Utilization of Corncob Hydrolysate Enables 2,3-Butanediol Production in *Enterobacter cholerae*

Lizhou Ma,[a,b,#] Jieming Wang,[a,b,#] Rui Zhi,[a,b,#] Lucian A. Lucia,[c,d] Guohui Li [a,b,*] and Yu Deng [a,b,*]

2,3-Butanediol (2,3-BDO) is an important industrial diol that could function in various fields. Currently, there are many substrates used for 2,3-BDO biosynthesis, but studies using green carbon sources such as corncob hydrolysate as a substrate are lacking. As a widely distributed waste lignocellulose-derived substrate, corncob hydrolysate is nutrient-rich and cost-effective. The present study evaluated 2,3-BDO production *via* an *Enterobacter cholerae* strain using corncob hydrolysate as carbon source. Chemical component analysis showed that concentrated corncob hydrolysate contained 233 g/L total sugar and showed no inhibitory effect, but it was beneficial for 2,3-BDO synthesis. Optimization experiments for fermentation resulted in a titer of 47.23 g/L 2,3-BDO with a yield of 0.30 g/g and a productivity rate of 0.66 g/L•h. This study is expected to provide insights for large-scale bioproduction of bulk chemicals utilizing corncob hydrolysates.

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Keywords: 2,3-Butanediol; Enterobacter cholerae; Corncob hydrolysate; Two-stage fermentation; Lignocellulose

*Contact Information: a: National Engineering Research Center of Cereal Fermentation and Food Biomanufacturing, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China; b: School of Biotechnology, Jiangnan University, 1800 Lihu Rd, Wuxi, Jiangsu 214122, China; c: Fiber and Polymer Science Program, North Carolina State University, 2401 Research Drive, Campus Box 8301, Raleigh, NC 27695-8301; d: Department of Forest Biomaterials, North Carolina State University, 2820 Faucette Drive, Campus Box 8005, Raleigh, NC 27695-8005; *Corresponding authors: guohuili@jiangnan.edu.cn, dengyu@jiangnan.edu.cn, # Lizhou Ma, Jieming Wang and Rui Zhi contributed equally*

INTRODUCTION

2,3-Butanediol (2,3-BDO) is an important platform compound with applications in aerospace, food, pharmaceuticals, chemicals, and fuels (Priya and Lal 2019). Along with the increasing problems of crude oil scarcity, rising fossil fuel prices, and the ever-increasing-environmental pollution, the synthesis of 2,3-BDO by biological methods has regained widespread attention (Zhang *et al*. 2016). The main substrates used for the biosynthesis of 2,3-BDO are glucose and glycerol. The problem associated with utilizing glycerol as carbon source is that the metabolic flux through the glycolysis decreases because the uptake rate of the strain for glycerol is slow. The fermentation time is significantly extended, which greatly increases the costs (Aristidou *et al.* 1999; Zawada and Swartz 2005; Zhang and Ye 2009). In contrast, microorganisms utilize glucose more quickly and can produce more of the target product in a shorter period of time. However, the main problem of this method regarding the utilization of glucose is that the price of the raw material is costly, which is not feasible for further large-scale production.

On account of its low cost, sustainability, and vast distribution, lignocellulose is considered to be one of the most promising and environmentally friendly renewable feedstocks to produce bulk chemicals and biofuels (Zhou *et al.* 2021). Lignocellulose generally refers to wood, grasses, and agricultural waste residues (Alonso *et al.* 2012). As the second most abundant agricultural residue in the world (Kumar *et al.* 2018), corncob is rich in cellulose and lignin. Corncob is widely distributed and is of low cost with an annual production of 250 million tons as an agricultural waste (Cao *et al.* 2004; Liu *et al.* 2020). The hydrolysate derived from corncob is a nutrient-rich, cheap, and sustainable carbon source. Corncob hydrolysate (CCH) has been utilized for production of many bulk chemicals such as D-xylonate, succinic acid, *etc*. (Zhao *et al.* 2016; Zhang *et al.* 2019). However, most of the corncobs are either burnt or dumped, causing significant environmental burden (Liu *et al.* 2020). Thus, large-scale utilization of corncob will mitigate the environmental burden and generate considerable economic value. As the global demand of 2,3-BDO is increasing annually, if the large-scale production of 2,3-BDO from lignocellulose-derived feedstock is enabled, it is expected to mitigate the greenhouse gases emission.

