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Using a combined oxygen-supply and substrate-feeding strategy to improve 2,3-butanediol production by metabolically engineered *Klebsiella oxytoca* KMS005

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Abstract

BACKGROUND: There is much demand for and extensive application for 2,3-butanediol (2,3-BD) in various fields, and micro-aerobic and substrate-feeding conditions greatly affect microbial growth and production. The theoretical maximum of 2,3-BD fermentative yield has rarely been reported. Therefore, our study aimed to develop an efficient combined oxygen-supply and substrate-feeding strategy to improve 2,3-BD production yield in metabolically engineered *Klebsiella oxytoca* KMS005.

RESULTS: The optimized oxygen consumption for 2,3-BD production by strain KMS005 was demonstrated at 9.2 g for 1 L working volume corresponding to KLa of 25.2 h^{-1}. During fed-batch, a glucose feeding rate of 2 g h^{-1} starting at the end of the growth phase for 48 h followed by a final batch phase of 40 h was found likely to be satisfactory for 2,3-BD production by the strain KMS005. A final 2,3-BD concentration was obtained at 74.7 g L^{-1} with few by-products formation. A theoretical maximum of 2,3-BD production yield of 0.5 g g^{-1} substrate used was also approached.

CONCLUSION: Our oxygen-supply strategy with the specific feeding pattern developed in this study allowed the highest fermentative production yield of 2,3-BD ever reported. The KMS005 strain may be used as a biocatalyst for cost-effective 2,3-BD production from renewable substrates. In addition, the outcome might bring a message for further developments of simple fed-batch fermentation under micro-aeration conditions into larger scales for 2,3-BD production by *K. oxytoca* KMS005 or even other microorganisms.

Keywords: biocatalysis; aeration; fermentation; metabolic engineering

INTRODUCTION

Bio-based bulk chemicals have been intensively developed to replace fossil-based chemicals owing to gradually increasing prices of petrochemical feedstocks and their diminishing availability.[1] Many derivatives of 2,3-butanediol (2,3-BD) have been recognized on the global market in the forms of methyl ethyl ketone (MEK), diacetyl, polyurethane-melamides, and others. Their applications include fuel, additives and flavoring agents for food and fragrances, pharmaceutical products, bio-plastics, and solvent productions.[2] 2,3-BD production via fermentation from biomass is a good option to supply the increasing global market demand. Fermentative 2,3-BD production is required to meet high final concentrations and yields but to minimize residual sugars and by-products to limit the cost of downstream processes. Some key factors of 2,3-BD production via fermentation process, including microorganisms, inexpensive substrates and media, simple modes of operation, and low costs of separation and purification steps, need to be considered for the making of a profit on a commercialized scale.

2,3-BD can be produced by anaerobic and facultative aerobic microorganisms. Oxygen supply is the most important parameter since the 2,3-BD pathway participates in the regulation of the $NADH/NAD^+$ ratio to maintain the redox balance in the cells. Under less than fully aerobic cultivation, lactate dehydrogenase, pyruvate-format lyase and α-acetolactate synthase enzymes act upon pyruvate (Fig. 1). 2,3-BD is generally produced under micro-aerobic conditions under slightly acidic pH.[3] Several 2,3-BD production runs were performed to optimize the oxygen supply by various control strategies, including oxygen mass transfer coefficient ($k_L a$), respiratory quotient (RQ), or oxygen uptake

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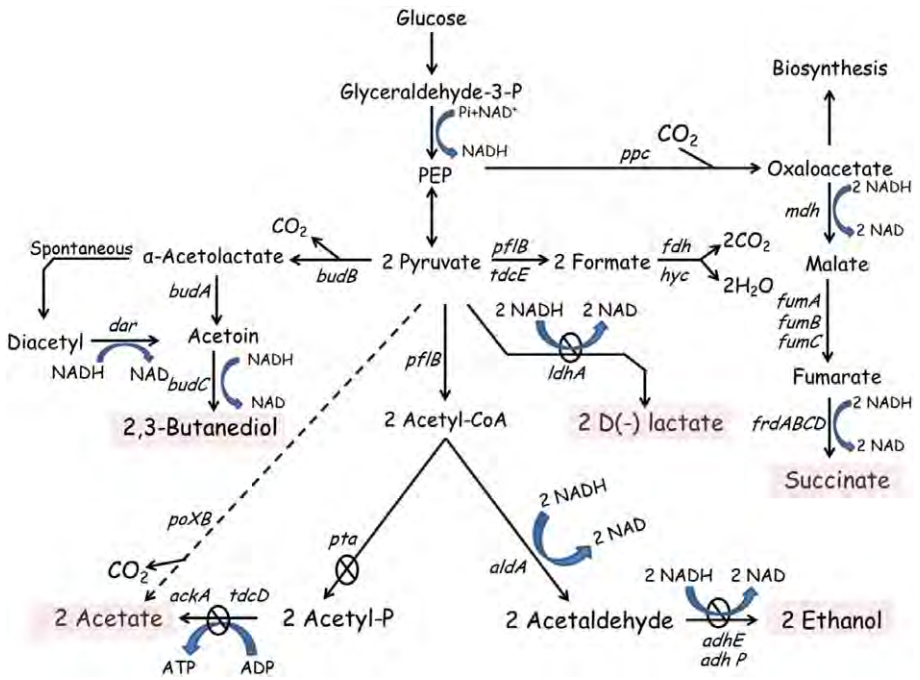


Figure 1. Fermentation pathways in *K. oxytoca* KMS005 under micro-aerobic conditions. Central metabolism indicating genes deleted in the engineered strain for 2,3-BD production. Solid arrows represent central fermentative pathways. Dashed arrow represents alternative acetate-producing pathway via pyruvate oxidase (*poxB*). Circled-crosses represent the gene deletions performed to obtain KMS005. Genes and enzymes: *ldhA*, lactate dehydrogenase; *pflB*, pyruvate formate-lyase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *tdcD,* propionate kinase; *tdcE*, threonine decarboxylaseE; *adhE*, alcohol dehydrogenase; *aldA,* acetaldehyde dehydrogenase; *ppc*, phosphoenol- pyruvate carboxylase; *mdh*, malate dehydrogenase; *fumABC*, fumaraseisozymes; *frdABCD*, fumaratereductase; *fdh*, formate dehydrogenase; *poxB*, pyruvate oxidase*; budA*, α-acetolactate decarboxylase; *budB,* α-acetolactate synthase; *budC*, butanediol dehydrogenase and *dar,* diacetylreductase (modified from Jantama *et al.*[10]).

