Butanol Fermentation by *Clostridium saccharobutylicum* Based on Poplar Wood

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As a potential source of liquid fuels, lignocellulosic material is an alternative to plant-derived starch and sugar, which are urgently needed to meet global demands for food. The utilization of wood as feedstock for bioconversion to biobutanol fuel not only could reduce production costs, but also could increase raw material supply. However, little is known about biobutanol fermentation based on lignocellulosic material from wood. In this paper, biobutanol fermentation from poplar wood hydrolysates by *Clostridium saccharobutylicum* was investigated under three different conditions. The desired biobutanol and ABE yields reached 6.98 and 9.64 g/L, respectively, and 69.8 g biobutanol and 96.4 g ABE per kg of poplar wood were achieved. Fermentation of hydrolysates with no additives and with extra mixed carbon sources to biobutanol was also studied. The predicted results were confirmed: in the former, the production of biobutanol and ABE were 4.88 and 6.63 g/L, respectively; in the latter, the biobutanol and ABE yields reached 7.28 and 10.18 g/L, respectively. The results indicated that poplar wood is a potential renewable raw material suitable for biobutanol production, and that *Clostridium saccharobutylicum* BAA-117 is a promising biobutanol producer for such conversion.

Keywords: Lignocellulose; Poplar wood; Anaerobic fermentation; Biobutanol

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INTRODUCTION

Due to sharply rising energy demands, the rapid depletion of non-renewable fossil fuels, serious environmental pollution, and global warming, the development of a renewable and alternative liquid biofuels is important. Butanol has numerous advantages over ethanol: it is less caustic to motor parts, has lower saturated vapor pressure, has similar energy density (29 MJ/L) as that of gasoline (32 MJ/L), and has much higher energy density than ethanol (16 MJ/L). It can be added into a gasoline engine directly without any alteration and can be transported in existing pipelines (Lee *et al*. 2008). Moreover, it can reduce hydrocarbon and nitrogen oxides emissions by 95 and 37% relative to gasoline, respectively (Bellido *et al*. 2014). At present, utilizing accessible, renewable, low-cost substrate sources for the fermentative production of biobutanol has been a primary goal of industrial biotechnology research (Tippkötter *et al*. 2014). A variety of renewable feedstocks such as sugar cane, corn stover, wheat stalk, barley straw, straw, and wood can be used to produce biobutanol (Sanchez and Cardona 2008). Wood has been identified as a promising potential feedstock because of its wide availability (Tippkötter *et al*. 2014).

China is a country with abundant forestry resources where forested area covers 208 million hectares (Tan *et al*. 2015). Poplar is one of the main plantation-planted tree species in China, wherein the planting scale is the largest in the world (Liang *et al*. 2006). Poplar wood is an abundant, cheap, and readily available source for the production of biofuels. At present the poplar price is about $30/dry ton in China.

Whether or not biobutanol production *via* microbial fermentation can replace chemical synthesis processes depends on the cost of the raw materials and the efficiency of the conversion technology, including materials and solvents (Qureshi *et al*. 2008; Sun and Liu 2012). Biobutanol is produced by *Clostridia* in an acetone-butanol-ethanol fermentation process, so-called "ABE fermentation". The ratio of A-to-B-to-E is 3:6:1, and butanol is typically a major product.

Two of the most common and thoroughly researched *Clostridia* are *Clostridium acetobutylicum* and *Clostridium beijerinckii*, which are recognized as high biobutanol producers (Rajagopalan *et al*. 2013). There is an added advantage that *Clostridia* can utilize both hexoses and pentoses to produce ABE. The main sugars released from lignocellulosic biomass after pretreatment and hydrolysis fall into these two classes. *Clostridium saccharobutylicum* is the fourth *clostridial* species, which was first used for ABE production in the late 1970s. It can use a wider range of substrates to produce ABE. The complete genome sequence of the representative strain *Clostridium saccharobutylicum* NCP262 (ATCC BAA-117) was determined (Poehlein *et al*. 2013). Bellido *et al*. (2011) considered that the efficient utilization of the fermentable sugars from lignocellulosic biomass is the key of an economic feasibility of biofuel production method. *Clostridial* species cannot directly transform lignocellulosic biomass into biobutanol until the poplar wood has been pretreated and subjected to hydrolysis techniques to degrade the polymeric structure and its original composition, releasing various organic compounds that can be further used as the fermentation substrate to produce biofuels.

