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Reduction of Acetate and Lactate Contributed to Enhancement of a Recombinant Protein Production in *E. coli* BL21

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Acetate and lactate in growth media are detrimental to the production of *Thermus* maltogenic amylase (ThMA), a heterologous protein, as well as to the growth of recombinant *Escherichia coli*. Only 50 mM of acetate or 10 mM of lactate reduced 90% of specific ThMA activity. In this study, mutant *E. coli* strains blocked in the *ackA-pta* or *ackA-pta* and *ldh* pathways were created, characterized, and assessed for their culture performace in 300 L-scale fermentation. The *ackA-pta* and *ldh* double-mutant strain formed significantly less lactate and acetate, and produced a concomitant increase in the excretion of pyruvate (17.8 mM) under anaerobic conditions. The *ackA-pta* mutant strain accumulated significant acetate but had an approximately 2-fold increase in the formation of lactate. The *ackA-pta* and *ldh* double-mutant strain had superior overall performance in large-scale culture under suboptimal conditions, giving 67% higher cell density and 66% higher ThMA activity compared with those of the control strain. The double-mutant strain also achieved a 179% improvement in volumetric ThMA production.

Keywords: Large-scale bioreactor, maltogenic amylase, metabolically engineered, production, recombinant protein

Introduction

Escherichia coli is used extensively in industry as a host cell for recombinant protein production. However, high-density cultures of *E. coli* result in the accumulation of acetate and the cessation of growth. Acetate accumulation lowers cell yield and recombinant protein productivity [14, 36]. Reducing the acetate production is a primary objective in fermentation and recombinant protein production by *E. coli*. Acetate formation is caused by an excess influx of carbon from glucose, resulting in repression of TCA cycle enzymes by glucose or uncoupled metabolism (Fig. 1) [13]. In addition, the production of acetate represents a loss of carbon flux for cell growth and a loss of recombinant protein production. Acetate can repress the synthesis of DNA, RNA, proteins, lipids, and peptidoglycans [6, 7, 18]. Lactate accumulation also has harmful effects on the cell growth and protein production [23].

A thermostable *Thermus* maltogenic amylase (ThMA) isolated from *Thermus* strain IM6501 has a higher optimal temperature (60°C) than those of other maltogenic amylases reported so far [20]. It has both hydrolytic and transglycosylation activities. It hydrolyzes starch and cyclodextrin to yield maltose. During large-scale production of recombinant protein (ThMA) in high cell density cultures of *E. coli*, it is very difficult to attain high protein yields because of difficulties in precisely controlling the environmental factors. The most important factor is dissolved oxygen tension (DOT), especially maintaining a constant DOT. A gradient in DOT exists in large-scale bioreactor cultures, and cells are inevitably exposed to transient anaerobic conditions, resulting in activation of the anaerobic pathway and accumulation of acetate, lactate, and other acid by-products [2, 27, 28]. For example, transient exposure to

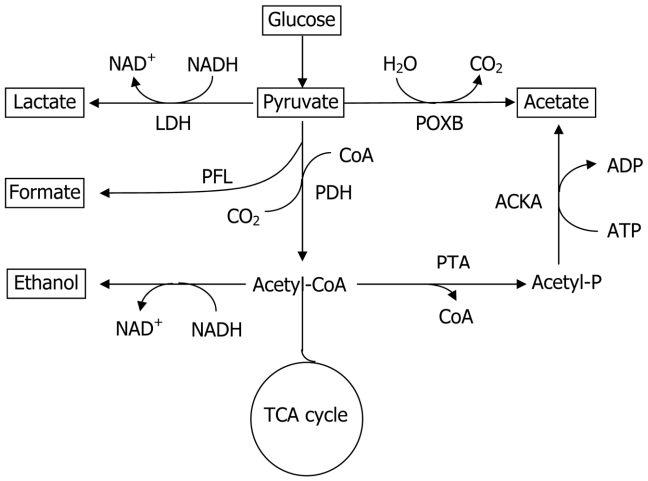

Fig. 1. Central metabolic pathway of *E. coli* around the pyruvate and acetyl-CoA nodes.

ACKA: Acetate kinase; LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase; PFL: pyruvate formate lyase; POXB: pyruvate oxidase; PTA: phosphotransacetylase.

anaerobic conditions of even a few seconds is detrimental to *E. coli*, as expression of genes from the anaerobic pathway is rapidly induced [24, 32, 33].

