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Application of a Pyruvate-Producing *Escherichia coli* Strain LAFCPCPt-accBC-aceE: A Case Study for D-Lactate Production

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Abstract: Pyruvate, a potential precursor of various chemicals, is one of the fundamental chemicals produced by the fermentation process. We previously reported a pyruvate-producing *Escherichia coli* strain LAFCPCPt-accBC-aceE (PYR) that has the potential to be applied to the industrial production of pyruvate. In this study, the availability of the PYR strain for the production of pyruvate-derivative chemicals was evaluated using a D-lactate-producing strain (LAC) based on the PYR strain. The LAC strain expresses a D-lactate dehydrogenase-encoding gene from *Lactobacillus bulgaricus* under the control of a T7 expression system. The D-lactate productivity of the LAC strain was further improved by limiting aeration and changing the induction period for the expression of D-lactate dehydrogenase-encoding gene expression. Under combined conditions, the LAC strain produced D-lactate at 21.7 ± 1.4 g·L^{-1}, which was compatible with the pyruvate production by the PYR strain (26.1 ± 0.9 g·L^{-1}). These results suggest that we have succeeded in the effective conversion of pyruvate to D-lactate in the LAC strain, demonstrating the wide versatility of the parental PYR strain as basal strain for various chemicals production.

Keywords: *Escherichia coli*; pyruvate; fermentation; D-Lactate; NADH/NAD$^+$ ratio

1. Introduction

Pyruvate is one of the key metabolites in the central metabolism of all known organisms, and has been studied extensively for industrial use. The typical example of the use of pyruvate itself is the compound's use in human health, for instance, in protecting cells against oxidative stress [1]. In addition, pyruvate is used as a precursor to various other chemicals, such as amino acids [2], alcohols [3], plastic monomers [4], and pharmaceuticals [5–7] (Figure 1). Three methods of pyruvate production are known: chemical synthesis, fermentation, and enzymatic production [2]. Among the three, the fermentation method is known to have the lowest costs of raw materials, and commercial pyruvate production by fermentation has been developed in various organisms [2,8–10]. These facts indicate that it is possible to construct various chemical-producing strains based on a pyruvate-producing strains.

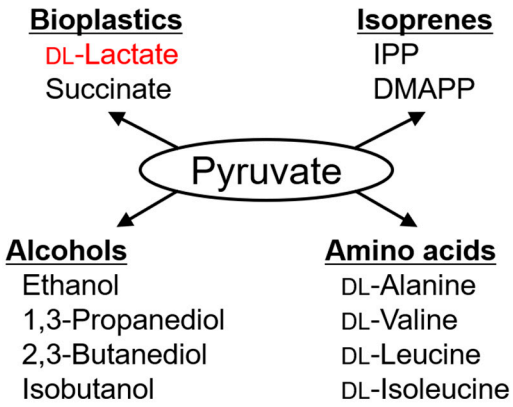


Figure 1. Derivatives of pyruvate. IPP and DMAPP indicate isopentenyl diphosphate and dimethylallyl pyrophosphate, respectively.

To enhance the productivity by strains of various chemicals, including derivatives of pyruvate, a range of metabolic modifications of the producer microbes have been attempted. The regulation of intracellular redox balance is one of the key strategies, given that cofactors NAD(P)$^+$ and NAD(P)H play important roles in microbial metabolism [11]. For example, the inactivation of aldehyde dehydrogenase to prevent competition for NADH with ethanol-forming reactions improved 1,3-propanediol production with *Klebsiella pneumoniae* by 2-fold [12]. In another example, the aeration control to prevent competition for NADH with the respiratory chain improved L-valine production with *Corynebacterium glutamicum* by 20-fold [13]. Therefore, the regulation of intracellular redox balance is important for the microbial production of chemicals, although the specific modifications needed depend on the production conditions, including the nature of the host strains and the targeted products.

