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A novel strategy for integrated utilization of Jerusalem artichoke stalk and tuber for production of 2,3-butanediol by *Klebsiella pneumoniae*

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ABSTRACT

Jerusalem artichoke stalk and tuber can serve as a low cost feedstock for the production of 2,3-butanediol. However, like other lignocellulosic materials, the sugar concentration in the hydrolysate prepared from Jerusalem artichoke stalk is too low to be utilized effectively by microorganisms. In this paper a novel strategy was proposed to increase the sugar concentration by adding Jerusalem artichoke tuber into the hydrolysate of the stalk. The sugar was then biotransformed into high-valued 2,3-butanediol by *Klebsiella pneumoniae*. Fed-batch simultaneous saccharification and fermentation (SSF) was effectively performed and 901.2 mmol/l (80.5 g/l) target products (2,3-butanediol plus acetoin) was obtained in 68 h by a stage-shift aeration strategy. The concentration, yield and productivity of target products were 16.9%, 16.8% and 23.4%, respectively, higher than the best results obtained with SSF operated under constant aeration. This showed that adding tuber to the stalk hydrolysate was a useful strategy for increasing the production of 2,3-butanediol from Jerusalem artichoke via fermentation.

1. Introduction

Lignocellulosic materials, such as wood, agricultural residues and herbaceous energy crops, have long been recognized as primary raw materials for biorefinery (Himmel et al., 2007; Ragauskas et al., 2006). The critical issues in the utilization of lignocellulosic materials for the production of biofuels and bio-based chemicals are low concentration of reducing sugars and the presence of toxic compounds in the hydrolysate of lignocellulose (Karimi et al., 2006). Generally, the concentration of total reducing sugar obtained from pretreatment of lignocellulose is about 20–30 g/l, whereas enzymatic hydrolysis of cellulose would yield about 40–50 g/l total reducing sugar (Chandel et al., 2007; Guo et al., 2008; Saha et al., 2005). Although most of the toxic compounds (furfural, 5-hydroxymethyl furfural (HMF) and acetic acid) can be removed by overliming (Carvalheiro et al., 2005; Saha et al., 2005), the detoxification would increase the complexity of the hydrolysis process and result in the loss of sugars (Amartey and Jeffries, 1996; Purwadi et al., 2004). So increasing the reducing sugar concentration in lignocellulose hydrolysate is one of the key problems for high production of cellulose-derived chemicals.

2,3-Butanediol (2,3-BD) is one of the important bio-based chemicals and liquid fuels that exhibits a wide range of potential utilizations in chemicals, foods, medicines, as well as cosmetics (Clinska and Grajek, 2009; Xiu and Zeng, 2008). Acetoin, the precursor of 2,3-BD, can be oxidized to diacetyl which is a high-valued flavoring agent in food products, giving a buttery taste (Clinska and Grajek, 2009). Currently, the microbial production of 2,3-BD from renewable resources is attracting more attention, especially with regard to biorefinery and white biotechnology. Although 2,3-BD production from lignocelluloses has been subjected to much study, only relatively low concentration of 2,3-BD (10–30 g/l) could be obtained by solely using the lignocellulosic materials (Clinska and Grajek, 2009; Grover et al., 1990).

Jerusalem artichoke is one of the low-requirement sugar crops that contain cellulose and hemicellulose in the stalk and a high content of inulin in the tuber, when harvested at maturity (Baldini et al., 2004). Jeruslem artichoke tuber is an excellent source of sugar for the production of ethanol and 2,3-BD, with a product concentration reaching 100–190 g/l for ethanol (Ge and Zhang, 2005; Szambelan et al., 2004) and 44–92 g/l for 2,3-BD (Fages et al., 1986; Sun et al., 2009), much higher than those produced from cellulosic materials. As for the lignocellulosic components in Jerusalem artichoke stalk, there has been little attempt to use it for the production of ethanol and 2,3-BD. In this paper, an integrated utilization of Jerusalem artichoke stalk and tuber for the production of 2,3-BD by *K. pneumoniae* is described. A novel strategy to increase the concentration of sugars in the hydrolysate of Jerusalem artichoke (a lignocellulosic material) was proposed, which combined the tuber with the stalk hydrosylate and used this as a fermentation medium. With this medium as a carbon source, and the use of stage-shift aeration, the final concentration of 2,3-BD was

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greatly improved in fed-batch SSF compared to those obtained from lignocellulosic materials reported in the literature.