Although plenty of studies have already been performed using a wide range of agro-industrial residues, such as corn stover, wheat straw, rice straw, and sweet sorghum bagasse (Cai *et al*. 2013; Chen *et al*. 2013; Ranjan *et al*. 2013; Bellido *et al*. 2014; Qureshi *et al*. 2014; Wen *et al*. 2014), in butanol fermentation processes, the use of wood as a feedstock for the production of biobutanol has been little studied. Sun *et al*. (2012) used concentrated sugar maple hemicellulosic hydrolysate to produce n-butanol by ATCC 824. Tippkötter *et al*. (2014) utilized enzymatic hydrolysis of beech wood lignocellulose as a substrate for biobutanol production.

There has been a general lack of reporting about using poplar wood to produce biobutanol *via* ABE fermentation. Cho *et al*. (2013) used yellow poplar as a raw material through alkaline prehydrolysis, enzymolysis, and fermentation to produce ABE. Sultanova *et al*. (2011) converted willow and poplar waste into the butanol by three cultures of solventogenic *Clostridia*. The aim of this study was to further determine whether or not poplar wood could be utilized as a feedstock for conversion to biobutanol through ABE fermentation by *Clostridium saccharobutylicum*. The study was focused on comparing butanol and ABE production under different conditions by *Clostridium saccharobutylicum* BAA-117, which is able to ferment sugars from both cellulosic and hemicellulosic fractions.

EXPERIMENTAL

Raw Material

Poplar wood was acquired from a forestry area in the north of Hunan, China. The poplar wood was cut into small pieces of 3 to 5 cm long, 3 to 5 cm wide, and 1 to 2 cm thick. Then these small pieces were slurried using steam explosion pretreatment. The moisture content of raw materials was about 30% at the time of steam explosion. Cellulose content is about 35% in pretreated biomass. The slurry was collected and dried as the raw material for enzymatic hydrolysis.

Enzymatic Hydrolysis

Enzymatic hydrolysis of pretreated poplar wood was performed in stirred tank reactors containing 10% (w/w) dry solids. A mixture of cellulase (Celluclast 1.5 L, enzyme activity 75.8 FPU/mL, β-glucosidase enzyme activity 38.5 IU/mL) and β-glucosidase (Novozym 188, enzyme activity 421.0 IU/mL) were added, for which the enzyme concentrations were 3.79 FPU/g and 10.34 U/g of pretreated biomass, respectively. The enzymatic hydrolysis conditions were 150 rpm mechanical agitation, 50 °C, pH 5.5, and 72 h of reaction time. The pH was adjusted by the addition of 1-M NaOH. After hydrolysis, samples were withdrawn, centrifuged, filtered, and stored for fermentation and HPLC analyses.

Microorganism

The organism used in this study was *Clostridium saccharobutylicum* BAA-117, obtained from the American Type Culture Collection. The strain was maintained on Reinforced Clostridial Medium in Hungate tubes in spore form and conserved at -80 °C.

Media

The growth medium used for the pre-cultures contained 10 g/L of beef extract, 10 g/L of tryptone, 3 g/L of yeast extract, 5 g/L glucose, 3 g/L $C_2H_9NaO_5 \cdot 3H_2O$, 5 g/L NaCl, 0.5 g/L L-cysteine hydrochloride, and 1 g/L soluble starch. The medium was autoclaved at 121 °C for 15 min.

The modified TYA medium was used as a seed medium and contained 25 g/L glucose, 3.0 g/L tryptone, 1.5 g/L yeast extract, 3.0 g/L CH_3COONH_4, 0.37 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L KH_2PO_4, 0.015 g/L $FeSO_4 \cdot 7H_2O$, and 4.45 g/L $CaCO_3$. The medium was autoclaved at 121 °C for 15 min.

There are three different fermentation media: (1) the poplar hydrolysates, which contain 50 g/L of total reducing sugars; (2) the poplar hydrolysates, which contain 50 g/L of total reducing sugars and other medium components: (I) nitrogen source: 1.5 g/L yeast extract; (II) mineral solutions 3.0 g/L CH_3COONH_4, 0.37 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L KH_2PO_4, 0.015 g/L $FeSO_4 \cdot 7H_2O$, and 4.45 g/L $CaCO_3$; and (3) the poplar hydrolysates, which contain 30 g/L of total reducing sugars with an extra 20 g/L of mixed sugars (11 g/L glucose, 7.8 g/L xylose, and 1.2 g/L arabinose) and other medium components. The carbon source and nitrogen source were sterilized separately at 121 °C for 15 min. Mineral solutions were filter-sterilized using sterile 0.22-μm membrane filters.