In a previous report, we described the construction of a pyruvate-producing *E. coli* strain, LAFCPCPt-accBC-aceE (designated here as PYR) [14]. The genes *accBC* and *aceE*, encoding acetyl-CoA carboxylase and pyruvate dehydrogenase, respectively, are known to be essential for cell growth when utilizing glucose as the sole carbon source [15]. Hence, in the PYR strain, the expression of *accBC* and *aceE* was controlled by a doxycycline-inducible system [14,16]. The silencing of *accBC* and *aceE* expression using this regulatory system was an effective mechanism for inducing pyruvate production [14,15]. Furthermore, in the PYR strain, several genes were disrupted to enhance pyruvate productivity, with targets including: (i) biosynthetic genes involved in the production of ethanol, lactate, acetate, and formate; and (ii) *cra*, a gene that encodes a global transcription regulator (Figure 2). In simple batch culture, the PYR strain showed high pyruvate productivity (26.1 ± 0.9 g·L^{-1}), demonstrating 56% efficiency per consumed glucose when glucose was used as the sole carbon source [14]. These results indicated that PYR is a promising foundation strain for the production of pyruvate derivatives. As a first example of the application of the PYR strain, a D-lactate-producing strain (designated here as old-LAC) was constructed by introducing a *Lactobacillus bulgaricum* gene encoding NAD$^+$-dependent D-lactate dehydrogenase (DLDH) into the PYR background [17]. The D-lactate production pathway was shown in Figure 2. However, the D-lactate production of the old-LAC strain was low level (1.1 ± 0.0 g·L^{-1}) compared with pyruvate production by the PYR strain (26.1 ± 0.9 g·L^{-1}), implying that the expression of *DLHD* in the old-LAC strain was insufficient. These results indicate that there is room for further improvement of the D-lactate production by the PYR-derived strain.

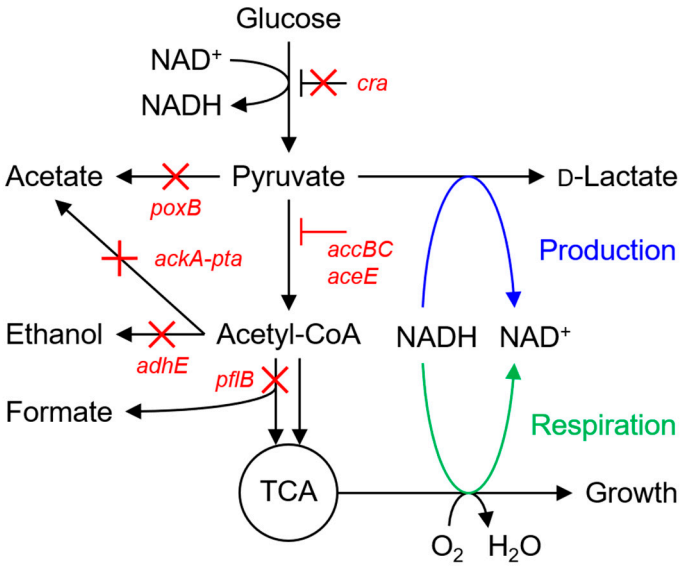


Figure 2. Schematic metabolic pathway of the D-lactate-producing (LAC) strain. The cross-marks denote gene inactivation. D-lactate production (blue) competes with respiration (green) for NADH utilization.

The purpose of this study was to evaluate the availability of the PYR strain for the production of pyruvate-derivative chemicals by using a new D-lactate-producing strain (LAC) based on the PYR strain. The results of our study demonstrate the utility of the PYR strain for the development of fermentation processes of pyruvate-based chemicals production.

2. Materials and Methods

2.1. Construction of Bacterial Strains and Plasmids

2.1.1. Plasmids

E. coli HIT-DH5α (SciTrove, Tokyo, Japan) was used for DNA manipulation. Ampicillin (100 mg·L^{-1}) and kanamycin (20 mg·L^{-1}) were added as necessary. In order to construct the expression vector containing the *L. bulgaricus* gene encoding NAD^+-dependent DLDH under control of the T7 promoter, the *DLDH* in pMALc5x/D-LDH [17] was excised with NdeI and BamHI and subcloned between the NdeI and BamHI sites of pETIK (BioDynamics Laboratory Inc., Tokyo, Japan) to generate pETIK-*DLDH*.

