Kafkas Univ Vet Fak Derg
24 (5): 735-742, 2018
DOI: 10.9775/kvfd.2018.19884

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

Optimization of Culture Conditions for High Cell-Density Fermentation of Bovine *Escherichia coli*

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Article Code: KVFD-2018-19884 Received: 31.03.2018 Accepted: 01.07.2018 Published Online: 02.07.2018

How to Cite This Article

Liu J, Li S, Xia X, Hu H, Fu Q, Xiao Y, Qu G, Shen Z, Cheng L: Optimization of culture conditions for high cell-density fermentation of bovine *Escherichia coli*. *Kafkas Univ Vet Fak Derg*, 24 (5): 735-742, 2018. DOI: 10.9775/kvfd.2018.19884

Abstract

Bovine *Escherichia coli* infection, which causes major economic losses to the cattle industry each year, can be prevented by administering formalin-inactivated vaccine. However, to enhance the application of this vaccine, the cell density of the formalin-inactivated *E. coli* should be boosted. This can be achieved by reducing the accumulation of acetate, a primary inhibitory metabolite in *E. coli* fermentation. To this end, the present study investigated the effect of pH, dissolved oxygen (DO) levels, and feeding methods on bovine *E. coli* fermentation, and developed two-stage pH and DO control strategies and a combined pH- and DO-mediated feeding strategy for the fermentation. The optimized conditions for Bovine *E. coli* were pH 7.0 at 0-10 h, 6.5 at 10-24 h; DO 50% at 0-10 h, 30% at 10–24 h; pH and DO feedback feeding at 0-10 h and 10-24 h, respectively. With Bovine E. coli fermentation under the optimized conditions, the acetate accumulation was 1.12 g/L and the cell density was 36.47 (OD600), which were 59.12% lower and 77.29% higher than these with the original conditions (pH 7.0; DO 20%; residual glucose concentration maintained at 2.0 g/L). After analyzing the main nodes of acetate synthesis, it was found that the lower carbon flux enters the Embden-Meyerhof pathway. Under the optimized conditions, the pyruvate flux and acetyl-CoA synthesis were low, and much of the acetyl-CoA participated in the tricarboxylic acid cycle. The extracellular acetate flux was 8.3%, which was 65.13% lower than in the original conditions.

Keywords: *Escherichia coli, Acetate, Dissolved oxygen, Feeding strategy, Metabolic flux distribution*

Sığırlarda *Escherchia coli*'nin Yüksek Hücre Yoğunluğunda Fermentasyonu İçin En Uygun Kültür Koşullarının Sağlanması

Öz

Sığırlardaki *Escherchia coli* enfeksiyonları her yıl sığırcılık sektörünü büyük çaplı maddi zarara uğratmakla birlikte, formalinle etkisizleştirilmiş bakteriden yapılan aşıyla önlenebilmektedir. Ancak, bu aşının etkisini artırmak için formalinde etkisizleştirmiş *E. coli* hücre yoğunluğunun artırılması gerekmektedir. Bunu elde etmek için *E. coli* fermentasyonunu baskılayan birincil bir metabolik ürün olan asetatın birikimi azaltılmalıdır. Bu amaçla, mevcut çalışmada pH, çözünmüş oksijen (ÇO) düzeyleri ve beslenme şeklinin sığırlardaki *E. coli* fermentasyonuna etkileri araştırılmış ve iki aşamalı bir pH ve ÇO kontrolü stratejisi geliştirilerek pH ve ÇO düzeylerini birlikte değerlendiren bir beslenme planı ile fermentasyonun artırılması yoluna gidilmiştir. Sığır *E. coli* için optimize edimiş koşullar pH için 0-10 saat aralığında 7.0, 10-24 saat aralığında 6.5; ÇO için 0-10 saat aralığında %50, 10-24 saat aralığında %30, pH ve ÇO temelinde besleme için sırasıyla 0-10 saat ve 10-24 saat olarak belirlendi. Optimize koşullar altında sığır *E. coli* fermentasyonu ile birlikte asetat birikimi 1.12 g/L olup hücre yoğunluğu 36.47 (OD600) olarak tespit edildi. Bu değerler orjinal koşullardan (pH 7.0; ÇO %20; kalıntı glukoz derişimi 2.0 g/L'de tutulmuş) sırasıyla %59.12 daha düşük ve %77.29 daha yüksek bulunmuştur. Asetat sentezinin temel unsurları incelendiğinde, daha az olan karbon akışının Embden-Meyerhof yolağına kaydığı saptanmıştır. En uygun koşullarda pirüvat akımı ve asetil CoA sentezi düşük iken, asetil CoA'nın büyük çoğunluğu trikarboksilik asit döngüsüne katılmıştır. Hücre dışı asetat akımı %8.3 olup, özgün koşullara göre %63.13 daha düşük çıkmıştır.