rate (OUR). Fages *et al.*[4] attempted to use k_La to optimize 2,3-BD production by *Bacillus polymyxa*. High (R,R)-2,3-BD concentration of 44 g L^{-1} was obtained with a productivity of 0.79 g L^{-1} h^{-1} by varying the values of k_La during fermentation. A two-stage air supply strategy by varying agitation speeds and aeration rates was also reported. For example, a two-stage agitation speed strategy was performed to supply different oxygen levels for optimizing cell growth and 2,3-BD production by *Klebsiella oxytoca*.[5] RQ was also assigned to determine optimal oxygen supply. Zeng *et al.*[6] employed an optimum RQ values ranging between 4 and 4.5 to obtain a high 2,3-BD concentration of around 96 g L^{-1}. Zhang *et al.*[7] also demonstrated 2,3-BD production at the concentration of 140 g L^{-1} by *Serratia marcescens* by switching RQ values from 1–1.5 to 1.8–2.0.

A fed-batch strategy was also superior compared with batch and continuous cultivations to obtain high concentrations of 2,3-BD. This improves the final product concentration thus avoiding the effect of substrate inhibition by the maintenance and supply of additional sugar at a relatively low level.[8] A fed-batch strategy providing a constant feeding rate is commonly considered as the simplest method, due to the lack of requirements for a computer coupled with a peristaltic pump, substrate sensors, and other sophisticated equipments for monitoring the fermentation condition. However, the feeding pattern to maintain a constant sugar residue concentration was likely to be preferential for 2,3-BD production by *Klebsiella* spp.[9]

The aim of this study was to improve the 2,3-BD production yield by a metabolically-engineered strain of *K. oxytoca* KMS005. In this work, the oxygen supply for efficient 2,3-BD batch production under micro-aerobic conditions was first optimized by varying k_La values under conditions of either constant or two-stage aeration. Then, several fed-batch conditions were carried out to investigate the optimized feeding strategy for maximizing 2,3-BD yield and concentration but minimizing substrate inhibition.

EXPERIMENTAL

Microorganism

A previously metabolically-engineered *K. oxytoca* KMS005[10] was used throughout this study. The strain KMS005 was developed by deleting genes involved in NADH competing pathways, including *adhE* (alcohol dehydrogenase E), *ackA*/*pt*a (acetate kinase A/phosphotransacetylase), and *ldhA* (lactate dehydrogenase A). Luria-Bertani (LB) agar composed (per liter) of 5 g yeast extract, 5 g NaCl, 10 g peptone, and 20 g agar was used to maintain the culture at 4 °C.

Culture method

For seed preparation, KMS005 was cultured on a Luria-Bertani (LB) agar. The plate was incubated at 37 °C for 24 h. A full single loop of the fresh seed was inoculated into 250 mL Erlenmeyer flasks containing 60 mL LB medium. The inoculum was incubated at 37 °C, 250 rpm for 16–18 h. A simple AM1 mineral salts medium (adapted from Martinez *et al.*[11]) in which KCl was excluded, was used as the fermentation medium throughout this study. The optimized glucose concentration at the concentration of 90 g L^{-1} was estimated from our previous study in 2,3-BD production from maltodextrin.[12]

Batch fermentations

Fermentations were carried out in 2 L bioreactors (BIOFLO 110, New Brunswick Scientific, USA) containing 1 L of AM1 medium

supplemented with 14% (w/v) glucose in duplicate. The seed culture was inoculated to the fermentation medium at the final OD_{550} of 0.1. The temperature was regulated at 37 °C. The pH of the medium fermentation was maintained after being dropped to 6.0 by the automatic addition of 3 mol L^{-1} KOH. The inlet gas flow rate was controlled at 0.8 or 1 vvm. Two Rushton turbines were agitated at speeds of 250 or 400 rpm. The bioreactor was equipped with an automatic gas analyzer (SERVOMEX 4100, UK) for on-line measurement of CO_2 and O_2 concentrations in the exit gas. Dissolved oxygen (DO) was measured by DO probe (Mettler Toledo 6800, USA). Cell dry weight, and concentrations of glucose, 2,3-BD, succinate, acetate, and ethanol samples were determined every 24 h.

Batch operations (B) were performed to study effects of different oxygen levels supplied during both growth and stationary phases of 2,3-BD production. For one-stage oxygen supply (1), oxygen was provided at the same level throughout the fermentation time with either low (L: 400 rpm and 0.8 vvm; k_La: 25.2 h^{-1}) or high (H: 400 rpm and 1.0 vvm; k_La: 36.1 h^{-1}) aeration. The conditions were designated as B1L and B1H, for batch-one stage oxygen supply under low and high aeration, respectively. For two-stage oxygen supply (2), levels of oxygen were provided at the same level as conditions of one-stage oxygen (low or high) supply during the growth phase. After that, levels of oxygen provided were switched to lower values for both low aeration (L: 250 rpm and 0.8 vvm; k_La: 19.6 h^{-1}) and high aeration (H: 400 rpm and 0.8 vvm; k_La: 25.2 h^{-1}) during the stationary phase. The conditions were designated as B2L and B2H, for batch-two stage oxygen supply under low and high aeration, respectively.

Fed-batch fermentations

At first, the fed-batch (FB) fermentation was carried out in the same way as batch mode except for the initial sugar concentrations. Initial glucose concentrations (S) were varied among experiments (90, 120, or 140 g L^{-1}). Then, the bioreactor was fed with a glucose stock solution (800 g L^{-1} of glucose in a diluted AM1 medium (1:4)) at constant flow rates depending on the targeted glucose concentration in the medium. The starting and duration of the feeding phase were varied among experiments. The feeding rate was initially estimated based on the optimum sugar consumption rate obtained from the batch condition, and was varied at rates of 1.25, 2.0, or 2.5 mL h^{-1}. The feeding rate at 2 g glucose h^{-1} was equivalent to 2.5 mL h^{-1} in this study. Each fed-batch condition with different initial sugars and feeding patterns is indicated in Table 2.

