2,3-Butanediol and Acetoin Production from Enzymatic Hydrolysate of Ionic Liquid-pretreated Cellulose by *Paenibacillus polymyxa*

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A safe microorganism (class 1), *Paenibacillus polymyxa,* was used for 2,3-butanediol and acetoin production, which could make the fermentation process cheaper and less complex. It showed a broad substrate spectrum, such as mannose, galactose, cellobiose, glycerol, the mixture of glucose and xylose, and the mixture of glucose and cellobiose. In addition, the strain can utilize highly concentrated glucose that was obtained by enzymatic hydrolysis of ionic liquid-pretreated cellulose. With a 15% initial cellulose consistency, the final glucose concentration was 109.5 g/L with 65.7% glucose yield. Without any treatment, the hydrolysate was successfully used to produce 2,3-butanediol and acetoin with a yield of 81.7% and a productivity of 0.7 g/(L·h) by *Paenibacillus polymyxa*. Higher concentration and higher productivity with relatively high yield, compared with previous works by acid hydrolysis, of 2,3-butanediol and acetoin were achieved. All these novel improvements offer significant opportunities to further decrease the cost of large-scale 2,3-butanediol and acetoin production.

Keywords: Paenibacillus polymyxa; 2,3-Butanediol; Fermentation; Enzymatic hydrolysis; Ionic liquid

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INTRODUCTION

Currently, using biomass to produce chemicals and fuels has received a significant amount of interest due to the forthcoming scarcity of traditional fuels as well as the need for more reasonable uses of food sources (Chen *et al*. 2014; Luterbacher *et al*. 2014). With a heating value of 27,200 J/g, 2,3-butanediol compares favorably with ethanol (29,100 J/g) and methanol (22,100 J/g) for use as a liquid fuel or fuel additive (Xiao *et al*. 2012). 2,3-butanediol also has potential industrial applications in the manufacture of printing inks, perfumes, fumigants, spandex, moistening and softening agents, explosives, plasticizers, foods, and pharmaceuticals as a promising bulk chemical (Garg and Jain 1995). Biological production of 2,3-butanediol on an industrial scale is still in its early stage but with strong prospects of growth (Celińska and Grajek 2009). Nevertheless, it requires efficient and economical fermentation processes and needs improvements based on current research. The strain and raw material utilized in the process are key problems for the high efficient production of biomass-derived 2,3-butanediol.

So far, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*) are absolutely unbeatable with respect to the efficient production of 2,3-butanediol (Ji *et al*. 2011). The biggest advantage of *Klebsiella* sp. is that

it has a broad substrate spectrum, including glucose, mannose, galactose, xylose, arabinose, cellobiose, and lactose. Hence, almost all of the sugars present in hemicellulose and cellulose hydrolysates can be converted to 2,3-butanediol. In addition, *K. pneumoniae* is able to convert glycerol (the main byproduct in biodiesel production) to 2,3-butanediol (Petrov and Petrova 2009, 2010). However, it is important to note that *Klebsiella* sp. is a class 2 (pathogenic) microorganism, which makes it unsuitable for industrial-scale fermentation because of the strict safety regulations and increasing cost of the process (Jurchescu *et al.* 2013; Li *et al.* 2013). Therefore, there is an urgent need for class 1 (nonpathogenic) microorganisms. *P. polymyxa* is a nonpathogenic strain and known to produce 2,3-butanediol. Apart from monosugars present in biomass, *P. polymyxa* can ferment carbohydrate polymers such as xylan, inulin, and starch, and it can secrete xylanase, inulase, and α-amylase, which could degrade those polymers to monosugars simultaneously (Kawazu *et al.* 1987; Hespell 1996; Gao *et al.* 2010). *P. polymyxa* has a wide range of properties, including nitrogen fixation, plant growth promotion, and soil phosphorus solubilisation. It also helps in bioflocculation and in the enhancement of soil porosity (Lal and Tabacchioni 2009). It has many applications after fermentation. Therefore, *P. polymyxa* could be the most suitable choice for commercial production of 2,3-butandiol.

