphate (S-6-P). S-6-P is cleaved by sucrose-6-phosphate-hydrolase into glucose-6-P (G-6-P) and fructose. If fructose is phosphorylated by fructokinase, the yielding fructose-6-phosphate (F-6-P) will be metabolized via the glycolytic pathway as same as G-6-P. Alternatively in a non-PTS permease system, sucrose transportation by a solute-cation symport system by sucrose permease followed by sucrose phosphorylase by sucrose phosphorylase yielding glucose-1-phosphate (G-1-P) and fructose. Then, the generated G-1-P and fructose can be readily converted to G-6-P and F-6-P by the bacterial cell and incorporated into the glycolytic pathway (Gänzle & Follador, 2012; Reid & Abratt, 2005). Although, sucrose phosphorylase exhibit a higher specificity for sucrose as a substrate than sucrose hydrolase, a less frequently identified in genome of lactobacilli was reported (Reid & Abratt, 2005). In homofermentative lactobacilli, the catabolism of G-6-P primarily proceeds via the Embden-Meyerhof pathway, leading to pyruvate. As a result, the end-products of sucrose fermentation in most

homofermentative lactobacilli are two moles of lactate per mole of glucose derived from sucrose, and theoretically, per mole of fructose derived from sucrose as well. In addition to environmental factors such as temperature and pH that affect lactic acid production, the choice of media composition is also relevant. In this study, we investigated the use of sucrose as a cheaper carbon source for lactic acid production and attempted to increase the sugar content in the culture media during fed-batch fermentation. However, the results showed that increasing the sugar content in the media above 100 g/L did not improve lactic acid production. Lactic acid titers of 94.0 g/L and 97.9 g/L were obtained when 200 g/L of glucose and sucrose were used as carbon sources, respectively (Fig. 3A and B, Tables S2 and S3). These correspond to lactic acid productivity of 1.85 g/L/h and 1.91 g/L/h, respectively. This may be due to end-product inhibition (Aguirre-Ezkauriatza et al., 2010; Luedeking & Piret, 1959) as a lower rate of lactic acid production was observed when the accumulation of produced lactic acid exceeded 80 g/L in the culture. Additionally, excess glucose and sucrose were detected (50–58 g/L) after the fermentation was finished at 72 h, resulting in a decrease in production yield and overall process productivity compared to using lower sugar concentrations.

It has been reported that the concentration of lactic acid that causes a 50% inhibition of growth is around 420 mM, while the critical concentration is 700 mM (Loubiere et al., 1997). This is thought to be due to the solubility of undissociated lactic acid within the cytoplasmic membrane and the insolubility of dissociated lactate. The latter causes acidification of the cytoplasm, leading to a failure of the proton motive forces (Othman et al., 2017). Despite being effective in countering end-product inhibition, pH control during fed-batch fermentation can lead to issues with osmotic pressure

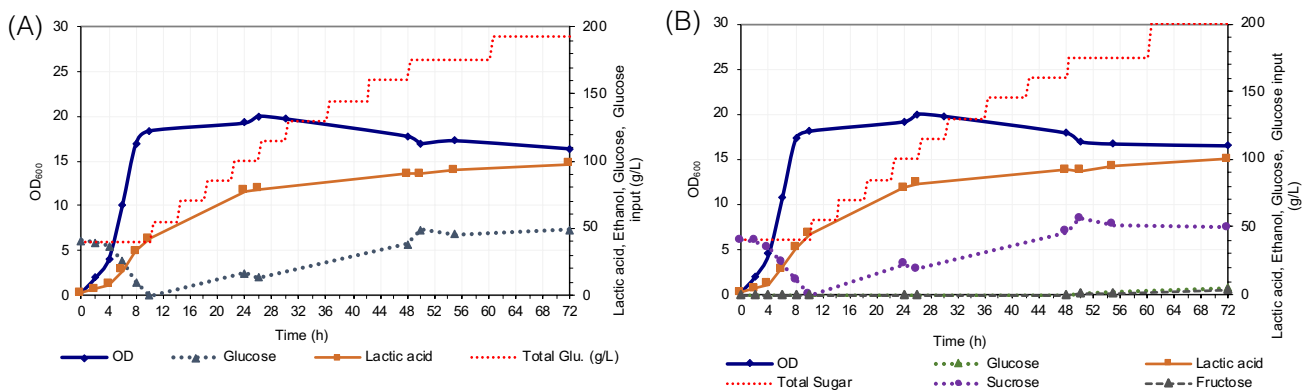


Fig. 3 Fermentation profiles of *L. saerimmeri* TBRC 5746 in a 5-L fermenter at 42 °C using different carbon sources (glucose **A** and sucrose **B**). Fed-batch fermentation was performed with an initial volume of 2 L semi-defined medium. Fed-batch fermentation using intermittent feeding of glucose stock solution (500 g/L, **A**) or sucrose