Hence, several strategies for reducing acetate accumulation have been suggested. Industrial strategies tend toward modification of the external or environmental conditions through medium selection, glucose limitation, and aeration. The internal genotype of the host cell can also be altered. Some of these approaches involve engineered bacterial strains with modification of the glucose uptake rate [8], redirection of the carbon flux to a less inhibitory by-products [3, 11, 16], the storage of excess carbon as glycogen [10], or elimination of the major acetate formation pathway [4]. However, no studies have reported on the design and use of engineered strains that can better cope with suboptimal conditions, including insufficient DOT, resulting in transient anaerobic conditions in large-scale bioreactors. In this study, we prepared *E. coli* mutant strains mutated for the acetate and lactate pathways and investigated the effect of the mutation on the production of recombinant protein (ThMA) using high cell density culture in a large-scale bioreactor.

Materials and Methods

Microorganisms and Plasmids

The strains used are listed in Table 1. The *ackA-pta* or *ackA-pta* and *ldh* deficient derivatives of BL21(DE3) were kindly provided by Prof. S. C. Kim (Korea Advanced Institute of Science and Technology). The plasmid p6xHTMK119, which was constructed in our laboratory, contains the structural gene encoding ThMA under the promotor pBLMA from *Bacillus licheniformis* maltogenic amylase [21].

Media and Culture Conditions

Cells were maintained as a 20% (v/v) glycerol stock at −80°C after growing in Luria-Bertani medium. The chemically defined medium (DM) (pH 6.9) for cultures contained the following per liter: KH_2PO_4, 20 g; $(NH_4)_2HPO_4$, 3 g; K_2HPO_4, 29 g; citric acid, 0.8 g; inositol, 30 mg; Ca-pantothenate, 1 mg; pyridoxine-HCl, 0.25 mg; folic acid, 25 mg; and trace element, 5 ml. The trace element metal solution contained the following per liter of 5 M HCl: $FeSO_4 \cdot 7H_2O$, 10.0 g; $CaCl_2$, 2.0 g; $ZnSO_4 \cdot 7H_2O$, 2.2 g; $MnSO_4 \cdot 4H_2O$, 0.5 g; $CuSO_4 \cdot 5H_2O$, 1.0 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.1 g; and $Na_2B_4O_7 \cdot 10H_2O$, 0.02 g. Separately sterilized glucose was supplemented to the medium to a final concentration of 20 g/l.

Fed-batch culture was carried out at 37°C in a 7 L bioreactor (Kobiotech Co., Incheon, Korea) containing 2 L of DM. The pH was controlled at 6.8 by ammonia-water (28% (v/v)). The dissolved oxygen concentration was controlled at 20% by automatic control of the agitation speed and aeration (up to 3 vvm). Antifoam (0.05% (v/v)) (antifoam 289; Sigma Chemical Co., MO, USA) was added at the onset of cultivation. The feeding solution used for the fed-batch culture contained the following per liter: glucose, 700 g; $MgSO_4 \cdot 7H_2O$, 15 g; and trace metal solution, 20 ml. The culture broth was centrifuged (Hanil Micro-12, Inchon, Korea) at 6,000 ×*g* for 15 min.

Pilot-Scale Culture

In a 300 L fermentor (KoBioTech), the first seed culture (500 ml) was prepared in the same manner as previously described [30]. For the second seed culture, the first seed of 150 ml was inoculated to a 7 L jar containing 2.5 L of seed medium. Then, the culture broth was cultivated for 12 h at 37°C with 500 rpm and 1.0 vvm. The second seed of 10 L, obtained from four 7 L jar fermentors, was transferred to the 300 L fermentor containing 160 L of production medium, and followed by cultivation for 120 h at 37°C. The dissolved oxygen concentration was controlled as the

Table 1. Bacterial strains used.

Strains	Characteristics	Source
BL21	*E. coli B, F*$^-$, *dcm, ompT, hsdS*($r_B^-m_B^-$), *gal*	Promega
Δ*ackA-pta*	BL21, Δ(*ackA-pta*)	This study
Δ*ackA-pta-ldh*	BL21, Δ(*ackA-pta-ldh*)	This study

desired 20% by automatic control of the agitation speed (up to 500 rpm) and aeration (up to 1 vvm). Sixty liters of feeding solution was fed by the pump coupled with pH probe automatically.

