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Engineering *Lactococcus lactis* for D-Lactic Acid Production from Starch

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Abstract

Bioprocess development is a current requirement to enhance the global production of D-lactic acid. Herein, we report a new bioprocess for D-lactic acid production directly from starch using engineered *Lactococcus lactis* NZ9000. To modify *L. lactis* as a D-lactic acid producer, its major endogenous L-lactate dehydrogenase (L-Ldh) gene was replaced with a heterologous D-Ldh gene from *Lactobacillus delbrueckii* subsp. *lactis* JCM 1107. The resulting strain AH1 showed a somewhat slower growth rate but similar lactic acid production compared to those of the intact strain when cultivated with glucose as a carbon source. The chemical purity of D-lactic acid produced by *L. lactis* AH1 was 93.8%, and the enzymatic activities of D- and L-Ldh in AH1 were 1.54 U/mL and 0.05 U/mL, respectively. Next, a heterologous α-amylase gene from *Streptococcus bovis* NRIC 1535 cloned into an expression vector pNZ8048 was introduced into AH1. The resulting strain AH2 showed an amylolytic activity of 0.26 U/mL in the culture supernatant. Direct production of D-lactic acid from starch as the carbon source was demonstrated using *L. lactis* AH2, resulting in D-lactic acid production at a concentration of 15.0 g/L after 24 h cultivation. To our knowledge, this is the first report on D-lactic acid production in engineered *L. lactis*.

Introduction

Lactic acid bacteria (LAB) fermentatively produce lactic acid, which is a renewable versatile chemical [1–3]. Lactic acid shows two optically active isomers, D-lactic acid and L-lactic acid. L-lactic acid has been used as a building block for the well-studied bioplastic poly(L-lactic acid) that possesses unique properties such as transparency, biocompatibility, and biodegradability [4]. The melting temperature (Tm) of crystallized poly(L-lactic acid) is around 180 °C whereas that of crystallized stereocomplex poly(lactic acid) consisting of poly(L-lactic acid) and poly(D-lactic acid) at a ratio of 1:1 is around 230 °C [5–7], meaning that the stereocomplex polymer is a preferable commodity plastic. However, global production of poly(D-lactic acid) is very low because the global market for D-lactic acid is currently less than that for L-lactic acid. To promote the stereocomplex poly(lactic acid) as a commodity bioplastic, development of a bioprocess to enhance the global production of D-lactic acid is highly desired.

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LAB are classified into two groups, homofermentative and heterofermentative LAB, and these are further divided into subgroups harboring D-lactate dehydrogenase (D-Ldh) and/or L-Ldh [8]. Homofermentative LAB produce two molecules of lactic acid from one molecule of glucose without any by-product, whereas heterofermentative LAB produce one molecule of lactic acid from one molecule of glucose with by-products such as acetic acid and ethanol. LAB harboring D- and L-Ldh produce D- and L-lactic acids respectively, but LAB harboring both enzymes can produce both lactic acids. Therefore, it is desired that industrial production of lactic acids is performed using homofermentative LAB harboring either D- or L-Ldh [9–12]. Some homofermentative LAB harboring only L-Ldh have been found in nature, such as *Lactococcus lactis*, where there is no homofermentative LAB harboring only D-Ldh except for *Lactobacillus delbrueckii*.

L. lactis is one of the most well-studied LAB. To date, many reports on genetic engineering of *L. lactis* have been published as *L. lactis* can be easily transformed using appropriate vectors with very high efficiency [13] and many genetic tools for gene expression in *L. lactis* [14–17]. The NICE system is one of the best known gene expression systems in *L. lactis* based on a combination of *L. lactis* hosts encoding *nisRK* genes with the expression vectors harboring a nisin-inducible strong promoter [18–21]. On the contrary,

there are only a few reports on genetic engineering of *L. delbrueckii* because it can be transformed with low efficiency compared to *L. lactis* [22–24]. These data suggest that *L. lactis* is preferable for D-lactic acid production rather than *L. delbrueckii*. Because homofermentative LAB can produce lactic acid without by-products and show tolerance to lactic acid produced by themselves, it is reasonable to engineer homofermentative LAB for lactic acid production compared to non-LAB such as *Escherichia coli* [25, 26]. For example, Okano et al. [27] have demonstrated production of D-lactic acid at 86.0 g/L from 100 g/L glucose for 36 h cultivation in a fermenter using engineered L-Ldh gene-deficient *Lactobacillus plantarum*.

