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Regulation of Metabolic Flux in *Lactobacillus casei* for Lactic Acid Production by Overexpressed *ldhL* Gene with Two-Stage Oxygen Supply Strategy

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This study describes a novel strategy to regulate the metabolic flux for lactic acid production in *Lactobacillus casei*. The *ldhL* gene encoding L-lactate dehydrogenase (L-LDH) was overexpressed in *L. casei*, and a two-stage oxygen supply strategy (TOS) that maintained a medium oxygen supply level during the early fermentation phase, and a low oxygen supply level in the later phase was carried out. As a consequence, a maximum L-LDH activity of 95.6 U/ml was obtained in the recombinant strain, which was over 4-fold higher than that of the initial strain. Under the TOS for *L. casei* (pMG-*ldhL*), the maximum lactic acid concentration of 159.6 g/l was obtained in 36 h, corresponding to a 62.8% increase. The results presented here provide a novel way to regulate the metabolic flux of *L. casei* for lactic acid production in different fermentation stages, which is available to enhance organic acid production in other strains.

Keywords: *Lactobacillus casei*, lactic acid, oxygen supply, L-lactate dehydrogenase

Introduction

Lactic acid bacteria (LAB) are industrially important microorganisms that are used worldwide in the manufacture of fermented foods and beverages. Recently, the sharply enlarged manufacture of the biodegradable polylactide polymer has increased global interest in the production of L-lactic acid using the fermentative route [2, 9, 20, 24, 25, 27]. Previous studies indicated that *Lactobacillus casei* is a homofermentative lactic acid bacterium and can produce L(+)-lactic acid with an optical purity of over 95% [10], making it essential to improve the metabolic flux of *L. casei* by genetic manipulation. Nevertheless, available work mainly focused on the improvement of lactic acid production in *L. lactis* using genetically modified organisms and multistage fermentation [1, 26]. Still, the regulation of the main metabolic flux in *L. casei* is lacking.

L-Lactate dehydrogenase (L-LDH) is the key enzyme for lactic acid production in *L. casei*, which catalyzes 1 molecule of pyruvic acid to L-lactic acid [3]. However, it was found that overexpressed *ldhL* has little influence on the lactic acid production, which mainly resulted from *Lactobacillus* sp. lacking an efficient respiration metabolism and major metabolic energy was gained from glycolysis [7, 8]. Therefore, to improve lactic acid production, the L-LDH activity and glycolysis flux should be enhanced synergistically.

Recently, the two-stage oxygen supply strategy (TOS) was proved to be an effective strategy for enhancement of enzymes and organic acids production [6, 18, 23]. Previous reports signified that the growth of some *L. lactis* strains can benefit from the presence of low oxygen levels. However, the oxygen supply has been shown to shift the sugar catabolism toward mixed/flavor fermentation with formation of acetate and acetoin in detriment of lactate production. This shift is not only related to the induction of the O_2-consuming oxidizing enzymes, but also to the competition for NADH between LDH and/or acetaldehyde and ethanol dehydrogenases and NADH-oxidases [17]. In

this study, with the target of improvement of L-lactic acid production, TOS was proposed and applied for the genetically modified *L. casei*, and the relevant metabolic flux was analyzed.

Materials and Methods

Biological Material

The genotypes of the microbial strains and plasmids used in the present study are summarized in Table 1. *L. casei* G-02 is deposited in the China Center of Type Culture Collection (CCTCC) as CCTCC M 208232. LA Taq DNA polymerase, T4 DNA ligase, DNA Marker, and restriction enzymes *Xba*I and *Pst*I were obtained from Takara (Dalian, China). Tris, NADH, X-gal, FBP, IPTG, valinomycin, ampicillin, kanamycin, erythromycin, and nigericin were obtained from Sigma-Aldrich (Steinheim, Germany). All inorganic compounds were of reagent grade or higher quality.