Analysis of Glucose and Organic Acids

The concentration of glucose was determined using a HPLC system (BioLC; Dionex, CA, USA) coupled to an electrochemical detector (ED40; Dionex, CA, USA) and CarboPac MA-1 column [25]. A 600 mM NaOH solution was used as the eluent. Organic acids were measured by high-pressure liquid chromatography over an organic acid column (Aminex HPX-87H; Bio-Rad) at 60°C with 0.1 N sulfuric acid as the eluent and a flow rate of 0.6 ml/min. The peaks were detected by determining the UV A_{210}.

Enzyme Assay

The cells were disrupted using an ultrasonic processor (Bandelin GM2200, Berlin, Germany) and centrifuged at 12,000 ×*g* for 15 min [29, 38, 39]. ThMA activity was determined as previously described [5, 21]. Acetate kinase (ACK) assay is based on the formation of hydroxamate acetate (A_{540}) [12]. Crude extract (100 µl) was added to 1 ml of reaction mixture and incubated for 5 min at room temperature; 1 ml of 10% trichloroacetic acid was then added, followed by 1 ml of 1.25% $FeCl_3$ in 1 N HCl. Phosphotransacetylase (PTA) activity was assayed as described in detail previously [31]. Lactate dehydrogenase (LDH) activity was determined based on oxidation of NADH [1]. One unit of activity is defined as the number of µmol of NADH oxidized per minute at 37°C [17, 35, 37].

Results

Effect of Acetate and Lactate on ThMA Production

The effect of organic acids on ThMA production was analyzed in the batch cultivation of *E. coli* BL21(DE3) harboring the ThMA expression vector (p6xHTMK119). Supplementation with acetate above 50 mM significantly inhibited cell growth and reduced the ThMA production by 90% (Fig. 2). Lactate in the medium was even more harmful to ThMA production. Only 10 mM of lactate in the DM could inhibit the cell growth completely and lower the ThMA production by approximately 90%. The results confirm that both acetate and lactate are detrimental to ThMA production as well as to the cell growth.

Effects of Alterations to the Acetate and/or Lactate Synthesis Pathway

Mutations in *ackA-pta* or *ackA-pta* and *ldh* were created by one-step chromosomal inactivation [9]. The assays of acetate kinase, phosphotransacetylase, and lactate dehydrogenase were carried out to confirm the mutants (Table 2). Under anoxic conditions, cell growth of the *ackA-pta* strain was 65% of the wild type (Fig. 3) and the consumption of glucose was 36% less than the control (Table 3). Acetate formation by the mutant strain was also significantly

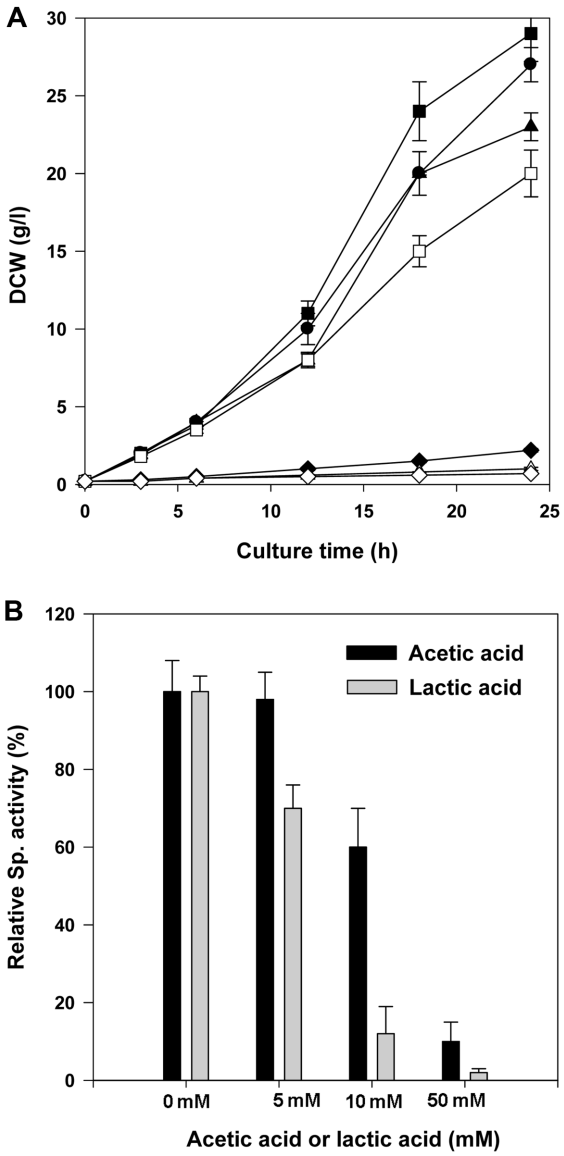

Fig. 2. Effect of acetate and lactate on growth and the production of recombinant protein (ThMA) by *Escherichia coli* BL21(DE3).