In this study, we developed a new bioprocess for D-lactic acid production using engineered *L. lactis*. The engineered strain producing D-lactic acid was constructed by replacement of its major endogenous L-Ldh gene with a heterologous D-Ldh gene from *L. delbrueckii* subsp. *lactis* JCM 1107. The cell growth and lactic acid production of the resulting strain *L. lactis* AH1 were characterized. To reduce production costs, it is beneficial if D-lactic acid can be directly produced using starch as the substrate, instead of glucose. In fact, some related studies on lactic acid production directly from starch by the expression of a heterologous amylase from *Streptococcus bovis* 148 (corresponding to NRIC 1535) in LAB have been published [27–29]. In this study, we demonstrated the direct production of D-lactic acid from starch using a further engineered strain based on the NICE system. A heterologous α-amylase gene from *S. bovis* NRIC 1535 was introduced into the *L. lactis* AH1 strain, resulting in *L. lactis* AH2, and the amylolytic activity and D-lactic acid production of *L. lactis* AH2 from starch were then characterized.

Materials and Methods

Strains, Media, and Culture Conditions

L. lactis NZ9000 (NIZO Food Research, Netherlands) [30] and *S. bovis* NRIC 1535 [31] were grown in M17 medium (Difco Laboratories, MI, USA) supplemented with 0.5% glucose (GM17) at 30 °C. *L. delbrueckii* subsp. *lactis* JCM 1107 (corresponding to CNRZ 327) was grown in MRS medium (Oxoid, Hampshire, UK) at 30 °C. *E. coli* JM109 (Toyobo, Osaka, Japan)) and TOP10 (Thermo Fisher Scientific, MA, USA) were grown in LB medium at 37 °C. For fermentation tests, the recombinant *L. lactis* were grown in 5 mL M17 medium with 2% glucose or soluble starch in the presence of 0.5 M 3-(*N*-morpholino)propanesulfonic acid) (MOPS, pH 7.0) at 30 °C for 24 h. When required, 50 mg/L kanamycin and 20 mg/L chloramphenicol were added to the media for *E. coli* and *L. lactis* transformants, respectively.

Genetic Engineering

Restriction and ligation enzymes were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Toyobo, respectively. Genomic DNA isolation from LAB was performed using ISOPLANT (Nippon gene, Tokyo, Japan). Plasmid DNA isolation from *E. coli* and *L. lactis* was performed using the MagExtractor Plasmid (Toyobo) and a protocol described elsewhere [32], respectively.

Replacement of the L-Ldh gene encoded on the genome of *L. lactis* NZ9000 with the D-Ldh gene from *L. delbrueckii* subsp. *lactis* JCM 1107 by homologous recombination is illustrated in Fig. 1. The *pfk-pyk* and the *nupC-nupB* regions from *L. lactis* NZ9000 were amplified by polymerase chain reaction (PCR) with primer sets, pfk-F1 (5′-ctggtggtgatgccccagggatgaatgcgg-3′) and pyk-R1