Construction of Strains and Plasmids

Isolation of chromosomal DNA from *L. casei* and standard recombinant DNA techniques were performed as described by Sambrook *et al*. [21]. The gene was amplified by using *L. casei* G-02 chromosome DNA as a template and two primers designed according to the published nucleotide sequence of the *ldhL* gene from *L. casei* BL23 [14]: 5′-GCTCTAGAGTGGCAAGTATTAC-3′(containing a *Xba*I site (underlined)) and 5′-AACTGCAGTTAC TGACGGGTTT-3′(containing a *Pst*I site (underlined)). The PCR-amplified *ldhL* gene fragment digested by *Xba*I-*Pst*I was inserted into the pMG36e vector to create plasmid pMG-*ldhL*. The plasmid pMG-*ldhL* was extracted after transforming into *E. coli* to culture the transformants and then introduced into *L. casei* G-02 by electrotransformation (voltage 1.5 kV, time 2.0 msec), yielding *L. casei* (pMG-*ldhL*). The plasmid pMG-*ldhL* was extracted from *E. coli* transformants, and the inserted *ldhL* gene was sequenced by Sangon Biotechnology Company (Shanghai, China). Analysis revealed that the amino acid sequence encoded by the *ldhL* gene from *L. casei* (pMG-*ldhL*) was identical to that of the template strain *L. casei* BL23 [14].

Batch Fermentations

E. coli JM109 was used for the construction of the plasmid pMG-*ldhL*, grown in LB/Em (5 μg/ml) media at 200 rpm and 37°C. *L. casei* (pMG-*ldhL*) was grown in MRS medium at 140 rpm and 37°C.

Lactic acid fermentation medium contained (g/L) glucose, 170; yeast extract, 10; trypton, 15; beef extract, 10; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.2; and CaCO, 100. The initial pH of all media was adjusted to 6.0. $CaCO_3$ was sterilized by dry heat sterilization at 160°C for 30 min before being added to the medium. Fermentations were carried out in a 7 L jar fermentor (KF-7 l; Korea Fermentor Co., Inchon, Korea), containing 3.5 L of medium with an inoculum size of 10% (v/v) from the seed culture grown to exponential phase (OD_{600} = 15). Two oxygen supply methods determined by controlling the aeration rate at 0.5 and 1.0 vvm were classified as low oxygen supply (LOS) level and medium oxygen supply (MOS) level, respectively. Without oxygen supply (NOS) level condition was used as a control. DO concentrations under different aeration conditions were expressed in terms of DO saturation level (%), whereas 100% DO concentration level corresponded to actual DO saturation at 37°C, 1 atm, and the agitation speed was controlled at 140 rpm. All experiments were done in triplicate, and the values are expressed as the means of duplicate measurements on three independent samples.

Influence of Overexpressed *ldhL* Gene on Metabolism

To determine the concentrations of the intracellular metabolites, the culture of *L. casei* was filtrated on cheese-cloth to get a clear supernatant, and then centrifuged at 5,000 ×*g* for 15 min at 4°C to harvest the cells. The cell pellets were washed twice with ice-cold saline (0.85% NaCl (w/v)) and resuspended in 25 ml of 200 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA. The

Table 1. Strains, plasmids, and oligonucleotides used in this study.

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence	Reference or purpose
Strains		
L. casei G-02	Wild-type *L. casei*	CCTCC M 208232
E. coli JM109	recA1 endA1 gyrA96 *thi*-1 hsdR17 supE44 relA1 Δ(*lac-proAB*)/ F′(traD36 proAB+ lac^q lacZ ΔM15)	Stratagene
L. casei (pMG-*ldhL*)	G-02 harboring pMG-*ldhL*	This work
Plasmids		
pMG36e	*E. coli*-*L. casei* shuttle vecter	Invitrogen
pMG-*ldhL*	Plasmid carrying the *ldhL* genes	This work
Oligonucleotides	5′ → 3′ sequence	
ldhL -F	GCTCTAGAGTGGCAAGTATTAC	Amplifying *ldhL*
ldhL-R	AACTGCAGTTACTGACGGGTTT	Amplifying *ldhL*