This study aimed to produce 2,3-BDO *via* CCH using an *Enterobacter cholerae* strain. The composition of the CCH was analyzed, and the inhibitory effect of the CCH was evaluated. Notably, CCH did not show any negative effect but increased the 2,3-BDO production instead. The initial total concentration was optimized for fermentation, and 10 g/L CCH was selected for further study. Fed-batch fermentation was conducted to scale up the production. After a series of experiments and optimizations, a two-stage feeding strategy was adopted to produce 2,3-BDO. Finally, 47.23 g/L 2,3-BDO was obtained in 72 h. This is the first study to produce 2,3-BDO *via Enterobacter cholerae* strain utilizing CCH as carbon source.

EXPERIMENTAL

Strain and Chemicals

Enterobacter cholerae used in this study was a previously characterized strain (Wang *et al.* 2022). Non-concentrated and concentrated CCH were kindly provided by ECO Environmental Investments Limited (Hong Kong, China).

Culture Medium and Culture Conditions

The media used for shaking flask fermentation were TB medium and LB medium. LB medium used for the seed culture consisted of 10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract. The seed was incubated at 37 °C with agitation at 250 r/min in a 250 mL Erlenmeyer flask with a total volume of 50 mL. TB medium was used for shake flask and fed-batch fermentation, and the composition of this medium was as follows: 12 g/L tryptone, 24 g/L yeast extract, 2.31 g/L KH_2PO_4, and 16.43 g/L $K_2HPO_4{\cdot}3H_2O$. The inoculum volume concentration of shake flask fermentation and seed culture were both 2%. All chemicals were of analytical grade from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Fed-batch Fermentation

The fed-batch fermentation experiments were carried out in a 5 L fermenter (Baoxing, Shanghai, China) with 2 L liquid loading. The fermentation medium was TB

medium, incubation temperature was 37 °C, and stirring speed was 400 r/min. The aeration was 1 vvm, inoculum was 5%, and the pH in the fermenter was adjusted with pure ammonia to keep it between 7.0 and 7.2 during the fermentation.

Analytical Methods

Optical density was measured by UV-visible spectrophotometer (UV-1800, AOE Instruments, Shanghai) at a wavelength of 600 nm (OD_{600}). The supernatant was collected and filtered through an aqueous membrane of 0.22 μm diameter, and the pretreatment filtrate was analyzed *via* high performance liquid chromatography (Agilent, Santa Clara, USA) with an Aminex HPX-87H (300 mm x 7.8 mm) column. The method for analyzing the concentration of each substance in the culture was described previously (Nielsen *et al.* 2010). The detector was a refractive index detector, and the column temperature was kept at 35 °C. The mobile phase was 5 mM H_2SO_4, and the flow rate was 0.6 mL/min.

RESULTS AND DISCUSSION

Evaluation of the Inhibitory Effect of the CCH

Because CCH is a mixture of various compounds, its composition was determined *via* high performance liquid chromatography (HPLC). As shown in Table 1, the CCH was comprised of glucose, xylose, and several fermentation inhibitors including acetate, formic acid, and 2-furfuraldehyde (FUR). These results were similar to other studies (Kim *et al.* 2015; Jampatesh *et al.* 2019). Because fermentation in large fermenters requires a higher content of sugar, a concentrated version of CCH was investigated. It contained a high concentration of glucose and xylose that was comparable to other studies (Kim *et al.* 2015; Suo *et al.* 2019). The high content of glucose provides an advantage when utilizing CCH as carbon source. This is because *Enterobacter cholerae* can mainly consume glucose other than xylose for energy generation and 2,3-BDO biosynthesis. Furthermore, the presence of FUR and acetate in the concentrated CCH was notable, as these inhibitors can severely inhibit cell growth and product synthesis (Jampatesh *et al.* 2019). Besides, there were two unknown compounds present in the CCH at trace level with an estimate total amount of 760 mg/L based on the integrated areas of their peaks in HPLC results.