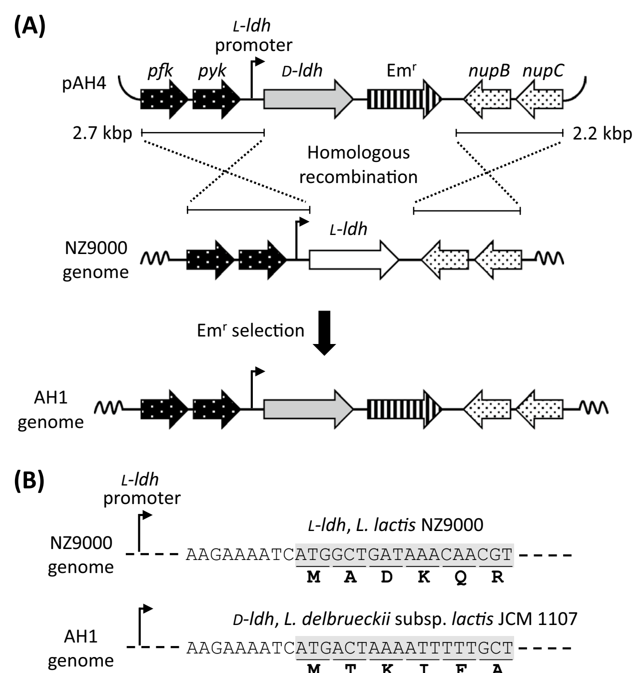

Fig. 1 **a** A scheme of replacement of the L-Ldh gene encoded on the genome of *L. lactis* NZ9000 with the D-Ldh gene from *L. delbrueckii* subsp. *lactis* JCM 1107 by homologous recombination using the pAH4 plasmid. *pfk*, 6-phosphofructokinase gene; *pyk*, pyruvate kinase; *nupB* and *nupC*, ABC transporter permease gene. D-Ldh gene and erythromycin resistance gene (Em^r) were cloned into the intergenic region between *pfk-pyk* and *nup-nupC* on pAH4. **b** Comparison of nucleotide and amino acid sequences between the 5′ flanking regions of L-Ldh and D-Ldh genes of *L. delbrueckii* subsp. *lactis* JCM 1107 and *L. lactis* NZ9000, respectively. By homologous recombination, the D-Ldh gene was inserted in-frame downstream of the endogenous L-Ldh gene encoded on the genome of *L. lactis* NZ9000, resulting in AH1. The shaded sequences are nucleotides encoding D-/L-Ldh and identical amino acids are indicated in bold letters

(5′-agatctgtcgacatatgtttctttaattcctttcaaattataaacg-3′); and nupB-F1 (5′-acaagatctgtcctcgttcctctcattgcc-3′) and nupC-R1 (5′-aagtcgacagagttagtagaaaatttcaac-3′), respectively, with *L. lactis* NZ9000 genomic DNA (accession no. CP002094) as a template, and KOD plus DNA polymerase (TOYOBO). The amplified *pfk*-*pyk* fragment was cloned into a plasmid pCR-BluntII-TOPO in the same direction as the *lacZ* gene via TOPO cloning using *E. coli* JM109 as the host, followed by cloning of the amplified *nupC*-*nupB* fragment into the resulting plasmid at the *Sal*I and *Bgl*II restriction enzyme sites, resulting in the pAH2 plasmid. To clone the D-Ldh gene from *L. delbrueckii* subsp. *lactis* JCM 1107 and the erythromycin resistance gene (Em^r) into pAH2, these genes and the pAH2 fragment were amplified by PCR using primer sets D-ldh-F1 (5′-aggaattaaagaaaatcatgactaaaatttttgcttacgc-3′) and D-ldh-R1 (5′-tatctgtgtcgacaaagcggctagattagccaacc-3′); Em-F1 (5′-ttgtcgacacagatagcgacagagaaggcg-3′) and Em-R1 (5′-ctgtcgacctctttagctccttggaagctg-3′); and pAH2-F1 (5′-ttttctttaattcctttcaaattataaacg-3′) and pAH2-R1 (5′-taaagaggtcgacagagagttagtagaaaatttcaac-3′), respectively, with *L. delbrueckii* subsp. *lactis* JCM 1107 genomic DNA (accession no. CCDV01000001), pNZ9520 plasmid [33], and pAH2 as respective templates, and KOD plus DNA polymerase, followed by fragment assembling using the Generates Seamless Cloning and Assembly Kit (Thermo Fisher Scientific) with *E. coli* TOP10 as a host, resulting in the pAH4 plasmid.