Restriction sites (*Xba*I, *Pst*I) are underlined.

cells were disrupted ultrasonically at 4°C for 40 cycles of 5 sec (ACX 400 sonicator at 20 kHz; Sonic and Materials, Newton, MA, USA). Cell debris was removed by centrifugation (10,000 ×*g* for 10 min at 4°C). The extracellular and intracellular metabolites (glucose, lactate, and pyruvate) were analyzed using high performance liquid chromatography (HPLC; Waters Associates model 209, equipped with a differential refractive index R1401 detector) using a Merck Lichrosorb-NH_2 column (4.6 × 250 mm, 5 µm), at a temperature of 30°C. A mixture of acetonitrile-water (60:40 (v/v)) was used as the mobile phase at a flow rate of 1.0 ml/min). All samples were injected twice. To detect the concentration of NADH and NAD^+, the cel1 samples were removed from the culture, frozen immediately in liquid nitrogen for 60 sec, and stored at −20°C for tests.

Analytical Methods

The intracellular L-LDH activity assay was a modification of the methods of Li *et al*. [13]. It is based on the conversion of NADH and pyruvate to lactic acid and NAD^+ by L-LDH, and the maximum absorption peak of NADH was 340 nm. The reaction was initiated by the addition of 2.9 ml of sodium pyruvate, 0.1 ml of NADH, and 2 µl of crude extract to the 5 ml cuvette and was incubated at the temperature of 40°C with pH 6.7. The absorbance of the reaction mixture was measured at 340 nm. One unit of L-LDH activity was defined as the amount of enzyme required to oxidize 1 µmol of NADH per minute.

L-Lactic acid and glucose were determined by a SBA-40E immobilized enzyme biosensor. The pH was measured using a pH meter. The biomass concentration was determined by measuring the OD_{600} or dry cell weight (DCW) per liter, where 5 ml of the culture broth was centrifuged (10,000 ×*g* for 10 min), washed twice with distilled water, and dried at 105°C until achieving a constant weight. Under these experimental conditions, an OD_{600} of 1.0 was equal to 0.41 g DCW/l. The protein concentrations of the cells mass were determined using the Bradford method with bovine serum albumin as the standard [4].

Results

Effect of Overexpressed *ldhL* Gene on Growth and Lactic Acid Production of *L. casei*

To enhance the L-LDH activity, the plasmid pMG-*ldhL* was introduced into *L. casei* G-02. SDS-PAGE analysis of the protein in the original and the recombinant strains were compared, which signified that the *ldhL* gene was overexpressed in the recombinant strain, with a distinct protein band at 37 kDa (Fig. 1). Further investigation found that the maximum L-LDH activity of 95.6 U/ml was obtained in the recombinant strain, which was over 4-fold higher than that of the original strain (22.1 U/ml) (Fig. 2). These results demonstrated that, as a constitutive expression vector, the plasmid pMG36e was favorable for use in

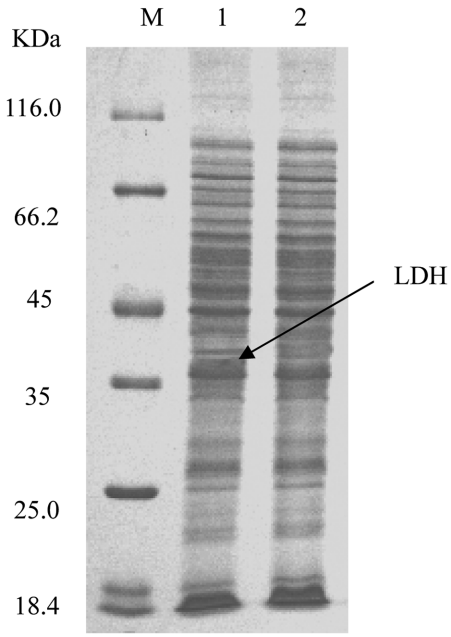

Fig. 1. SDS-PAGE analysis of pMG-*ldhL* overexpressed in recombinant strain of *Lactobacillus casei*.
M: Markers; 1: *L. casei* (pMG-*ldhL*); 2: *L. casei* G-02.