2.1.2. Bacterial Strains

The LAC strain was constructed based on the PYR strain [14]. The λDE3 lysogenization kit (Novagen, Madison, WI, USA) was employed to introduce a T7-driven expression system into the chromosome of the PYR strain according to the kit manufacture's protocol. The insertion into the PYR strain chromosome of the gene encoding T7 RNA polymerase was confirmed by the PCR-based screening of candidate strains using primers 5′-ATGAACACGATTAACATCGC-3′and 5′-TTACGCGAACGCGAAGTC-3′. The selected strain (designated PYR(DE3)) then was transformed with pETIK (the empty vector) or pETIK-*DLDH*, yielding strains designated NULL or LAC, respectively (Table 1). The NULL strain was used as a control.

Table 1. *E. coli* strains used in this study.

Strains	Relevant Descriptions [1]	References
PYR	Derived of the MG1655; Δ*ldhA* Δ*adhE* Δ*pflB* Δ*(ackA-pta)* Δ*poxB* Δ*cra* P_{accBC}::P_{tet}-*accBC* P_{aceEF}::P_{tet}-*aceE*	[14]
old-LAC	Derived of the PYR strain; [pMAL-c5X-*DLDH*]	[17]
PYR(DE3)	Derived of the PYR strain; λ(DE3)	This study
NULL	Derived of the PYR(DE3) strain; [pETIK] [1]	This study
LAC	Derived of the PYR(DE3) strain; [pETIK-*DLDH*]	This study

[1] [] indicates plasmid-carrier state.

2.2. Culture Conditions for D-Lactate Production

All *E. coli* strains were pre-cultured for 12 h at 37 °C with 250 rpm in LB medium (5 g·L^{-1} yeast extract, 10 g·L^{-1} tryptone, and 10 g·L^{-1} NaCl). Main cultures were generated by an inoculation of pre-cultures into N5G medium (1 g·L^{-1} K_2HPO_4, 10 g·L^{-1} $(NH_4)_2SO_4$, 2 g·L^{-1} NaCl, 0.25 g·L^{-1} $MgSO_4{\cdot}7H_2O$, 15 mg·L^{-1} $CaCl_2{\cdot}2H_2O$, 60 g·L^{-1} glucose, and 0.2 g·L^{-1} Adekanol LG-294 as an antifoam agent) at 600 nm (OD_{600}) of 0.05. Culturing for D-lactate production was performed by growth for 72 h in a Bio Jr. 8 small-scale bioreactor (ABLE & Biott, Tokyo, Japan). The basal conditions were as follows: volume, 80 mL; temperature, 35 °C; aeration, agitation at 1800 rpm and airflow at 150 mL·min^{-1}; pH 5.7 (as adjusted using 4 M NaOH); induction point, 12 h by 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Dry cell weight was calculated using a conversion coefficient of 0.3 gDCW·L^{-1} $OD_{600}{}^{-1}$ [18].

2.3. Quantification of Metabolites

2.3.1. Extracellular Metabolites

The concentrations of extracellular metabolites (glucose, pyruvate, DL-lactate, formate, acetate, and ethanol) in the filtered culture supernatants were determined using an high-performance liquid chromatography (HPLC) system (JASCO, Tokyo, Japan) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA), a UV-2070 Plus UV/vis detector, and a RI-2031 Plus refractive index detector. The chromatographic conditions were as follows: mobile phase, 1.5 mM H_2SO_4; flow rate, 0.6 mL·min^{-1}; column oven temperature, 65 °C. The purity check of the produced D-lactate was performed using an HPLC system equipped with a CHIRALPAK MA(+) column (Daicel, Osaka, Japan). The chromatographic conditions for purity check were as follows: mobile phase, 0.5 mM $CuSO_4$; flow rate, 0.4 mL·min^{-1}; column oven temperature, 30 °C.