Anahtar sözcükler: *Escherichia coli, Asetat, pH, Çözünmüş oksijen, Besleme stratejisi, Metabolik akım dağılımı*

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INTRODUCTION

Bovine *Escherichia coli*, which is present in most cattle producing countries, is the causative agent of septicemia, enterotoxemia and meningitis, leading to major economic losses to the cattle industry each year [1]. Protection against bovine *E. coli* infection is provided by formalin-inactivated bacterins [2], but the low cell density of bovine *E. coli* inhibits the application of this vaccine. Improving the cell density of bovine *E. coli* would enhance the applicability of the vaccine to the cattle industry. The cell density is limited by acetate, a primary inhibitory metabolite that accumulates in *E. coli* cultures, which was caused of acetate inhibition for DNA transcription, RNA translation, protein stability and compound synthesis [3]. The acetate accumulation can be reduced by optimizing the culture conditions and genetically modifying the cells [4-6]. In *E. coli*, acetate is formed via two pathways: the phosphotransacetylase-acetate kinase (Pta-AckA) pathway and the pyruvate oxidase B (PoxB) pathway, which use acetyl-CoA and pyruvate as substrate, respectively [7,8]. Conversely, reducing the carbon flux in the Embden-Meyerhof pathway (EMP) reducing of the acetate excretion by lowering the pyruvate production [3,9].

Acetate synthesis and its metabolic flux distribution are affected from the values of the culture parameters [8,10]. At the pH extremes of *E. coli* growth, (4.4 and 9.2; note the wide range), the *E. coli* cells release several enzymes related to acetate formation, including the proteins encoded by pta, sucB, and sucC [11]. Acetate secretion by *E. coli* is 6 g/L at pH 6.0 and 6.5, increasing to 12 g/L at pH 7.5 [12]. The dissolved oxygen (DO) level critically determines the acetate formation and cell density in *E. coli* cultivations. Increasing the DO level decreases the acetate accumulation by enhancing the TCA cycle activity and altering the transcription levels of genes associated with glucose and acetate metabolism, whereas low DO levels increase the acetate accumulation [5]. However, increasing the DO concentration during *E. coli* growth stimulates the intracellular accumulation of reactive oxygen species (ROS). High ROS levels are known causes of stress in *E. coli*, causing irreversible damage to their cellular components [13]. By adjusting the glucose-feeding rate in a fed-batch fermentation process, the glucose concentration can be maintained below the critical value that triggers acetate formation [14,15]. The pH-stat activates the addition of a nutrient when the pH increases, maintaining the growth rate and glucose concentration significantly below the threshold for acetate production [16,17] An appropriate feeding method is important for reducing the acetate accumulation and increasing the cell density. The present study investigated the effect of pH, DO, and feeding methods on bovine *E. coli* fermentation, then developed two-stage pH and DO control strategies and a combined pH- and DO-mediated feeding strategy in bovine *E. coli* fermentation. Finally, to elucidate the driving mechanisms of low acetate accumulation and high cell density, it analyzed the flux distribution of the important nodes of acetate synthesis under the original and optimized culture conditions.

MATERIAL and METHODS

Microorganism and Medium

The bovine *E. coli* strain used in this study was obtained in an earlier study in our laboratory, and stored at the Culture Collection of Shandong Binzhou Animal Science and Veterinary Medicine Academy (Collection number: TCCC17023). During storage, the organism was maintained on Luria-Bertani agar.

The seed medium contains the following ingredients: glucose 5.0 g/L, yeast extract 10.0 g/L, $(NH_4)_2SO_4$ 4.0 g/L, sodium citrate 2.0 g/L, $MgSO_4{\cdot}7H_2O$ 1.5 g/L, KH_2PO_4 2.0 g/L, $FeSO_4{\cdot}7H2O$ 0.15 g/L, vitamin B_1 0.05 g/L. The bovine *E. coli* was cultivated in fermentation medium with the following constituents: glucose 5.0 g/L, yeast extract 2.0 g/L, $(NH_4)_2SO_4$ 3.0 g/L, sodium citrate 1.5 g/L, $MgSO_4{\cdot}7H_2O$ 1.5 g/L, KH_2PO_4 2.0 g/L, $FeSO_4{\cdot}7H_2O$ 0.1 g/L. The pH of both seed and fermentation media was adjusted to 7.0 using 4 mol/L NaOH.