2. Methods

2.1. Materials

Jerusalem artichoke stalks were harvested in maturity from Dalian in China at the end of November. Jerusalem artichoke tubers were purchased from a local market. Jerusalem artichoke stalks and tubers were naturally air-dried, milled and then passed through a 60 mesh sieve. The powder obtained was stored at ambient temperature, and used with a stalk to tuber ratio of 3:4 (w/w) in all experiments. Cellulase and xylanase were purchased from Wuxi Xuemei Enzyme (Xuxi, China), and inulinase was donated by Dalian Institute of Chemical Physics, CAS (Dalian, China).

2.2. Dilute acid pretreatment of Jerusalem artichoke stalks

Jerusalem artichoke stalks were soaked in 1% sulfuric acid solution at room temperature for 14 ± 2 h using a stalk to acid ratio of 1:16. The mixture was then pretreated in an autoclave set at 130 °C for 1.5 h, and then filtered to separate the liquid from the solid residue.

2.3. Enzymatic saccharification of Jerusalem artichoke stalks

Water was added to the solid residue above at a ratio of 16:1 (w/w) and the mixture was adjusted to pH 5.0 with $NH_3{\cdot}H_2O$, followed by addition of enzymes (40 U cellulase + 28 U xylanase/g stalk). The mixture was incubated at 50 °C for 24 h with shaking at 100 rpm.

2.4. Dilute acid and enzymatic hydrolysis of Jerusalem artichoke tubers

The acid and enzymatic hydrolysis of Jerusalem artichoke tuber were carried out according to a method optimized by Sun et al. (2009). Acid hydrolysis was conducted with 7% (w/w) H_2SO_4 at 80 °C for 30 min. Enzymatic hydrolysis was carried out for 12 h under the optimum temperature of 55 °C, pH 5.0, and an inulinase concentration of 2.0 U/g substrate.

2.5. Preparation of sugar solutions

Jerusalem artichoke tuber powder and H_2SO_4 were added to the above filtrate (Section 2.2) to further hydrolyze the inulin in the tuber powder (conditions as shown in Section 2.4) to yield sugar solution A. Enzymatic hydrolyis of Jerusalem artichoke stalk was carried out as described in Section 2.3 to yield sugar solution B. Jerusalem artichoke tuber powder and inulinase were added to sugar solution B to hydrolyze the inulin in the tuber powder (conditions as shown in Section 2.4) to yield sugar solution C.

2.6. Microorganism and media

K. pneumoniae CICC 10011 was obtained from China Center of Industrial Culture Collection (Beijing, China). The strain was previously acclimatized with the hydrolysate of combined Jerusalem artichoke stalk and tuber. The acclimatized strain was maintained at −20 °C in the presence of 20% (w/w) glycerol. The medium used for seed culture was the filtrate of sugar solution A, and those used for fermentation experiments were prepared from a combination of sugar solutions (A, B and/or C) without filtration.

