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Enhanced acetone-butanol-ethanol production from lignocellulosic hydrolysates by using starchy slurry as supplement

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Abstract:

This study aims to improve acetone-butanol-ethanol production from the hydrolysates of lignocellulosic material by supplementing starchy slurry as nutrients. In the fermentations of glucose, xylose and the hydrolysates of *Salix schwerinii*, the normal supplements such as buffer, minerals, and vitamins solutions were replaced with the barley starchy slurry. The ABE production was increased from 0.86 to 14.7 g/L by supplementation of starchy slurry in the fermentation of xylose and the utilization of xylose increased from 29% to 81%. In the fermentations of hemicellulosic and enzymatic hydrolysates from *S. schwerinii*, the ABE yields were increased from 0 and 0.26 to 0.35 and 0.33 g/g sugars, respectively. The results suggested that the starchy slurry supplied the essential nutrients for ABE fermentation. The starchy slurry as

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supplement could improve the ABE production from both hemicellulosic and cellulosic hydrolysate of lignocelluloses, and it is particularly helpful for enhancing the utilization of xylose from hemicelluloses.

Keywords: acetone-butanol-ethanol; lignocelluloses; starchy slurry; xylose

1. Introduction

The climate change has raised the concerns of biofuels production from renewable biomasses. Biobutanol, produced through acetone, butanol and ethanol (ABE) fermentation by *Clostridium* strains, is considered as a potential biofuel. The main advantage of ABE fermentation is that *Clostridium* has the ability to consume a wide variety of substrates such as glucose, sucrose, lactose, xylose and starch (Bankar et al., 2013). Lignocellulosic materials consisted of a variety of monosaccharides represent the most abundant, sustainable and cost-effective biomass for biofuels production. To date, a number of lignocellulosic materials such as wheat straw, sugar maple wood, corncob, swichgrass, eucalyptus and corn stalk have been investigated for ABE fermentation (Qureshi et al., 2008; Sun and Liu, 2012; Zhang et al., 2013; Liu et al., 2015; Zheng et al., 2015; Cai et al., 2016).

The commercial production of biobutanol is limited by various factors such as the low productivity, yield and selectivity in butanol fermentation due to the lack of an efficient butanol-producing host strain. Moreover, the production of biobutanol from the lignocelluloses is not cost effective due to the complicated lignocellulosic material processing for example pretreatment and hydrolysis. Pretreatment disrupts the heterogeneous structure of lignocellulosic material, removes hemicelluloses and/or lignin, increases the surface area and porosity of biomass, and reduces the crystallinity of cellulose, thus increasing the accessibility of cellulose to enzymes (Wyman et al.,

2005). However, nearly 18-20% of the total project cost is attributed to the pretreatment in biofuels production (Yang et al., 2007). Many pretreatment technologies including physical, chemical, physico-chemical and biological methods have been developed (Wyman et al., 2005).

Enzymatic hydrolysis of lignocelluloses into fermentable sugars is one of the most expensive steps in biobutanol production due to the high cost of the enzymes. There are a number of obstacles which affect the performance of enzymes, including 1) product inhibition of enzymes; 2) unproductive binding of enzymes on substrates; 3) the hinder of enzymes to cellulose by hemicelluloses and lignin; 4) the denaturation or inactivation of enzymes during hydrolysis (Jørgensen et al., 2007). Thus, the enzymes should be used efficiently during the hydrolysis. As reported earlier, the addition of hemicellulose-degrading enzymes could improve the performance of cellulases during the hydrolysis of lignocelluloses (Zhang et al., 2011). Simultaneous saccharification and fermentation (SSF) process reduces the inhibition of end products of hydrolysis and requires relatively low amounts of enzyme (Wingren et al., 2003). Certain additives, in particular surfactants such as Tween and polyethylene glycol (PEG) have shown the potential to increase the hydrolysis efficiency by affecting the hydrophobic interaction between lignin surfaces and enzymes (Eriksson et al., 2002).

In addition to the lignocellulosic materials processing, nutrients supplementation during the fermentation is critical to enhance the ABE production by *Clostridium* strains. The nutrients including nitrogen sources, buffer reagents, metal ions, and vitamins are usually supplemented to the medium to compensate the nutritional deficiencies of lignocellulosic hydrolysates (Zheng et al., 2015). Many studies supplement the modified P2 stock solutions containing buffer, minerals, and vitamins to lignocellulosic hydrolysates for the onset and maintenance of ABE production (Qureshi et al., 2008;

Liu et al., 2010; Zhang et al., 2013). However, the P2 solutions need to be added separately prior to the inoculation of *Clostridium* strains, which may cause contamination of the medium and introduces additional cost. It would be preferable to avoid nutrient supplementation and simplify the medium composition in order to reduce the overall production cost.