L. casei to enhance the L-LDH activity. However, it was found that the cell growth and lactic acid production of the recombinant strain were not enhanced significantly, which was consistent with the reports indicating the metabolic bottleneck at the level of pyruvate kinase [15].

To determine the influence of pyruvic acid concentrations on the intracellular L-LDH activity, the enzyme activities

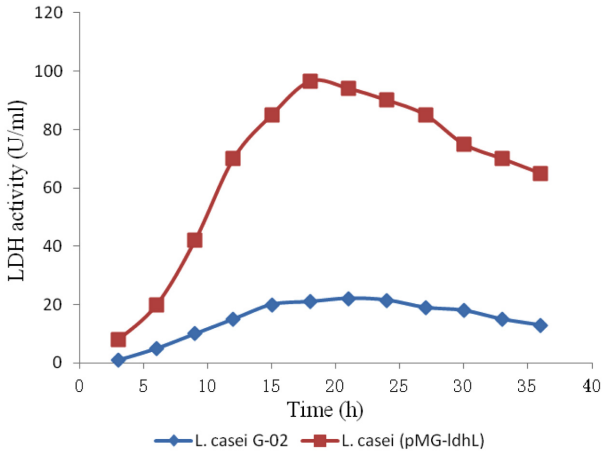

Fig. 2. Time course of intracellular L-LDH activity of *L. casei* G-02 and *L. casei* (pMG-*ldhL*).
The experiments were carried out in a 7-liter jar fermentor containing 3.5 liters of MRS liquid culture with an inoculum size of 10% (v/v) at 150 rpm and 37°C.

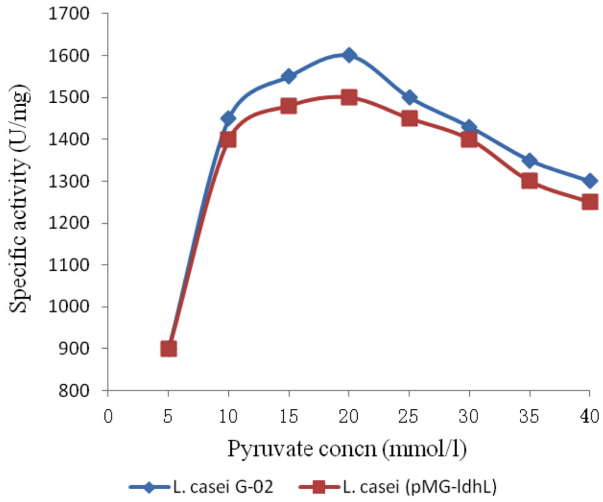

Fig. 3. Effect of pyruvate on the enzyme activity of L-LDH.
The L-LDH activity assay were carried out in a 5 ml cuvette and incubated as described in Materials and Methods, supplemented with 5 to 40 mmol/l pyruvate.

were measured under various concentrations of pyruvic acid (0–40 mmol/l). As shown in Fig. 3, the enzyme activity was significantly influenced by the pyruvate concentrations, and maximum enzyme activity was obtained in the system with 20 mmol/l pyruvate.