Analytical methods

Cell dry weight

The optical density (OD) at 550 nm of a 1 mL sample was measured by a spectrophotometer Spekol®1500. Cell mass was estimated from a linear equation derived from a plot of OD versus cell dry weight (CDW).

Glucose, 2,3-BD, acetate, succinate, and ethanol concentrations

The fermentative culture broth was centrifuged at 13 500 rpm for 4 min to separate cells and supernatant. The supernatant was filtrated through a 0.2 µm filter membrane. Twenty-five micro-liters of injection volume were automatically analyzed by HPLC (Thermo Scientific, France) equipped with a column, Rezex ROA organic acid H^+ (8%), 250×4.6 mm phase–reverse column (Phenomenex, France) thermostated at 30 °C, and associated with a Refractive Index detector in series with a UV detector. The elution with an aqueous solution of 10 mmol L^{-1} H_2SO_4 was performed at 170 µL per min.

Determination of volumetric oxygen mass transfer coefficient (k_La)

The k_La values were estimated by a dynamic gassing-out method[13] by monitoring an increase in DO concentration in AM1 medium containing 14% (w v^{-1}) glucose monohydrate without inoculation at the working volume of 1 L in the bioreactor. Different gas flow rates (between 0.8 and 1 vvm) and stirring speeds (between 250 and 400 rpm) were used as parameters for determining k_La values.

Calculation of respiratory quotient

The gas balancing method was used to calculate OUR (oxygen uptake rate) and CPR (CO_2 production rate) by an assumption of well-mixed bioreactor under steady-state conditions.[14] Concentrations of O_2 and CO_2 in the inlet gas were approximately constant and considered equal to 21% and 0%, respectively. The respiratory quotient (RQ) was then calculated as:

$$RQ = CPR/OUR \ \left(\text{mol mol}^{-1}\right)$$

Calculation of total O_2 consumed during batch fermentation

Mass balance for the DO can be written as:

$$dC_L/dt = k_La \ \left(C_L^* - C_L\right) - OUR$$

where C_L is the concentration of DO in the liquid phase and C_L^* is the concentration at which the DO was saturated ($C_L^* = 8\times10^{-3}$ g L^{-1}). Since C_L is constant and close to zero after approximately 8 h of fermentation, it can be written as:

$$k_La\left(C_L^*\right) = OUR$$

$$O_2\text{consumed (g)} = k_La.C_L^*.t.V \quad \text{for } 8 < t < 52 \text{ h}$$

where V is the volume of the liquid phase and *t* is the duration of the oxygen limitation phase. For the first 8 h incubation, the oxygen consumed was calculated by integration. Therefore, the total amount of consumed oxygen could be estimated.

Carbon balances

Quantities (Q in gram unit) of glucose consumed and fermentative products produced (2,3-BD, acetate, succinate, ethanol, CO_2, and biomass) were calculated during fermentation. For biomass, the calculation was based on the average formula of *K. oxytaca* equivalent to $CH_{1.73}O_{0.43}N_{0.24}$.[15] The percentage of carbon recovery was calculated as:

$$\%C \text{ recovery} = \Sigma \ \left[n_i \left(Q_i/M_i\right)\right] / \left[6 \ \left(Q_{glucose}/M_{glucose}\right)\right]$$

where index i refers to fermentative products, n_i is equal to the number of carbon mole by mole of compound i, Q_i is the mass of compound i produced during fermentation, and M_i is the molecular weight of compound i. $Q_{glucose}$ (g) is the glucose consumed during fermentation and $M_{glucose}$ is the molecular weight of glucose.

Statistical analysis

The mean values and the standard errors were expressed from the data obtained with duplicate experiments. Differences between the sample means were analyzed by Duncan's Multiple Range tests at $\alpha = 0.05$, and 95% ($P < 0.05$) significance level by SPSS software version 17.0.

Table 1. Comparison of the performance of batch fermentation for 2,3-BD production by the strain KMS005 from initial glucose concentration of 140 g L^{-1} with different aeration strategies

		One-stage aeration		Two-stage aeration	
	Phases	B1L (Low aeration)	B1H (High aeration)	B2L (Low aeration)	B2H (High aeration)
k_La (h^{-1}) [rpm, vvm] (min^{-1})	Growth (0–24 h)	25.2 [400, 0.8]	36.1 [400, 1.0]	25.2 [400, 0.8]	36.1 [400, 1.0]
	Stationary (24–52 h)	25.2 [400, 0.8]	36.1 [400, 1.0]	19.6 [250, 0.8]	25.2 [400, 0.8]
Max CDW[a] (g L^{-1})		$4.9 \pm 0.1^{e,\mu}$	$4.6 \pm 0.2^{\mu}$	$5.1 \pm 0.4^{\mu}$	$5.0 \pm 0.4^{\mu}$
2,3-BD (g L^{-1})		$49.4 \pm 3.0^{\mu}$	$45.1 \pm 2.0^{\alpha}$	$44.8 \pm 5.5^{\alpha}$	$50.2 \pm 0.1^{\mu}$
Yield[b] (g g^{-1})		$0.37 \pm 0.01^{\mu}$	$0.37 \pm 0.01^{\mu}$	$0.36 \pm 0.01^{\mu}$	$0.37 \pm 0.01^{\mu}$
Av. Prod[c] (g L^{-1} h^{-1})		$0.95 \pm 0.06^{\mu}$	0.86 ± 0.04	$0.78 \pm 0.10^{\alpha}$	$0.96 \pm 0.01^{\mu}$
%Sugar consumed		93	85	87	97
%Carbon balance		96	93	99	95
By-products[d] (g L^{-1})					
Succinic acid		$0.4 \pm 0.0^{\mu}$	$0.4 \pm 0.0^{\mu}$	$6.1 \pm 0.1^{\alpha}$	$0.3 \pm 0.1^{\mu}$
Ethanol		$0.1 \pm 0.1^{\mu}$	$0.1 \pm 0.0^{\mu}$	Not detected	$0.1 \pm 0.1^{\mu}$
Acetic acid		$1.8 \pm 0.6^{\mu}$	$2.8 \pm 0.1^{\alpha}$	$1.9 \pm 0.0^{\mu}$	$2.8 \pm 0.1^{\alpha}$

[a] Cell dry weight (CDW) was determined at the end of growth phase.
[b] Yield was calculated as grams of 2,3-BD produced divided by grams of glucose consumed.
[c] Average productivity was calculated at 52 h incubation.
[d] No lactate was found in all tested conditions.
[e] All data represent the averages of two fermentations with standard errors. Values bearing different Greek symbols were significantly different between columns ($P < 0.05$).