2.3.2. Intracellular NAD^+ and NADH

The intracellular NAD^+ and NADH were extracted and quantified using an EnzyChrom™ NAD^+/NADH Assay Kit (BioAssay Systems, Hayward, CA, USA) and a Synergy HTX fluorescence plate reader (BioTek, Winooski, VT, USA) according to the kit manufacture's protocol.

2.4. Enzyme Assays of DLDH

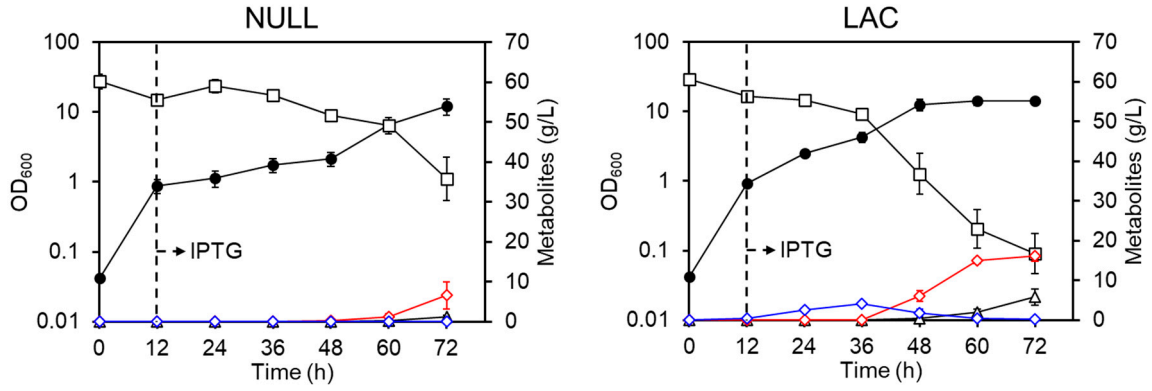
The old-LAC and the LAC strain cells under the basal conditions were collected at 36 h. All procedures after cell collection were performed on ice. The collected cells were washed twice with 9 g·L^{-1} NaCl, resuspended with 150 mM potassium phosphate (pH 6.5), homogenized by ultrasonication, and centrifuged. The resultant supernatants were used as crude extract. The protein concentration in the crude extract was determined by using the Bradford protein assay kit (Bio-Rad, CA, USA). The DLDH activity was measured spectrophotometrically at 25 °C by monitoring NADH consumption at 340 nm with GeneQuant 1300 (GE Healthcare, Chicago, IL, USA). The reaction mixture (1 mL) contained 150 mM potassium phosphate (pH 6.5), 30 mM pyruvate, 2.5 mM $MgCl_2$, 0.3 mM NADH, and the crude extract. The reaction was initiated by the addition of the crude extract. The extinction coefficient of NADH was 6.22 $mM^{-1}{\cdot}cm^{-1}$.

3. Results

3.1. Culture Profiles of the LAC Strain under Basal Conditions

In our previous study, the relatively low production of D-lactate by the old-LAC strain suggested the possibility that the expression of *DLDH* in the old-LAC strain was limiting. The previous report showed that the T7 promoter was more effective for the targeted protein production in *E. coli* [19]. Hence, we replaced the *tac* promoter originally used to drive *DLDH* expression on pMAL-c5X with the T7 promoter on pETIK, resulting in the LAC strain (Table 1).