2.7. Fermentation experiments

The seed culture was prepared by inoculating 2% (v/v) cell stock into 250-ml shake flasks each containing 80 ml medium. The flasks were incubated at 37 °C and 200 rpm for 24 h (average OD_{650} of about 10). Fermentation experiments included SHF (separate hydrolysis and fermentation) and SSF (simultaneous saccharification and fermentation). The general procedures of fermentation are shown schematically in Fig. 1 and Fig. 2. In SHF processes, Jerusalem artichoke stalk and tuber were first hydrolyzed to sugar solution A, B or C, and then subjected to batch and fed-batch fermentations. In SSF processes, Jerusalem artichoke stalks were first pretreated, followed by partial enzymatic hydrolysis, and then subjected to fed-batch fermentations. All fermentation experiments were carried out in a 5 l stirring bioreactor (BIOTECH-5JG, Shanghai, China) with a working volume of 1.5 l. The seed culture prepared previously was inoculated (5%, v/v) into the fermentation medium, and fermentation was carried out at 37 °C and pH 5.8, invariably maintained by automatic addition of 10% $NH_3{\cdot}H_2O$. The agitation speed was 300 rpm for SHF and 450 rpm for SSF, while the aeration rate was kept at 0.1 vvm for SHF and varied for SSF to investigate the effects of different aeration rates on 2,3-BD fermentation.

The cultivation conditions for SSF with stage-shift aeration were the same as those for SSF with constant aeration, except the aeration rate.

2.8. Analytical methods

The hemicellulose, cellulose, and lignin fractions of Jerusalem artichoke stalk were determined according to the method presented by Van Soest et al. (1963, 1991). Reducing sugars were quantified by the 3,5-dinitrosalicylic acid method (Miller, 1959). Glucose was assayed by a glucose analyzer (Biosensor SBA-50, Shandong, China). Acetic acid, furfural and HMF in the pretreated solution of Jerusalem artichoke stalk were analyzed by high-performance liquid chromatography (HPLC) equipped with an Aminex HPX-87 H column maintained at 55 °C, and eluted with 5 mmol/l sulfuric acid at a flow rate of 0.4 ml/min. The compounds, which were detected by RI detector, were identified and quantified by

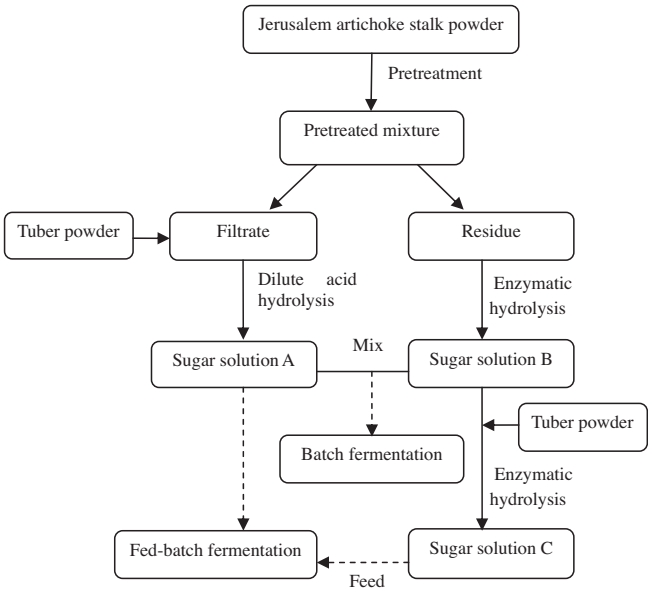

Fig. 1. General procedure for SHF.

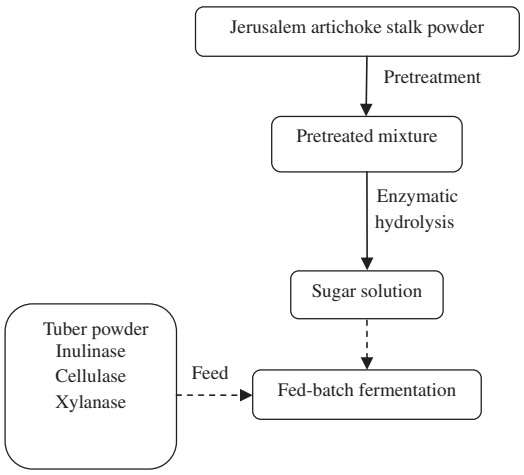

Fig. 2. General procedure for SSF.

comparing with standards. 2,3-BD, acetoin and ethanol were determined by gas chromatography (GC) system (SHIMADZU GC-2010, Japan) with a FID detector. Chromatography was performed using a ϕ5 mm $\times$ 2 m glass column packed with Chromosorb 101 and operated with N_2 as carrier gas at a flow rate of 50 ml/min, and a temperature of 170 °C. The temperature of the vaporizing chamber and detector were set at 200 °C and 220 °C, respectively. Lactic acid, acetic acid and succinic acid in the fermentation broth were analyzed by HPLC (Mu et al., 2006). The viscosity of fermentation broth was quantified by the NDJ-1 rotational viscometer (Shanghai, China).