In this study, fast growing willow *Salix schwerinii* was used as a lignocellulosic feedstock for ABE production by *Clostridium acetobutylicum* DSM 1731. To avoid the nutrients supplementation, heat-treated starchy biomass barley was used as a supplement to replace the P2 solutions of the normal used medium. Barley has been regarded as an energy crop and used for bioethanol production (Nghiem et al., 2010). It was reported that the integration of the first generation feedstock (wheat) with the second generation feedstock (wheat straw) for bioethanol production showed the positive effect on product yields (Erdei et al., 2012). In previous study, co-fermentation of barley grain slurry and barley straw hemicellulosic hydrolysate improved the ABE production from hemicelluloses, however, P2 stock solutions were added (Yang et al., 2015). Choi et al. (2013) found that the yield and productivity of ABE in the fermentation of 6% corn steep liquor-containing medium were 32 and 26% higher than that in the fermentation of Clostridial growth medium, respectively. The aim of this study is to evaluate the feasibility of using treated barley grain slurry to replace the nutrients of the fermentation medium and to improve the ABE production from lignocelluloses. Dilute acid pretreatment was employed to solubilize the hemicelluloses from lignocelluloses. The pure glucose and xylose medium, the hemicellulosic hydrolysate and the hydrolysate derived from enzymatic hydrolysis of cellulose were fermented respectively by using starchy slurry as supplement.

2. Materials and methods

2.1 Materials

Six-year-old *S. schwerinii* was harvested in the beginning of June in 2013 from the field in North Karelia, Finland. After harvesting, the material was debarked, chipped, and transported to the laboratory to be stored at 4°C. Before further analysis, the chipped *S. schwerinii* were air-dried, milled to a particle size of 1 mm, and kept in paper bags. Barley grain was collected in 2011 from a field in North Karelia, Finland. It was air dried, milled to a particle size of 0.25 mm, and kept in paper bags. The barley grain contained 55.2% (w/w) starch, 6.2% glucan, 11.6% xylan and 3.1% arabinan (Yang et al., 2013).

2.2 Pretreatment

Air-dried *S. schwerinii* material was pretreated using 0.1% (w/v) H_2SO_4 with the ratio of 1:10 in a steel cylinder. After heating to 200 °C, the cylinder was left to cool to the room temperature. The liquid was separated by a filter paper (Whatman® 589/1, Schleicher&Schuell) from the solid. The pretreated liquid defined as hemicellulosic hydrolysate was used for the ABE fermentation and the analysis of carbohydrates and degradation products. The pretreated solid defined as cellulosic solid was washed with water and stored in the freezer (-18°C) for enzymatic hydrolysis and composition analysis. Barley grain material was suspended in water with the solid content of 9.1% in 125-mL screw-capped bottle and heat-treated at 121 °C for 30 min. The slurry defined as starchy slurry was used as the supplement for the ABE fermentations.

2.3 Enzymatic hydrolysis

The enzymes Celluclast 1.5L and Novozyme 188 (Novozymes A/S, Bagsværd, Denmark) were used as cellulase preparation (CEL). PEG 4000 was used as a surfactant to improve the performance of enzymes. The enzymes and PEG 4000 were all

purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzymatic hydrolysis of pretreated *S. schwerinii* with 2% (w/v) dry matter (DM) loading was carried out in tubes with a working volume of 3 mL in 0.05 M sodium citric buffer (pH 5.0). Hydrolysis was performed in a shaker with stirring at 200 rpm at 50 °C for 48 h. Prior to hydrolysis, 10 FPU/g biomass of Celluclast 1.5L (70 FPU/ml) and 400 nkat/g biomass of Novozyme 188 (6000 nkat/ml) were added as one dosage of CEL to the slurry for enzymatic hydrolysis. PEG 4000 was also added to the slurry with the dosage of 2 g/100 g DM. After enzymatic hydrolysis, the samples were boiled for 10 min to stop the enzymatic hydrolysis and then centrifuged for 10 min at 12,000 rpm. The supernatant was collected for sugar analysis. Enzymatic hydrolysis of 6% (w/v) pretreated *S. schwerinii* was performed in a 100 ml blue cap bottle to get the cellulosic hydrolysate for ABE fermentation.