stock solution (40 g/L, **B**) started after 10 h at 0, 4, 8, 12, 16, 20, 26, 32, 38 and 50 h time points. Total lactic acid and other metabolites were determined using HPLC equipped with an Aminex HPX-87H ion exchange column

and result in bacteriostatic phenomena. To overcome these challenges, a fermentation process using an extractive fermentation system has been developed to overcome end-product inhibition and improve product recovery (Bai et al., 2003; Othman et al., 2017).

Bioprocess Development for High Titer Production of D-Lactic Acid Using Inexpensive, Renewable Carbon Sources

To enhance the feasibility of the fermentation process for industrial application, inexpensive, renewable carbon sources such as raw sugar and enzyme-hydrolyzed cassava chips were tested as alternatives to glucose during fed-batch fermentation. Before testing, sugar analysis was performed on both substrates using HPLC. The results showed that both substrates contained glucose, fructose, and sucrose at different concentrations and ratios (Table 2).

Table 2 Sugar concentration and solid content in raw sugar and enzyme-hydrolyzed cassava chip

| Carbon source | Sugar type | Sugar concentration (%w/w) | Solid content (%w/w) |
|---|------------|----------------------------|----------------------|
| Raw sugar | Sucrose | 4.69 | 74.74 |
| | Glucose | 34.42 | |
| | Fructose | 33.35 | |
| | Total | 72.46 | |
| Enzymatic hydrolysis cassava chip (Supernatant) | Sucrose | 0.27 | 22.38 |
| | Glucose | 15.68 | |
| | Fructose | 0.21 | |
| | Total | 16.16 | |

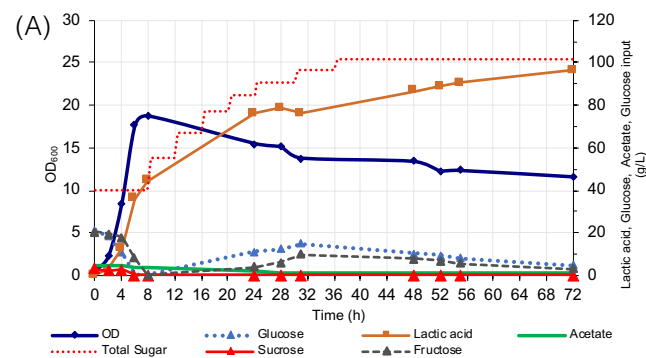
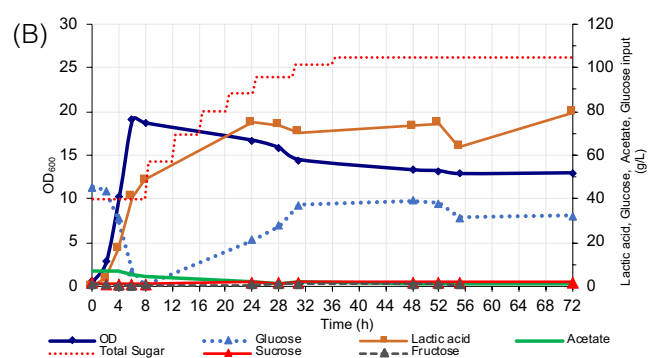


Fig. 4 Fed-batch fermentation profiles of *L. saerimneri* TBRC 5746 in a 5-L fermenter at 42 °C using cheap renewable carbon sources (raw sugar **(A)** and enzyme-hydrolyzed cassava chip **(B)**). Fed-batch fermentation was performed with an initial volume of 2 L semi-defined medium. Fed-batch fermentation using intermittent feeding of raw sugar solution (223 ml of 159.424 g/L total sugar equivalent, **A**)

To investigate the suitability of raw sugar and enzyme-hydrolyzed cassava chips as alternative carbon sources for *L. saerimneri* TBRC 5746, fed-batch fermentation was conducted at 42 °C, pH 6, and without aeration (Fig. 4A and B). The results showed that the strain was able to grow and produce lactic acid, achieving maximum titers of 96.3 g/L and 79.5 g/L using raw sugar and hydrolyzed cassava chips, respectively. These alternative carbon sources have potential as cost-effective and sustainable options for lactic acid production, however, residual sugar was detected. It is possible that inhibitors or toxic substances present in the hydrolysates may impede efficient substrate utilization and result in glucose accumulation. The possibility of contamination and the cost of substrate pretreatment and downstream processing should be considered when scaling up the fermentation process for industrial use with alternative carbon sources.