RESULTS AND DISCUSSION

Effect of oxygen supply strategy in batch fermentation

Oxygen transfer rate (OTR) is considered the most important operating parameter for 2,3-BD production. High levels of OTR usually result in high growth of cells leading to an increase in 2,3-BD productivities. In contrast, even though the limitation in oxygen increases 2,3-BD yield, a decrease in overall production rate of 2,3-BD is observed due to a lower cell density. In addition, at an extremely low oxygen level, equal molar amounts of 2,3-BD and ethanol are regularly formed by natural 2,3-BD producers.[16] Hence, conditions providing high 2,3-BD concentration and productivity do not guarantee the high production yield of 2,3-BD. With these considerations, several oxygen supply strategies were initially screened in batch fermentations to obtain an optimized oxygen level suitable for efficient 2,3-BD production. Table 1 shows the k_La values at different aeration and stirring rates. Values of k_La of 36.1, 25.2, and 19.6 h^{-1} were observed at stirring and aeration conditions of 400 rpm with 1.0 vvm, 400 rpm with 0.8 vvm, and 250 rpm with 0.8 vvm, respectively.

In one-step aeration experiments, low (B1L) and high (B1H) OTR conditions were applied (Table 1). For both conditions, the growth phase of the strain occurred at 24 h (Fig. 2(a), (b)). At the beginning, oxygen transfer was not a limiting parameter since DO values did not reach zero. In addition, the specific growth rate reached maximum (data not shown). After 8 h incubation, oxygen transfer became limiting and the rate became linear. Meanwhile, 2,3-BD production started (likely growth-associated production). During the stationary phase (from 24 to 52 h), 2,3-BD production did not follow the growth-associated pattern. The 2,3-BD productivity during the growth phase (1.3 g L^{-1} h^{-1} for both B1L and B1H) was also higher than those observed during the stationary phase (1.0 g L^{-1} h^{-1} for B1L and 0.9 g L^{-1} h^{-1} for B1H). No significant difference was observed in the maximum CDW but there was significance in the final 2,3-BD concentration and the 2,3-BD productivity between B1L and B1H conditions (Table 1). The significantly lower productivity observed during the stationary phase for B1H may be explained by the accumulation of more by-products and lower consumption of sugar. In addition, high percentages of carbon balance (93–99%) obtained for each tested condition revealed that the carbon distribution through the 2,3-BD production pathway of KMS005 strain was nearly conserved in which all consumed glucose was almost totally dedicated to the combined concentrations of 2,3-BD, cell biomass, by-products and carbon dioxide (Table 1). RQ profiles were similar for both conditions (B1L and B1H). They reached a maximum value during the growth phase then started to decrease when oxygen started to deplete. Noticeably, the RQ value of the B1H condition was constant (around 3.5) until the end of the growth phase. In contrast, lower RQ values during the stationary phase were in the range of 1–2. It was more likely that 2,3-BD was produced during both growth and non-growth phases regardless of RQ values. However, higher RQ values during the growth phase caused higher 2,3-BD production rate compared with that of lower RQ values observed in the stationary phase.

In two-step aeration experiments, decreased OTR conditions during the stationary phase were applied. These were B2L with lower aeration and B2H with higher aeration (Table 1). The B2L condition resulted in decreased 2,3-BD concentration compared with the one-stage lower aeration (B1L). However, the B2H condition significantly improved 2,3-BD production and productivity compared with observations under B2L and B1H conditions. No significant differences in the 2,3-BD production yield were observed for all conditions except for B2L where the yield was lower. Results confirmed that oxygen supply during two-stage experiments may be suitable for mixed growth-associated 2,3-BD production. The accumulation of by-products significantly