2.4 Microorganism cultivation and fermentation

Clostridium acetobutylicum DSM 1731 was obtained from DSMZ, Braunschweig Germany (German Collection of Microorganisms and Cell Cultures). Freeze-stored culture was inoculated to 50 mL of Reinforced Clostridial Medium (Hirsch and Grinsted, 1954) for 14–16 h. Then, 1 mL of active growing cells was inoculated into 50 mL of sterilized pre-fermentation P2 media prepared in a 125-mL screw-capped bottle. The pre-fermentation P2 medium contained glucose 30 g/L and yeast extract 1 g/L. Before inoculation, 0.5 mL each of the filter-sterilized stock solution (buffer: KH_2PO_4 50 g/L, K_2HPO_4 50 g/L, ammonium acetate 220 g/L; mineral: $MgSO_4 \cdot 7H_2O$ 20 g/L, $MnSO_4 \cdot H_2O$ 1 g/L, $FeSO_4 \cdot 7H_2O$ 1 g/L, NaCl 1 g/L; and vitamin: para-aminobenzoic acid 0.1 g/L, thiamin 0.1 g/L, biotin 0.001 g/L) was added into the P2 media. The culture was allowed to grow for approximately 16 h at 37 °C before

inoculation into the ABE fermentation media. All fermentations were conducted at least twice to ensure the reproducibility.

The ABE fermentations were conducted in 125-mL screw-capped bottles containing 50 mL fermentation media. The glucose 50g/L, xylose 50g/L and the hemicellulosic and cellulosic hydrolysates of *S. schwerinii* were used as carbon sources of the fermentation media, in which 1 g/L yeast extract was added. The pH of the hemicellulosic hydrolysate and enzymatic hydrolysates were adjusted to 6.5 with NaOH. For the fermentations using P2 solutions as nutrients, each of P2 stock solution (buffer, mineral and vitamin) was added into the media prior to the inoculation of *C. acetobutylicum*. For the fermentations without adding P2 solutions, different volumes of starchy slurry were mixed into the media prior to sterilization. The glucose and xylose solutions were mixed with starchy slurry with the volume ratio of 1:4, 2:3 and 3:2, respectively. The hemicellulosic and cellulosic hydrolysates were mixed with the starchy slurry with the ratio of 3:2, respectively. All the media were purged with N_2 for 10 min to maintain an anaerobic condition and were sterilized at 121 °C for 20 min. Fermentation started at 37 °C when inoculated into the *C. acetobutylicum* DSM 1731 culture (10%, v/v). Fermentation samples were taken at 24-h intervals.

2.5 Chemical analysis

The chemical compositions of raw and pretreated *S. schwerinii* were analysed according to the method in literature (Hayes et al., 2012). Materials weighing 300 mg were treated with 72% H_2SO_4 for 1 h at 30 °C in a 100-mL triangular flask and then diluted to 4% H_2SO_4 with deionized water and autoclaved for 1 h at 121 °C. The slurry was neutralized with solid $CaCO_3$ to pH 4-5 and centrifuged for 10 min at 12,000 rpm. Sugar composition and acid soluble lignin were analyzed on a DIONEX ICS-3000 ion

chromatography system consisting of an electrochemical detector (using pulsed amperometricdetection), a gradient pump, a temperature-controlled column and detector enclosure, and an AS50 autosampler with injection volume of 10 μL.

The starch content in fermentation residues was determined with Total Starch Assay Kit (Megazyme International Ireland Ltd.). The solvents (acetone, butanol, ethanol, acetic acid, butyric acid), sugars (glucose and xylose), and other compounds (furfural, 5-hydroxymethylfurfural (HMF), formic acid, levulinic acid and citric acid) in the samples were analyzed with Nuclear Magnetic Resonance (NMR). The NMR spectra for quantification of these compounds were recorded on a Bruker AVANCE 500 DRX NMR spectrometer equipped with a 5 mm QNP SB probe. Above mentioned compounds were identified from routine two-dimensional proton-proton and proton-carbon correlated spectra. Quantitative 1H NMR spectra were collected with water presaturation (zgcppr) by using a 90° pulse angle, 48 dB presaturation power, 40 s relaxation delay, and 16 scans at 300 K. Prior to the NMR measurements, 200 μL of sample liquid was transferred to a 5 mm NMR tube followed by addition of deuterium oxide (D_2O, 275 μL) and 3-(trimethylsilyl)-propionic-d4 acid (25 μL, 20 mM) in D_2O as an internal standard of known concentration.

The sugar production yields after enzymatic hydrolysis were calculated as follows:

$$\text{Glucose yield (\%)} = \frac{\text{Glucose in enzymatic hydrolysate (g)} \times 0.9}{\text{Cellulose in pretreated substrates (g)}} \times 100$$

$$\text{Xylose yield (\%)} = \frac{\text{Xylose in enzymatic hydrolysate (g)} \times 0.88}{\text{Xylan in pretreated substrates (g)}} \times 100$$

The sugar consumption, butanol and ABE yields were all calculated according to the concentration of original sugars and the residual sugars in the fermentation medium. Due to the complex sugar components of the starting medium, the total sugar input was used as the original sugars to make all the batches comparable.