Cells were cultivated in a 5 L bioreactor charged with 2 L of defined medium supplemented with acetate or lactate at 28°C and under aerobic condition. (**A**) The growth of *E. coli* BL21(DE3) with no addition of acetate or lactate (●), with 5 mM acetate (■) or lactate (□), 10 mM acetate (▲) or lactate (△), and 50 mM acetate (◆) or lactate (◇) in terms of dry cell weight (DCW). (**B**) Relative specific ThMA activity of cells taken from the culture (**A**) with acetate (black bar) or lactate (grey bar) at 24 h. Each value represents the mean of triplicate experiments.

reduced and ethanol was not detected. The formation of lactate by the *ackA-pta* mutant under anoxic conditions was 86% higher than that of the wild type. However, the formation of acetate and lactate was significantly decreased in the double mutant (*ackA-pta*, *ldh*), while 9.5 mM ethanol and up to 17.8 mM pyruvate were produced (Table 3). However, under aerobic conditions, lactate, ethanol, and pyruvate were less than 0.5 mM (Table 4). Furthermore, deleting the *ackA-pta* pathway only reduced acetate production by 10%, and deleting both the *ackA-pta* and *ldh* pathways lowered acetate production by 18% compared with the wild type (Table 4). Pyruvate accumulation was less than 0.5 mM for all strains under aerobic conditions.

Enhanced ThMA Production by Deleting Acetate and Lactate Formation

Using a pH-stat feeding strategy, the growth rate of the wild-type strain was maintained at 0.2–0.3 h^{-1} for 48 h, resulting in a final DCW of about 80.9 g/l (Fig. 3). However, with the accumulation of acetate (65.4 mM) and lactate (9.5 mM) after a 48 h culture, the ThMA activity and growth

Table 2. Acetate kinase, phosphotransacetylase, and lactate dehydrogenase activities.[a]

Strains	Specific activities (U/mg protein)		
	ACK[b]	PTA[c]	LDH[d]
BL21	0.80 ± 0.04	1.16 ± 0.11	15.4 ± 1.3
Δ*ack-pta*	0.00	0.00	15.5 ± 1.4
Δ*ack-pta-ldh*	0.00	0.00	0.020 ± 0.001

[a]Acetate kinase, phosphotransacetylase, and lactate dehydrogenase activities were assayed by the method of [12], [31], and [1], respectively. The data shown are means of three replicate experiments.

[b]One unit of ACK activity is defined as 1 μmol hydroxamate formed per minute.

[c]One unit of PTA activity is defined as 1 μmol NADH formed per minute.

[d]One unit of LDH activity is defined as 1 μmol NADH oxidized per minute.