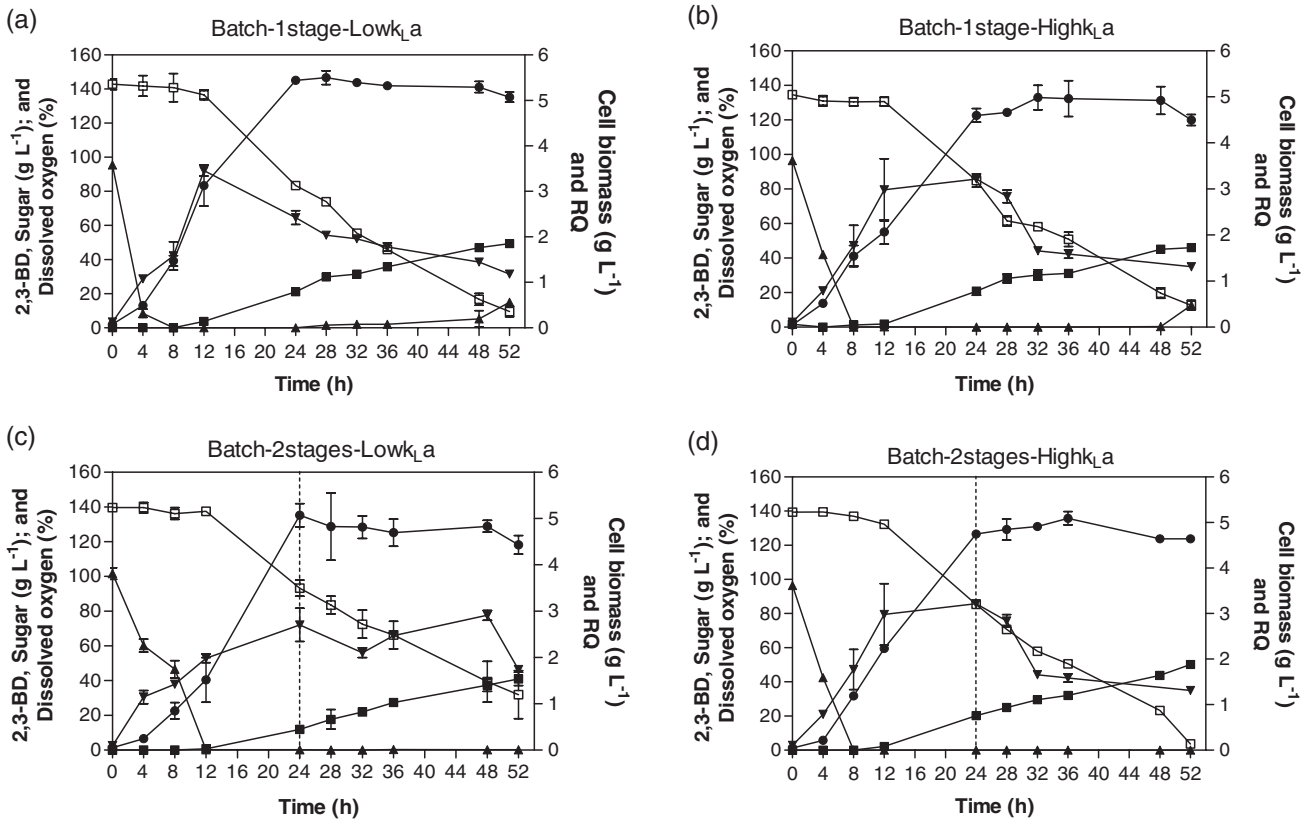


Figure 2. Time course of 2,3 BD production by *K. oxytoca* KMS005 during batch fermentation with different aeration strategies: one-step aeration with (a) low aeration and (b) high aeration, and two-stage aeration with (c) low aeration and (d) high aeration. The symbols represent: 2,3-BD (■), dissolved oxygen (▲), RQ (▼), cell biomass (●), sugar consumed (□). The vertical dashed line represents the change in aeration rate.

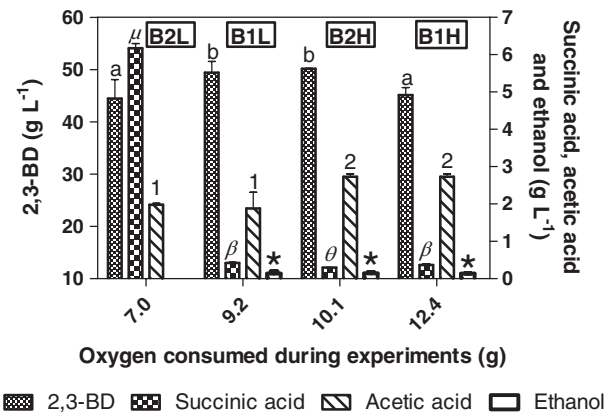


Figure 3. Effect of oxygen consumption on final concentrations of 2,3-BD, succinate, ethanol and acetate during batch fermentation (7.0 for B2L, 9.2 for B1L, 10.1 for B2H, and 12.4 for B1H). Bar graphs bearing different lower case letters, Greek symbols, numbers and asterisks were significantly different between treatments ($P < 0.05$) for 2,3-BD, succinic acid, acetic acid and ethanol, respectively.

increased during the B2L two-stage aeration experiment compared with those in the one-stage aerations.

Figure 3 shows variations in final concentrations of 2,3-BD and by-products (mainly acetate and succinate) depending on total O_2 consumption by the strain KMS005 in batch fermentation. According to these results, B1L appeared to be the best condition among others due to minimizing formation of by-products but maximizing 2,3-BD concentration. However, no significant difference in 2,3-BD production yield (around 0.37 g g^{-1} glucose used) was observed among conditions. For the B1L condition, the OUR value was 6.3 mmol L^{-1} h^{-1}, which was equivalent to a total oxygen consumption of 9.2 g. A k_La of 25.18 h^{-1} was obtained under stirring and aeration of 400 rpm and 0.8 vvm, respectively. The condition obtained in this study was comparable with the findings of Ji *et al.*,[5] in which a k_La value of 26.7 h^{-1} was obtained under stirring and aeration of 300 rpm and 1.0 vvm, respectively. Our findings were also supported by previous reports of the effect of aeration strategy for improvement of 2,3-BD production.[4,5,7] As shown in Table 1, B2H resulted in the highest 2,3-BD concentration at 50.2 ± 0.1 g L^{-1}, while low initial aeration conditions in B2L significantly decreased 2,3-BD production to the lowest concentration of 44.8 ± 5.5 g L^{-1}. Interestingly, the highest 2,3-BD concentration was achieved when total transferred oxygen supplied was only in the range 9.2 to 10.1 g (Fig. 3). The RQ was able to reach the range from 3.5 to 4.5 at 12 h during the growth phase and between 1 and 2 during the stationary phase (Fig. 2(c), (d)). Our results were in an agreement with Zeng *et al.*[6] who reported that the optimum 2,3-BD production was observed at an RQ between 4.0 and 4.5. However, Zhang *et al.*[7] claimed that the RQ should be separately controlled at 1.0–1.5 for cell growth and at 1.8 to 2.0 for 2,3-BD production by *S. marcescens* H30 based on the stoichiometric calculation of sucrose metabolism under anaerobic or micro-aerobic conditions. The different RQ values found in our study and the work of Zhang *et al.*[7] may result from the strains used. In addition, Zhang *et al.*[7] performed 2,3-BD production from sucrose in a complex medium supplemented with peptone and yeast extract while our study employed *K. oxytoca* KMS005 to utilize glucose to

2,3-BD in a simple mineral salts medium without supplementation of complex and rich nutrients. Metabolic responses among strains to different supplemented nutrients might result in different RQ values during 2,3-BD production.