The substrate accounts for more than half of the total production cost of 2,3-butanediol (Celińska and Grajek 2009; Ji *et al.* 2011). Consequently, it is necessary to develop an economical fermentation process based on low-cost raw materials. On the other hand, separation of 2,3-butanediol from fermentation media is also one of the economic barriers to the commercial microbial production of 2,3-butanediol (Ji *et al.* 2011). Highly concentrated 2,3-butanediol can cut the cost of downstream separation. Because the final 2,3-butanediol concentration is directly proportional to the initial sugar concentration, highly concentrated fermentable sugars are required for practical applications. As the most abundant, cheap, and renewable potential source of sugars, lignocellulose is a promising feedstock for a biorefinery. Acid hydrolysates of lignocelluloses (*e.g.*, corncob, *Jatropha* hulls, and sugarcane bagasse) as raw materials have been used for 2,3-butanediol production (Cheng *et al.* 2010; Jiang *et al.* 2012; 2013a,b). However, the total reducing-sugar concentration obtained from lignocelluloses is about 20 to 30 g/L (Guo *et al.* 2008; Cheng *et al.* 2010; Jiang *et al.* 2012). Therefore, this process is always accompanied by a concentration step with high energy consumption to achieve relative highly concentrated reducing-sugar (about 80 g/L) and 2,3-butanediol (about 35 g/L). This process also needs detoxification before fermentation. To obtain biomass hydrolysates with high sugar concentration without inhibitors is the key for the efficient production of biomass-derived 2,3-butanediol. Many studies have indicated that enzymatic hydrolysates are free of inhibitors of microbial metabolism. By combined pretreatments of dilute acid and ionic liquid (IL), the glucose yield of sugarcane bagasse reached 95.5% after enzymatic saccharification (Jiang *et al.* 2013b). The enzymatic hydrolysate of IL-pretreated cellulose thus seems to be a suitable candidate for 2,3-butanediol fermentation.

In this work, *P. polymyxa* (a class 1 microorganism) was selected for the production of 2,3-butanediol and its precursor acetoin, which could make the fermentation process cheaper and less complex. Fermentation of different widely used sugars and glycerol were investigated to assess their feasibility for 2,3-butanediol and acetoin production. For practical uses, the enzymatic hydrolysis of IL-pretreated cellulose was further studied. The hydrolysates obtained were subsequently fermented to 2,3-butanediol and acetoin.

EXPERIMENTAL

Materials

Microcrystalline cellulose was purchased from Sigma-Aldrich (Shanghai), milled to a size of less than 150 mesh, and dried in an oven at 60 °C for 24 h before use. 1-Butyl-3-methylimidazolium chloride ([BMIM]Cl) (purity 99%) was purchased from Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences (Lanzhou, Gansu). Ionic liquid-pretreated cellulose was obtained as follows: first, 4 g (4 wt%) of microcrystalline cellulose was added to a 500 mL flask containing 100 g of [BMIM]Cl. Then, without removing air, the flask was sealed with a cork and placed in an oil bath at 110 °C with stirring at 400 rpm for 1 h. Deionized water at 90 °C (400 mL) was added to the mixture to precipitate cellulose with vigorous shaking for 10 s. The regenerated cellulose was then transferred to a 1000-mL beaker, washed with 75 °C deionized water (700 mL) thoroughly 15 times by filtration to remove IL, freeze-dried (EYELA 1200 freeze dryer, Tokyo Rikakikai Co., Ltd.), and recovered as IL-pretreated cellulose for hydrolysis. Glucose, xylose, mannose, galactose, arabinose, and cellobiose (purity > 98%) were purchased from Bomei Biotech Co., Ltd., Heifei, Anhui. Glycerol, 2,3-butanediol, acetoin, and acetic acid (purity > 98%) were purchased from Sigma-Aldrich (Shanghai). The strain *Paenibacillus polymyxa* (CICC 10010), used for fermentation, was purchased from China Center of Industrial Culture Collection (Beijing).