Influence of Oxygen Supply on Lactic Acid Production of *L. casei*

The effect of oxygen supply levels on intracellular carbon flux distributions of strains *L. casei* G-02 and *L. casei* (pMG-*ldhL*) were investigated by metabolic flux analysis under low oxygen supply (LOS) level, medium oxygen supply (MOS) level, and control conditions. The cell growth and lactic acid production were observed mainly within 36 h, and the results are shown in Fig. 4. For strain *L. casei* G-02, maximum cell growth and lactic acid production were obtain under LOS condition, which were increased by 25.1% and 28.5% compared with those under the control condition. Further increase in the oxygen supply level to

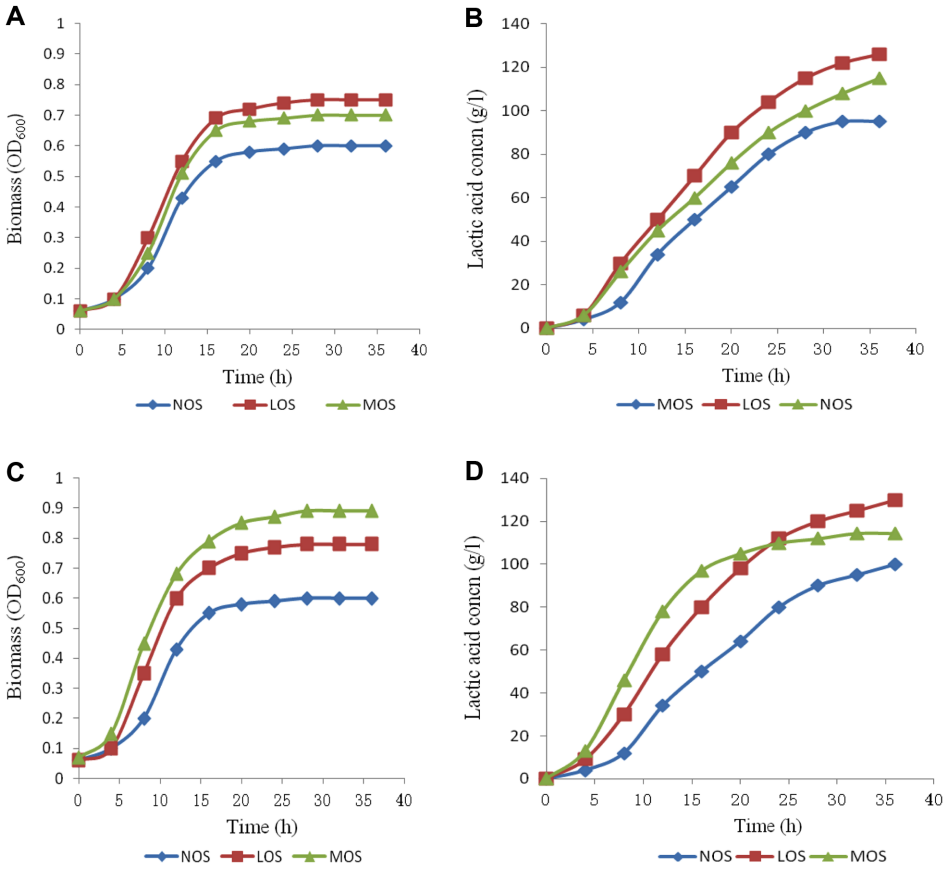

Fig. 4. Cell growth and lactic acid production of strain *L. casei* G-02 (**A** and **B**) and strain *L. casei* (pMG-*ldhL*) (**C** and **D**) under low oxygen supply (LOS) level, medium oxygen supply (MOS) level, and no oxygen supply (NOS) level.
OD_{600} of the liquid fermentation was measured after it was diluted 25 times.

MOS condition caused decreased cell growth and lactic acid synthesis. For strain *L. casei* (pMG-*ldhL*), maximum cell growth was obtained under MOS condition, which was increased by 48.3% compared with that under the control condition.