Culture Conditions

Fermentations were performed in 10-L fermenters (Biotech -2015 Bioprecess controller, Bailun, Shanghai, China). A 500-mL baffled flask (Shuniu, Chengdu, China) containing 100 mL seed medium was inoculated with a single colony of bovine *E. coli*, then cultivated at 35°C shaking at 200 rpm for 12 h. A 100-mL inoculum of this culture was inoculated aseptically (2% v/v) into 5-L of fermentation medium in a 10-L fermenter. The temperature was maintained at 35°C during the cultivation period. Unless otherwise specified, the pH was adjusted to 7.0 with 25% ammonium hydroxide (w/w), and the DO level was maintained at 20%. When the initial glucose supply was depleted, the residual glucose concentration was maintained at 2.0 g/L by adding glucose solution (50% w/v) to the fermenters.

pH Control Strategy

The pH levels in the fermentation process were measured automatically by pH electrodes attached to the fermenters, and were controlled at their designated values (6.0, 6.5, 6.8, 7.0, 7.2, and 7.5) using 25% ammonium hydroxide. By maintaining the pH at different levels, we could investigate the effects of pH on bovine *E. coli* fermentation. From the results, a two-stage pH control was developed for the bovine *E. coli* fermentation.

DO Control Strategy

The DO concentrations in the fermentation process were measured automatically by DO electrodes attached to

the fermenters, and were controlled at their designated values (5%, 20%, 30%, 50%, and 100%) by adjusting the agitation and aeration rates. By maintaining the DO at different levels, we could investigate the effects of DO on bovine *E. coli* fermentation. From the results, a two-stage DO control strategy was developed for the bovine *E. coli* cultivation.

Feeding Strategies

The feeding strategy in the bovine *E. coli* fermentation was varied as intermittent feeding, pH feedback feeding, DO feedback feeding and a glucose-stat feeding strategy. During intermittent feeding, glucose (3 g/L) was added to the fermenter every 2 h. In the pH and DO feedback feeding strategies, the glucose solution was fed when the pH or DO level rose above its set value. The glucose solution automatically entered the fermenter through a peristaltic pump. In the glucose feeding strategy, the glucose concentration was controlled at 0.5 g/L by adjusting the feeding rate of the glucose solution.

Analysis of Fermentation Products

The DO, pH and temperature were measured automatically by electrodes attached to the fermenters. The cell optical density (OD) was monitored at 600 nm in a spectrophotometer (722N, INESA, China) [18]. The glucose concentration was monitored using an SBA-40E biosensor analyzer (Biology Institute of Shandong Academy of Sciences, Jinan, China). The acetate concentration was determined by high-performance liquid chromatography using an Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA) equipped with an Aminex HPX-87H (Bio-Rad Laboratories, Inc, USA) [19].

Analysis of Metabolic Flux

The metabolic flux distribution of acetate synthesis during the later cultivation period of the bovine *E. coli* fermentation under different culture conditions was calculated by MATLAB 7.0. The results were based on the analysis of metabolic flux balance and stoichiometry [3,20].

Statistical Analysis

All experiments were conducted in triplicate, and the data were averaged and presented as the mean±standard deviation. One-way analysis of variance followed by Dunnett's multiple comparison test were used to determine significant difference [21]. Statistical significance was defined as $P<0.05$.

RESULTS

pH Control Strategy of Bovine E. coli Fermentation

Effect of pH: Fig. 1 compares the cell densities and accumulated acetate concentrations at different pH levels in the bovine *E. coli* fermentation. The acetate concentration increased with increasing pH, reaching 2.14 g/L at pH 6.5 and 3.53 g/L at pH 7.5. The cell density increased with pH up to 7.0, then decreased at higher pH. At pH 7.0, the acetate concentration was 2.74 g/L. The cell density was highest at pH 7.0 (20.57), 35.06% higher than at pH 6.5 (15.23), and 44.15% higher than at pH 7.5 (14.27).