3. Results and discussions

3.1 Effect of starchy slurry as supplement on ABE fermentation of pure glucose

3.1.1 Effect of P2 solutions on fermentation of glucose

The fermentation of pure glucose with P2 solutions (P2) as control produced 12.3 g/L ABE, which consisted of 9.3 g/L butanol, 2.5 g/L acetone and 1.2 g/L ethanol in 120 h (Fig. 1, Table 1, Batch 1). The sugar consumption was 82%, and the yields of butanol and ABE were 0.23 and 0.31 g/g sugar, respectively (Table 1). The product yields are in agreement with the study of Li et al. (2011), in which batch ABE fermentation of glucose was conducted with pH-control. However, without P2, only 1.2 g/L ABE containing 1.0 g/L butanol was produced (Batch 2). The sugar consumption, butanol and ABE yields were significantly lower than that in the fermentation with P2 (Table 1).

The results showed the significant positive effect of P2 on ABE fermentation by *C. acetobutylicum*. As reported earlier, P2 solutions including buffer, minerals and vitamins were essential ingredient for the growth and solvents production of *C. acetobutylicum* (Kheyrandish et al., 2015). The best concentrations of $MgSO_4$, $MnSO_4$, KCl, $FeSO_4$, ammonium acetate, para-amino benzoic acid and thiamin for ABE fermentation were obtained in the range of 50-200 mg/L, 0-20 mg/L, 0.015-8 g/L, 1-50 mg/L, 1.1-2.2 g/L, 1 mg/L and 0.01 mg/L, respectively (Monot et al., 1982). Biotin is also important for the solvent production in batch fermentation by *C. acetobutylicum*

(Oxford et al., 1940). Low ammonia concentration caused the decreased glucose utilization and residual glucose remained at the end of fermentation, because the *Clostridium* cells could not undergo a shift to solvent production at low concentration of ammonia (Long et al., 1984). The failure to produce solvents under the conditions of nutrients limitation was probably due to the failure to generate threshold concentrations of acids for the conversion to solvents (Jones and Woods, 1986). In this study, the concentrations of acetic acid and butyric acid produced in the fermentation without P2 were 0.7 and 1.1 g/L, respectively, which were probably too low to result in the shift from acids to solvent production by *C. acetobutylicum*.

3.1.2 Supplementation of starchy slurry to replace P2 solutions

Different dosages of starchy slurry as a replacement of P2 were added to the pure glucose medium (Fig. 1). When both P2 and 10 mL starchy slurry were added to glucose medium, 15.3 g/L ABE was produced, in which 9.6 g/L was butanol (Batch 3). After fermentation, 87% of total sugars were consumed. The butanol and ABE yields were 0.23 and 0.36 g/g sugars, respectively (Table 1). Correspondingly, without addition of P2 in Batch 4, significant lower concentration of total ABE and butanol were obtained. The yields of ABE and butanol were also decreased, and only 49% of sugars were utilized (Table 1). When the dosage of the starchy slurry increased to 20 mL, the fermentations produced similar concentrations of ABE and butanol compared to Batch 3 even without adding P2 (Batch 5 and 6). There were no further increase of butanol concentrations and yields when 30 mL starchy slurry was added to glucose medium regardless of P2 (Batch 7 and 8, Table 1). The ABE yields of fermentations with additive starchy slurry were higher than that in the fermentation of glucose as the former fermenations produced more amount of acetone (Fig. 1).

The results showed that lower amount of starchy slurry did not have the same effect with P2 solutions on solvent production. However, P2 solutions could be replaced by addition of relatively higher amount of starchy slurry. The reasons behind the improved solvent production probably are attributed to the components of grain slurry, which provided the essential nutrients for ABE fermentation. Survase et al. (2013) suggested that market refused vegetables as a supplement could be used for improving ABE production by *C. acetobutylicum* DSM 792. The content of various amino acids, vitamins and proteins in vegetables can be responsible for improving the solvent production (Survase et al., 2013). Although the model components such as vitamins, tricarboxylic acid intermediates and pyrroloquinoline quinone were not found to improve the solvent production, it was infered that high content of protein, provided electron carriers, minerals (manganese and iron) or antioxidants (lycopene, vitamin A and E, flavonoids) etc. probably help in improving the solvent production (Tashiro et al., 2007; Survase et al., 2013). In this study, with the addition of starchy slurry, more acetone was produced. It was reported that nutritional factors such as the presence of citrate, addition of lactate or a growth-limiting iron supply could increase the ratio of butanol to acetone during ABE fermentation by *C. acetobutylicum* (Bahl et al., 1986). The starchy slurry probably provided different nutrition formula with P2 solutions.