The culture profiles of the NULL (used as control because of same genetic background with the LAC strain except for *DLDH*) and LAC strains under the basal conditions used in the previous study [14] are shown in Figure 3. The final cell density and glucose consumption of the LAC strain were 1.2-fold (=4.23 gDCW·L^{-1}/3.64 gDCW·L^{-1}) and 1.8-fold (=43.83 g·L^{-1}/24.32 g·L^{-1}) those of the NULL strain, respectively. The pyruvate produced by the NULL strain is lower than that by the PYR strain [14]. The unexpected product from multi-cloning site of pETIK might be affected to pyruvate production by the NULL strain. As expected, D-lactate production was observed only in the LAC strain. The D-lactate produced by the LAC strain demonstrated over 99% optical purity (data not shown). The highest concentration of D-lactate produced by the LAC strain was 4.1 ± 0.4 g·L^{-1} at 36 h, which was approximately 9.0-fold (=4.07 g·L^{-1}/0.45 g·L^{-1}) that of the old-LAC, at 36 h [17]. The DLDH activity at 36 h of the LAC strain was 33-fold (=292.40 μmol·mg^{-1}·min^{-1}/9.01 μmol·mg^{-1}·min^{-1}) that of the old-LAC strain. From these results, it seemed that the DLDH activity was correlated with the D-lactate productivity. In addition, the amount of glucose consumed through until 72 h by the LAC strain was 11-fold (= 43.83 g·L^{-1}/3.99 g·L^{-1}) that of the old-LAC strain [17]. Most notably, however, the D-lactate concentration in the culture medium decreased gradually between 36 h and 72 h (from 4.1 ± 0.4 g·L^{-1} to 0.2 ± 0.1 g·L^{-1}, respectively), accompanied by an increase in the pyruvate concentration (0 ± 0 g·L^{-1} to 16.1 ± 1.4 g·L^{-1}, respectively). These data suggest that the D-lactate that was produced after 36 h was subsequently reconverted to pyruvate.



(**a**) (**b**)

Figure 3. Growth characteristics of the NULL and LAC strains grown under the basal conditions. The (**a**) NULL and (**b**) LAC strains were cultured in N5G medium under the basal conditions [14]. Parameters: temperature, 35 °C; aeration, 1800 rpm (agitation) and 150 mL·min^{-1} (airflow rate); pH, 5.7; induction point, 12 h (vertical dashed line). Symbols: filled circles, OD_{600}; squares, glucose; triangles, acetate; red diamonds, pyruvate; blue diamonds, D-lactate. Data shown are the mean ± SD (n = 3).

3.2. Regulation of Aeration Improves D-Lactate Production

The production of pyruvate from D-lactate was expected to be accompanied by NADH generation (D-lactate + NAD^+ => pyruvate + NADH). The generated NADH presumably then would be used for O_2-respiration, given that the basal conditions were aerobic (Figure 2). Hence, to prevent the O_2-respiration, we limited the aeration after 36 h by decreasing the agitation and air-flow rate from

1800 rpm and 150 mL·min^{-1} (respectively) to 300 rpm and 0 mL·min^{-1} (termed air-regulated conditions) (Figure 4).