Two-stage pH Control Strategies: Based on the varying pH results, we applied four two-stage pH control strategies in the bovine *E. coli* fermentation: strategy I (6.5 at 0-10 h, 7.0 at 10-24 h), strategy II (6.5 at 0-10 h, 7.2 at 10-24 h), strategy III (7.0 at 0-10 h, 6.5 at 10-24 h), and strategy IV (7.2 at 0-10 h, 6.5 at 10-24 h). *Fig. 2* shows the cell densities and acetate concentrations under the different pH controls in the bovine *E. coli* fermentation. When the pH was controlled at a higher level during the early fermentation stage than during the late stage, the cell growth rate was improved and the cell density was increased, but the acetate accumulation was high. The final cell densities (acetate accumulations) in strategies III and IV were 23.47 (2.45 g/L) and 20.58 (2.66 g/L), respectively. When the pH was lower during the early cultivation stage than during the late stage, less acetate was excreted, but the growth rate and cell density were comparatively low. The cell densities (acetate

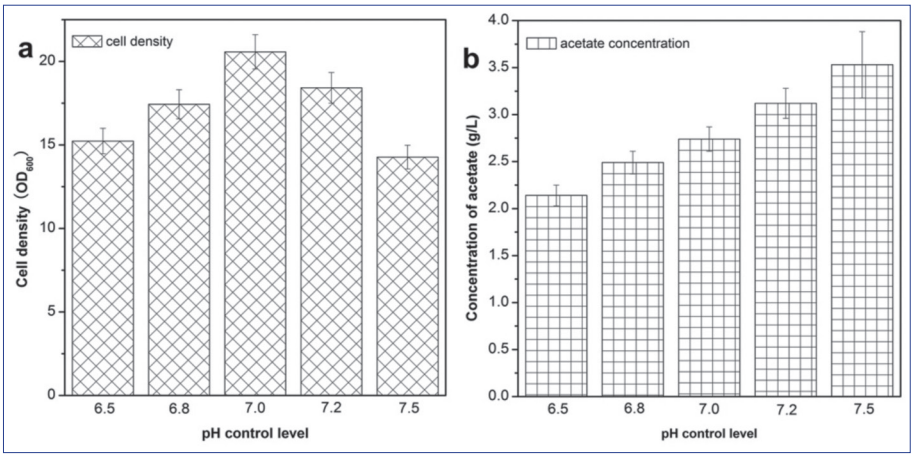


Fig 1. Effect of pH on cell density (a) and acetate accumulation (b) in bovine *E. coli* fermentation ($P<0.05$)

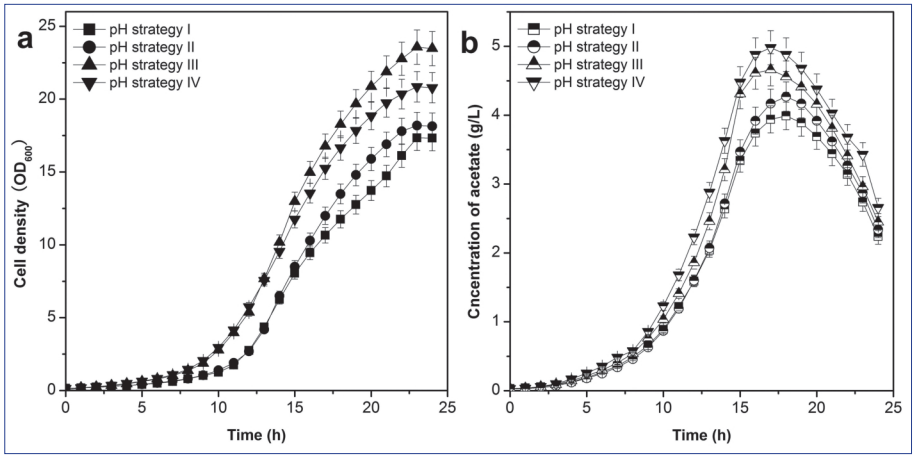
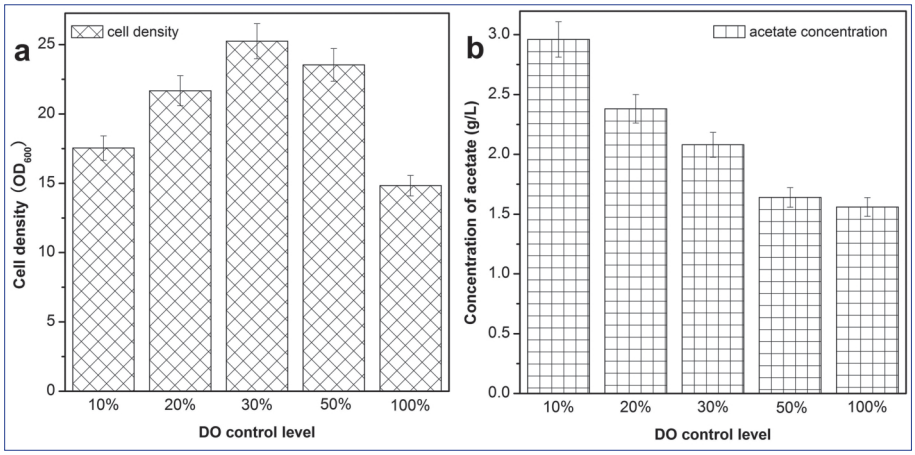