3.2 Effect of starchy slurry as supplement on ABE fermentation of pure xylose

3.2.1 Effect of P2 solutions on fermentation of xylose

The fermentations of pure xylose both with and without P2 solutions produced a small amount butanol and total ABE (Fig. 2, Table 2, Batch 9 and 10). The low xylose consumption (25-29%) resulted in low butanol and ABE yields, which were 0.04 and 0.05 g/g sugars, respectively (Table 2). Although similar concentrations of ABE were

produced in Batch 9 and 10，the concentrations of by-products acetic acid and butyric acid in Batch 10 (with P2) were 1.73 g/L and 2.63 g/L, respectively, which were considerably higher than that in Batch 9 (without P2).

Clostridium is able to ferment both hexose and pentose sugars. However, the xylose utilization of *Clostridium* is not efficient, as observed in the fermentation of xylose by *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 (Gu et al., 2009; Xiao et al., 2012). Gu et al. (2009) found that *C. acetobutylicum* ATCC824 utilized 86% of glucose within 40 h, whereas only 6% of xylose was consumed even after an elongated incubation time. In this study, the results also suggested that xylose was undesired for *C. acetobutylicum* to produce ABE. It seems that xylose medium without P2 did not initiate the ABE production because of the low concentrations of acids (acetic acid and butyric acid) found in the medium. In contrast, with P2, more acids were produced, which suggested that the ABE production has been initiated but the transfer of acids to solvent was not maintained. This is probably due to that the nutrients are not enough for the shift from acids to solvents pathway of *C. acetobutylicum* during the fermentation of xylose. In batch culture fermentations used for the industrial production of solvents, nutrients were normally present in excess (Jones and Woods, 1986).

3.2.2 Supplementation of starchy slurry to replace P2 solutions

Different dosages of starchy slurry were also added to the pure xylose medium to replace P2 (Fig. 2). With the addition of P2 and 10 mL starchy slurry, 13.4 g/L ABE containing 8.4 g/L butanol was produced (Batch 11). The butanol and ABE yields were 0.19 and 0.31 g/g sugars, respectively (Table 2). The sugar consumption was 88%, and only 6.1 g/L xylose was left after fermentation. Correspondingly, without addition of P2

in Batch 4, significant lower concentrations and yields of butanol and ABE were obtained (Batch 12, Table 2). The sugar consumption was 44%, and the residual xylose was 24.9 g/L (Table 2). Interestingly, the addition of 20 mL starchy slurry produced similar amount of ABE and butanol compared to Batch 11, even without adding P2 (Batch 13 and 14). The sugar consumption increased to 81-90% (Table 2). When 30 mL starchy slurry was added to xylose medium with or without P2, the concentrations and yields of butanol and ABE relatively increased (Batch 15 and 16, Table 2). The butanol and ABE yields of fermentations with additive starchy slurry were significantly higher than that in the fermentation of pure xylose.

The results showed that P2 solutions could also be replaced by addition of relatively higher amount of starchy slurry in xylose fermentation. The additive starchy slurry significantly improved the xylose utilization and ABE production by *C. acetobutylicum*. This is in agreement with the study of Amartey and Jeffries (1994) that corn steep liquor is a good source of nutrients that can support growth and fermentation activity of the xylose fermenting yeast. The results indicated that the starchy slurry may supply enough nutrients for the utilization of xylose by *C. acetobutylicum*, or the nutrition formula provided for xylose fermentation is possibly different with P2 solutions.

Xylose is catabolized via pentose phosphate pathway (PPP) in most microorganisms. The rate-limiting enzymes in the PPP of *C. acetobutylicum* are responsible for the xylose uptake. However, the xylose utilization was repressed when the organism was cultivated on a mixture of glucose and xylose, which is called carbon catabolite repression (Ounine et al., 1985). Grimmler et al. (2010) identified that two putative operons involved in PPP of *C. acetobutylicum* were subjected to the catabolite repression by glucose. In this study, it seems that there was no carbon catabolite

repression when the *C. acetobutylicum* was cultivated on the mixture of xylose and starch, but whether the additive starchy slurry promoted the metabolism of xylose is not clear. Thus, the further study related to the metabolic intermediates and enzymes in PPP affecting by addition of starchy slurry would be of interest.