Genome Sequencing and Identification of Putative *L-ldh* Genes in *L. saerimneri* TBRC 5746

Although preliminary fermentation studies of *L. saerimneri* TBRC 5746 discussed above confirmed that the strain exhibits homofermentative metabolism. A 5-L fed-batch fermentation of *L. saerimneri* TBRC 5746 resulted in lactic acid production at a titer of 99.4 g/L and yield of 0.90 g/g glucose or 90% of the theoretical yield. However, employing this fermentation condition at elevated temperature, we also observed L-lactic acid production, which reduced the product's optical purity. In order to improve the production titer and optical purity of D-lactic acid from *L. saerimneri* TBRC 5746 as well as to understand the basis of the strain's L-lactic acid production, we performed genome sequencing using the Illumina platform. The genome of *L. saerimneri* TBRC 5746 was found to be 1.6 Mb in size with a GC content of 42%.



or enzyme-hydrolyzed cassava chip solution (223 ml of 169.81 g/L total sugar equivalent, **(B)**) started after 8 h at 0, 4, 8, 12, 16, 20 and 24 h time points. Total lactic acid and other metabolites were determined using HPLC equipped with an Aminex HPX-87H ion exchange column

We identified putative *Dldh* and *Lldh* genes in the genome of *L. saerimneri* TBRC 5746, including D-lactate dehydrogenase *Dldh* on Scaffold1:13,437:14,438:- and L-lactate dehydrogenase *Lldh* on Scaffold9:17,046:18,014:+ and Scaffold15:547:1455:-. Our fermentation results were consistent with the presence of two *Lldh* genes in the genome of *L. saerimneri* TBRC 5746 (Fig. 5). To improve D-lactic acid production and optical purity, we used CRISPR/dCas9-assisted transcriptional regulation to repress these genes.

CRISPR-Cas9-based Repression of Putative *Lldh* Genes to Improve D-Lactic Acid Optical Purity

The presence of two *Lldh* genes in the genome of *L. saerimneri* TBRC 5746 provides clues to the production of

both D- and L-lactic acid at the bioreactor scale. In order to improve the production titer and optical purity of D-lactic acid, we employed the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease (Cas9) system to repress the expression of the putative *Lldh* genes in *L. saerimneri* TBRC 5746 (Dominguez et al., 2016). Despite efforts to delete the L-LDH genes from *L. saerimneri*, no viable colonies were observed post-transformation, possibly due to essential roles of these genes or cellular stress from double-strand breaks in a strain with limited homology-directed repair capacity. The CRISPR-Cas9 system, which has been widely used in recent years, allows for efficient and precise editing of the bacterial genome, including those from LAB (Doudna & Charpentier, 2014; Jinek et al., 2012; Wu et al., 2021). For example,