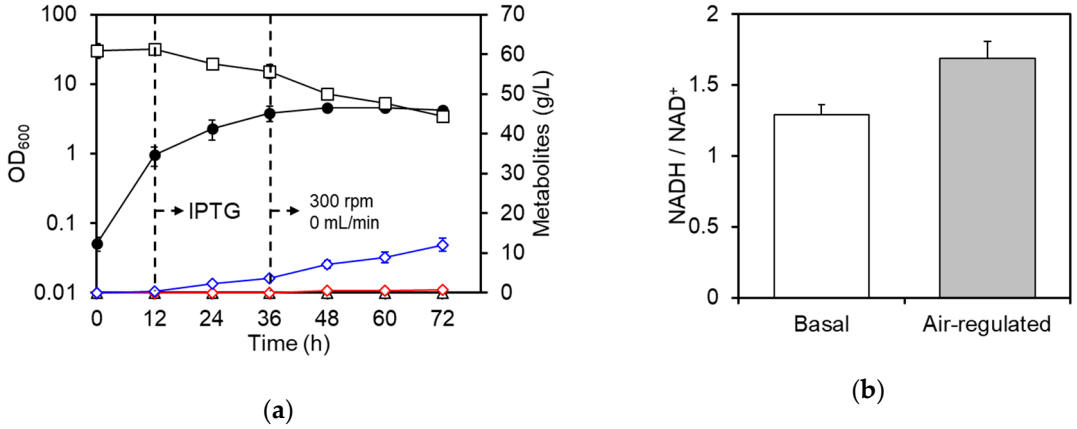


Figure 4. D-Lactate production under air-regulated conditions. The values of aeration parameters were changed at 36 h (central vertical dashed line) from 1800 rpm and 150 mL·min^{-1} to 300 rpm and 0 mL·min^{-1}, respectively. (**a**) The culture profile of the LAC strain and (**b**) NADH/NAD$^+$ ratio for cultures grown under the basal (white) and the air-regulated (gray) conditions. Symbols: filled circles, OD600; squares, glucose; triangles, acetate; red diamonds, pyruvate; blue diamonds, D-lactate. The data shown are the mean ± SD at 72 h (n = 3); values in (**b**) are at 72 h.

The culture profiles of LAC strain until 36 h were similar to those for cells growing under the basal conditions (Figures 3b and 4a). However, the cell density reached a plateau under the air-regulated conditions and was 3.2-fold (=4.23 gDCW·L^{-1}/1.28 gDCW·L^{-1}) lower than that under the basal conditions at 72 h. On the other hand, the concentration of D-lactate increased gradually through 72 h, reaching 12.1 ± 1.6 g·L^{-1}, which was 53-fold (=12.1 g·L^{-1}/0.23 g·L^{-1}) that obtained under the basal conditions. The pyruvate production was lower under the air-regulated conditions (0.7 ± 0.6 g L^{-1}) compared to that obtained under the basal conditions. These data indicated that changing the aeration parameters at 36 h was an effective method for enhancing D-lactate production. The intracellular NADH/NAD$^+$ ratio at 72 h under the air-regulated conditions was 1.3-fold (=1.69/1.29) that obtained under the basal conditions (Figure 4b), suggesting that the decreased aeration provided an intracellular redox state suitable for the conversion from pyruvate to D-lactate [20].

3.3. Improvement of D-Lactate Production and Cell Density by Pre-Induction of DLDH

To further increase the productivity, the influence of the induction period of *DLDH* was investigated. When 0.1 mM IPTG was included in the pre-culture medium, D-lactate production at 72 h under the air-regulated conditions (termed combined conditions) was 1.8-fold (=21.72 g L^{-1}/12.09 g L^{-1}) that obtained from a main culture initiated using IPTG-naïve pre-culture (Figure 5). In addition, the glucose consumption under the combined conditions was 2.1-fold (=35.0 g L^{-1}/16.4 g L^{-1}) that under the air-regulated conditions. The maximum cell density under the combined conditions at 36 h was 2.4-fold (=2.78 gDCW L^{-1}/1.16 gDCW L^{-1}) that under the air-regulated conditions. The specific D-lactate production rates after 36 h by the LAC strain under the air-regulated and the combined conditions were same level (Figure S1). These data suggested that the increase of the cell density by pre-induction of *DLDH* had a positive effect on D-lactate production.

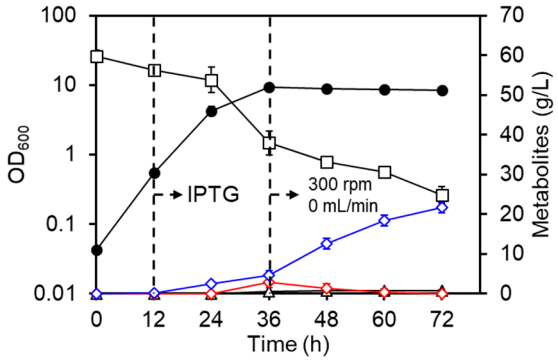


Figure 5. Culture profiles of the LAC strain under the combined conditions. IPTG was added to the pre-culture at 0 h and to the main culture at 12 h. The values of the aeration parameters were changed at 36 h from 1800 rpm and 150 mL min^{-1} to 300 rpm and 0 mL min^{-1}, respectively. Symbols: circles, OD_{600}; squares, glucose; triangles, acetate; red diamonds, pyruvate; blue diamonds, D-lactate. Data shown are the mean ± SD (n = 3).