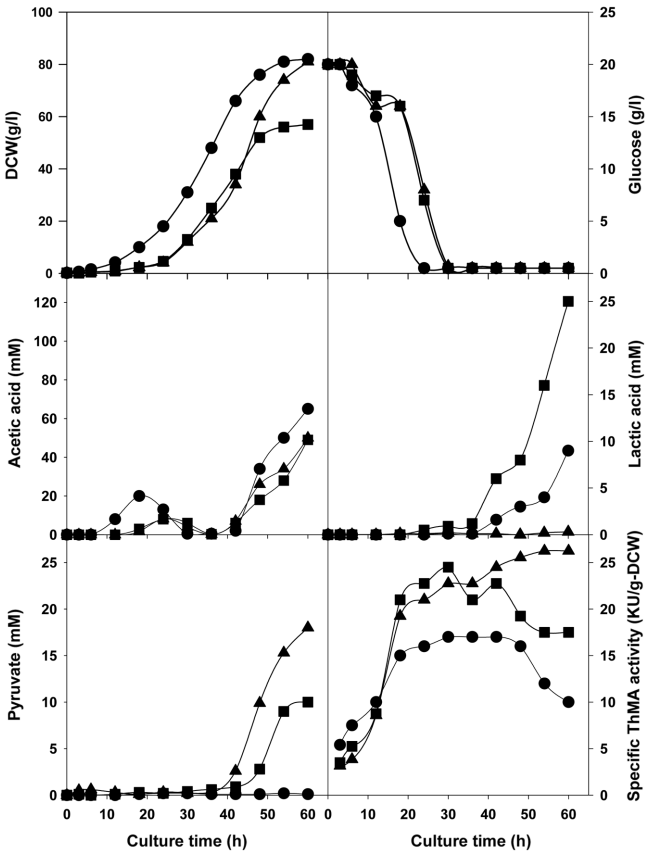

Fig. 3. Typical fermentation profiles for recombinant protein (ThMA) production by *Escherichia coli* BL21(DE3), *ackA-pta* mutant strain, and *ackA-pta* and *ldh* double-mutant strain.

Cells were cultivated in a 7 L bioreactor charged with 2 L of defined medium at 28°C and under aerobic condition. The typical profiles of growth, glucose consumption, acetate excretion, lactate excretion, pyruvate excretion, and specific ThMA activity are shown with symbols of the control strain (●), *ackA-pta* mutant strain (■), and *ackA-pta* and *ldh* double-mutant strain (▲). Each value represents the mean of triplicate experiments and varied from the mean by not more than 15%.

Table 3. Comparison of growth, glucose consumption, and the accumulation of extracellular metabolites by *E. coli* strains under anaerobic conditions.

Strains	Glucose[a] (mM)	DCW[b] (g/l)	Extracellular metabolites (mM)			
			Lactate	Acetate	Ethanol	Pyruvate
BL21(DE3)	28.8 ± 2.4	5.8 ± 0.5	24.8 ± 1.8	20.4 ± 1.4	23.8 ± 1.8	0.62 ± 0.04
Δ*ackA-pta*	18.4 ± 1.3	3.6 ± 0.3	46.2 ± 3.1	0.81 ± 0.05	ND	1.6 ± 0.1
Δ*ackA-pta-ldh*	9.7 ± 0.8	2.0 ± 0.1	0.90 ± 0.08	1.2 ± 0.1	9.5 ± 0.7	17.8 ± 1.5

The strains were grown in LB + 20 g-glucose/l-medium. The cultures were sampled 24 h after inoculation for analysis. The data shown are means of three replicate experiments.

[a]Amount of glucose consumed.

[b]Dry cell weight.

ND: not detectable.

Table 4. Comparison of growth, glucose consumption, and the accumulation of extracellular metabolites by *E. coli* strains under aerobic conditions.

Strains	Glucose[a] (mM)	DCW[b] (g/l)	Extracellular metabolites (mM)			
			Lactate	Acetate	Ethanol	Pyruvate
BL21(DE3)	36.2 ± 2.1	7.2 ± 0.5	0.5 ± 0.04	92.5 ± 8.4	ND	0.4 ± 0.03
Δ*ackA-pta*	38.5 ± 2.8	7.5 ± 0.8	0.3 ± 0.02	83.2 ± 7.1	ND	0.3 ± 0.01
Δ*ackA-pta-ldh*	32.7 ± 3.1	6.5 ± 0.6	0.2 ± 0.02	76.5 ± 5.1	ND	0.4 ± 0.02

The strains were grown in LB + 20 g-glucose/l-medium. The cultures were sampled 24 h after inoculation for analysis. The data shown are means of three replicate experiments.

[a]Amount of glucose consumed

[b]Dry Cell Weight

ND: not detectable

Table 5. Summary of results from fed-batch fermentation using a 7 L bioreactor.

Strains	DCW (g/l)	Acetate (mM)	Lactate (mM)	Pyruvate (mM)	ThMA	
					kU/g-DCW	kU/l
Control	80.9 ± 7.4	65.4 ± 5.7	9.5 ± 0.8	ND	10.2 ± 0.7	809 ± 58
Δ*ackA-pta*	57.2 ± 4.6	49.7 ± 2.5	25.3 ± 1.4	12.6 ± 0.9	17.4 ± 1.3	972 ± 36
Δ*ackA-pta-ldh*	81.0 ± 7.1	48.9 ± 4.3	0.5 ± 0.03	18.2 ± 1.7	26.1 ± 1.7	2,110 ± 120

Effect of the reduction of acetate and lactate on overall fermentation performance in *E. coli* BL21(DE3) and *ackA-pta* or *ackA-pta-ldh* deficient derivatives. The data shown are means of three replicate experiments.