Fig 2. Effect of two-stage pH control strategy on cell density (a) and acetate accumulation (b) in bovine *E. coli* fermentation (P<0.05)

Fig 3. Effect of DO level on cell density (a) and acetate accumulation (b) in bovine *E. coli* fermentation (P<0.05)



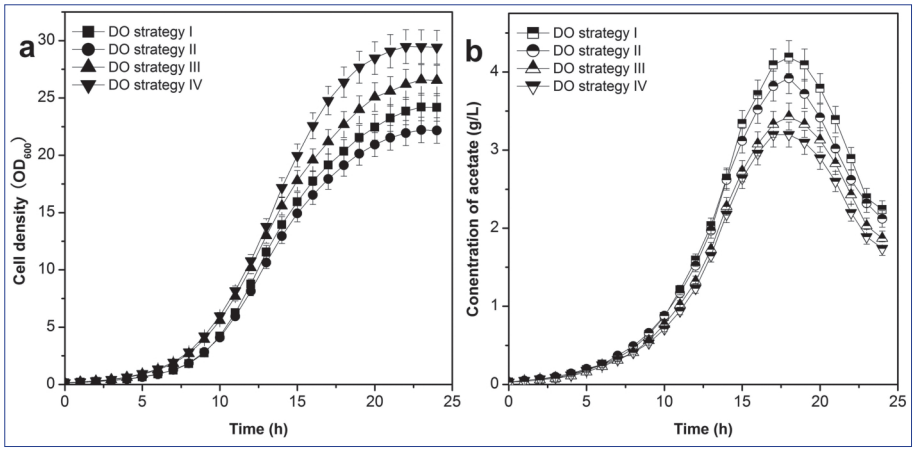


Fig 4. Effect of two-stage DO control strategy on cell density (a) and acetate accumulation (b) in bovine *E. coli* fermentation (P<0.05)

accumulations) in strategies I and II were 17.34 (2.96 g/L) and 18.14 (2.34 g/L), respectively. Strategy III achieved the highest cell density, but accumulated more acetate than strategies I and II.

DO Control Strategy of Bovine E. coli Fermentation

Effect of DO Level: The cell densities and acetate accumulations at different DO levels in the bovine *E. coli* fermentation are displayed in *Fig. 3*. The acetate accumulation decreased with increasing DO level, and the cell density was maximized at 30% DO. Comparatively, the cell density at 30% DO (25.24) was 43.89% and 70.19% higher than that at 10% DO (17.54) and 100% DO (14.83), respectively. The acetate accumulation at 30% DO was 2.08 g/L, 42.31% lower and 33.33% higher than that at 10% DO (2.96 g/L) and 100% DO (1.56 g/L), respectively. A relatively high DO level boosted the cell density while also reducing the acetate excretion.

Two-stage DO Control Strategies: Based on the above results, we trialed four two-stage control strategies in the