Our finding may imply that the strain KMS005 preferred an oxygen supply at the optimal level ($k_L a \approx 25.2\ h^{-1}$) to drive the 2,3-BD production pathway and to maintain proper regulation of NAD^+/NADH ratio inside the cells. Proper regulation was observed when minimized concentrations of by-products were obtained under appropriate micro-aerobic conditions. The 2,3-BD metabolism by the strain KMS005 become a partially growth-associated product similar to those reported by Wong *et al.*[17] and Chan *et al.*[12] In addition, 2,3-BD was produced at different rates during growth and stationary phases. These observations may be explained with an understanding of enzymatic pathways via mixed acid fermentation between wide type and metabolically engineered strain KMS005. *Klebsiella* strains are microorganisms growing and obtaining energy by respiration and fermentation. Under excessive oxygen supply, the only products from its metabolism are mostly CO_2 and biomass. However, under anaerobic conditions, mixed organic acids and ethanol serve as major products. The strain KMS005 was metabolically engineered via deletions of genes encoding alcohol dehydrogenase (*adhE*), acetate kinase-phosphotrans acetylase (*ackA/pta*), and lactate dehydrogenase (*ldhA*) enzymes. Thus, there are few amounts of by-products such as lactic acid, acetic acid, and ethanol.[10] Even though the transferred oxygen amount was increased to over 9.2 g, increased acetate formations were observed in both B1H and B2H conditions. Syu[16] also reported that further increase in O_2 availability caused higher production of acetic acid. Furthermore, Jantama *et al.*[10] claimed that the production of acetic acid was still detectable, even though acetate kinase-phosphotrans acetylase (*ackA/pta*) genes were successfully deleted in the strain KMS005. This result may postulate the activation of other acetate-producing pathways under micro-aerobic conditions instead. Abdel-Hamid *et al.*[18] stated that pyruvate oxidase (POXB encoded by *poxB*) was necessary for cell survival during the stationary phase under micro-aerobic conditions (Fig. 1). Therefore, this may imply that POXB compensated ACKA-PTA activities and high oxygen supply over 9.2 g stimulated this pathway for acetic acid production in the KMS005 strain.

Unlike acetate, succinate sharply increased when the oxygen amount consumed was lowered to 7.0 g in B2L (the lowest aeration and $k_L a$). In contrast, the succinate level was constant at very low concentration when the amount of total oxygen consumption increased from 9.2 to 12.4 g in the other three conditions (B1L, B2H, and B1H). It suggested that low oxygen level in the B2L condition was preferable to succinate production compared with other conditions, and thus might activate enzymes involving succinate production in the strain KMS005. It was likely that the more aeration increased, the more succinate decreased. Levanon *et al.*[19,20] revealed that malate dehydrogenase (MDH) was usually responsible for succinate production from oxaloacetate by the reductive branch of TCA cycle under anaerobic and micro-aerobic conditions in *E. coli* and other bacteria in the family of *Enterobacteriaceae* including *K. oxytoca*. Then, lower succinate detection in the strain KMS005 might result from very low malate dehydrogenase (MDH) activity under high oxygen supply conditions. In addition, Guest *et al.*[21] suggested that putative fumarate-nitrate reductase (FNR) proteins regulate transcription levels of functional genes in 2,3-BD-producing pathways (*bud* operon). The operon contains a cluster of genes encoding for α-acetolactate synthase, α-acetolactate decarboxylase, and acetoin reductase. FNR also activated genes involved in succinate production anaerobically. In the absence or with low amounts of oxygen (lower than 7.0 g), FNR may down-regulate the expression of *budABC* genes resulting in the highest production of succinate. By increasing the consumed oxygen amount until micro-aeration (9.2 g), more oxygen stimulated α-acetolactate synthase while FNR was down-regulated resulting in low expression for genes involved in succinate production. On the other hand, aerobic supply (more than 10.1 g oxygen) resulted in the succinate production pathway being less active. However, the conditions were more preferable for the activation of acetate-producing pathways while 2,3-BD production declined because of the inactivation of BudABC enzymes by high O_2 levels.

Effects of feeding pattern in fed-batch fermentation

A series of fed-batch experiments was carried out with the objective to increase the final 2,3-BD concentration in comparison with B1L batch experiment by extending the stationary phase beyond 52 h. During all these experiments, aeration was constant ($k_L a = 25.2\ h^{-1}$) while sugar feeding rates, feeding times, and initial sugar concentrations were varied (Table 2).

The first experiment (FB-S140-2.5) was similar to batch B1L except that after 48 h, sugar started to be fed to the culture at a flow rate of 2 g h^{-1} (2.5 mL h^{-1} of stock solution of 800 g L^{-1} of glucose). This corresponded with the sugar consumption rate observed in batch B1L during the stationary phase. The production of 2,3-BD was similar to the B1L condition in the first 48 h (Fig. 4(a)). After sugar feeding, the 2,3-BD production rate was maintained at 0.8 g L^{-1} h^{-1} until 76 h while sugar and biomass concentrations were constant. After 76 h, a decrease in 2,3-BD production rate (0.4 g L^{-1} h^{-1}) was observed. Simultaneously, sugar and DO concentrations increased (data not shown). This indicated that the metabolic activity of cells decreased. Consequently, the global productivity of 2,3-BD was lower than that observed in the batch experiment even though the final concentration and yield were higher (Table 2).

In FB-S140-2.5b, sugar feeding began earlier (at 36 h) and the flow rate was decreased when sugar concentration in the medium increased (Table 2). This resulted in higher production of 2,3-BD during the first part of feeding (until 75 h) thus providing a higher final concentration (77 g L^{-1}) compared with FB-S140-2.5. However, the global productivity was still lower because of decreasing metabolic activity of the cells after 75 h. By calculating the global production rate at time 75 h, when the final concentration was similar to that obtained in FB-S140-2.5 (65 g L^{-1}), the productivity was 0.88 g L^{-1} h^{-1}. However, a 2,3-BD yield of 0.49 g g^{-1} glucose used, which is close to the theoretical maximum of 0.5 g g^{-1}, was obtained. Besides, residual sugar concentrations in these two fed-batches were very high (Table 2). This may cause a decrease in the efficacy of the down-stream process.