Methods

Fermentation of different substrates and highly concentrated glucose

The culture was maintained on a Luria-Bertani agar slant at 4 °C. The seed medium was composed of glucose (10 g/L), peptone (10 g/L), beef extract (10 g/L), and NaCl (5 g/L) with a pH of 6.8 to 7.0. For seed preparation, a full loop of strain from a fresh slant tube was inoculated in a 250-mL flask containing 50 mL of fresh seed medium in a rotary shaker at 200 rpm for 12 h at 30 °C. Seed culture (5%, v/v) was then inoculated into the fermentation medium. The fermentation of different substrates was carried out in the following medium: substrates (24 to 36 g/L), peptone (5 g/L), beef extract (5 g/L), K_2HPO_4 (12.55 g/L), and KH_2PO_4 (3.9 g/L). Highly concentrated glucose was fermented in the following medium: glucose (80 to 160 g/L), peptone (8 g/L), beef extract (8 g/L), K_2HPO_4 (12.55 g/L), and KH_2PO_4 (3.9 g/L). All substrates were sterilized at 115 °C for 15 min alone. All fermentation experiments were carried out in 250 mL flasks loaded with 50 mL of sample solutions at 200 rpm and 30 °C.

Enzymatic hydrolyses

IL-pretreated cellulose was sterilized at 115 °C for 20 min. Enzymatic hydrolyses were carried out in a 100-mL Erlenmeyer flask containing sodium citrate (50 mM, pH 4.8) reaction buffer at 50 °C with shaking at 90 rpm in a rotary shaker. The samples were hydrolyzed with the mixture of cellulase (Celluclast 1.5L®) and cellobiase (Novozyme 188) (Sigma-Aldrich) at a weight ratio of 4:1. The activity of the mixture (cellulose:cellobiase = 4:1) was determined according to the National Renewable Energy Lab method (Andey and Baker 1996). Enzymatic saccharification experiments were carried out at different IL-pretreated cellulose concentrations (5 to 15% w/v) with 50 FPU/g cellulose.

Fermentation of enzymatic hydrolysate

The enzymatic hydrolysate was harvested and centrifuged to remove the un-hydrolyzed residue, and the supernatant was used for fermentation studies. The pH was adjusted to 7.0 with H_3PO_4 or KOH. Then, the hydrolysate was supplemented with 8 g/L peptone, 8 g/L beef extract, 12.55 g/L K_2HPO_4, 3.9 g/L KH_2PO_4, and 5% (v/v) seed culture.

Analytical methods

The samples were withdrawn at regular intervals and centrifuged at 10,000 rpm for 5 min, and the supernatant was analyzed. Concentrations of glucose, xylose, 2,3-butanediol, acetoin, acetic acid, and ethanol were measured using high-performance liquid chromatography (LC-20A, Shimadzu; Japan) fitted with a refractive index detector and Aminex HPX-87H column (Bio-Rad; USA) at 60 °C, with 0.005 M H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. Each product was calibrated by its standard solutions with four different concentrations (*e.g.*, 1, 2, 3, and 4 g/L). All the standard calibration curves obtained had R-squared values > 0.998. Glucose yield (wt%) was defined as:

$$\text{Glucose yield (wt\%)} = (\text{mass of glucose/mass of cellulose}) \times 0.9 \times 100\% \quad (1)$$

Both 2,3-butanediol and its precursor acetoin were considered the target products in 2,3-butanediol production and expressed as target product yield (%):

$$\text{Target product yield (wt\%)} = \frac{\text{mass of 2,3 butanediol} + \text{mass of acetoin}}{\text{mass of initial substrate} - \text{mass of final substrate}} \times 2 \times 100\% \quad (2)$$

Three parallel replicates were performed, and the reported values are averages.