Table 1. Composition of CCH

Composition	Non-concentrated CCH	Concentrated CCH
Glucose	107.22 g/L	212.35 g/L
Xylose	11.25 g/L	20.65 g/L
FUR	1.72 g/L	4.38 g/L
Acetate	1.59 g/L	8.92 g/L
Formic acid	0.14 g/L	1.53 g/L

FUR is formed during dehydration of pentose, which is the hydrolyzation product of lignocellulosic biomass (Jönsson *et al.* 2013). Acetate is formed by the dehydration of the acetyl group in hemicellulose (Kumar *et al.* 2020). Several studies have demonstrated the negative effect of FUR and acetate during fermentation process (Bernal *et al.* 2016; Suo *et al.* 2019; Watanabe *et al.* 2019). FUR can inhibit cell growth by damaging DNA or by inhibiting the Embden-Meyerhof-Parnas (EMP) pathway through disrupting its associated enzymes. After permeating the cell membrane, acetate can reduce the

intracellular proton gradient, which uncouples the proton-motive force, therefore inhibiting ATP synthesis and cell growth. Additionally, due to high chemical activity of the aldehyde group of FUR, it can be further converted to formic acid by chemical catalysis. Therefore, the inhibitory effect of the CCH was evaluated.

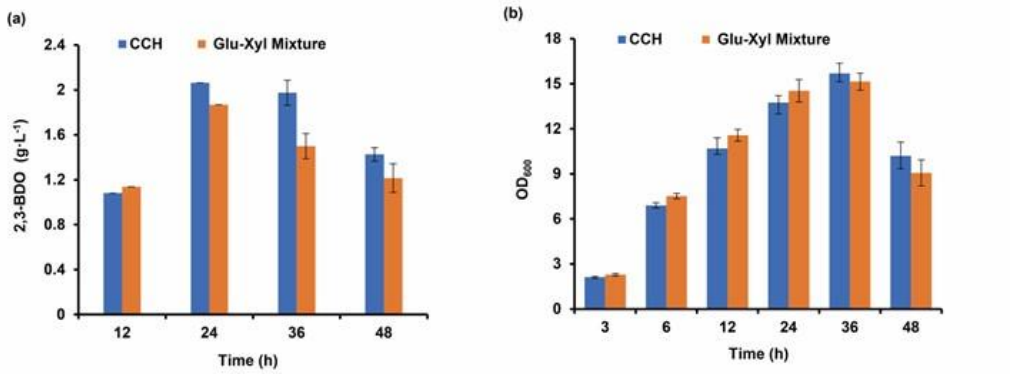

Fig. 1. (a) 2,3-BDO production of CCH (the total sugar concentration of CCH was 10.13 g/L, the concentration of glucose and xylose was 8.88 g/L and 1.25 g/L, respectively.) and control group (Glu-Xyl) (b) OD_{600} of CCH and control group (Glu-Xyl)

Shake-flask fermentation of CCH was conducted utilizing 10 g/L CCH with 10 g/L glucose-xylose (Glu-Xyl) mixture as control. As shown in Fig. 1, the maximum titer of 2,3-BDO and OD_{600} utilizing CCH as carbon source were higher than those of the control. Furthermore, during the process of fermentation, the OD_{600} of CCH group was not lower than the pure glucose-xylose group, suggesting that the inhibitors in CCH did not affect the growth of the strain greatly. Combining these results and recent studies, it can be assumed that the acetate in CCH may be dissimilated by the strain for cell growth, which promotes the synthesis of 2,3-BDO (Bernal *et al.* 2016), or there may be other unknown nutrients that the strain could use. In sum, the results suggest that the inhibitors in CCH did not affect cell growth or 2,3-BDO production, and CCH used in this study could be used for further study.