3.4. Summary of Culture Profiles

The values of the culture profiles are summarized in Table 2. The final concentration of D-lactate (21.7 ± 1.4 g L^{-1}) obtained with the LAC strain under the combined conditions approached that of pyruvate (26.1 ± 0.9 g L^{-1}) obtained with the PYR strain under basal conditions [14]. In addition, the D-lactate productivity was increased 20-fold (=21.72 g L^{-1}/1.07 g L^{-1}) compared to that in our previous study using the old-LAC strain [17]. The yield of D-lactate production per consumed glucose under the combined conditions was 2.3-fold (=(21.72 g L^{-1}/35.0 g L^{-1})/(1.07 g L^{-1}/3.99 g L^{-1})) that obtained in the previous study [17]. These results suggested that we succeeded in our goal of efficiently producing D-lactate as a pyruvate derivative by combining the construction of a new strain and changes in culture conditions.

Table 2. Summary of culture profiles at 72 h.

Strains	Conditions	Consumed Glucose ($g \cdot L^{-1}$)	Produced D-Lactate ($g \cdot L^{-1}$)	Produced Pyruvate ($g \cdot L^{-1}$)	Yields [1] ($g \cdot g^{-1}$)	References
PYR	Basal	48.0 ± 4.7	N/A [2]	26.1 ± 0.9	54 ± 2	[14]
old-LAC	Basal [3]	4.0 ± 1.9	1.1 ± 0.0	N/A	33 ± 12	[17]
LAC	Basal	43.8 ± 4.7	0.2 ± 0.1	16.1 ± 1.4	1 ± 0	This study
LAC	Air-regulated	16.4 ± 2.6	12.1 ± 1.6	0.7 ± 0.6	74 ± 8	This study
LAC	Combined	35.0 ± 0.8	21.7 ± 1.4	0.1 ± 0.0	62 ± 3	This study

[1] Produced pyruvate by PYR strain or D-lactate by LAC series strains per consumed glucose. [2] N/A indicates not applicable. [3] The pH value and the concentration of NH_4Cl were set to 6.0 and 20 g L^{-1}, respectively.

4. Discussion

In this study, to evaluate the availability of the PYR strain for the production of pyruvate-derivative chemicals, we investigated the D-lactate production ability of the LAC strain, which is based on the PYR strain. The maximum D-lactate productivity in this study was 20-fold greater than that obtained in our previous study [17]. Furthermore, the D-lactate productivity of the LAC strain (21.7 ± 1.4 $g \cdot L^{-1}$) was comparable with the pyruvate productivity of the PYR strain (26.1 ± 0.9 $g \cdot L^{-1}$) [14], indicating that most of the produced pyruvate was converted to D-lactate. Since lactate is a monomer of poly-lactic acid, which is one of the biodegradable and biocompatible plastic polyesters, it may contribute to the production of D-lactate by the LAC strain because of low by-product production.

This work represents the first study demonstrating the versatility of our pyruvate-producing PYR strain. To enhance the D-lactate productivity of the LAC strain, we modified the culture conditions in two steps. First, aeration was limited after 36 h of cultivation (Figure 4a). Our previous study showed that pyruvate production by the PYR strain was favored under more aerobic conditions [14], suggesting that the NADH generated in glycolysis was consumed by O_2-respiration to maintain intracellular redox

homeostasis. Other pyruvate production studies have also employed aerobic conditions, irrespective of the bacterial species [8–10]. On the other hand, a shift to D-lactate production was expected to create competition for NADH consumption between the pyruvate to D-lactate reaction and O_2-respiration. Therefore, the limiting aeration after 36 h of cultivation was expected to favor D-lactate production over O_2-respiration. Second, we changed the induction period of *DLDH* expression (Figure 5). In the PYR strain, the NADH generated by glycolysis was not consumed by O_2-respiration during the early phase of cultivation because the expression of the pyruvate dehydrogenase gene has been suppressed at early time points [14]. In fact, initial cell growth by the PYR strain was observed to be repressed until 12 h [14]. On the other hand, the initial cell growth of the LAC strain was enhanced by the expression of *DLDH* starting in the pre-culture (Figure 5), suggesting that the NADH produced in the early-phase culturing was consumed by DLDH. However, glucose remained in the culture at 72 h of growth under the combined conditions (Figure 5). In addition, specific D-lactate production rates by the LAC strain in the air-regulated and the combined conditions were same level (Figure S1). These results implied that D-lactate production by the LAC strain might be improved by further increases in DLDH activity.