3. Results and discussion

3.1. Analysis of by-products in pretreated solution of Jerusalem artichoke stalk

Jerusalem artichoke stalk used in this investigation contained (25.6 ± 0.4)% hemicellulose and (43.7 ± 0.6)% cellulose, which accounts for about 70% of the dry weight (Table 1). After pretreatment, 4.01 g/l acetic acid and 0.05 g/l furfural were produced, and the yield of reducing sugar was 40% of the total sugar, and HMF was not detected. It has been reported that 2,3-BD production is stimulated by lower levels of acetic acid, but is inhibited if the levels of acetic acid become relatively high (Nakashimada et al., 2000; Stormer, 1968; Yu and Saddler, 1982). The highest 2,3-BD production was obtained when the culture media contained 5 g/l acetic acid, whereas at 20 g/l acetic acid, growth of *K. pneumoniae* was completely inhibited (Yu and Saddler, 1982). Therefore, 4.01 g/l acetic acid produced during the pretreatment of Jerusalem artichoke stalk might have promoted the production of 2,3-BD in this work. As for furfural, a concentration of 0.2–0.4 g/l, which is about 4–8 times higher than the concentration detected in this pretreated solution of Jerusalem artichoke stalk (0.05 g/l), has been shown to cause a dramatic drop in growth and solvent production (Nishikawa et al., 1988). Thus inhibition of 2,3-BD fermentation by furfural was not observed in this study.

Table 1
Composition of Jerusalem artichoke stalk on the basis of dry solid.

Constituents	% of dry solid
Hemicellulose	25.6 ± 0.4
Cellulose	43.7 ± 0.6
Lignin	14.1 ± 0.3
Extractable constituents	10.7 ± 0.7
Ash	5.9 ± 0.3

3.2. Production of 2,3-BD from Jerusalem artichoke stalk and tuber by SHF

Jerusalem artichoke stalk (240 g) and tuber (320 g) were hydrolyzed to prepare sugar solution A and B, respectively (Fig. 1). Batch SHF was carried out with a mixture of sugar solution A and B as initial carbon sources, and the initial reducing sugar concentration in this mixture was 906.3 mmol/l. By 52 h, 521.6 mmol/l (46.9 g/l) 2,3-BD and 131.0 mmol/l (11.8 g/l) acetoin were obtained from 893.7 mmol/l reducing sugar (Fig. 3). The productivity of the target products (2,3-BD plus acetoin) was 12.6 mmol/(l h) and the yield was 0.73 mol/mol. The yield of products with respect to the combined Jerusalem artichoke stalk and tuber was 0.164 g/g.

Fed-batch SHF was conducted to overcome the inhibition by initial substrate concentration and improve the final concentration of

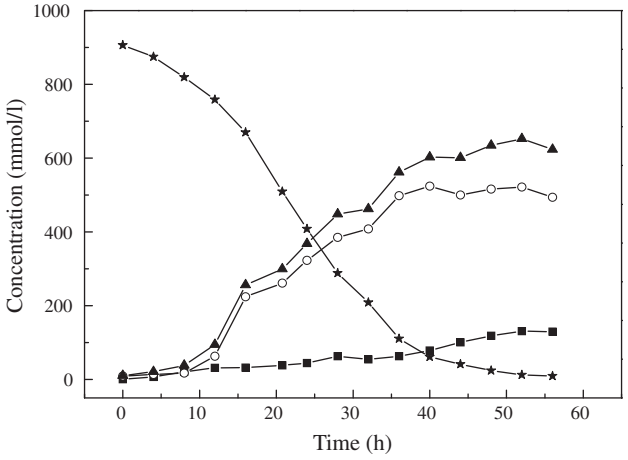

Fig. 3. Time course of batch SHF for 2,3-BD production from Jerusalem artichoke stalk and tuber. Symbols: reducing sugar concentration (★); total concentrations of 2,3-BD and acetoin (▲); concentration of 2,3-BD (○); concentration of acetoin (■).