RESULTS AND DISCUSSION

Fermentation of Substrate Solutions

Solutions (24 g/L) of glucose, xylose, mannose, galactose, arabinose, cellobiose, and glycerol were fermented for 20 h to assess the capacity for 2,3-butanediol and acetoin production. As summarized in Table 1, the strain had a wide substrate range and could utilize almost all of these single substrates to produce 2,3-butanediol and acetoin. A small amount of residual xylose (5.5 g/L) and glycerol (2.3 g/L) and a large amount of residual arabinose (22.8 g/L) were found after fermentation. The highest 2,3-butanediol and acetoin production (7.2 g/L) was obtained from glucose, and the lowest (0.3 g/L) was from arabinose. Acetic acid and ethanol were the main by-products. It is important to note that the strain can utilize glycerol as raw material to produce 2,3-butanediol and acetoin (3.8 g/L). A large amount of glycerol is produced during the manufacturing of biodiesel from plant oils in a weight ratio of about 10% biodiesel (Yang *et al.* 2012). It was projected that the world biodiesel market would reach 37 billion gallons by 2016, which implies that approximately 4 billion gallons of crude glycerol would be produced (Anand and Saxena 2012). The strain could ferment glycerol to 2,3-butanediol and acetoin (3.8 g/L) and ethanol (5.2 g/L). The mixture of ethanol and 2,3-butanediol could be a good liquid fuel (*e.g.*, heating value of 27,660 J/g for their equimolar mixture). Therefore, the presence of ethanol would not affect the use of 2,3-butanediol as fuel (Yu and Saddler 1982).

Table 1. Fermentation of Different Single Sugars

Substrates	Residual substrates (g/L)	2,3-Butanediol and Acetoin(g/L)	Acetic acid (g/L)	Ethanol (g/L)
Glucose	0	7.2±0.08	1.0±0.04	1.7±0.05
Xylose	5.5±0.4	4.8±0.05	2.1±0.03	1.1±0.08
Galactose	0	6.5±0.03	0.6±0.1	1.8±0.03
Mannose	0	6.9±0.03	1.1±0.1	1.5±0.03
Arabinose	22.8±0.1	0.3±0.01	0.05±0.01	0
Cellobiose	0	6.9±0.07	1.4±0.07	1.6±0.03
Glycerol	2.3±1.3	3.8±0.3	0.3±0.05	5.2±0.6