Recombinant protein (ThMA) yields are reported as either specific (kU/g-DCW) or volumetric (kU/l) activities.

rate were significantly decreased. The *ackA-pta* mutant had a longer lag phase than the wild type, and lactate accumulated significantly (25.3 mM) after 45 h of culture, although acetate production was below 50 mM. Pyruvate accumulation was observed up to 12.6 mM after a 40 h culture. The specific activity of ThMA had a maximum value of 23 kU/g-DCW and gradually decreased to 17 kU/g-DCW following the accumulation of lactate and acetate (Table 5).

The *ackA-pta* and *ldh* double-mutant exhibited a similar growth profile to the wild type, although the lag phase was about 10 h longer. The double-mutant accumulated a similar amount of acetate (~49 mM) as the *ackA-pta* mutant, but excreted only 0.5 mM lactate. More interestingly, the double-mutant achieved a significant increase in ThMA production, up to 26 kU/g-DCW. The specific ThMA activity was maintained during the stationary phase in the double-mutant, while it was significantly decreased in both the wild type and *ackA-pta* mutant (Fig. 3). The final volumetric ThMA yields reached 2,110, 972, and 809 kU/l for the *ackA-pta* and *ldh* double-mutant strain, *ackA-pta* mutant strain, and control, respectively. Hence, the engineered *ackA-pta* and *ldh* double-mutant strain achieved a 156% improvement in ThMA production.

Fed-Batch Cultivation Scaled Up to a 300 L Bioreactor

Fed-batch experiments in a 300 L fermentor were conducted to assess the ability of the *ackA-pta* and *ldh* double-mutant strain compared with the control strain in producing ThMA under industrial conditions. Fig. 4 compares the performance of the *ackA-pta* and *ldh* double-mutant strain with that of the control strain using pH-stat feeding. The control strain reached a final DCW of 31 g/l and a ThMA activity of 12 kU/g-DCW. Acetate and lactate accumulated from 35 h of culture, resulting in a significant reduction in the specific activity of ThMA. The *ackA-pta* and *ldh* double-mutant excreted a similar amount of acetate and lactate to those in the 7 L fermenter, but achieved 20 kU/g-DCW of specific ThMA activity, which was 76% of the value from the laboratory-scale experiments. The final volumetric ThMA yields reached 884 and 372 kU/l for the *ackA-pta* and *ldh* strain and the control, respectively.

Discussion

High-density cell cultures inevitably result in the accumulation of acetate and/or lactate, which lead to reduced recombinant protein yields [34, 41]. Several approaches, such as optimization of the growth medium,