As glucose is the main carbon source, the EMP pathway was activated under oxygen supply condition to produce precursors, such as G6P, F6P, T3P, PG3, and PEP, for amino acid production and cell synthesis. Although the metabolic flux of EMP under MOS was more active than that under LOS and control, maximum lactic acid production in the G-02 strain was obtained under LOS condition. Nevertheless, for strain *L. casei* (pMG-*ldhL*), as the L-LDH activity was increased over 4-fold higher than that of the original strain, maximum lactic acid synthesis was obtained under MOS in the early fermentation phase (beginning 20 h). Nevertheless, compared with lactic acid production under LOS condition (maximum lactic acid concentration of 130 g/l in 36 h), the lactic acid synthesis activity for strain *L. casei* (pMG-*ldhL*) under MOS decreased in the later phase (maximum lactic acid concentration of 114.5 g/l in 36 h).

These results signified that overexpressed L-LDH resulted in enhanced cell growth and lactic acid synthesis under MOS condition in the beginning of the fermentation, which was consistent with the reports that lactic acid synthesis was accompanied with cell growth. It was also found in Table 3 that the *in vivo* [NAD^+]/[NADH] was increased from 4.61 to 13.63 by overexpressed L-LDH under MOS condition, which decreased the intracellular pyruvate level from 4.53 μmol/g DCW to 1.98 μmol/g DCW. These results were mainly attributed to the oxygen induction under the MOS condition. Therefore, to improve the lactic acid production in the later phase of the fermentation, as the cell growth reached a stable stage, the oxygen-induction level should be attenuated.

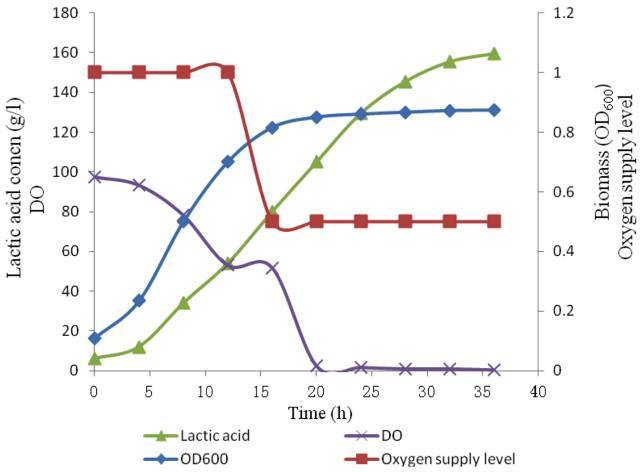

Fig. 5. Cell growth and lactic acid production of strain *L. casei* (pMG-*ldhL*) under a two-stage oxygen supply strategy (TOS) that maintained MOS during the early fermentation phase (beginning 20 h), and LOS in the later phase (following 16 h).

Improve Lactic Acid Production by Two-Stage Oxygen Supply Strategy

To further enhance the lactic acid production in *L. casei* (pMG-*ldhL*), a two-stage oxygen supply strategy that maintained MOS during the early fermentation phase (beginning 20 h), and then step-wisely reduced aeration to the LOS profile in the later phase was carried out. As shown in Fig. 5, the lactic acid productivity throughout the fermentation was maintained at 4.43 $g \cdot l^{-1} \cdot h^{-1}$, and the maximum lactic acid concentration of 159.6 g/l was

Table 2. Comparison of parameters of lactate fermentation by *L. casei* G-02 and *L. casei* (pMG-*ldhL*) without oxygen supply.

Parameters	*L. casei* G-02	*L. casei* (pMG-*ldhL*)
Fermentation time (h)	36	36
Maximum dry cell mass (g/l)	4.71 ± 0.11	4.66 ± 0.12
Total consumed glucose (g/l)	114.50 ± 2.21	115.00 ± 2.48
Maximum lactate concentration (g/l)	95.16 ± 2.06	100.08 ± 2.32
Lactate productivity (g/l/h)	1.98 ± 0.09	2.11 ± 0.10
Intracellular NADH level (μmol/g dry cell weight)[a]	1.21 ± 0.08	1.02 ± 0.06
Intracellular NAD^+ level (μmol/g dry cell weight)[a]	1.36 ± 0.05	2.68 ± 0.07
[NAD^+]/[NADH] ratio	1.12	2.62
Intracellular pyruvate level (μmol/g dry cell weight)[a]	4.69 ± 0.10	4.48 ± 0.10
LDH activity (U/ml)[a]	22.10 ± 0.19	95.60 ± 3.82