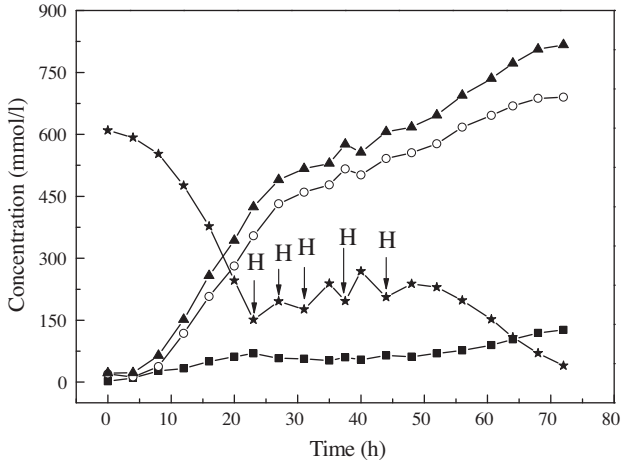

Fig. 4. Time course of fed-batch SHF for 2,3-BD production from Jerusalem artichoke stalk and tuber. H, hydrolysate. Symbols: reducing sugar concentration (★); total concentrations of 2,3-BD and acetoin (▲); concentration of 2,3-BD (○); concentration of acetoin (■).

target products. Jerusalem artichoke stalk (390 g) and tuber (520 g) were hydrolyzed to obtain sugar solution A and C. The concentrations of reducing sugar were 609.4 mmol/l for sugar solution A and 1903 mmol/l for sugar solution C (Fig. 1). Fed-batch SHF was conducted with sugar solution A as an initial carbon source. During the fermentation, 170 ml sugar solution C was fed to the system when the residual reducing sugar concentration was below 250 mmol/l. By 72 h, 690.1 mmol/l (62.1 g/l) 2,3-BD and 126.6 mmol/l (11.4 g/l) acetoin were obtained (Fig. 4). The residual sugar concentration was 40.0 mmol/l and the productivity of target products was 11.3 mmol/(l h). The yield of products with respect to Jerusalem artichoke stalk and tuber was 0.194 g/g.

It was previously reported that 2,3-BD production from lignocellulose hydrolysate was very low. Using the reducing sugar in wood hydrolysate, the concentration of 2,3-BD achieved by *K. pneumoniae* NRRL B-199 was 133 mmol/l (about 0.54 mol/mol yield) (Grover et al., 1990). The initial sugar concentration could be increased by concentrating the wood hydrolysate, but this also led to increased concentration of toxic compounds (Grover et al., 1990). Production of 2,3-BD was most efficient at a concentrations of 56–112 mmol/l for sugars prepared from wood, and decreased when hydrolysates having higher sugar levels were used (Yu et al., 1982). In this study, the initial sugar concentration used in batch fermentation of the combined Jerusalem artichoke stalk and tuber hydrolysate was 906.3 mmol/l, and no inhibition was observed, indicating that it was not essential to perform the detoxification prior to fermentation. The combined hydrolysis of Jerusalem artichoke stalk and tuber not only increased the sugar concentration of the hydrolysate prepared from Jerusalem artichoke stalk, but also save time and energy needed to concentrate the sugar