Fermentation Assays

Pre-cultures were grown in 150-mL serum bottles under anaerobic conditions. The serum bottles were filled with preboiled growth medium and flushed with N_2. Before

inoculating the preculture with the first post-sporal Hungate-tube culture, a heat-shock was performed for 90 s at 80 °C to stimulate the germination of spores, and the contents were then transferred into fresh growth medium at 37 °C for 16 to 18 h. After microorganism growth, the samples were transferred into the seed culture medium. Subsequently, 15 mL of active cultures were inoculated into the fermentation medium. Unless otherwise stated, all experiments were carried out in 250-mL screw-capped bottles containing 150 mL of the fermentation medium or different concentrations of carbon sources. The headspace of the screw-capped bottles was purged with filtered nitrogen to remove traces of dissolved oxygen. The pH of the medium was adjusted to 6.0 using 1-M NaOH or 1-M HCl. The fermentation culture was initiated by inoculating 15-mL active cells previously grown in the seed medium. Cultures were incubated at 37 °C without shaking in an incubator, and samples were collected regularly for the analysis of sugar, butyric acid, acetic acid, and ABE contents. All experiments were carried out as biological triplicates. The whole cultivation process was under anaerobic conditions.

Analytical Methods

The concentrations of sugars and acids were determined by HPLC (HPLC-1210, Shimadzu, Japan). The detector was based on refractive index measurement. An Aminex HPX-87H column was used to quantify the glucose, xylose, acetic acid, and butyric acid concentrations. 0.2-M H_3PO_4 was used as the mobile phase at a flow rate of 1 mL/min and the temperature was maintained at 30 °C.

ABE contents were determined using the internal standard method with a gas chromatograph (GC) system (GC-2010, Shimadzu, Japan) equipped with a flame ionization detector (FID). A, B, and E were separated in an Agilent HP-INNOWAX capillary column (30 mm×0.25 mm×0.25 μm). The column temperature was maintained at 45 °C for 1 min and increased in increments of 5 °C/min to 155 °C and the final temperature was held for 2 min. The injector and detector temperatures were maintained at 180 and 200 °C, respectively. Biobutanol was used as the internal standard and the carrier gas was nitrogen gas. All samples were centrifuged at 5000 rpm for 10 min and filtered before being analyzed.

RESULTS AND DISCUSSION

Direct Butanol Fermentation from Poplar Wood Hydrolysates without Additive

Lignocellulose includes mainly cellulose, hemicellulose, and lignin. The cellulose and hemicellulose can be degraded into pentose and hexose, respectively (glucose, xylose, arabinose, *etc*.), for use in fermentation. Shake-flask anaerobic fermentation with *Clostridium saccharobutylicum* BAA-117 was carried out to produce ABE without additives and with only poplar wood hydrolysates with a total reducing sugar concentration of 50 g/L as the sole substrate.

The fermentative butanol production reaction process over 72 h is shown in Fig. 1, and the concentrations of the solvents, sugars, and organic acids during fermentation are illustrated. As can be observed, the productions of butanol and ABE were low, at 4.8 and 6.6 g/L, respectively. The total reducing sugar concentration was consumed quickly within 48 h and remained almost constant afterwards. The residual sugar concentration was 11.78 g/L at the end of fermentation, and the utilized sugar percentage was 74.83%. Acetic acid

was present in the medium at a concentration of 0.73 g/L after 24 h, and its concentration remained the same after 72 h of fermentation. Butyric acid was also detected in the medium at the end of the process at a concentration of 0.85 g/L.

The direct fermentation of poplar wood hydrolysate to biobutanol was successful, and *Clostridium saccharobutylicum* BAA-117 could be used as a promising biobutanol producer. However, the yield of biobutanol and ABE was not high through this type of direct fermentation. The limited nutrients available in the poplar wood hydrolysates influenced the growth of the microbial cell (Fig. 4), such that the fermentation process was slow and the substrate (reducing sugars) could not be fully utilized. Similarly, it has been shown that yeast extract and mineral supplements (such as K_2HPO_4, NaCl, and $MgSO_4$) have significant positive effects on biobutanol production by *Clostridium* species (Survase *et al*. 2012; Al-Shorgani *et al*. 2013). From the results presented, it was deduced that biobutanol production is dependent on some essential nutritional components in addition to the carbon source in the fermentation medium.