[a]After incubation for 18 h, 20 ml of culture was removed from the fermentation vessel, frozen immediately in liquid nitrogen for 60 sec, filtrated on cheese-cloth, and centrifuged at 5,000 ×*g* for 15 min at 4°C to get a clear supernatant as described under Materials and Methods. Measurements are averages of three supernatants. The standard deviations were lower than 10% of the values.

Table 3. Comparison of parameters of lactate fermentation by *L.casei* G-02 and *L.casei* (pMG-*ldhL*) under TOS.

Parameters	*L. casei* G-02	*L. casei* (pMG-*ldhL*)
Fermentation time (h)	36	36
Maximum dry cell mass (g/l)	7.68 ± 0.38	9.13 ± 0.35
Total consumed glucose (g/l)	140.52 ± 2.45	166.50 ± 3.01
Maximum Lactate concentration (g/l)	126.50 ± 3.28	159.60 ± 3.45
Lactate productivity (g/l/h)	3.51 ± 0.08	4.43 ± 0.10
Intracellular NADH level (μmol/g dry cell weight)[a]	0.53 ± 0.06	0.27 ± 0.02
Intracellular NAD^+ level (μmol/g dry cell weight)[a]	2.21 ± 0.02	3.68 ± 0.09
[NAD^+]/[NADH] ratio	4.61	13.62
Intracellular pyruvate level[a] (μmol/g dry cell weight)[a]	4.53 ± 0.12	1.98 ± 0.47
LDH activity (U/ml)[a]	22.40 ± 0.88	101.10 ± 1.21

[a]After incubation for 18 h, 20 ml of culture was removed from the fermentation vessel, frozen immediately in liquid nitrogen for 60 sec, filtrated on cheese-cloth, and centrifuged at 5,000 ×*g* for 15 min at 4°C to get a clear supernatant as described under Materials and Methods. Measurements are averages of three supernatants. The standard deviations were lower than 10% of the values.

obtained in 36 h.

To determine the influence of oxygen supply strategy on the metabolic flux of *L. casei*, the fermentation parameters of G-02 strain and *L. casei* (pMG-*ldhL*) under the control and TOS conditions were summarized (Tables 2 and 3). It was found that, without oxygen supply, the cell growth and lactic acid production were not influenced significantly by the overexpressed *ldhL* gene. Moreover, for the G-02 strain, the influence of oxygen supply on the lactic acid synthesis and cell growth strategy was not significant. However, for *L. casei* (pMG-*ldhL*), under TOS condition, lactic acid productivity was enhanced from 2.11 $g \cdot l^{-1} \cdot h^{-1}$ to 4.43 $g \cdot l^{-1} \cdot h^{-1}$, a 109.2% increase.

The results also signified that without oxygen supply, the concentrations of intracellular pyruvate in strain G-02 are basically compatible with that in *L. casei* (pMG-*ldhL*). Nevertheless, under TOS, the accumulation of pyruvate in *L. casei* (pMG-*ldhL*) was decreased to 1.98 μmol/g DCW, corresponding to a 55.8% decrease. Interestingly, it was found that *in vivo* [NAD^+]/[NADH] ratios under TOS condition were higher than that without oxygen supply, and overexpressed *ldhL* resulted in a high ratio under whatever oxygen supply strategy.

Discussion

Previous reports signified that under growing conditions, glycolysis is already running at a maximal rate. The glycolytic flux in non-growing cells is lower because there are fewer ATP-consuming processes. Expression of ATPase resulted in increased glycolytic flux [15]. Therefore, it can be accepted that LDH has virtually no flux control in *L. lactis* [19], which was consistent with the conclusion obtained in this study when the strains were cultured under LOS.