3.3 Effect of starchy slurry as supplement on ABE fermentation of hemicellulosic hydrolysate

The raw *S. schwerinii* contains 44.0% glucose, 17.6% xylose, and other sugars (Table 3). Pretreatment of *S. schwerinii* with dilute sulfuric acid solubilized 16.3 g/L xylose and 3.6 g/L glucose to the hemicellulosic hydrolysate, and 41.1% glucose was left in the pretreated solid. There was no significant change on the contents of total lignin in raw material. The hemicellulosic hydrolysate was used for ABE fermentation as control experiment. However, *C. acetobutylicum* did not grow at all and no ABE production was detected (Table 4). The hydrolysate then was mixed with 20 mL starchy slurry and fermented for 120 h (Fig. 3). Total 10.6 g/L ABE was produced, which consisted of 6.7 g/L butanol, 3.4 g/L acetone and 0.6 g/L ethanol. The concentrations of acetic acid and butyric acid were 3.9 g/L and 1.6 g/L, respectively. The glucose and xylose were consumed simultaneously, and most of sugars including starch were utilized after the fermentation (Fig. 3, Table 4). The butanol and ABE yields were 0.22 and 0.35 g/g sugars, respectively.

Dilute acid pretreatment solubilizes hemicellulose from lignocellulose and incereases the digestibility of cellulose (Wyman et al., 2005). However, during the pretreatment of this study, 4.9 g/L acetic acid, 0.3 g/L formic acid, 0.4 g/L levulinic acid, 1.45 g/L furfural, and 0.36 g/L HMF were formed. These compounds were considered to be inhibitory for *Clostridium* fermentation (Qureshi et al., 2010; Liu et al., 2015).

This is probably the reason why *C. acetobutylicum* could not grow in the hemicellulosic hydrolysate of *S. schwerinii* used directly as medium. The inhibitory compounds affect the microbial cell growth, glycolytic and fermentative enzymes in the central metabolic pathways (Ibraheem and Ndimba, 2013). These also affect the redox and energy metabolism of the organism (Ask et al., 2013). In previous studies, the inhibitors were removed from hemicellulosic hydrolysate of sugar maple wood for ABE fermentation (Sun and Liu, 2012). Qureshi et al. (2010) diluted barley straw hydrolysate to decrease the concentration of inhibitors for successful fermentation. In this study, the additive starchy slurry promoted the fermentability of the hemicellulosic hydrolysates, which could be due to the dilution of inhibitors. On the other hand, the starchy slurry replaced the P2 nutrients, which would be cost-effective for industrial ABE production. Furthermore, the *C. acetobutylicum* would be more robust under the condition of preferable nutrients to tolerate the inhibitory compounds.

3.4 Effect of starchy slurry as supplement on ABE fermentation of cellulosic hydrolysate

3.4.1Enzymatic hydrolysis of pretreated cellulosic residues

With the increasing dosages of cellulase (from half to double dosages of CEL), more glucose was released from pretreated *S. schwerinii* during the enzymatic hydrolysis (Fig. 4A). The maximum glucose yield of 80% was achieved with double dosage of CEL in 48 h. By addition of 2 g/100 g DM PEG 4000 in the hydrolysis, considerable increases of glucose and xylose yields from pretreated Salix were achieved (Fig. 4B). With half dosage of CEL, the glucose and xylose yields increased to 75.8% and 60.7%, respectively. With a dosage of CEL, the cellulose was completely hydrolyzed to glucose, and the xylose yield increased to 89%. There was no further increase of the hydrolysis yields when using more dosages of CEL. The increases of

sugar yields by PEG 4000 are in agreement with the results obtained by previous study (Yang et al., 2015). Also, Kristensen et al. (2007) found that surfactants increased cellulose conversion by up to 70% from acid- and steam-treated wheat straw.

The positive effects of non ionic surfactants on enzymatic hydrolysis have been extensively studied. Surfactants adsorb to lignin surface and significantly reduce unproductive binding of enzyme, thus enhancing the cellulose hydrolysis (Eriksson et al., 2002). The surfactants could also increase the cellulase activity and enzymes stabilities. According to a study of Hsieh et al. (2015), the hydrolysis boosting effect of PEG is specific for exo-cellulase cellobiohydrolase (CBHI), but not for endoglucanase (EG). The degree of increased free cellulase activity obtained by PEG addition is in connection with the amount of phenolic hydroxyl groups in various substrates; the phenolic hydroxyl groups exposed on the lignin surface interact with PEG through hydrogen bonding, forming a layer of PEG on lignin surface, which prevents unproductive binding of cellulases on lignin (Sipos et al., 2011). Li et al. (2012) confirmed that PEG 4000 prevents cellulase deactivation induced by cellulose, and promotes the removal of amorphous cellulose. Another mechanism related to PEG-water interaction rather than PEG-substrate or PEG-protein interactions was also proposed; the activity of enzyme on the substrate surface was increased by addition of PEG that is due to the increase of relaxation time of the liquid-phase water (Hsieh et al., 2015).