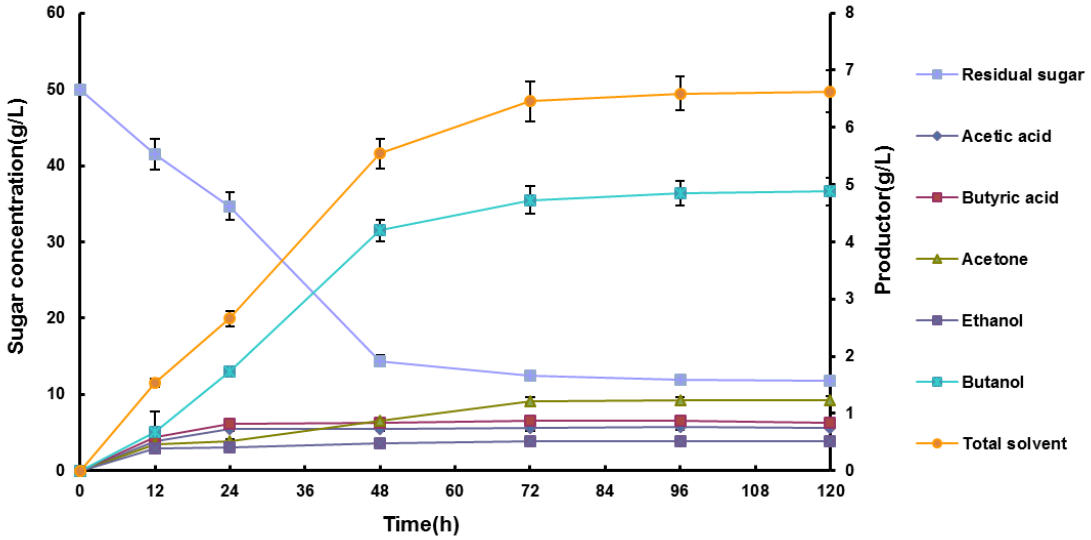

Fig. 1. Direct fermentation of poplar wood hydrolysates to biobutanol with no additives (First mode). The figure shows the concentration of organic acids and solvents (acetone, butanol, ethanol) as a function of fermentation time.

Fermentation of Poplar Wood Hydrolysates with Other Medium Components

In contrast to the direct fermentation of poplar wood hydrolysates, adding a nitrogen source and mineral solutions to the fermentation medium increased the accumulated biobutanol in the 250-mL broth to 6.98 g/L following the 72-h fermentation. As shown in Fig. 2, the acidogenic phase was observed within the initial 24 h culture, during which acetic acid and butyric acid were predominantly produced. Biobutanol and total solvent rapidly accumulated in the beginning of the solventogenic phase. Previous studies have shown that the beginning of the solventogenic phase is triggered by butyric acid when its titer reaches 2.0 g/L, which is characterized by the organic acids' reassimilation and ABE formation (Cheng *et al*. 2012; Liu *et al*. 2013). At the end of the solventogenic phase, 9.64 g/L of ABE was obtained, containing 1.73 g/L of acetone, 0.93 g/L of ethanol, and 6.98 g/L of biobutanol. This is equivalent to 69.8 g of biobutanol and 96.4 g of ABE per 1 kg of poplar wood raw material. The proportions of ethanol, acetone, and biobutanol were 1:1.8:7.5. The residual sugar concentration was 7.1 g/L, and its

utilized percentage was 85.8%, far higher than found by previous experiment (74.83%). The yield of total solvent and biobutanol were 0.19 g/g and 0.14 g/g, respectively, and the concentration of total acid was 1.37 g/L (0.69 g/L butyric acid and 0.68 g/L acetic acid) (Fig. 2, Table 1). Previous studies have indicated that the nitrogen source and mineral solutions (containing buffer medium) can enhance carbohydrate utilization and the final biobutanol concentration (Bryant and Blaschek 1988). $CaCO_3$ had a notably positive effect on the production of ABE, which could be ascribed to the buffering effect of carbonate. Further, Ca^{2+} was found to promote biobutanol production (Isar and Rangaswamy 2012; Han *et al*. 2013). Concerning the boosting effects of $CaCO_3$ on biobutanol production, it may also be that $CaCO_3$ could enhance the stability of membrane proteins due to the presence of bivalent ions (Ca^{2+}) (Richmond *et al*. 2011). In this study, it was found that when the acetic acid and butyric acid concentrations were low, excellent biobutanol and ABE production were obtained. This indicates that more butyric acid was converted into biobutanol under optimized conditions, especially when 4.45 g/L of $CaCO_3$ was present in the fermentation broth.