The demand for ATP seems to exert some control when the glycolytic flux is much lower than the maximal capacity [11, 12, 16]. The effect of oxygen on the distribution of endproducts has long been disclosed. Neves *et al*. [17] reported the *in vivo* ^{13}C NMR analysis of non-growing cell suspensions to obtain a reliable picture of the oxygen-induced changes in glycolytic metabolite pools. It was found that, under aerobic conditions, no NADH accumulation was observed by *in vivo* NMR at the onset of glucose exhaustion [17]. As in *L. lactis*, ATP was formed by substrate level phosphorylation and reducing equivalents (NADH) through the glycolytic pathway. These results signified that oxygen increases the carbon metabolism flux in *L. lactis* by improved ATP demands.

In the *Lactobacillus* sp. strain, lactate is formed by reduction of pyruvate *via* L-LDH maintaining the redox balance by regenerating NAD^+. The influences of oxygen on the carbon metabolism of *L. lactis* has already been reported [17]. Neves *et al*. [17] reported that upon supply of glucose, the *in vivo* [NAD(+)]/[NADH] ratio was positively affected by the L-LDH activity. The oxygen supply strategy can enhance the glycolytic flux, even in LDH-deficient strains [11, 16, 17]. Under anaerobic conditions, sugars may be converted to lactate or, alternatively, to the mixed-acid products formate, ethanol, and acetate. Nevertheless, oxygenation of cultures results in an altered redox state and greater NADH oxidase activity. The catabolic carbon fluxes and the redox metabolism become uncoupled owing to operation of an extra pathway for NAD^+ regeneration

involving NADH oxidases. As a consequence, sugar catabolism is shifted toward mixed/flavor fermentation with formation of acetate and acetoin in detriment of lactate production. This shift is not only related to the induction of the O_2-consuming oxidizing enzymes, but also to the competition for NADH between LDH and/or acetaldehyde and ethanol dehydrogenases and NADH-oxidases.

A deviation from homolactic fermentation has also been reported under aerobic conditions [15]. The regulation of this shift from homolactic to mixed-acid fermentation was originally explained with models based on the allosteric modulation of two enzymes competing for pyruvate: LDH and PFL. The oxygen-induced metabolic shift would be explained by activation of the pyruvate dehydrogenase complex under aerobic conditions [5, 15]. However, some *L. lactis* strains can benefit from the presence of oxygen, and their growth is positively affected by low oxygen levels. Sensitivity to aeration is extremely variable and strain dependent, and oxygen can partially or completely inhibit the growth of some *L. lactis* strains [22].

In this study, maximum growth and lactic acid production in *L. casei* were obtained under LOS. Further increase of the oxygen supply level resulted in lower cell growth and lactic acid, attributing to the metabolic shift caused by oxygen induction. To enhance the metabolic flux for lactic acid synthesis, enhancement of the LDH activity was essential under high oxygen supply level. Nevertheless, for strain *L. casei* (pMG-*ldhL*) under MOS, maximum cell growth was obtained in 20 h of the fermentation, but the lactic acid synthesis ratio decreased in the later fermentation. The lowered carbon flux for lactic acid synthesis mainly resulted from the increasing metabolic shift caused by oxygen induction. Under this stage, LOS was more favorable for lactic acid synthesis than MOS.

In the present study, the *ldhL* gene in the *L. casei* strain was overexpressed, which found that although the intracellular L-LDH activity was enhanced significantly, lactic acid synthesis increased indistinctively. Therefore, to enhance the precursor accumulation, TOS was applied to increase the glycolytic pathway flux. As a consequence, during the early fermentation phase of *L. casei*, under cell growing conditions, as glycolysis is running at a maximal rate, high LDH activity is favorable to compete for pyruvate in the lactic acid synthesis process under MOS. In the later fermentation, under the cell non-growing stage, as acid and osmotic stress are increased, LOS is appropriate to decrease the metabolic shift caused by oxygen induction.

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