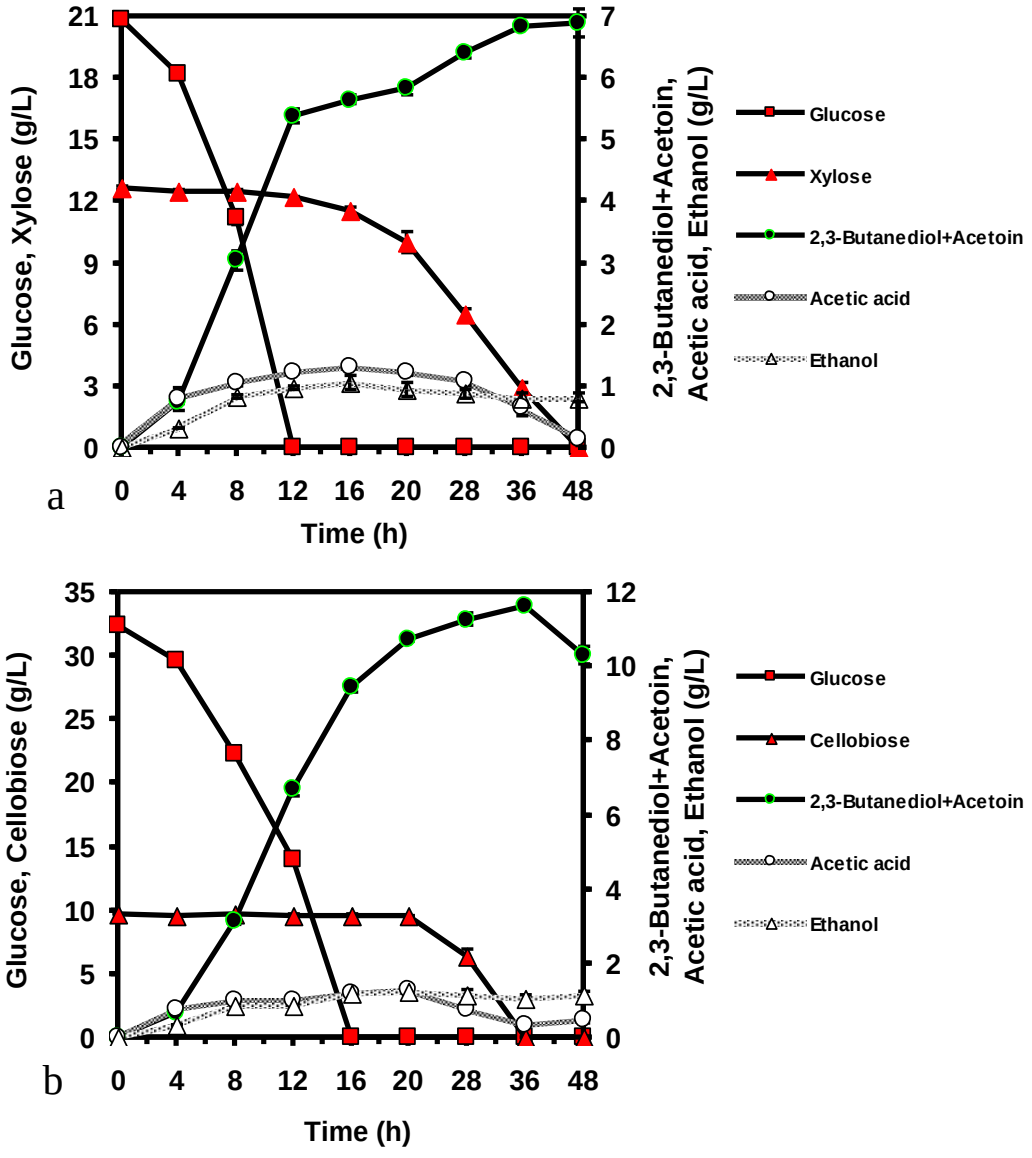

Fig. 1. Fermentation of sugar mixtures as model hydrolysate of hemicellulose and cellulose: (a) glucose and xylose mixtures; and (b) glucose and cellobiose mixtures

Glucose and xylose are the main components of lignocellulose hydrolysate. Glucose and cellobiose are the main components of cellulose hydrolysate. Therefore, the mixture of glucose (20.8 g/L) and xylose (12.6 g/L) and the mixture of glucose (32.4 g/L) and cellobiose (3.3 g/L) as their model hydrolysates were also evaluated for their feasibility for fermentation. In Fig. 1a, all glucose was consumed after 12 h of fermentation, after which the strain began to utilize xylose. In Fig. 1b, all glucose was consumed after 16 h of fermentation, after which the strain began to utilize cellobiose from 20 to 36 h. The strain gave priority to glucose consumption before xylose and cellobiose. Hence, the strain has a broad substrate spectrum and almost all of the sugars and their mixtures present in biomass hydrolysate were converted to 2,3-butanediol and acetoin.

Fermentation of Highly Concentrated Glucose

The above experiments showed that glucose was the most suitable raw material for 2,3-butanediol production. The concentration and yield changed with initial glucose concentrations ranging from 80 to 160 g/L (Fig. 2). As the amount of glucose increased, an increase in the yield was observed. A relatively high 2,3-butanediol and acetoin concentration was obtained (about 53 g/L) after 72 h with initial glucose concentrations of 120 or 140 g/L. A further increase in glucose concentration to 160 g/L led to a decrease in target product production (50.8 g/L). The lowest yield (77.4%) was obtained from 160 g/L glucose after 48 h. However, yields and productivities were always relatively high (77.4% to 90.1%) (Fig. 2). Taking 120 g/L glucose as an example, after 48 h of fermentation, 52.6 g/L total concentration of 2,3-butanediol and acetoin was obtained, giving a yield of 90.1% and a productivity of 1.1 g/(L·h). To make the fermentation process economical and practical, the fermentation of cellulose hydrolysate with highly concentrated glucose was further studied.