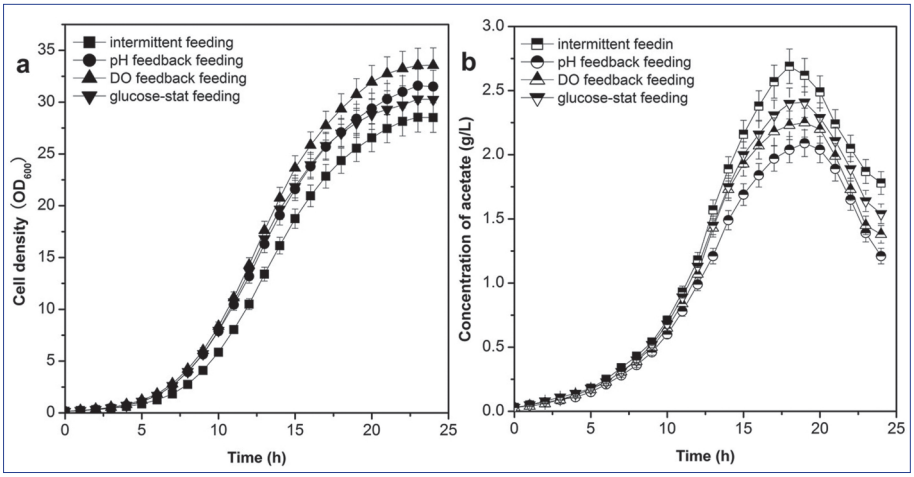


Fig 5. Effect of feeding strategy on cell density (a) and acetate concentration (b) in bovine *E. coli* fermentation ($P<0.05$)

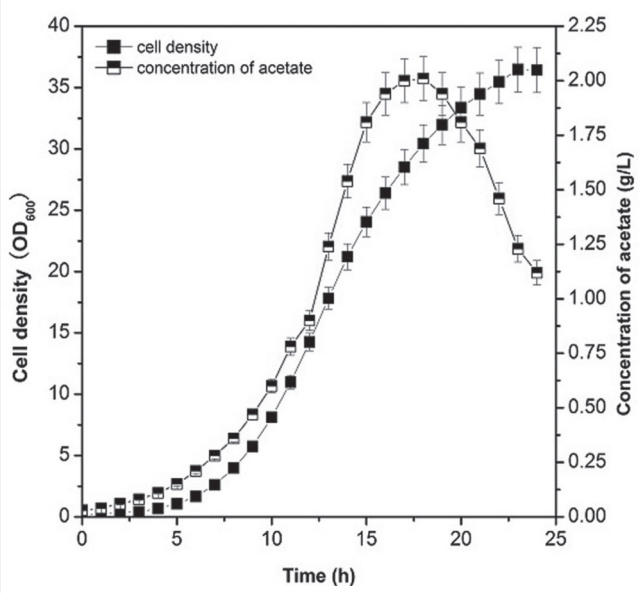


Fig 6. Application of combined pH and DO feedback feeding in the bovine *E. coli* fermentation ($P<0.05$)

in *Fig. 4*. When the DO level was higher during the early fermentation stage than during the later stage, the cell density was increased and the acetate accumulation was reduced. DO strategy IV achieved the highest cell density (29.47, 10.89% higher than in Strategy III) and lowest acetate accumulation (1.74 g/L, 6.95% lower than in strategy III). The cell densities (acetate accumulations) in DO strategies I and II were 24.21 (2.24 g/L) and 22.21 (2.12 g/L), respectively.

Feeding Strategy of Bovine E. coli Fermentation

Effect of Feeding Strategy: Fig. 5 shows the cell densities and acetate concentrations in bovine *E. coli* fermentation under four feeding strategies: intermittent feeding, pH feedback feeding, DO feedback feeding and glucose-stat feeding. Both the growth and acetate excretion depended on the feeding strategy. DO feedback feeding maximized the cell density (33.56), but did not minimize the acetate accumulation (at the end of the fermentation, the acetate concentration was 1.38 g/L). The pH feedback strategy minimized the acetate excretion (1.21 g/L), but reduced

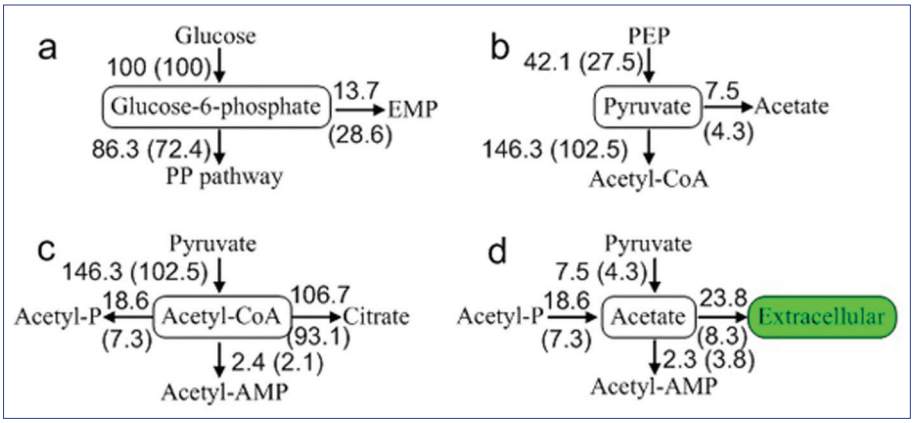


Fig 7. Metabolic flux distributions of important nodes for acetate synthesis under the original and optimized conditions during the later fermentation phase (20–24 h) of bovine *E. coli* fermentation