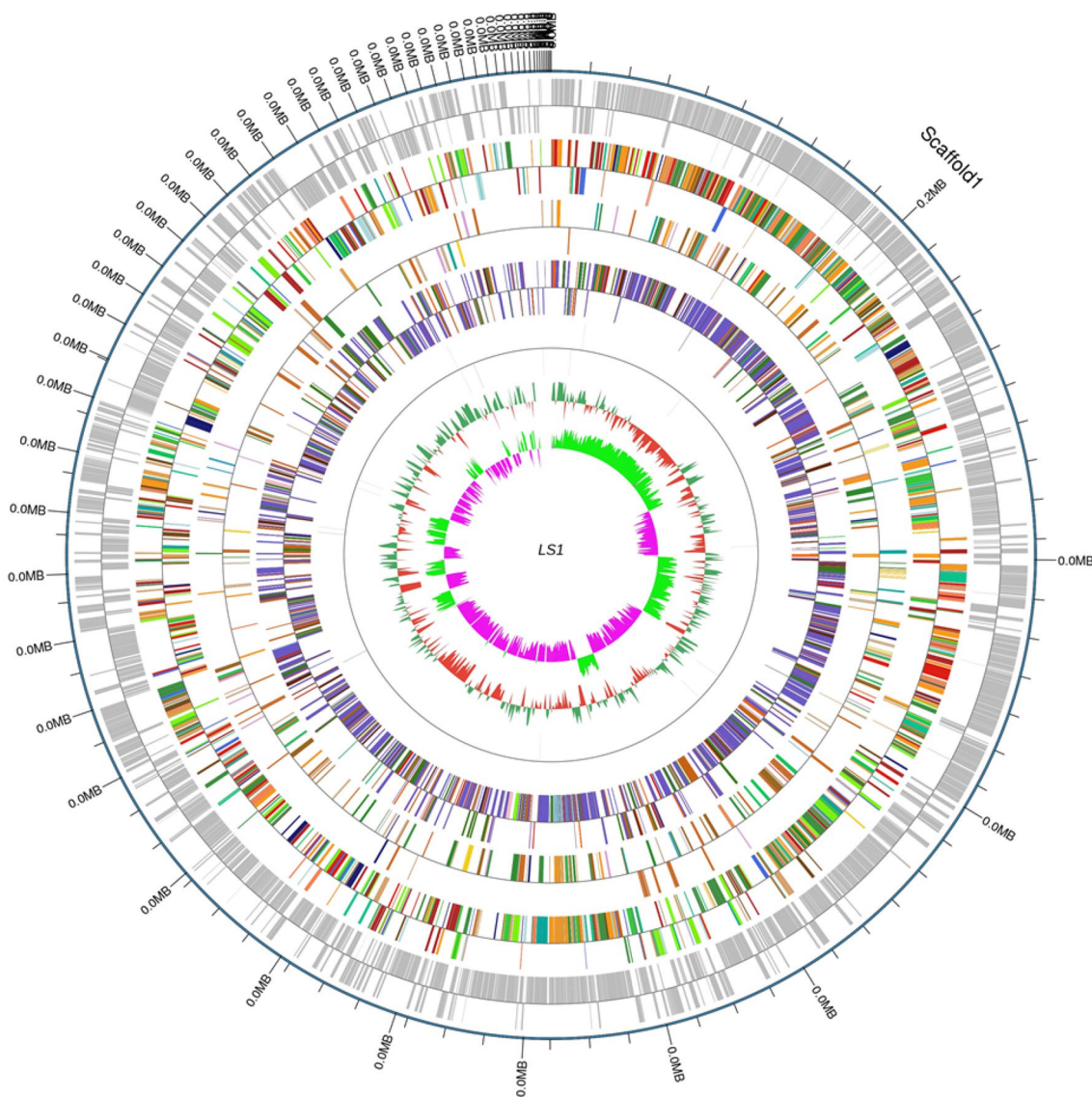


Fig. 5 Genome information of *L. saerimneri* TBRC 5746

Song and coworkers developed a RecE/T-assisted CRISPR/Cas9 genome editing system in *L. plantarum* and *L. brevis* that utilizes the phage-derived recombination system to overcome the challenges posed by the sensitivity of some *Lactobacillus* species to double-strand breaks (Huang et al., 2019). Leenay and coworkers demonstrated CRISPR-Cas9 editing in three strains of *L. plantarum* and observed variable editing outcomes that appeared to be strain-specific (Leenay et al., 2019).

Based on these studies, we developed a CRISPR/dCas9-assisted transcriptional repression system in *L. saerimneri* TBRC 5746 that targets the two *Lldh* genes (named *Lldh_Scaffold9* and *Lldh_Scaffold15*). The CRISPR/dCas9 plasmids contain a gene encoding the nuclease-deficient Cas9 from *Streptococcus pyogenes* (*SpCas9_{D10A,H840A}* or *SpdCas9*) as well as the crRNA that targets the individual *Lldh* genes. Three *L. saerimneri* TBRC 5746 transformants harboring each CRISPR/dCas9 plasmid were selected for real-time PCR (RT-PCR) analysis of *Dldh* and *Lldh* gene expression in test tube cultures (Fig. 6). In the *L. saerimneri* TBRC 5746 strains harboring the CRISPR/dCas9 plasmid targeting *Lldh_Scaffold9*, we observed decreased expression of *Lldh_Scaffold9* in two (clone #1 and clone #3) out of the three selected clones. Similarly, in strains harboring the CRISPR/dCas9 plasmid targeting *Lldh_Scaffold15*, we observed decreased expression of *Lldh_Scaffold15* in two (clone #2 and clone #14) out of the three selected clones. These RT-PCR experiments using small-scaler cultures provide a preliminary and qualitative indication of the CRISPR/dCas9 system's ability to downregulation gene expression in *L. saerimneri* TBRC 5746.