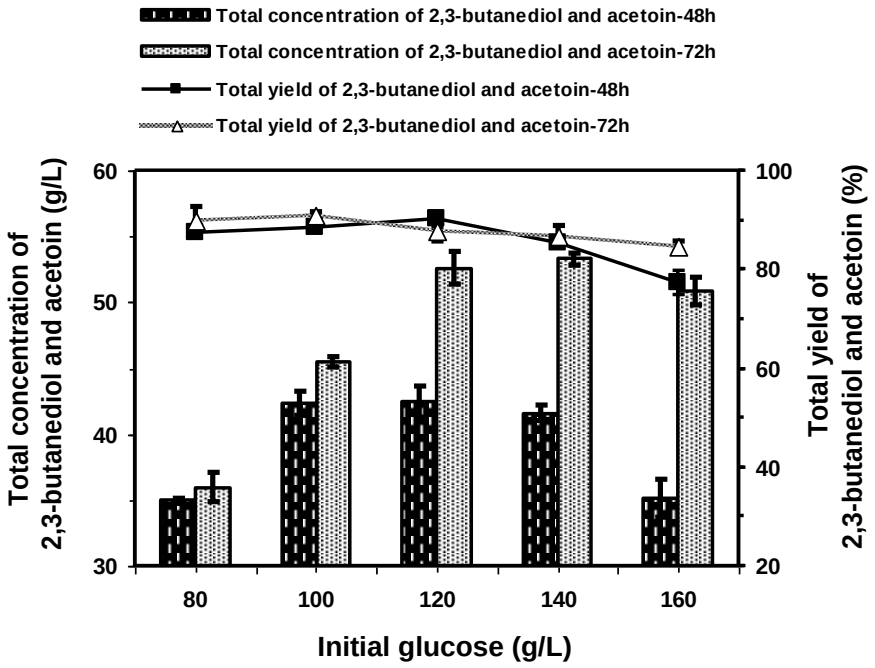

Fig. 2. Fermentation of highly concentrated glucose for 2,3-butanediol and acetoin production

Enzymatic Hydrolyses of Cellulose

Higher concentration and higher yield of glucose were achieved from IL-pretreated cellulose, compared with un-treated cellulose by enzymatic hydrolysis in previous work (Jiang *et al.* 2013b). To obtain cellulose hydrolysate with high glucose concentration without inhibitors, high IL-pretreated cellulose concentration was used for enzymatic hydrolysis. A series of batch experiments was performed using IL-pretreated cellulose with concentrations of 5%, 10%, and 15% (Figs. 3a-3c).