bovine *E. coli* fermentation: strategy I (20% at 0-10 h, 30% at 10-24 h), strategy II (20% at 0-10 h, 50% at 10-24 h), strategy III (50% at 0-10 h, 20% at 10-24 h), and strategy IV (50% at 0-10 h, 30% at 10-24 h). The results are presented

the cell density to 31.59. The cell densities (acetate accumulations) in the intermittent and glucose-stat feeding strategies were 28.54 (1.78 g/L) and 30.29 (1.54 g/L), respectively.

Application of a Combined Feeding Strategy: Based on the analysis of the fermentation process and the results of different feeding strategies, we applied a combined feeding strategy of pH (0-10 h) and DO (10-24 h) feedback feeding in the bovine *E. coli* fermentation. The time-dependent cell density and acetate concentration in this combined feeding strategy are presented in *Fig. 6*. The combined feeding reduced the acetate accumulation to 1.12 g/L and increased the cell density to 36.47.

Metabolic Flux Distribution of Main Nodes for Acetate Synthesis: After analyzing the effect of pH, DO and feeding strategy on bovine *E. coli* fermentation, the optimized fermentation conditions were obtained as follows: pH 7.0 at 0-10 h and 6.5 at 10-24 h; DO 50% at 0-10 h and 30% at 10-24 h; pH feedback feeding at 0-10 h and DO feedback feeding at 10-24 h. *Fig. 7* shows the flux distributions of the important nodes for acetate synthesis under the original conditions (pH 7.0; DO 20%; residual glucose concentration maintained at 2.0 g/L) and the optimized conditions. Under the optimized conditions, less carbon flux entered the EMP pathway, so the pyruvate flux (27.5%) was 34.68% lower than under the original conditions. Under the optimized (original) conditions, the flux of acetyl-CoA from pyruvate was 102.5% (146.3%), and 90.74% (72.93%) of the acetyl-CoA entered the TCA cycle. The flux of acetate synthesis from pyruvate and acetyl-CoA was 11.6% under the optimized conditions, 55.56% lower than under the original conditions; consequently, the acetate accumulation was lowered. The extracellular acetate flux under the optimized conditions was 8.3%, 65.13% lower than under the original conditions.

DISCUSSION

pH exerts complex effects because it influences the solubility of nutrients and trace elements, and the cellular metabolism in general [22]. pH homeostasis is important for the function and stability of all cellular enzymes [23]. The main regulator of pH is the *pta* gene, which is involved in acetate synthesis. At high pH, this gene is induced [11], and its product accumulates to high acetate concentrations. Components of the TCA cycle, such as *suc*B and *suc*C, are induced at low pH in culture, encouraging exploitation of the high proton potential and increasing the capacity of the TCA cycle. Consequently, the concentrations of acetyl-CoA and acetate decrease [10,11]. In the present study, the acetate concentration was lower in strategies I and II than in strategies III and IV because the pH was maintained at low levels during the early cultivation period, but the cell density was also low. High H^+ concentration inhibits the activity of phosphofructokinase, interdicting the EMP pathway and ultimately disrupting the cell growth [24]. Controlling the pH at 7.0 (0-10 h) and 6.5 (10-24 h) increased the cell density of bovine *E. coli*.

Raising the DO level reduced the acetate accumulation in the bovine *E. coli* fermentation. The transcription levels of the gluconeogenesis (*pck*A, *pps*A) and anaplerotic pathway (*ppc*, *sfc*A) genes are reportedly lower at low DO levels than at high DO levels, favoring the accumulation of pyruvate and acetyl-CoA, and increasing the acetate accumulation through the Pta-AckA and PoxB pathways [5]. At high DO levels, the high transcription levels of gluconeogenic genes increase the conversion of pyruvate to glucose by gluconeogenesis, reducing the pyruvate concentration and consequently reducing the acetate accumulation [4]. The acetate concentration was minimized at 100% DO, but the cell density was lowest under this condition, possibly because the high ROS contents at 100% DO caused irreversible damage to the cellular components [13]. Maintaining a moderately high DO level can both decrease the acetate accumulation and increase the cell density. Among the two-stage DO control strategies, the acetate excretion was suppressed by maintaining the DO level at 50% during the early fermentation phase, whereas DO levels of 20% and 30% avoided the formation of ROS. The highest cell density was obtained in strategy IV (50% at 0-10 h, 30% at 10-24 h).