To replace the L-Ldh gene with the D-Ldh gene, *L. lactis* NZ9000 was transformed with pAH4 by electroporation according to a protocol described elsewhere [15]. The cells were subsequently incubated at 30 °C for 18 h on GM17 agar medium supplemented with 1 mg/L erythromycin, resulting in *L. lactis* AH1. To confirm the desired mutant, agarose gel electrophoresis was performed using PCR amplicons of respective deleted regions using mutant genomic DNA as the template with the following primer sets: L-ldh-F1 (5′-agttatccttgttggtgacggtgctgtagg-3′) and L-ldh-R1 (5′-gtaatacgagcaagggctacagccacaccg-3′), and D-ldh-F1 and D-ldh-R1.

Plasmid construction for heterologous α-amylase expression in *L. lactis* NZ9000 was performed in the following manner. The α-amylase gene *amyA* from *S. bovis* was amplified by PCR using KOD plus DNA polymerase, *S. bovis* NRIC 1535 genomic DNA (accession no. AB000829) as a template, and a primer set of amyA-F1 (5′-ccctcatgacatttcagaataaagtaaatt-3′) and amyA-R1 (5′-ctgctgcagaagctacttcttagggaaagg-3′). After The amplified *amyA* fragment containing the region encoding the leader peptide was digested with *Pag*I and *Pst*I, the resulting fragment was cloned into pNZ8048 at *Nco*I and *Pst*I sites, resulting in pNZ8048-amyA. *L. lactis* NZ9000 was transformed with pNZ8048-amyA by electroporation, resulting in *L. lactis* AH2.

Characterization of the Engineered *L. lactis*

The chirality of lactic acid in the supernatant of *L. lactis* cells (optical density at 600 nm (OD_{600}) of 1) incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 1% glucose at 30 °C for 24 h was determined using a Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a MCI GEL CRS10 W column (Mitsubishi Chemical, Tokyo, Japan) and monitored at 254 nm using 1 mM copper(II) sulfate solution as the mobile phase at a 0.5 mL/min flow rate. Commercial D-/L-sodium lactate reagents (Sigma-Aldrich, St. Louis, USA) were used as controls for lactic acid quantification. Lactic acid produced in the culture supernatant of *L. lactis* was quantified using the HPLC system equipped with a SCR-102H column (Shimadzu). Lactic acid was eluted using 0.1% perchloric acid solution. The flow rate was 0.9 mL/min, and the eluate was monitored by absorbance at 210 nm. D-lactic acid production in GM17 was obtained by multiplying the lactic acid concentration determined using HPLC by the chemical purity.

Glucose in the culture supernatant was measured using the commercial kit, Glucose CII-Test Wako (Wako Pure Chemical Industries, Osaka, Japan). Sugar concentration in the culture supernatant was determined by the phenol-sulfuric acid method [34].

Recombinant *L. lactis* cultivated in GM17 medium at 30 °C for 18 h was inoculated in fresh GM17 medium, and was cultured to an optical density at OD_{600} of 0.1. After the cultures were subsequently grown at 30 °C until they reached an OD_{600} of 0.5–0.7, the cells were washed twice with a resuspension buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5 mM PMSF) [35]. The washed cells were resuspended in 90 µL of resuspension buffer containing 0.5 g/L Labiase (Ozeki Corp., Hyogo, Japan) and lysozyme (Wako) and then incubated at 37 °C for 30 min. After adding 10 µL of BugBuster 10X Protein Extraction Reagent (Merck Millipore, MA, USA) to the cell suspension, the suspension was incubated at a room temperature for 20 min. The cell extract was centrifuged and its protein concentration was then measured using the protein assay reagent in the TaKaRa BCA Protein Assay Kit (Takara Bio Inc., Shiga, Japan), based on the BCA method [36], with bovine serum albumin as a standard. D-/L-Ldh specific activities in recombinant *L. lactis* were assayed in the following manner: 10 µL of the cell extract was incubated with 200 µL of substrate solution (100 mM Tris-HCl (pH 8.0), 15 mM NAD^+, 500 mM D-/L-sodium lactate, 10 mM fructose 1,6-bisphosphate) at 37 °C for 10 min. The released NADH was measured at 340 nm using a spectrophotometer. One unit (U) corresponds to 1 µmol of NADH formed per min.