3.4.2 ABE fermentation of cellulosic hydrolysate

The fermentation of the enzymatic hydrolysate of *S. schwerinii* with P2 produced 8.2 g/L ABE in 120 h, which consisted of 6.0 g/L butanol, 1.8 g/L acetone and 0.4 g/L ethanol (Fig. 5). The yields of butanol and ABE were 0.19 and 0.26 g/g sugars,

respectively (Table 4). When the P2 solutions were replaced with 20 mL starchy slurry, the mixture produced 12.4 g/L ABE, which consisted of 8.1 g/L butanol, 3.7 g/L acetone and 0.6 g/L ethanol. The yields of butanol and ABE correspondingly increased to 0.21 and 0.33 g/g sugars (Table 4).

The fermentation of cellulosic hydrolysate resulted in relatively lower butanol and ABE yields compared to that of glucose fermentation (Batch 1). This is probably due to the inhibitory factors for ABE fermentation in enzymatic hydrolysate. Besides the sugars found in the cellulosic hydrolysate, the concentration of citric acid before and after adding starchy slurry were 51.3 and 37.6 mM, respectively. Xue et al. (2016) found that the strengths of citrate buffer in the range of 20-100 mM had no effect on enzymatic hydrolysis, but greatly influenced the performance of ABE fermentation using corn stover hydrolysate. It was reported earlier, sodium sulfate and sodium chloride formed during the neutralization process are toxic to *C. beijerinckii* (Ezeji et al., 2007a). After the enzymatic hydrolysis, the cellulosic hydrolysate (citrate buffer) was neutralized with sodium hydroxide and more sodium citrate was formed, which might be one of the inhibitory factors. However, the additive starchy slurry increased the butanol and ABE yields, which was comparable to that of glucose fermentation. This is probably also due to the dilution of inhibitory factors and the contributed nutrient components.

3.5 Comparison to other studies

Various lignocellulosic feedstocks have been investigated for ABE production (Table 5). The concentrations of ABE and the production yields differ with different feedstocks, pretreatments, hydrolysis methods, fermentation technologies and *Clostridium* species. In this study, the concentration of butanol and ABE ranged from

6.2 to 7.8 and 9.0 to 13.5 g/L, respectively. These were comparable to some of the studies in which wheat bran, corncob and corn stalk etc. were used as feedstocks (Liu et al., 2010; Zhang et al., 2013; Cai et al., 2016). However, the concentrations of ABE were lower than those studies of butanol production by *C. beijerinckii* P260 from agricultural residues (Qureshi et al., 2008). *C. beijerinckii* P260 is a commercial strain which was used in South Africa in the early 1980s. *Clostridium* strain is the most important factor affecting the efficiency of biobutanol production process (Jones and Woods, 1986).

Fast growing Salix has been evaluated as a suitable feedstock for bioethanol production (Sassner et al., 2006). However, in the ABE production, only a butanol yield of 0.12 g/g sugars was reported from Salix hydrolysate after acid hydrolysis (Han et al., 2013). Potential inhibitors of ABE fermentation formed during the acid hydrolysis resulted in the failure of transition from the acid-producing pathway to the solvent-producing pathway of *C. beijerinckii*. In this study, the acid hydrolyzed hemicellulosic hydrolysate was fermented separately for ABE production. The higher concentrations and yields of ABE were obtained by using starchy slurry as nutrients compared to the study of Han et al. (2013). The sugar recovery from cellulosic solids was increased by addition of PEG4000 during the hydrolysis, which further increased the butanol production efficiency as well. This is in agreement with previous study of Yang et al. (2015). Fig. 6 presents the mass balance of the ABE production from Salix and barley grain slurry. When 1 kg *S. schwerinii* was used as the substrate alone, 114.9 g ABE was produced. This amount of ABE was only produced from cellulosic biomass. By using barley grain slurry alone, 217.8 g ABE could be produced. When the ABE production from *S. schwerinii* was integrated with that from barley grain slurry, total 539.0 g ABE was produced, which was increased by 62% of the sum of ABE produced from *S.*

schwerinii and barley grain separately. This is mainly due to the significant enhancement of ABE production from hemicellulosic hydrolysate with the addition of the starchy slurry. The results are in agreement with the study of Erdei et al. (2012) that co-fermentation of wheat meal and wheat straw could improve xylose utilization for ethanol production.