We then evaluated the effect of *Lldh* repression on D-lactic acid production in a 5-L bioreactor (Fig. 7). We hypothesized that the expression of *Ldh* genes would be

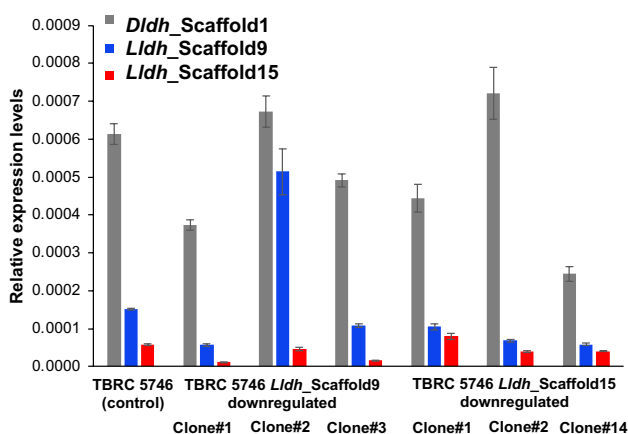


Fig. 6 Relative expression of putative *Lldh* and *Dldh* genes in engineered *L. saerimneri* TBRC 5746. *DldhSc1*, expression of *Dldh_Scaffold1*; *LldhSc9*, expression of *Lldh_Scaffold9*; *LldhSc15*, expression of *Lldh_Scaffold15*. Real-time PCR was performed in triplicates

up-regulated in fed-batch fermentation in the presence of high concentrations of glucose, making the effects of the CRISPR/dCas9-assisted transcriptional repression of *Ldh* genes more pronounced. Our observations supported this hypothesis: we saw a significant improvement in D-lactic acid production in the strain harboring the CRISPR/dCas9 plasmid targeting *Lldh_Scaffold9*, reaching a D-lactic acid titer of 78.5 g/L, a 38% increase over the titer observed in the control strain (*L. saerimneri* TBRC 5746; 57.0 g/L) (Fig. 7B vs. 7A). This increase in D-lactic acid production was also accompanied by a decrease in L-lactic acid production, with the engineered strain producing L-lactic acid at a titer of 21.9 g/L, a 24% decrease from the level observed in the control strain (28.8 g/L). In contrast, the strain harboring the CRISPR/dCas9 plasmid targeting *Lldh_Scaffold15* saw a more modest increase in D-lactic acid production (62.6 g/L) and negligible change in L-lactic acid production (31.8 g/L) (Fig. 7C). These results suggest that repression of *Lldh* genes using CRISPR/dCas9 system has the potential to improve D-lactic acid production in *L. saerimneri* TBRC 5746.

Our study showed that the CRISPR/dCas9 system can effectively improve the production of D-lactic acid, which is of great significance for the industrial production of lactic acid. D-lactic acid is widely used in the food and pharmaceutical industries, and high optical purity is often a requirement. However, there are certain applications where a mixture of L-lactic acid and D-lactic acid may be more desirable than a single isomer. One example is in the food industry, where a mixture of L-lactic acid and D-lactic acid can be used as a flavoring agent or a preservative. In this case, the mixture can provide a more complex and nuanced flavor profile than a single isomer. In future work, we will focus on optimizing the CRISPR/dCas9 system for improved efficiency and broader applicability to other lactic acid bacterial strains. Additionally, more research is needed to investigate the potential of this system for improving other fermentation processes and product yields.