Acetate excretion can be prevented by maintaining the glucose concentration below the critical level for acetate synthesis, and the acetate accumulation decreases as the glucose concentration lowers [25]. An intermittent feeding and glucose-stat feeding strategy increases the acetate accumulation by maintaining a high glucose concentration, and also reduces the cell density [15]. For reducing acetate formation during a fed-batch process, the changes in DO or pH are easily monitored online [17]. In a previous study, a pH-based or DO-based feeding strategy was found to maintain the glucose concentration and thereby reduce the acetate excretion [26]. The pH and DO feedback feeding strategies also lowered the acetate concentration and raised the cell density in the present study of bovine *E. coli* fermentation. Applying the DO feedback feeding in the later fermentation period satisfies the oxygen requirements of the cells, enhancing the balance between growth rate and oxygen consumption, and inhibiting the formation of acetate [3,27]. Consequently, when the pH and DO feedback feeding strategies were combined, the cell density increased to 36.47 while the acetate accumulation decreased to 1.12 g/L.

The metabolic flux distribution of acetate synthesis depends on the culture conditions [8]. Under the optimized conditions, less carbon flux enters the EMP pathway, avoiding "overflow" of the central metabolic pathway and decreasing the formations of pyruvate and acetyl-CoA, thereby reducing the acetate excretion [3]. The acetate-synthesis flux from pyruvate and acetyl-CoA was lowered by DO feedback feeding at 30% DO and pH 6.5. At pH 6.5, the acetyl-CoA flux mainly participates in the TCA cycle because the TCA cycle capacity is enhanced at this level [11]. The higher transcription levels of acetyl-CoA

synthetase (Acs) in *E. coli* significantly decrease the acetate accumulation and improve the efficiency of acetate assimilation. High DO level increases the expression of Acs and the optimal conditions enhance the flux from acetate to acetyl-AMP [28]. In the present study, the optimized conditions lowered the acetate-synthesis flux and raised the acetate-assimilation flux. Consequently, the flux of extracellular acetate was 8.3%, which was 65.13% lower than under the original conditions.

In this study, the acetate accumulation was decreased by optimizing the pH and DO levels and the feeding strategy in a bovine *E. coli* fermentation, achieving a high cell-density cultivation of bovine *E. coli*. Under the optimized conditions (pH 7.0 at 0-10 h, 6.5 at 10-24 h; DO 50% at 0-10 h, 30% at 10-24 h; pH and DO feedback feeding at 0-10 h and 10-24 h, respectively), the cell density reached 36.47 (OD_{600}) and the acetate accumulation decreased to 1.12 g/L. These values were 77.29% higher and 59.12% lower, respectively, than under the original conditions (pH 7.0; DO 20%; residual glucose concentration maintained at 2.0 g/L). In addition, the optimized conditions reduced the carbon flux entering the EMP pathway and minimized the fluxes of pyruvate and acetyl-CoA synthesis. The extracellular acetate flux (8.3%) was 65.13% lower than under the original conditions. By reducing the acetate accumulation and boosting the cell growth, we can improve the applicability of the bovine *E. coli* vaccine, and better protect the cattle industry from *E. coli* infection. The developed approach also provides a theoretical foundation for decreasing the acetate accumulation in high-cell density cultivations of other bacteria.

Acknowledgments

This work was supported by the <Development of Science and Technology Plan Program of Binzhou> under Grant <2015ZC0107>, and <Innovation Program of Modern Agricultural Industry System of Shandong> under Grant <SDAIT-09-12>. We also thank Leonie Z. Pipe, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Disclosure Statement

No potential conflict of interest was reported by the authors.

Funding

Development of Science and Technology Plan Program of Binzhou (No. 2015ZC0107), Innovation Program of Modern Agricultural Industry System of Shandong (No. SDAIT-09-12), the National Key Research and Development Program of China (No. 2016YFD0500708), the National Natural Science Foundation of China (No. 31702263), and the China Postdoctoral Science Foundation (No. 2017M622346).

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