Effect of Initial Total Sugar Concentration on 2,3-BDO Production

During fermentation, the initial sugar (glucose and xylose here) provides the carbon flux for energy generation, cell growth, and product synthesis (Li *et al.* 2014). Thus, the initial total sugar concentration influences the performance of the strain (Wang *et al.* 2010). The effect of the initial total sugar concentration for 2,3-BDO production and cell growth was investigated. The shake-flask experiment was conducted with different diluted CCH concentration ranging from 5 to 20 g/L. As shown in Fig. 2, with the increase of sugar concentration, the production of 2,3-BDO increased, peaking (3.15 g/L) at 20 g/L glucose. The maximum OD_{600} (17.45) was achieved at the total sugar concentration of 10 g/L and decreased thereafter. Though the maximum titer of 2,3-BDO was achieved at the initial concentration of 20 g/L, the maximum yield of 2,3-BDO was achieved at 10 g/L initial total sugar concentration (Fig. 2a). Taking fermentation cost and cell growth into consideration, the initial total sugar concentration of 10 g/L was selected for further study.

The xylose in CCH was consumed after glucose was completely depleted (Fig. 2b). Generally, without any metabolic engineering regarding xylose-utilizing pathway, *Enterobacter cholerae* could employ the xylose isomerase pathway (XIP) (Bañares *et al.* 2021) to convert xylose to dihydroxyacetone phosphate. This is an intermediate of the glycolysis, which could potentially increase the ability of the strain to produce 2,3-BDO.

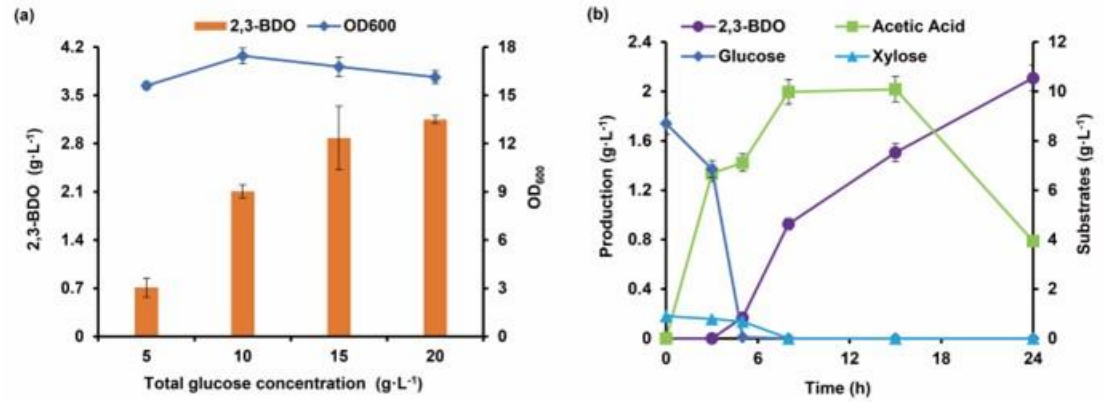

Fig. 2. (a) 2,3-BDO production and OD_{600} with 5-20 g/L CCH. (b) Shake-flask fermentation with 10 g/L CCH (the concentration of glucose and xylose was 8.8 g/L and 1.2 g/L, respectively)

Fed-batch Fermentation Using CCH as Carbon Source for 2,3-BDO

To scale-up the 2,3-BDO production, fed-batch fermentation of both groups was carried out. Glucose group was fed with 500 g/L glucose with a rate of 18 mL/h starting from 8 h. The CCH group was fed with 233 g/L CCH with a rate of 39 mL/h. The difference of the feeding rate between the two groups is to equalize the amount of sugar fed in the fermenter. The results of both groups are summarized in Fig. 3.