Recombinant *L. lactis* cultivated in GM17 medium at 30 °C for 18 h was inoculated in fresh GM17 medium,

and cultured to OD_{600} of 0.1. After the cultures were subsequently grown at 30 °C until they reached an OD_{600} of 0.4–0.6, 5 μg/L nisin A (Sigma-Aldrich) was added to the cultures and then incubated at 30 °C for 18 h for *amyA* expression. The α-amylase activity in the culture supernatant of recombinant *L. lactis* was determined using a commercial kit, α-Amylase Assay Kit (Kikkoman Corp., Chiba, Japan) with 2-chloro-4-nitrophenyl 6^5-azido-6^5-deoxy-β-maltopentaoside as the substrate according to the manufacturer's instructions. One unit (U) of α-amylase activity was defined as the release of 1 μmol of 2-chloro-4-nitrophenol within 1 min. The plate assay for α-amylase activity was performed as follows: *L. lactis* NZ9000 cells harboring pNZ8048-amyA or pNZ8048 were inoculated on GM17 agar plate supplemented with 1% soluble starch. After incubation for 18 h at 30 °C, the plate was stained with iodine solution (0.12% iodine + 0.4% potassium iodide). Appearance of a clear zone around the recombinant colony indicates starch hydrolysis.

Results and Discussion

Characterization of *L. lactis* AH1

By double crossover recombination between the *pfk-pyk* and *nupC-nupB* regions, the major L-Ldh gene on the genome of *L. lactis* NZ9000 was replaced with the fragment containing the D-Ldh gene from *L. delbrueckii* subsp. *lactis* JCM 1107, resulting in *L. lactis* AH1 (Fig. 1a). The D-Ldh gene was inserted in-frame downstream of the endogenous promoter of the L-Ldh gene (Fig. 1b). To check the gene replacement, the chirality of lactic acid produced by AH1 was analyzed. The cells were incubated in a phosphate buffer containing 1% glucose and the supernatant was then analyzed because the impurities from M17 medium affected detection of the chirality by HPLC (data not shown). After 24 h incubation, *L. lactis* AH1 and NZ9000 produced 1.5 ± 0.1 g/L and 0.1 ± 0.1 g/L, and 0.0 ± 0.0 g/L and 1.7 ± 0.1 g/L of D- and L-lactic acids, respectively (Fig. 2a). From this result, the chemical purity of D-lactic acid produced by *L. lactis* AH1 was calculated as 93.8% with 87.5% optical purity. This indicates that the introduced D-Ldh gene functions in *L. lactis* AH1 and that the production level of lactic acid was unaffected by the gene replacement. *L. lactis* AH1 produced 0.1 g/L of L-lactic acid; this is due to the presence of L-Ldh homologue genes that are identical to *ldhB*, *ldhX*, and *hicD* encoded on the genome of *L. lactis* strains [37–39]. This is in good agreement with the result that the enzymatic activities of D- and L-Ldhs in *L. lactis* AH1 were 1.54 ± 0.00 U/mL and 0.05 ± 0.00 U/mL, respectively (Fig. 2b). Since the titer of L-lactic acid produced by *L. lactis* AH1 was negligible, the recombinant strain was used as a D-lactic acid producer hereafter. The effect of gene replacement on the growth rate and lactic acid production was characterized by cultivation of the recombinant strain in GM17 at 30 °C. The turbidity of *L. lactis* AH1 and NZ9000 cultures at the stationary phase was almost the same (OD_{600} = 2.5), whereas they reached the stationary phase after 12 h and 6 h cultivation, respectively (Fig. 2c). This may be because the D-Ldh gene from *L. delbrueckii* showing an optimal growth at 40–44 °C [40] was introduced into *L. lactis* showing an optimal growth at 27–33 °C [41]. This suggested that D-Ldh showing an optimal temperature at 27–33 °C is preferable for cell growth of *L. lactis* AH1. *L. lactis* AH1 produced 6.6 ± 0.0 g/L of D-lactic acid after 24 h cultivation; this titer was similar to the L-lactic acid production of *L. lactis* NZ9000 (7.0 ± 0.0 g/L). This is in good agreement with the