For FB-S140-1.25, sugar feeding rate (started at 36 h) was decreased to 1.25 mL h^{-1} to avoid substrate accumulation in the bioreactor. During 36 to 56 h incubation, 2,3-BD production rate was lower than that observed in FB-S140-2.5b (0.66 g L^{-1} h^{-1} for FB-S140-1.25 and 0.9 g L^{-1} h^{-1} for FB-S140-2.5b). After 56 h, the sugar concentration was constant at 20 g L^{-1} (Fig. 4(a)) and the 2,3-BD production rate decreased to 0.25 g L^{-1} h^{-1} at 120 h. Jantama *et al.*[10] found that butanediol dehydrogenase (BudC) activity was up-regulated in the strain KMS005 in medium containing 100 g L^{-1} glucose compared with that in medium containing 50 g L^{-1} glucose. This was due to inactivation of other enzymes involved in NADH re-oxidation including alcohol dehydrogenase

Table 2. Comparison of the performance of fed-batch fermentation for 2,3 BD production with different sugar feeding strategies. All experiments were performed in duplicate

	FB-S140-2.5	FB-S140-2.5b	FB-S140-1.25	FB-S90-2.5	FB-S120-2.5
Max CDW, g L^{-1} (%g g^{-1})[a]	4.5 (3.1)	5.3 (3.4)	4.7 (3.0)	6.1 (3.5)	5.2 (3.5)
Initial sugar (g L^{-1})	140	140	140	90	120
Glucose	2.5	2.5 (36–75 h)	1.25	2.5	2.5
Feeding rate	(48–120 h)	2.0 (75–100 h)	(36–120 h)	(24–76 h)[c]	(20–68 h)[d]
(mL h^{-1})		1.25 (100–120 h)			
2,3-BD g L^{-1}, (%g g^{-1})	$65.3 \pm 2.1^{\beta}$ (45.0)	$77.3 \pm 1.2^{\mu}$ (49.0)	$60.4 \pm 0.9^{\rho}$ (39.0)	$66.6 \pm 3.2^{\beta}$ (37.9)	$74.7 \pm 0.6^{\alpha}$ (50.0)
2,3-BD Yield (g g^{-1})	$0.45 \pm 0.01^{\rho}$	$0.49 \pm 0.00^{\mu}$	$0.39 \pm 0.02^{\beta}$	$0.38 \pm 0.01^{e,\beta}$	$0.50 \pm 0.01^{\mu}$
Overall Prod (g L^{-1} h^{-1})	$0.56 \pm 0.01^{\mu}$	$0.54 \pm 0.02^{\mu}$	$0.51 \pm 0.01^{\rho}$	$0.65 \pm 0.01^{\beta}$	$0.64 \pm 0.00^{\beta}$
Residual sugar (g L^{-1})	$67.3 \pm 2.3^{\beta}$	$77.1 \pm 3.6^{\mu}$	$17.8 \pm 1.5^{\alpha}$	$4.9 \pm 0.1^{\pi}$	$32.4 \pm 0.3^{¥}$
By-product[b], g L^{-1} (%g g^{-1})					
Succinate	$0.4 \pm 0.2^{\mu}$ (0.2)	$0.2 \pm 0.1^{\alpha}$ (0.1)	$0.3 \pm 0.3^{\mu}$ (0.2)	$0.1 \pm 0.1^{\alpha}$ (0.1)	$0.5 \pm 0.1^{\mu}$ (0.3)
Ethanol	$0.6 \pm 0.0^{\alpha}$ (0.4)	$0.3 \pm 0.0^{£}$ (0.2)	$0.7 \pm 0.0^{\alpha}$ (0.4)	$0.1 \pm 0.0^{\mu}$ (0.1)	$0.2 \pm 0.0^{\pi}$ (0.1)
Acetate	$2.6 \pm 0.2^{\pi}$ (1.8)	$2.2 \pm 0.1^{\beta}$ (1.4)	$2.3 \pm 0.2^{\alpha}$ (1.5)	$3.8 \pm 0.1^{\mu}$ (2.2)	$4.3 \pm 0.1^{\rho}$ (2.9)
% Carbon balance	NA	NA	116	90	105

[a] Yields (%g g^{-1}) were calculated as grams of 2,3-BD, cells or by-products produced divided by grams of glucose consumed.
[b] By-products were ethanol, succinate, and acetate. No lactate was detected.
[c] Fermentation time from 76–100 h without sugar feeding.
[d] Fermentation time from 68–116 h without sugar feeding.
[e] All data represented the averages of two fermentations with standard errors. Values bearing different Greek symbols were significantly different between columns ($P < 0.05$).

and lactate dehydrogenase. This suggested that the 2,3-BD production rate may be increased at higher sugar concentrations. High yield and 2,3-BD concentration may be correlated with high residual sugar concentration in which the high glycolytic flux was triggered. Therefore, this might activate enzymes involving NAD^+ regeneration to relieve constraints to prevent halting glucose consumption.

The two last experiments (FB-S90-2.5 and FB-S120-2.5) were performed with the aim to decrease residual sugars. Two strategies were proposed to vary different sugar concentrations during the feeding phase (about 20 g L^{-1} for FB-S90-2.5 and 50 g L^{-1} for FB-S120-2.5). Also, feeding phases were shortened (52 h for FB-S90-2.5 and 48 h for FB-S120-2.5) in which the feeding was started at the end of the growth phase. The fermentation was prolonged to ensure the complete utilization of substrate (Fig. 5). Maximum biomass at 6.05 g L^{-1} was obtained in FB-S90-2.5 g L^{-1}. This was the highest cell concentration among all fed-batch experiments. During the feeding phase, 2,3-BD production rate was 1.0 g L^{-1} h^{-1} along with the residual glucose concentration of 50 g L^{-1} (FB-S120-2.5). In FB-S90-2.5, the 2,3-BD production rate was only 0.72 g L^{-1} h^{-1} while the sugar concentration was 20 g L^{-1}. Therefore, the production rate decreased to 0.69 g L^{-1} h^{-1} for FB-S120-2.5 and 0.4 g L^{-1} h^{-1} for FB-S90-2.5 during the last batch phase (Fig. 5). For FB-S90-2.5, the sugar was almost totally consumed (< 5 g L^{-1}) after 116 h whereas the residual sugar concentration of 32 g L^{-1} was still found in FB-S120-2.5 (Fig. 5; Table 2). Even though, 2,3-BD yield at FB-S120-2.5 approached the theoretical maximum of 0.5 g g^{-1} substrate used; the carbon balance was 105% for this experiment thus indicating a slight experimental error. The approaching of theoretical maximum may result from modification of the 2,3-BD metabolism in the modified strain KMS005 thus changing to partially growth-associated production.[17] A greater 2,3-BD production rate was observed when the sugar concentration in the medium was maintained at 50 g L^{-1} (1.0 g L^{-1} h^{-1}) rather than that at 20 g L^{-1} (0.72 g L^{-1} h^{-1}). Garge and Jain[22] reported that the most commonly-used initial sugar concentrations were in the range of 5 to 10%. Célinska *et al.*[3] also suggested that the range of sugars may be adjusted depending on the type of substrates, inhibitory compounds, and types of media. Remarkably, slight increases in acetate and higher biomass concentrations were also detected in FB-S90 and FB-S120 conditions (Table 2). It can be observed in Fig. 4(b) that the RQ profiles are similar to the batch experiments, with higher values during the growth phase, except for experiment FB-S140.2.5b with the highest residual sugar concentration. This suggested that the feeding substrate in the exponential phase may affect the metabolic activity of cells. This could be supported by the work of Jantama *et al.*[10] They reported that more activations of pyruvate oxidase (PoxB) in which CO_2 and acetyl-CoA are usually generated, and acetolactate synthase (BudB) with butanediol dehydrogenase (BudC) activities in the strain KMS005 were found when a higher sugar concentration was supplied under micro-aerobic conditions during mid and late exponential phases.