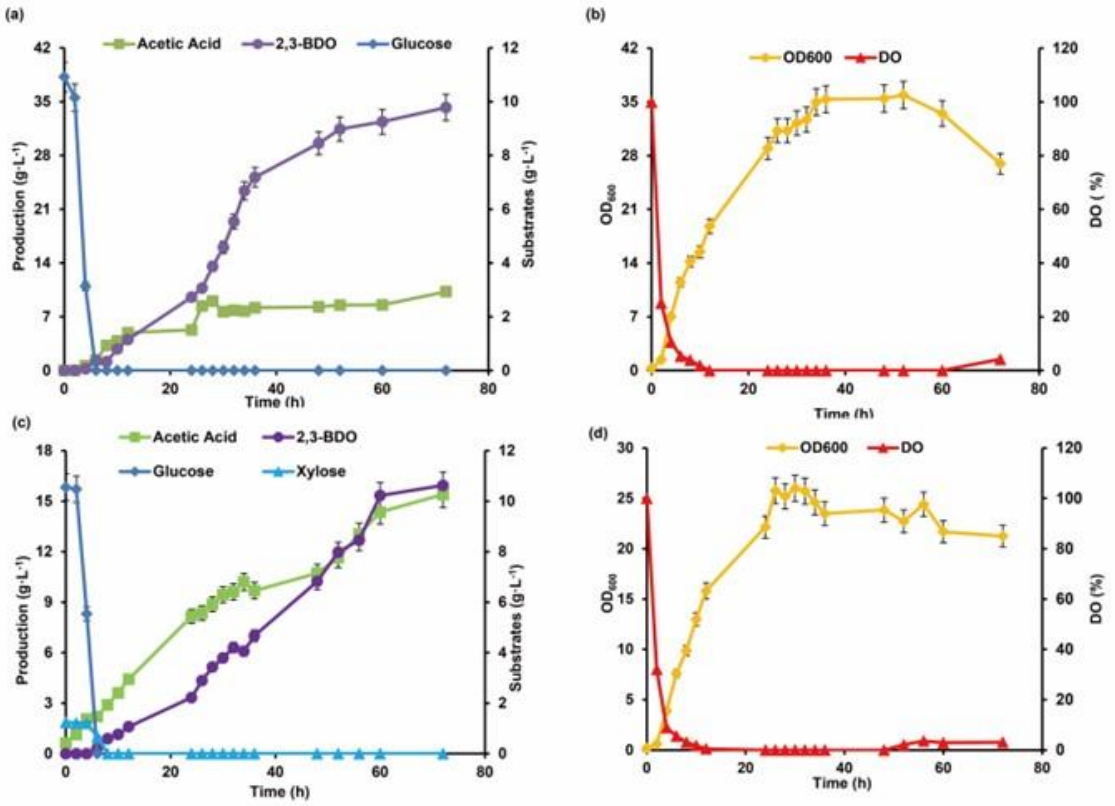

Fig. 3. Fed-batch fermentation of 2,3-BDO utilizing (a)(b) glucose and (c)(d) CCH (the initial total sugar concentration of CCH= 11.79 g/L, the concentration of glucose and xylose was 10.55 g/L and 1.24 g/L, respectively) as carbon source

The glucose group produced 34.23 g/L 2,3-BDO with 10.27 g/L acetate as by-product, while the CCH group produced 15.93 g/L 2,3-BDO, nearly two times less than the glucose group, plus the acetate production reached 15.4 g/L. As the accumulation of acetic acid in the CCH group was nearly 1.5 times greater than that in the glucose group, it is thought that one of the important reasons for the lower yield of target products in the CCH group was the higher concentration of acetic acid, which reduced the accumulation of 2,3-BDO and cell growth (Fig. 3b and 3d). With the preferable amount of sugar (*i.e*., glucose) being fed to the strain, the produced acetate cannot be totally consumed under the fed-batch fermentation (Fig. 3a and Fig. 3c). CCH originally contains acetate, and for these reasons, the acetate accumulation was higher in the CCH group than the glucose group. Therefore, in the fermentation of 2,3-BDO from CCH as a substrate, controlling the production of acetic acid during fermentation is important to improve the yield of 2,3-BDO.

Because high concentrations of acetate can be harmful to cells (Bernal *et al.* 2016), the accumulation of acetate was controlled by stopping the feeding of sugar once acetate exceeded a certain level (Fig. 4). While there was no significant decrease of acetate, less sugar fed in the fermenter caused an imbalance in the C/N ratio. When C/N is low, the strain tends to grow instead of synthesizing the target compound (Chen *et al.* 2009). This effect resulted in the 2,3-BDO titer decreasing to 8.01 g/L with OD_{600} increasing up to 68.6.