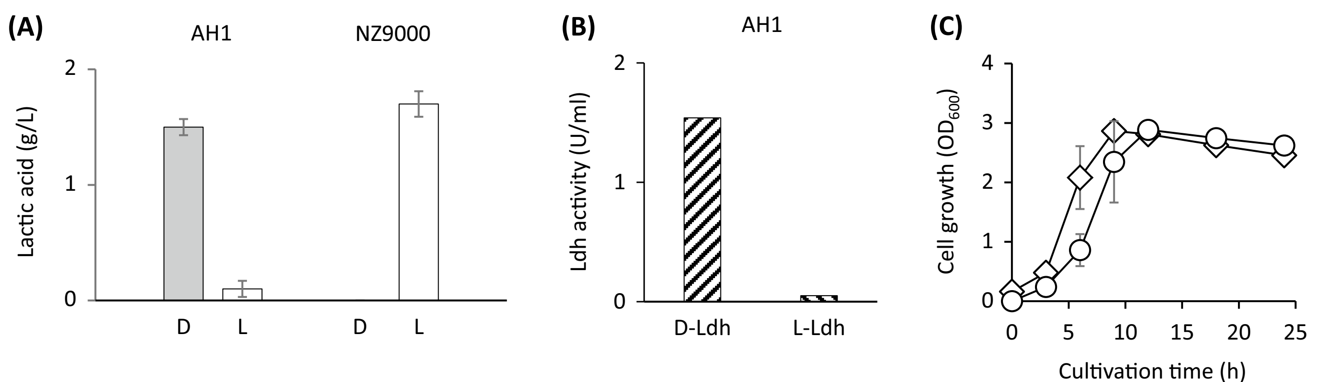

Fig. 2 **a** D-/L-lactic acid production by the cells of *L. lactis* AH1 and *L. lactis* NZ9000 incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 1% glucose at 30 °C for 24 h. **b** Specific D-/L-Ldh activities in the cell extract of *L. lactis* AH1. Ten microliters cell extract were incubated with 200 μL of substrate solution (100 mM Tris-HCl (pH 8.0), 15 mM NAD^+, 500 mM D-/L-sodium lactate, 10 mM fructose 1,6-bisphosphate) at 37 °C for 10 min. The released NADH was measured at 340 nm. **c** Cell growth of *L. lactis* AH1 (open circles) and NZ9000 (diamonds). These strains were cultivated in GM17 at 30 °C for 24 h. All experiments were performed in duplicate, and the average is represented with error bars