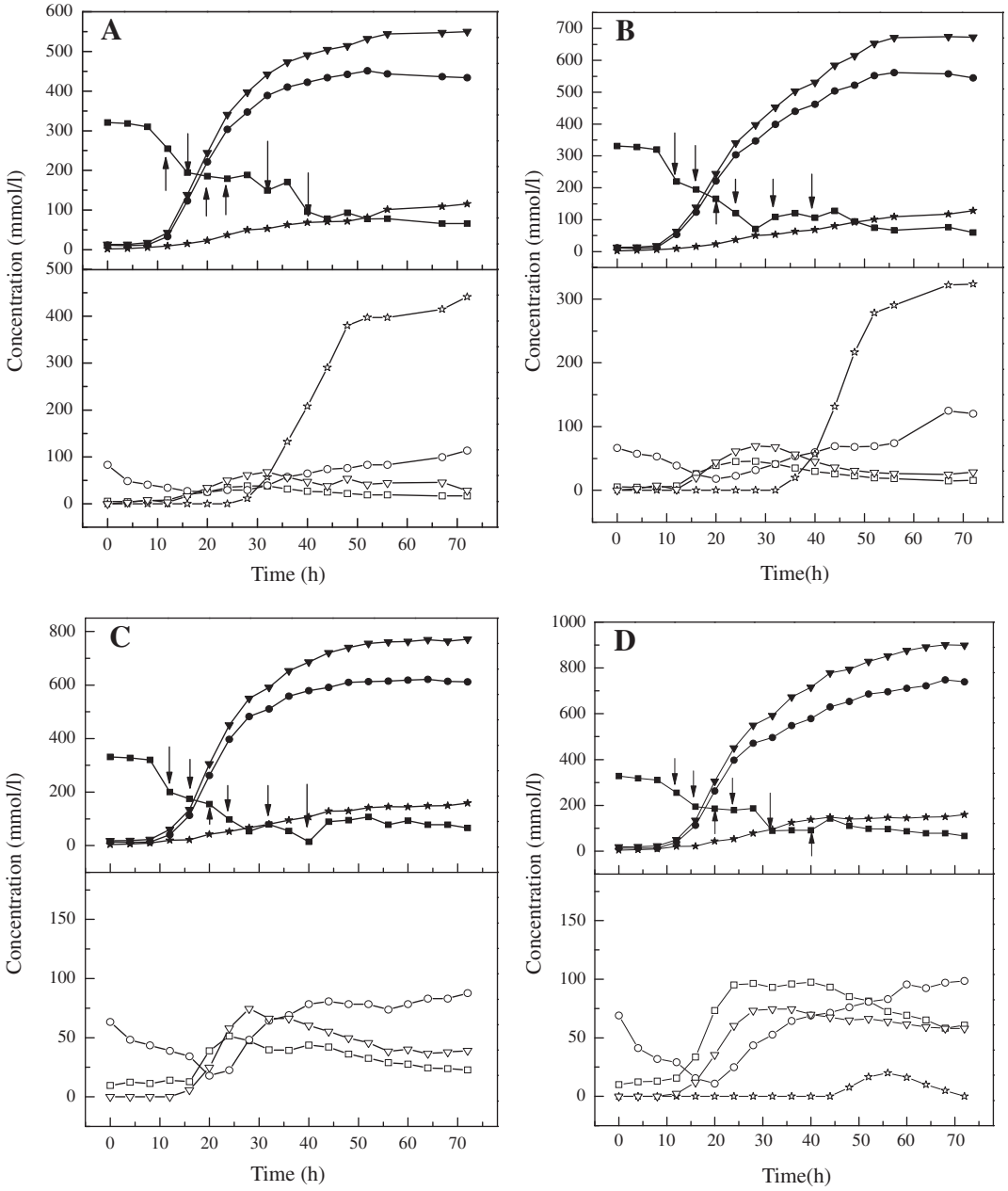

Fig. 5. Time courses of fed-batch SSF for 2,3-BD production from Jerusalem artichoke stalk and tuber at different aeration rates: (A) 0.2 vvm; (B) 0.3 vvm; (C) 0.5 vvm; (D) stage-shift aeration. Symbols: reducing sugar concentration (■); total concentrations of 2,3-BD and acetoin (▼); concentration of 2,3-BD (●); concentration of acetoin (★); concentration of ethanol (□); concentration of acetic acid (○); concentration of lactic acid (☆); concentration of succinic acid (▽).

solution. The results showed that such a strategy was very efficient for solving the issue of low sugar concentration in lignocellulose hydrolysate and low productivity of 2,3-BD from lignocellulose.