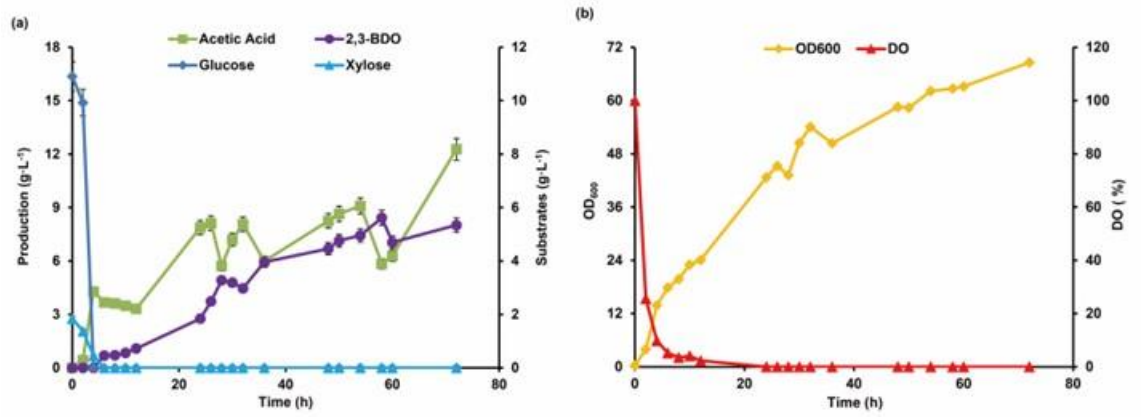

Fig. 4. Intermittent replenishment of controlled acetic acid fermentation (a) Substrate (the initial total sugar concentration of CCH was 12.85 g/L, the concentration of glucose and xylose was 10.91 g/L and 1.94 g/L, respectively.) and product concentrations (b)OD_{600} and DO during fermentation

Considering that reducing the specific growth rate also reduces the production of acetate, the stirring rate was changed at different times to change the specific growth rate and increase the efficiency of 2,3-BDO synthesis (Joshi *et al*. 2021). The culture was first fermented with a low stirring speed of 300 r/min for the first 24 h and the CCH. After 24 h of fermentation, the agitation speed was restored to 400 r/min, and the strain was fed with sufficient glucose. During the 24 h period, the bacteria are in rapid accumulation and sugar is used primarily for cell growth. Rapid bacterial growth leads to large specific growth rates, allowing for greater synthesis of the by-product acetate, so a lower agitation speed and feeding of CCH (total sugar concentration was 223 g/L as determined in the first section) was chosen to maintain normal bacterial growth (Ganjave *et al.* 2022). However, after this, the organism began to enter a formal 2,3-BDO production period, requiring higher agitation rates and the provision of sufficient sugar (500 g/L glucose) for rapid production and accumulation of 2,3-BDO. As shown in Fig. 5, under the above

fermentation strategy, 2,3-BDO production reached 47.23 g/L with a lower acetic acid content (10.84 g/L). At the same time, a productivity of 0.66 g/L/h was achieved, which is the highest reported for 2,3-BDO production by *Enterobacter cholerae* using CCH. Meanwhile, the yield and productivity of *Enterobacter cholerae* was both in the high-level compared with the reports using other microorganisms (Table 2). The results suggest that lower agitation rates and sugars are sufficient to bacterial growth during the cell accumulation and can reduce acetate accumulation throughout the fermentation process. The above fermentation strategy ultimately resulted in a 1.4-fold higher accumulation of 2,3-BDO. Most importantly, the fermentation control strategy has the advantage that the agitation rate and the sugar requirement of the bacterium can be reasonably controlled, so that the method can be used not only for large-scale bio-based chemical production using various lignocellulose hydrolysates, but also for significant energy savings when industrialized.