The LAC strain exhibited superior D-lactate productivity in the simple batch culture upon the modification of the culture conditions from those of our previous study [14]. Since the genes encoding enzymes related to the production of major organic acids (*pta-ackA* and *poxB*, acetate; *pflB*, formate; *ldhA*, D-lactate; *adhE*, ethanol) are deleted in the PYR strain, high-purity fermentation production is expected following the introduction of genes encoding enzymes for the synthesis of target products, as demonstrated in the present study. In fact, the production of formate and ethanol was not detected in the present study (data not shown). In the case of future industrial use of the LAC strain, this character will contribute to save the cost for the separation process of D-lactate. However, the titers and yields of D-lactate reported in other studies using glucose as a sole carbon source were greater than those obtained in the present study [21–23], indicating that further genetic modification of the PYR strain would facilitate increased productivity of various pyruvate derivatives. For example, the succinate biosynthetic pathway is still present in the PYR strain. Since succinate production is activated under anaerobic conditions to maintain redox balance [24,25], succinate might be produced as an unnecessary metabolite during the anaerobic production of pyruvate derivatives. Previous reports have demonstrated that the inactivation of *frd* suppresses the production of succinate under anaerobic conditions [21,26,27], suggesting that this strategy may lead to increased D-lactate production by derivatives of the LAC strain. Another route for improved productivity might be the inactivation of respiratory chain enzymes such as ATPase and cytochrome oxidases, which would lead to an increased rate of glucose uptake [28–30]. Since these modifications can be applied to the PYR strain, further improvements in the productivity of pyruvate derivatives can be expected.

5. Conclusions

In this study, we constructed a new D-lactate-producing strain, LAC, by modifying the pyruvate-producing PYR strain. The LAC strain expresses the *L. bulgaricus* DLDH-encoding gene under the control of a T7-driven expression system. The D-lactate productivity of LAC strain was increased from 0.2 ± 0.1 $g{\cdot}L^{-1}$ to 12.1 ± 1.6 $g{\cdot}L^{-1}$ by limiting the aeration of the culture after 36 h, suggesting that preventing NADH consumption by O_2-respiration was important. Furthermore, the cell density was increased by the pre-induction of the *DLDH* gene, resulting in an improvement in D-lactate productivity to 21.7 ± 1.4 $g{\cdot}L^{-1}$. The final concentration of D-lactate obtained in the present study (21.7 ± 1.4 $g{\cdot}L^{-1}$) was compatible with the pyruvate production by PYR strain (26.1 ± 0.9 $g{\cdot}L^{-1}$). These results suggested that we succeeded in the effective conversion of pyruvate to D-lactate in the LAC strain, demonstrating not only the versatility of the PYR strain but also the improvement of D-lactate productivity of the LAC strain when comparing with that of the old-LAC strain. Further engineered LAC strain may be used for applications such as industrial poly-lactic acid production.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/6/3/70/s1, Figure S1: Specific D-lactate production rates after 36 h by LAC strain under the air-regulated (red) and the combined (blue) conditions.

Author Contributions: Conceptualization, K.W., T.F., H.A., T.M.; methodology, K.W., T.F., H.I., H.A.; validation; K.W.; formal analysis, K.W.; investigation, K.W.; resources, K.W.; data curation, K.W., H.I., T.M., T.F.; writing—original draft preparation, K.W.; writing—review and editing, K.W., T.F., H.I., H.A., T.M., A.M.; visualization, K.W.; supervision, T.F.; funding acquisition, K.W. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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