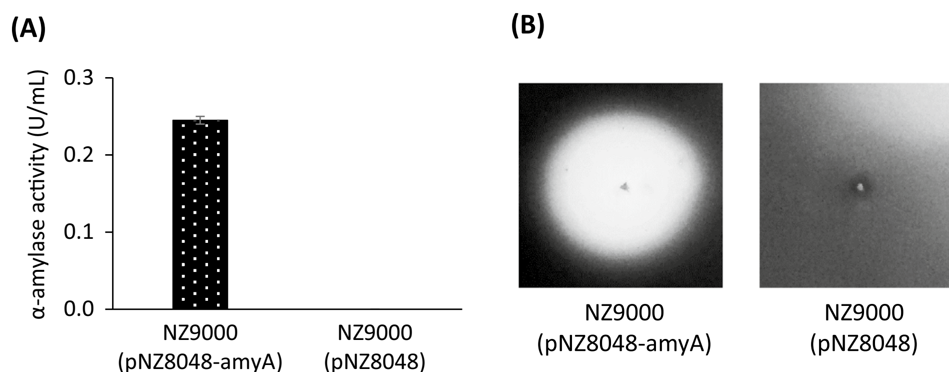

Fig. 3 Expression of AmyA from *S. bovis* NRIC 1535 in *L. lactis* NZ9000. **a** Specific AmyA activities in culture supernatants of *L. lactis* NZ9000 harboring pNZ8048-amyA or pNZ8048. This assay was performed at 30 °C in duplicate, and the mean value is presented with error bars. **b** Iodine staining of 1% starch-containing agar plates after inoculation with *L. lactis* NZ9000 harboring pNZ8048-amyA or pNZ8048. Clear zones indicate hydrolysis of starch around the recombinant colonies

result of lactic acid production by AH1 cells incubated in the buffer.

Characterization of *L. lactis* AH2

A functional assay to determine the activity of α-amylase expressed in *L. lactis* NZ9000 was demonstrated by introduction of pNZ8048-amyA into *L. lactis* NZ9000. The α-amylase from *S. bovis* NRIC 1535 showed amylolytic activity (0.26 ± 0.01 U/mL) in the culture supernatant of *L. lactis* NZ9000 harboring pNZ8048-amyA, whereas *L. lactis* NZ9000 harboring pNZ8048 as a control showed no amylolytic activity (Fig. 3a). A similar result (Fig. 3b) was observed on the plate assay, indicating that heterologous α-amylase was secreted from *L. lactis* NZ9000 harboring pNZ8048-amyA and starch was then utilized in the *L. lactis* strain after hydrolysis by α-amylase.

Lactococcus lactis AH2 was obtained by introduction of pNZ8048-amyA into *L. lactis* AH1. Using the resulting strain, the direct production of D-lactic acid from starch was demonstrated for 24 h with 2% soluble starch as the carbon source. To reduce the effect of pH decrease by lactic acid produced on cell growth, 0.5 M MOPS (pH 7.0) was supplemented in the culture medium. To normalize the effect of harboring the plasmid, *L. lactis* AH1 harboring pNZ8048 was used as a control. Cultivation of *L. lactis* AH1 harboring pNZ8048 with 2% glucose resulted in maximal cell growth ($OD_{600} = 5.10 \pm 0.53$) and maximal L-lactic acid production of 23.5 ± 0.2 g/L after 18 h cultivation, the levels of which remained unchanged thereafter (Fig. 4a). *L. lactis* AH2 showed a similar profile when cultivated with 2% glucose, showing the maximal cell growth ($OD_{600} = 6.85 \pm 0.25$; specific growth rate (μ) = 1.05) and maximal D-lactic acid production of 23.3 ± 0.1 g/L (D-lactic acid productivity (P) = 1.14 g/L/h) (Fig. 4b). Compared to a related report using engineered L-Ldh gene-deficient *L. plantarum* cultivated in the presence of 100 g/L glucose [27], the production level obtained was about four times lower, possibly because this study used 20 g/L glucose. In the presence of 2% soluble starch, *L. lactis* AH2 didn't reach a stationary phase even after 24 h cultivation ($OD_{600} = 2.78 \pm 0.06$, $\mu = 0.12$) but showed maximal D-lactic acid production of 15.0 ± 0.5 g/L ($P = 0.63$ g/L/h) (Fig. 4c). It has been reported that soluble starch was hydrolyzed to maltose (62.9%) and glucose (37.1%) when treated with α-amylase from *S. bovis* 148 [29, 42]. This is probably why the growth rate and D-lactic acid production rate were slower than when glucose was used. This suggests that maltose consumption was the rate-limiting step in starch fermentation. In fact, a similar phenomenon was observed in a previous related study [29], suggesting that D-lactic acid production by *L. lactis* from starch can be enhanced by improving this step (e.g., expression of maltase).