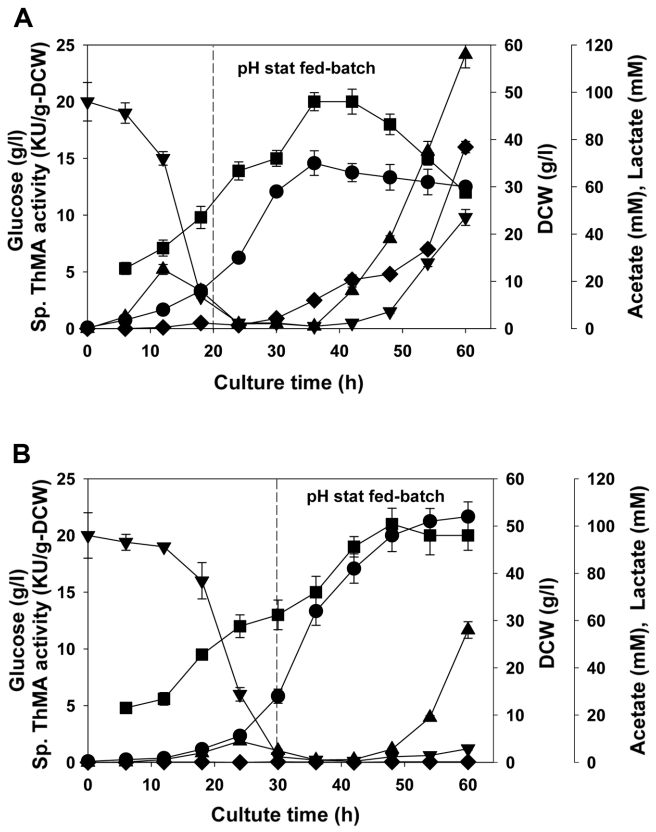

Fig. 4. Fed-batch cultures of *Escherichia coli* BL21(DE3) and *ackA-pta* and *ldh* double-mutant strain in a 300 L bioreactor.
Cells were cultivated in a 300 L bioreactor charged with 200 L of defined medium at 28°C. The pH was controlled in the range of 6.8–6.9 using the feeding solution and 28% NH_4OH. The rpm and air was changed within their operating limit to maintain the aerobic condition. The typical fermentation profiles of control strain (**A**) and *ackA-pta* and *ldh* double-mutant (**B**) are with the symbols of growth (●), glucose consumption (▼), specific ThMA activity (■), acetate accumulation (▲), and lactate accumulation (◆). The dotted line indicates the beginning of fed-batch culture after the depletion of glucose initially added to the medium.

glucose feeding strategy, and growth conditions, and metabolically engineered bacterial strains to reduce acetate accumulation in *E. coli* cultures have been reported [3, 20]. However, most reports have focused on the reduction of acetate during culture of *E. coli* in laboratory-scale experiments. DOT is considered as the most difficult factor to control in scaling from laboratory to industrial scale for production of recombinant proteins using high cell density culture of *E. coli.* In particular, the DOT gradient in large-scale cultures results in transient anaerobic conditions that induce mixed-acid fermentation pathways detrimental to the growth of *E. coli* [2, 27, 28].

In this study, two mutant strains were engineered at the level of the mixed-acid fermentation pathway to improve culture performance under transient anaerobic conditions. One was a single mutant with an inactivated *ackA-pta* pathway, and the other was a double-mutant blocking both the *ackA-pta* and *ldh* pathways. Interestingly, the accumulation of acetate was considerably reduced but not completely stopped, and the excretion of lactate was somewhat increased under anaerobic conditions when the *ackA-pta* pathway was inactivated. Pyruvate oxidase and *ack-pta* are two acetate-producing pathways in *E. coli* (Fig. 1). The *ackA-pta* pathway is active both aerobically and anaerobically and converts acetyl-CoA to acetate [15, 40]. Under aerobic conditions, *ackA-pta* mutant still consumed glucose to give acetate by pyruvate oxidase, which decarboxylates pyruvate to acetate and carbon dioxide.

Both the *ackA-pta* mutant and *ackA-pta* and *ldh* double-mutant strain showed a reduction in acetate accumulation and a significant increase in pyruvate accumulation compared with the control strain. In addition, the *ackA-pta* and *ldh* double mutant did not excrete lactate throughout the fermentation period, while the *ack-pta* single-mutant strain excreted more than 2.7-fold the lactate of the control strain. Lactate accumulation has serious detrimental effects on growth and production of ThMA, even at 10 mM, which could explain the poor ThMA production of the *ackA-pta* single-mutant strain. The *ackA-pta* and *ldh* double-mutant strain achieved an overall superior performance when compared with the other strains, indicating a synergistic effect of the mutations. More importantly, fed-batch experiments in a 300 L fermentor were conducted to assess the ability of the *ackA-pta* and *ldh* double-mutant strain to produce recombinant protein ThMA under industrial conditions. The engineered *ackA-pta* and *ldh* double-mutant strain showed superior performance and achieved a 179% improvement in volumetric ThMA production compared with that of the control strain.

Owing to recent advances in metabolic engineering, microorganisms have been used to produce a number of useful substances, including enzymes [19, 22, 42]. To improve the production efficiency of ThMA in microbial cells, several approaches, such as improvement of metabolic flux and supplementation with amino acids, have been used [20, 26]. In this study, mutant strains blocked in some of the mixed-acid fermentation pathways at the level of the pyruvate node (*ackA-pta*, *ackA-pta* and *ldh*) were constructed and assessed for their performance at larger scales. The *ackA-pta* and *ldh* double-mutant strain gave superior overall performance in large-scale culture under suboptimal

conditions, reaching a higher cell density and higher ThMA activity than the control strain. The engineered *ackA-pta* and *ldh* double-mutant strain achieved a 179% improvement in volumetric ThMA production.

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