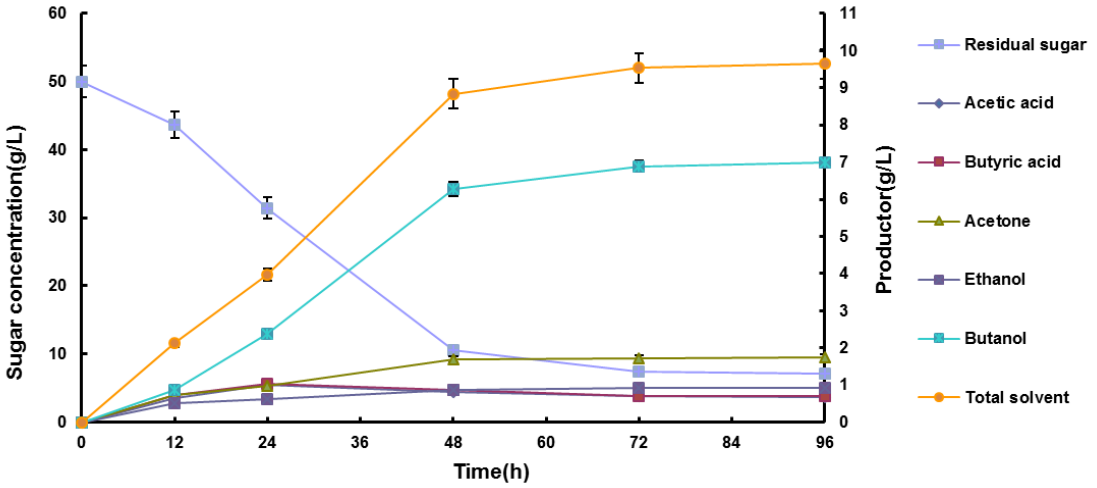

Fig. 2. Fermentation of poplar wood hydrolysates in the presence of other medium components (Second Mode). The figure shows the concentration of organic acids and solvents (acetone, butanol, ethanol) as a function of fermentation time.

Fermentation of Poplar Wood Hydrolysates with Extra Mixed Sugars and Other Medium Components

Poplar wood hydrolysates can contain inhibitors to the growth of the microbial strain and fermentation. Furthermore, biobutanol fermentation with 30 g/L of reducing sugars of poplar wood hydrolysates and 20 g/L of supplementary mixed sugars (11 g/L glucose, 7.8 g/L xylose, and 1.2 g/L arabinose) as the fermentation substrate was studied instead of poplar wood hydrolysates of total reducing sugar content 50 g/L. Figure 3 shows the fermentation products accumulated over 72 h. The total solvent was 10.18 g/L, including 7.28 g/L of biobutanol, 1.93 g/L of acetone, and 0.96 g/L of ethanol. The proportions of ethanol, acetone, and biobutanol were 1:2:7.5. Acetic acid and butyric acid were not fully metabolized; their concentrations were 0.89 and 0.93 g/L, respectively. At the end of fermentation, the residual sugar concentration only was 4.76 g/L, corresponding to a utilized percentage of 90.48%. Compared with the former two types of fermentation processes, the biobutanol and total solvent production were effectively improved. Biobutanol production was increased by 49.2% and 4.3%, respectively (Fig. 3, Table 1).

A certain proportion of external carbon promoted biobutanol production. It may be that the concentration of poplar wood hydrolysates in the fermentation broth was reduced and correspondingly, the inhibitor concentrations declined, improving cell growth (Fig. 4), metabolism, and metabolic product concentration. Cell metabolism is severely affected by toxic substances. Weak acids inhibit fermentation by either the uncoupling of cell metabolism or intracellular anionic accumulation.