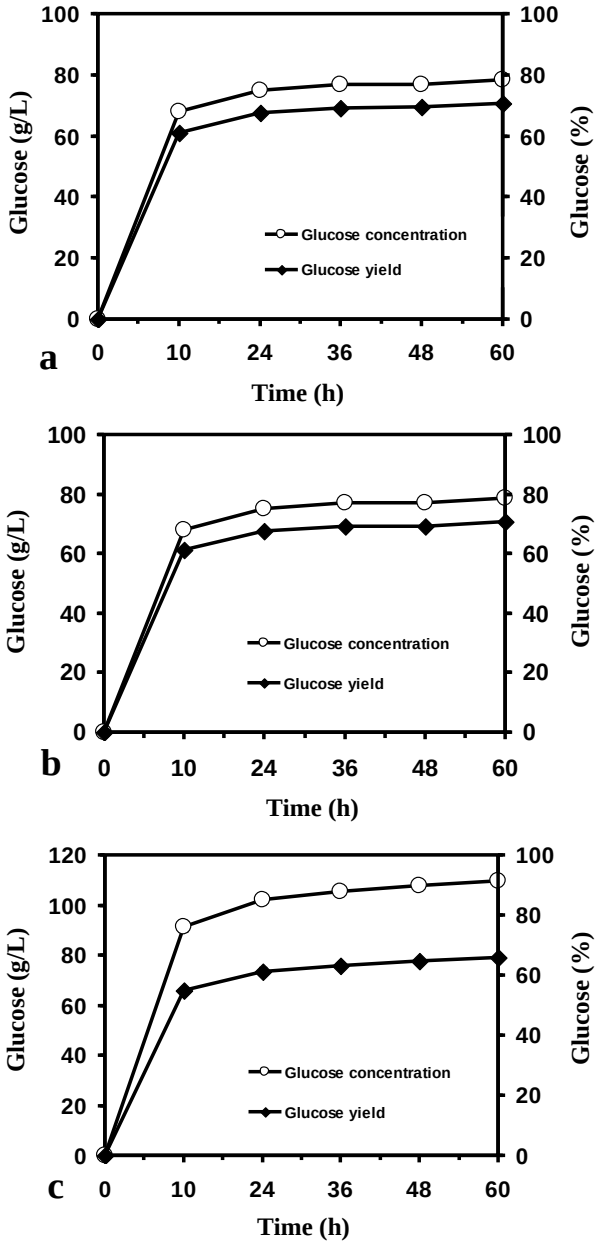

Fig. 3. Glucose concentration and yield released during the enzymatic hydrolyses of IL-pretreated cellulose at (a) 5%; (b) 10%; and (c) 15% initial substrate consistencies.

The maximum glucose concentration at 5% cellulose loading was 50.0 g/L with 90.0% glucose yield (Fig. 3a), that at 10% cellulose loading was 78.5 g/L with 70.7%

glucose yield (Fig. 3b), and that at 15% cellulose loading was 109.5 g/L with 65.7% glucose yield (Fig. 3c). The results indicated that as cellulose concentration increased, glucose concentration rose, but its yield decreased. This yield drop may be attributed to the product inhibition, improper heat and mass transfer, and the thermal deactivation of enzymes (Hodge *et al.* 2009). Similar results were reported in a previous work (Gupta *et al.* 2012) dealing with the enzymatic hydrolyses of delignified *P. juliflora* wood (with 4% sodium chlorite). With a substrate concentration of 15%, the maximum glucose concentration in that study was 90.07 g/L. In this work, with a substrate concentration of 15%, the maximum glucose concentration of hydrolysis was 109.5 g/L. Therefore, the glucose concentration and yield were obviously higher than those of the previous work.

Fermentation of Enzymatic Hydrolysate of Cellulose

Without concentration and detoxification, the enzymatic hydrolysate was fermented with *P. polymyxa*. With an initial glucose concentration of 97.9 g/L, a maximum of 40.0 g/L target product (2,3-butanediol and acetoin) was obtained after 60 h of fermentation, giving a yield of 81.7% and a productivity of 0.7 g/(L·h) (Fig. 4). With an initial glucose concentration of 100 g/L, 42.3 g/L target product was obtained after 48 h of fermentation, giving a yield of 88.6% and a productivity of 0.9 g/(L·h). The concentration and yield of the final product from enzymatic hydrolysate had similar results to that from glucose.

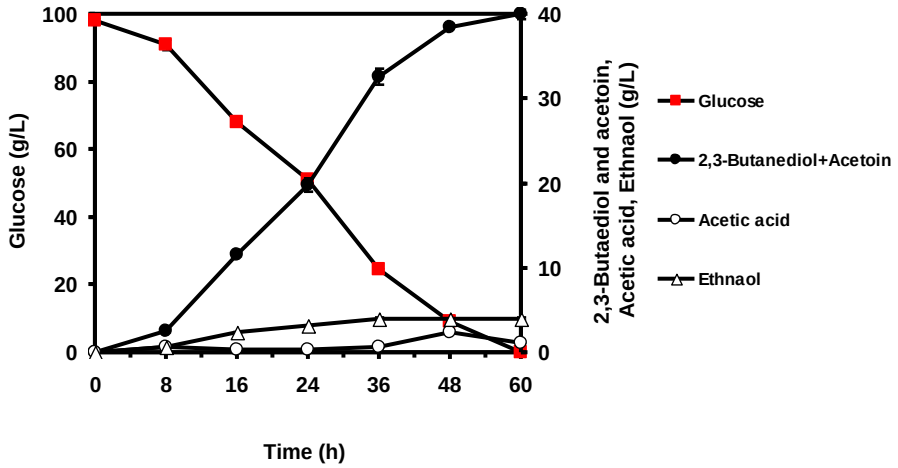

Fig. 4. Fermentation of enzymatic hydrolysate of IL-pretreated cellulose at 15% initial loading