Conclusion

In this study, we screened 600 lactic acid bacterial isolates for D-lactic acid production and identified twelve strains that produce high titers of exclusively D-lactic acid. Initial fermentation experiments showed that only *L. saerimneri* TBRC 5746 exhibited homofermentative metabolism. A 5-L fed-batch fermentation of *L. saerimneri* TBRC 5746 resulted in a lactic acid titer of 99.4 g/L, with a yield of 0.90 g/g glucose, or 90% of the theoretical yield. However, at this titer, we also observed the production of L-lactic acid, which lowered the product's optical purity. Genome sequencing of *L. saerimneri* TBRC 5746 revealed the presence of two putative *Lldh* genes, providing support for the

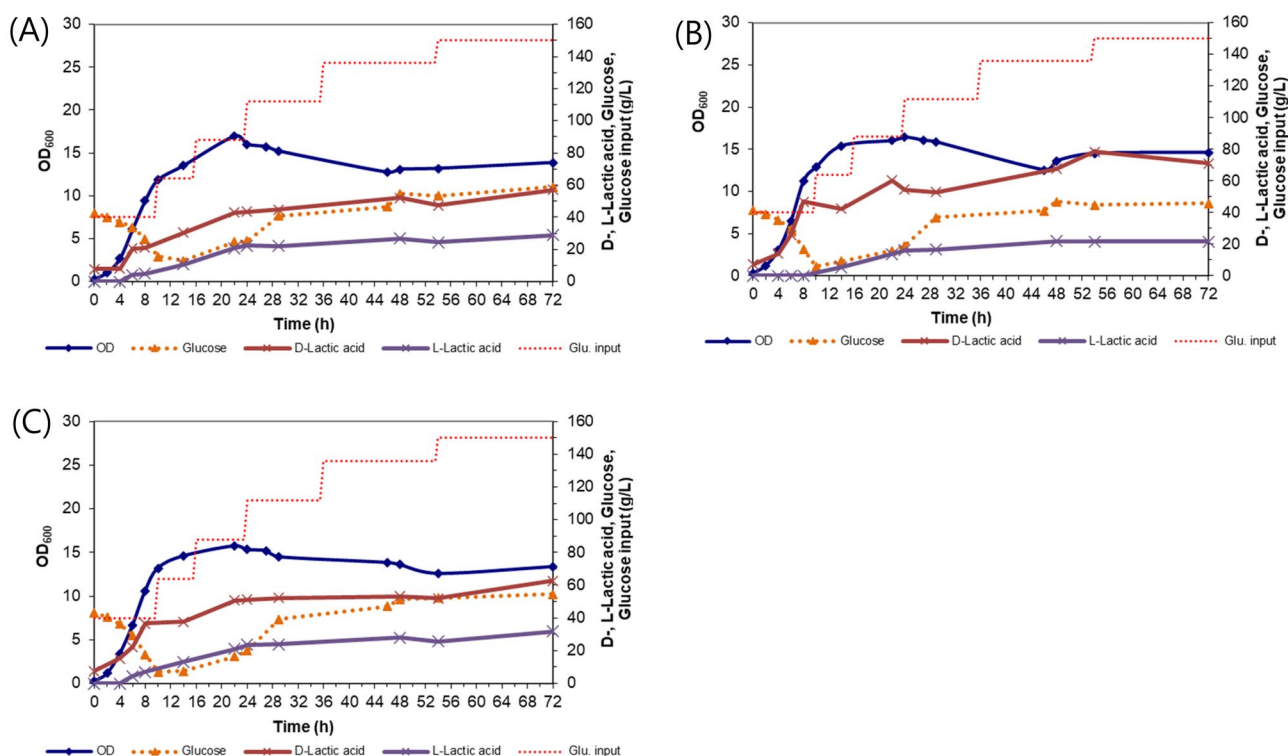


Fig. 7 Fed-batch fermentation of engineered *L. saerimneri* TBRC 5746 strains. **(A)** *L. saerimneri* TBRC 5746; **(B)** *L. saerimneri* TBRC 5746 harboring the CRISPR/dCas9 plasmid targeting *Lldh_Scaffold9*;

(C) *L. saerimneri* TBRC 5746 harboring the CRISPR/dCas9 plasmid targeting *Lldh_Scaffold15*

production of L-lactic acid as a minor product. Repressing these genes using CRISPR/dCas9-assisted transcriptional repression resulted in a 38% increase in D-lactic acid production and improved optical purity. To our knowledge, this is the first demonstration of CRISPR/dCas9-assisted transcriptional repression in *L. saerimneri*. Our work represents progress towards efficient microbial production of D-lactic acid.

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Declarations

Conflict of interest The authors declare no conflict of interest. The funder had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Ethical approval Not applicable.

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