3.3. Production of 2,3-BD from Jerusalem artichoke stalk and tuber by fed-batch SSF

Fed-batch SSF was an effective mode to make full use of raw materials and further improve the product concentration. In this work, the fermentation broth was quite viscous (viscosity of 72.8 mPa s) due to the residues from Jerusalem artichoke stalk and tuber. Under such condition, the solubility of oxygen in the fermentation broth is much lower than that in water. Several studies have demonstrated that the concentration of dissolved oxygen is the most crucial parameter for high level 2,3-BD production (Clinska and Grajek, 2009; Jansen et al., 1984; Ramachandran and Goma, 1987). To improve the amount of dissolved oxygen in the viscous fermentation broth, a strategy for enhancing the aeration of the fermentation broth was applied during fermentation.

3.3.1. Effect of different aeration rates on 2,3-BD production

Jerusalem artichoke stalks (210 g) were pretreated and then hydrolyzed by adding 6000 U cellulase and 4200 U xylanase, followed by incubation for 20–24 h. After inoculating with *K. pneumoniae*, 130 g tuber powder was added together with the required nutrients. During the fermentation, 100 U inulinase was added at 12, 16, 20, 24, 32 and 40 h, and 50 g tuber powder was added at 24, 32 and 40 h, and 2400 U cellulase and 1680 U xylanase were added at 12 h. The effects of aeration rates (0.2 vvm, 0.3 vvm and 0.5 vvm) on 2,3-BD fermentation were investigated. The results revealed that aeration rate played a vital role in 2,3-BD production. As shown in Fig. 5(A–C), the concentration of lactic acid changed significantly in this process, which greatly affected the production of target products. As aeration rate increased, the time at which lactic acid could be detected lagged gradually, and the final concentration of lactic acid decreased gradually (441.3 mmol/l at 0.2 vvm, 323.7 mmol/l at 0.3 vvm, not detected at 0.5 vvm), while the final concentration of target products increased gradually (549.8 mmol/l at 0.2 vvm, 674.6 mmol/l at 0.3 vvm, 771.0 mmol/l at 0.5 vvm).

2,3-BD is produced from pyruvate in a mixed acid fermentation process. The ratio of oxygen demand and supply can control the proportions of metabolites produced. Aeration (precisely microaerobic conditions) increases 2,3-BD production. When oxygen is limited, lactic acid and ethanol are usually produced (Converti et al., 2003; Zeng et al., 1990). Unlike lactic acid, the variation in ethanol production was less pronounced at different aeration rates in this study. This may be related to the preference of metabolites in *K. pneumoniae*. When the ethanol pathway was knocked-out in a 2,3-BD producing strain of *K. oxytoca*, the production of 2,3-BD only increased by 6.67% (Ji et al., 2010). By knocking-out the lactic acid pathway in a 1,3-propanediol producing strain of *K. oxytoca*, the production of 1,3-propanediol increased by 45% over that of wild type under both anaerobic and microaerobic conditions, while the production of 2,3-BD was 73% and 39% more than wild type under anaerobic and microaerobic conditions, respectively (Yang et al., 2007). If no lactic acid is produced, the carbon flux used to form lactic acid would switch to form target products. The concentrations of target products were improved by increasing the aeration rate as shown in this study, but higher aeration rates mainly led to the growth of cells at the expense of 2,3-BD production. It was obvious that high concentration and high yield of target products could not be achieved simultaneously by maintaining a constant aeration rate throughout the whole fermentation.