In past decades, dilute sulfuric acid hydrolysis has been the main technique for synthesis of fermentable substrates from lignocellulosic biomass for 2,3-butanediol and acetoin production (Table 2). In dilute acid, hemicellulose and amorphous cellulose are easily and nearly completely hydrolyzed to fermentable sugars accessible to microorganisms for biofuel production. Wood acid hydrolysate has been used for 2,3-butanediol production, obtaining 13.3 g/L with a yield of 58% and a productivity of 0.28 g/(L·h) (Grover *et al.* 1990). Corncob acid hydrolysate, after concentration and detoxification by boiling, overliming, and activated charcoal adsorption, has been used as a feedstock. After 60 h of fed-batch fermentation, a maximum of 35.7 g/L target product was obtained, giving a productivity of 0.59 g/(L·h) and the highest product yield (100%)

reported so far (Cheng *et al*. 2010). Microcrystalline cellulose, IL-pretreated cellulose, IL-pretreated *Jatropha* hulls, water-washed *Jatropha* hulls, and hemicellulose of sugarcane bagasse have all been hydrolyzed by dilute sulfuric acid; after concentration and detoxification by boiling, overliming, and activated charcoal adsorption, those hydrolysates were also utilized for 2,3-butanediol fermentation (Jiang *et al.* 2013a,b) with 57.2 to 83.2% yield. Unfortunately, during acid hydrolysis, secondary decomposition of sugars not only caused raw material waste but also brought some toxic derivatives (*e.g*., 5-hydroxymethylfurfural and furfural), which could seriously affect bacterial fermentation. Furthermore, the concentration of sugars contained in dilute acid hydrolysate was relatively low (about 20 to 30 g/L *vs.* 109.5 g/L in this work). Although sugars could be concentrated by vacuum evaporation, and inhibitors could be removed by detoxification, those processes consumed high energy, which would greatly increase the overall process cost. In this work, enzymatic hydrolysate from IL-pretreated cellulose with high glucose yield containing a high concentration of glucose (109.5 g/L) without inhibitors was utilized for 2,3-butanediol and acetoin production. Higher concentration and higher productivity with a relatively high yield were achieved. However, these advantages need to be counterbalanced by the high cost associated with ILs. To overcome this difficulty, it is necessary to develop effective recovery and recycling methods and reduce energy consumption for recycling ILs. Then, new ILs should be designed to improve the capacity of lignocellulose dissolution at lower temperatures within shorter times.

Table 2. 2,3-Butanediol and Acetoin Production by Fermentation using Various Biomass Hydrolysates

Substrates	2,3-Butanediol + Acetoin			References
	Concentration (g/L)	Productivity (g/(L·h))	Yield (%)	
Wood acid hydrolysate	13.3	0.28	58.0	Grover *et al.* 1990
Corncob acid hydrolysate	35.7	0.59	100.0	Cheng *et al.* 2010
Cellulose acid hydrolysate	32.41	0.54	81.22	Jiang *et al.* 2013a
Acid hydrolysate of IL-pretreated cellulose	33.49	0.56	83.20	Jiang *et al.* 2013a
Acid hydrolysate from water washed *Jatropha* hulls	20.70	0.35	57.20	Jiang *et al.* 2013a
Acid hydrolysate from IL-pretreated *Jatropha* hulls	24.13	0.40	66.58	Jiang *et al.* 2013a
Acid hydrolysate from sugarcane bagasse	26.2	0.6	72.2	Jiang *et al.* 2013b
Enzymatic hydrolysate from IL-pretreated cellulose	40.0	0.7	81.7	This study

CONCLUSIONS

1. *Paenibacillus polymyxa*, a safe microorganism, was selected and used successfully for the production of 2,3-butanediol and its precursor acetoin.
2. The strain showed a wide substrate spectrum for sugars, their mixtures, and glycerol. Furthermore, it could utilize highly concentrated glucose (120 g/L) with a high yield (90.1%).
3. Highly concentrated glucose (109.5 g/L) without inhibitors was obtained from ionic liquid-pretreated cellulose by enzymatic hydrolysis.
4. The cellulose hydrolysate without any concentration and detoxification was further successfully used as raw material for 2,3-butanediol and acetoin fermentation. Higher concentration and higher productivity with a relatively high yield were achieved.

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