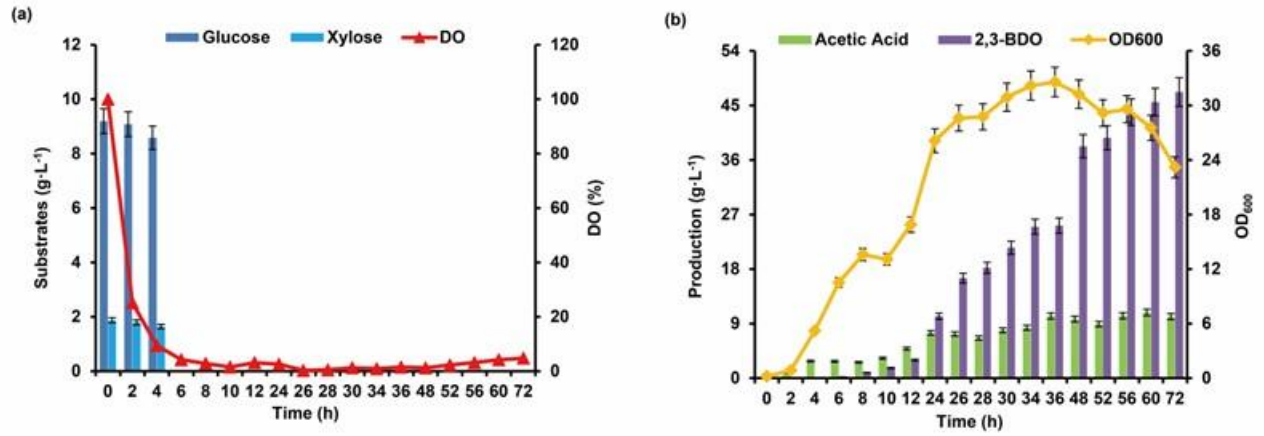

Fig. 5. 2,3-BDO production employing fermentation control strategy (a) glucose, xylose consumption and DO during fermentation, (the initial total sugar concentration of CCH=11.06 g/L, the concentration of glucose and xylose was 9.19 g/L and 1.87 g/L, respectively.) (b) 2,3-BDO, acetate accumulation and OD_{600}

Table 2. 2,3-BDO Production Using Different Substrates

Strain	Substrate	Production ($g.L^{-1}$)	Yield ($g \cdot g^{-1}$)	Productivity ($g \cdot L^{-1} \cdot h^{-1}$)	
Klebsiella pneumoniae	Wood	13.6	0.29	0.28	Grover *et al.* 1990
Klebsiella oxytoca	Corncob	25	0.31	0.36	Cao *et al.* 1997
Klebsiella oxytoca	Xylose	29.6	0.30	1.35	Jansen *et al.* 1984
Bacillus polymyxa ATCC 1232	Cheese whey	5.5	0.25	0.03	Speckman and Collins 1982
K. oxytoca ATCC 8724	Lactose	32.49	0.21	0.86	Ramachandran *et al.* 1990
Enterobacter cholerae	CCH	47.23	0.30	0.66	This study

CONCLUSIONS

1. The composition of corncob hydrolysate (CCH) included glucose, xylose, and a certain amount of fermentation inhibitors. Through comparison of sole glucose and CCH as carbon source for shake-flask fermentation, CCH was found to be beneficial for 2,3-butanediol (2,3-BDO) production.
2. The initial sugar for fermentation process is vital. Therefore, the optimal initial total sugar concentration for 2,3-BDO production was investigated. To balance the fermentation cost, cell growth and 2,3-BDO production, an initial total sugar concentration of 10 g/L with the highest 2,3-BDO yield was eventually selected for shake flask study.
3. In fed-batch fermentation, to increase the production of 2,3-BDO and decrease by-product (mainly acetate) accumulation, a series of strategies were employed. In the attempt to reduce the accumulation of acetate, it was found that the imbalance of C/N ratio can lead to rapid decrease of 2,3-BDO and high cell growth. Then, a titer of 47.23 g/L 2,3-BDO and a productivity of 0.66 g/L·h was obtained by maintaining an appropriate cell growth through adjusting the stirring speed and feeding strategies (*i.e.*, two-stage fermentation).

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