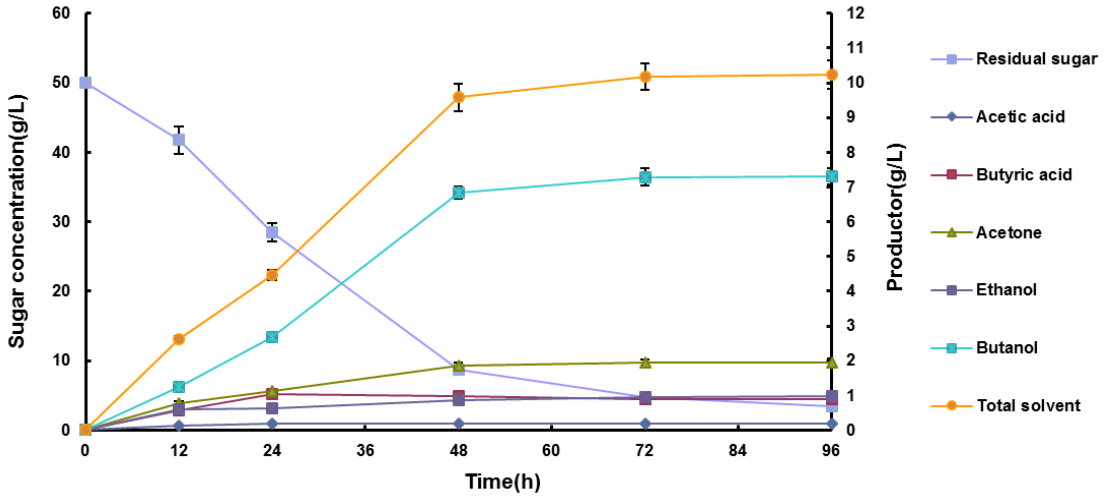

Fig. 3. Fermentation of poplar wood hydrolysates with additional mixed sugars (Third mode). The figure shows the concentration of organic acids and solvents (acetone, butanol, ethanol) as a function of fermentation time.

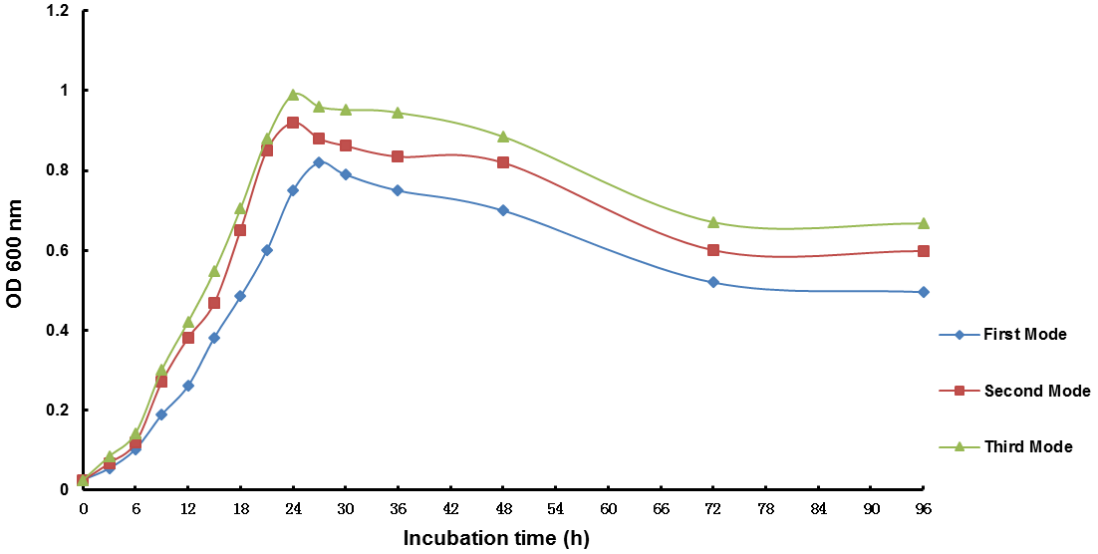

Fig. 4. Fermentation time and microbial growth curve

If high concentrations of toxic substances are present, NAD(P)Hs (nicotinamide adenine dinucleotide phosphate) can be used to produce less toxic compounds from these inhibitors. However, ethanol and butanol production is decreased. For instance, furfural and HMF were converted into their alcoholic forms using NAD(P)Hs (Ujor *et al*. 2014). Additionally, cell replication was also inhibited in the presence of furfural and HMF at higher concentrations. However, lower concentrations were beneficial to ABE fermentation (Ezeji *et al*. 2007). Furthermore, a study conducted by Cho *et al*. (2009) showed that phenolic compounds interfered with the metabolic pathway of the conversion

of acetyl-CoA to butyryl-CoA in the acidogenic phase, which affected solvent production during the phase. More importantly, phenolic compounds have also been reported to be more toxic, even at lower concentrations, than furfural and HMF (Ibraheem and Ndimba 2013).