Our 2,3-BD concentration was lower than, but 2,3-BD yield was superior to, those of other previous reports.[23,24] They were also higher than some using *K. oxytoca* as producers.[25,26] Nevertheless, all previous works in 2,3-BD production by *Klebsiella* species or other microorganisms were mostly conducted with rich media supplemented with EDTA, yeast extract and casamino acid. Media increased the fermentation cost and caused more steps in the purification process. Unlike the expensive complex nutrients, the simple mineral salts medium with less inorganic nitrogen sources and trace metals used in our study was expected to reduce some obstacles to product recovery.

CONCLUSION

Klebsiella oxytoca KMS005 was optimized to evaluate suitable fermentative parameters for 2,3-BD production under micro-aerobic cultivation. Optimum oxygen supply and sugar feeding profile improved the final concentration and yield of 2,3-BD. Employing a constant k_La at 25.2 h^{-1} under a constant feeding rate

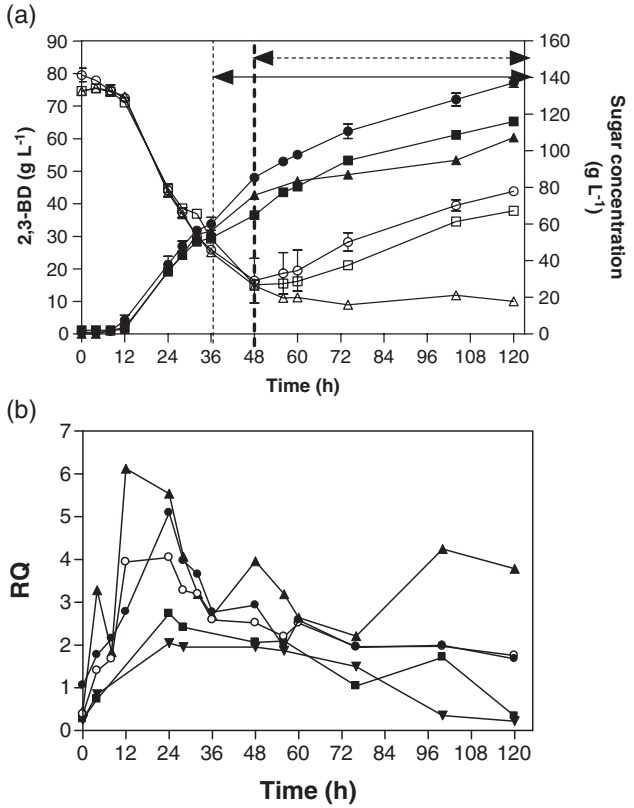


Figure 4. Fermentation profile of (a) 2,3-BD and sugar concentrations, and (b) RQ during fed-batch fermentation with initial sugar concentration of 140 g L^{-1}. The symbols represent (a): 2,3-BD for FB-S140-2.5 (■), FB-S140-2.5b (●), and FB-S140-1.25 (▲); sugar consumption for FB-S140-2.5 (□), FB-S140-2.5b (○), and FB-S140-1.25 (△); feeding time for FB-S140-1.25 and 2.5 b (↔); feeding times for FB-S140-2.5 (←→). The symbols represent (b): FB-S140-2.5 (○), FB-S140-2.5b (▲), FB-S140-1.25 (●), FB-S90-2.5 (▼), FB-S120-2.5 (■).

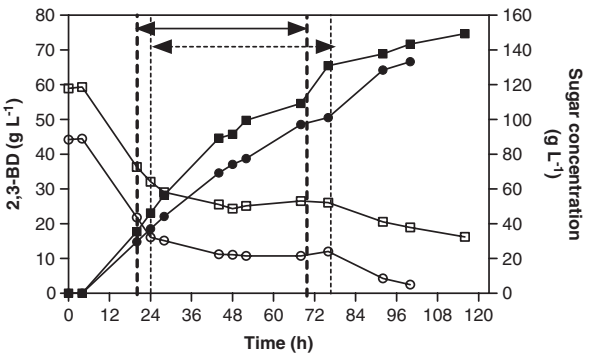


Figure 5. Fermentation profile of 2,3-BD and sugar concentrations during fed-batch fermentation with varied initial sugar concentration and feeding time. The symbols represent: 2,3-BD for FB-S90-2.5 (●), and FB-S120-2.5 (■); sugar consumption for FB-S90-2.5 (○) and FB-S120-2.5 (□); feeding period for FB-S90-2.5 (←→)., and FB-S120-2.5 (↔).

of 2 g h^{-1}, fed-batch fermentation by starting the feeding at the end of the growth phase (FB-S120-2.5) may enhance 2,3-BD production to reach 74.7 g L^{-1} with less residual sugar concentration. In addition, 2,3-BD yield approached the theoretical maximum of 0.5 g g^{-1} substrate consumed using this strategy. The use of a simple medium and fed-batch mode in this study may also reduce the 2,3-BD production costs related to medium preparation, process operation, and waste disposal. Therefore, this study may provide insights into development of fermentative conditions for producing other microbial products by engineered or non-engineered microorganisms under micro-aerobic conditions.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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