3.3.2. Effect of stage-shift aeration rate on 2,3-BD production

Based on the above results, a stage-shift aeration strategy was performed to reduce the production of lactic acid and maximize the production of 2,3-BD. Other experimental conditions were the same as those in constant aeration fermentation. Aeration rate was changed at the same time when the Jerusalem artichoke tuber power in the fermentation broth was replenished. The aeration rate changes at each time point were 0.2 vvm (0∼24 h), 0.3 vvm (24∼32 h), 0.4 vvm (32∼40 h) and 0.5 vvm (40∼72 h). The effects of stage-shift aeration on the productions of 2,3-BD, acetoin, lactic acid and succinic acid are shown in Fig. 5D.

When stage-shift aeration was adopted, a small amount of lactic acid (20 mmol/l) was produced. After 68 h of fermentation, the concentration, yield and productivity of target products were 901.2 mmol/l (80.5 g/l), 0.259 g/g Jerusalem artichoke and 13.2 mmol/(l h), respectively, which were 16.9%, 16.8%, and 23.4% higher than the best result obtained with constant aeration (Table 2). The results showed that the strategy of stage-shift aeration was efficient for enhancing 2,3-BD production in viscous fermentation broth.

Ji et al. (2009) controlled the oxygen supply in 2,3-BD fermentation by a two-stage agitation, and the concentration, yield and productivity of 2,3-BD were 6.2%, 6.2% and 22.1%, respectively, higher than the best results obtained with constant agitation speeds. However, such a strategy is only suitable for inviscid fermentation broth. For viscous fermentation broth, oxygen supply should be controlled by aeration so as to reach a high level of 2,3-BD production.

The comparison of 2,3-BD production from different modes of fermentation is shown in Table 2. SSF with stage-shift aeration greatly enhanced the concentration and yield of target products compared to SHF, leading to an improved efficiency of 2,3-BD production over conventional SSF, in which constant aeration rate was used throughout the fermentation process. The key feature to a more efficient production of 2,3-BD from Jerusalem artichoke

Table 2
Comparison of 2,3-BD production using different modes of fermentation.

Fermentation modes	Fermentation time (h)	$C^{a}_{2,3\text{-BD}}$ (mmol/l)	$C^{b}_{2,3\text{-BD+acetoin}}$ (mmol/l)	$C^{c}_{\text{lactic acid}}$ (mmol/l)	$P^{d}_{2,3\text{-BD+acetoin}}$ (mmol/l h)	$Y^{e}_{2,3\text{-BD+acetoin}}$ (g/3 g stalks + 4 g tubers)
SHF (batch)	52	521.6	652.6	ND	12.6	1.15
SHF (fed-batch)	72	690.1	816.7	ND	11.3	1.36
SSF (0.2 vvm)	72	433.5	549.8	441.3	7.6	1.11
SSF (0.3 vvm)	72	544.5	674.6	323.7	9.4	1.36
SSF (0.5 vvm)	72	611.7	771.0	ND	10.7	1.55
SSF (stage-shift aeration)	68	748.3	901.2	5.0	13.2	1.81

[a] The concentration of 2,3-BD.
[b] The total concentrations of 2,3-BD and acetoin.
[c] The concentration of lactic acid. ND, not detected.
[d] The productivity of 2,3-BD and acetoin.
[e] The yield of 2,3-BD and acetoin from Jerusalem artichoke stalks and tubers.

therefore, was the changes in aeration employed during the fermentation, which enhanced the dissolved oxygen in viscous fermentation broth.

4. Conclusion

In this study, an integrated utilization of Jerusalem artichoke stalk and tuber was adopted for the production of 2,3-BD by *K. pneumoniae* CICC 10011. By combining sugar materials with lignocellulose hydrolysate, the concentration of reducing sugar in the hydrolysate was greatly increased compared to that of lignocellulose hydrolysate alone. Improved production of target products was obtained, yielding 901.2 mmol/l (80.5 g/l) after 68 h fermentation using SSF process coupled to stage-shift aeration. This represents 16.9% increase over the best result achieved by using constant aeration. This study not only provided a novel strategy to increase the sugar concentration in lignocellulose hydrolysate, but also demonstrated the high effectiveness of stage-shift aeration in improving the efficiency of fermentation for viscous broth.

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