Table 1. Fermentation Assays of Poplar Wood Hydrolysates by *Clostridium saccharobutylicum*

Parameter/Performance	Fermentation Medium		
	First Mode	Second Mode	Third Mode
Initial Sugar (g/L)	50	50	50
Final Sugar (g/L)	11.78	7.1	4.76
Acetic Acid (g/L)	0.74	0.68	0.89
Butyric Acid (g/L)	0.84	0.70	0.93
Acetone (g/L)	1.23	1.73	1.93
Biobutanol (g/L)	4.88	6.98	7.28
Ethanol (g/L)	0.52	0.93	0.96
Total ABE (g/L)	6.63	9.64	10.18
Biobutanol yield(g/g)	0.10	0.14	0.15
Biobutanol productivity(g/L•h)	0.041	0.073	0.076
Proportion of Butanol	73.6%	72.4%	71.5%

Table 2. Production of ABE from Other Substrates

Reference	Microorganism	Substrates	Butanol (g/g)	ABE yield(g/g)
This work	*C. saccharobutylicum* BAA-117	100% poplar hydrolyzates	0.14	0.19
Kudahettige-Nilsson *et al*. (2015)	*C. acetobutylicum* ATCC 824	50% Kraft liquor-derived hydrolyzates	0.1	0.13
		100% Kraft liquor-derived hydrolyzates	0.1	0.12
Lu *et al.* (2013)	*C. beijerinckii* CC101	50% wood pulp hydrolyzates	0.31	0.38
		100% wood pulp hydrolyzates	0.19	0.29
Cho *et al.* (2013)	*C. beijerinckii*	50% yellow poplar hydrolyzates	0.16	0.25
Jesse *et al.* (2002)	*C. beijerinckii* BA101	Actual agriculture waste	0.19	0.28
Huesemann *et al.* (2012)	*C. acetobutylicum* ATCC 824	sea weed extract (glu and mannitol)	0.12	0.16
Ranjan *et al* (2013)	*C. acetobutylicum* NCIM 2337	Rice straw	0.34	0.58
Liu *et al.* (2010)	*C. beijerinkii* ATCC 55025	Wheat bran	0.41	0.54

Using renewable feedstock such as lignocellulosic feedstocks and other alternative feedstock for conversion to biobutanol are receiving much attention at present. Studies have reported an increase in biobutanol with the use of wood hydrolyzate substrate (Table 2). Table 2 compares the biobutanol and total ABE solvent yield reported for wood hydrolyzates and other agro residues fermentation obtained. Relative to hydrolyzates derived from agricultural residues (wheat, rice), fermentation with wood hydrolysates had lower biobutanol yield, possibly due to the considerable amount of inhibitors (*e.g.* phenolics) as recently reviewed by Koutinas *et al.* (2014). The biobutanol yield was changed with the concentrations of wood hydrolysate in fermentation medium, and the yield showed a negative correlation with the concentrations of wood hydrolysate.

CONCLUSIONS

1. Efficient biobutanol fermentation can be performed by *Clostridium saccharobutylicum* BAA-117 using poplar wood. The highest ABE production (9.64 g/L) and ABE yield (0.19 g/g) were achieved when the basic nutrient compositions and fermentation conditions were satisfied, in which the biobutanol content was 6.98 g/L and yield was 0.14 g/g (g butanol/g sugars), and biobutanol content was as high as 72.2% in the total solvent.
2. This study successfully demonstrated that *Clostridium saccharobutylicum* BAA-117 has a superior capacity to produce ABE from poplar wood, a low-cost raw material. It was proven that the production of biobutanol and ABE were increased with the improvement of fermentation conditions.
3. There is great potential for using poplar wood (or other wood materials) as feedstock to produce fuel biobutanol *via* fermentation.

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