In conclusion, this study showed that replacement of L-Ldh gene with D-Ldh gene is useful for engineering LAB for D-lactic acid production. To our knowledge, this is the first report on D-lactic acid production in engineered *L. lactis*. The technique described herein for D-lactis acid production from starch by *L. lactis* expressing α-amylase may reduce the total incurred costs including production costs due to the carbon source. As *L. lactis* can be genetically modified easily and the NICE system provides strong gene expression in *L. lactis*, it is possible to perform further metabolic engineering of recombinant *L. lactis* as an easily engineerable host such as *E. coli* for bioproduction of a series of versatile chemicals such as propionic acid [43] and 1,2-propanediol [44], using D-lactic acid as the platform chemical.

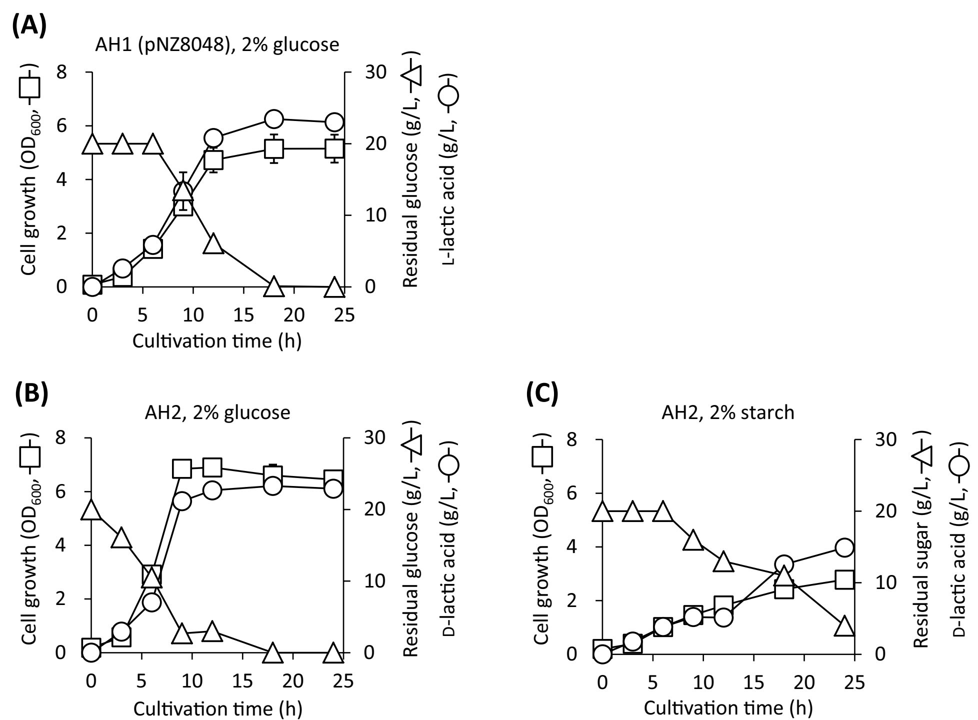

Fig. 4 Production of lactic acid in *L. lactis* AH1 harboring pNZ8048 and *L. lactis* AH2 in different culture media. **a** *L. lactis* AH1 harboring pNZ8048 was cultivated in M17 supplemented with 2% glucose, and **b** *L. lactis* AH2 was cultivated in M17 supplemented with **b** 2% glucose and **c** 2% soluble starch at 30 °C for 24 h. All media contain 0.5 M MOPS (pH 7.0). Open squares, cell growth; open triangles, residual glucose/sugar concentration; open circles, D-/L-lactic acid concentration. This cultivation was performed in duplicate, and the average is represented with error bars

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest for this study.

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