In most studies, the lignocellulosic hydrolysates used for ABE fermentation were supplemented with P2 stock solutions (Table 5). The advantage of this study was that the starchy slurry replaced P2 solutions while reasonable butanol and ABE yields were obtained. This is in agreement with that the fermentation of saccharified degermed corn does not require supplementation with normal P2 media, because of the presence of nutrients in corn (Ezeji et al., 2007b). In order to accomplish large scale utilization of biomass feedstocks to produce biobutanol, various sources of feedstocks are probably utilized. These are likely to include starch based materials such as barley, sorghum, potato and sweet potato. For example, barley has been regarded as a good supplement to corn biofuel production in anticipation of the commercialization of lignocellulosic biofuel (Nghiem et al., 2010). Cassava or waste starch stream from potato factories have been studied as suitable carbon sources for butanol production by *C. acetobutylicum* (Li et al., 2015; Kheyrandish et al., 2015). Thus, the starch-containing feedstocks possess large potentials as supplements for improved ABE production from lignocellulosic biomasses.

4. Conclusion

In the fermentations of glucose, xylose and the hydrolysates of *S. schwerinii*, the normally used supplements such as buffer, minerals, and vitamins solutions could be replaced with the starchy slurry. The starchy slurry ensures the essential nutrients for

ABE fermentations and improves the ABE production from both hemicellulosic and cellulosic hydrolysate of lignocelluloses, and it is particularly helpful for the utilization of xylose by *C. acetobutylicum*. In order to accomplish large scale utilization of biomass feedstocks for butanol production, various sources of feedstocks are probably utilized. The starch-containing feedstocks possess large potentials as supplements for improved ABE production from lignocellulosics.

Acknowledgements

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Figure Captions

Fig. 1. Fermentations of glucose by supplementation of different dosages of starchy slurry to replace the P2 solutions. Batch 1: Glucose+P2; Batch 2: Glucose; Batch 3: Glucose+P2+10 mL starchy slurry; Batch 4: Glucose+10 mL starchy slurry; Batch 5: Glucose+P2+20 mL starchy slurry; Batch 6: Glucose +20 mL starchy slurry; Batch 7: Glucose+P2+30 mL starchy slurry; Batch 8: Glucose+30 mL starchy slurry.

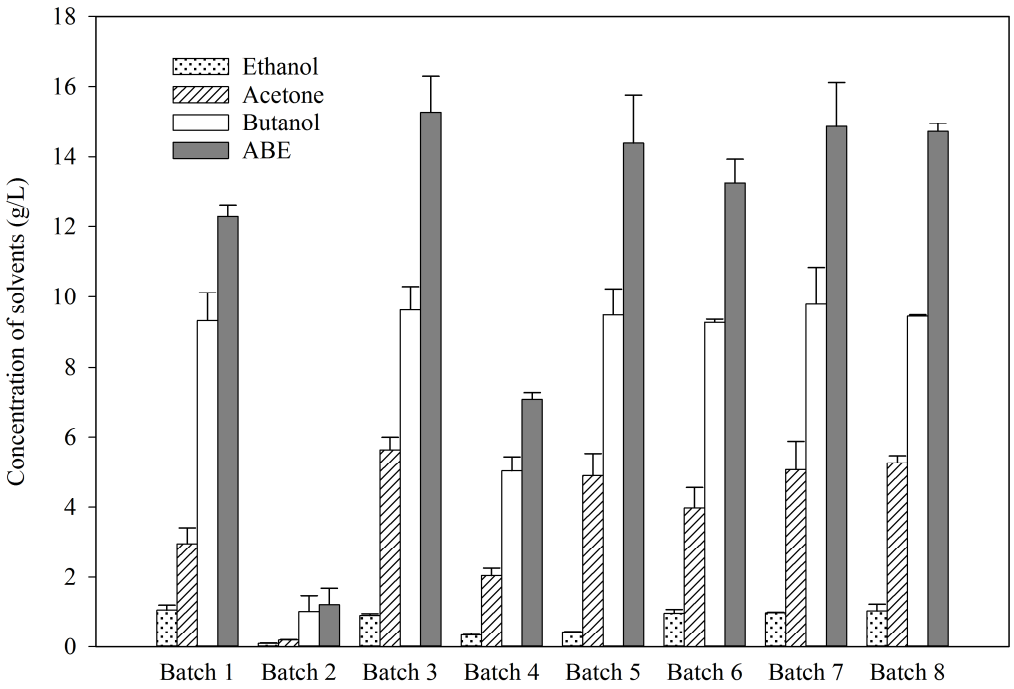
Fig. 2. Fermentations of xylose by supplementation of different dosages of starchy slurry to replace the P2 solutions. Batch 9: Xylose+P2; Batch 10: Xylose; Batch 11: Xylose+P2+10 mL starchy slurry; Batch 12: Xylose+10 mL starchy slurry; Batch 13: Xylose+P2+20 mL starchy slurry; Batch 14: Xylose+20 mL starchy slurry; Batch 15: Xylose+P2+30 mL starchy slurry; Batch 16: Xylose+30 mL starchy slurry.

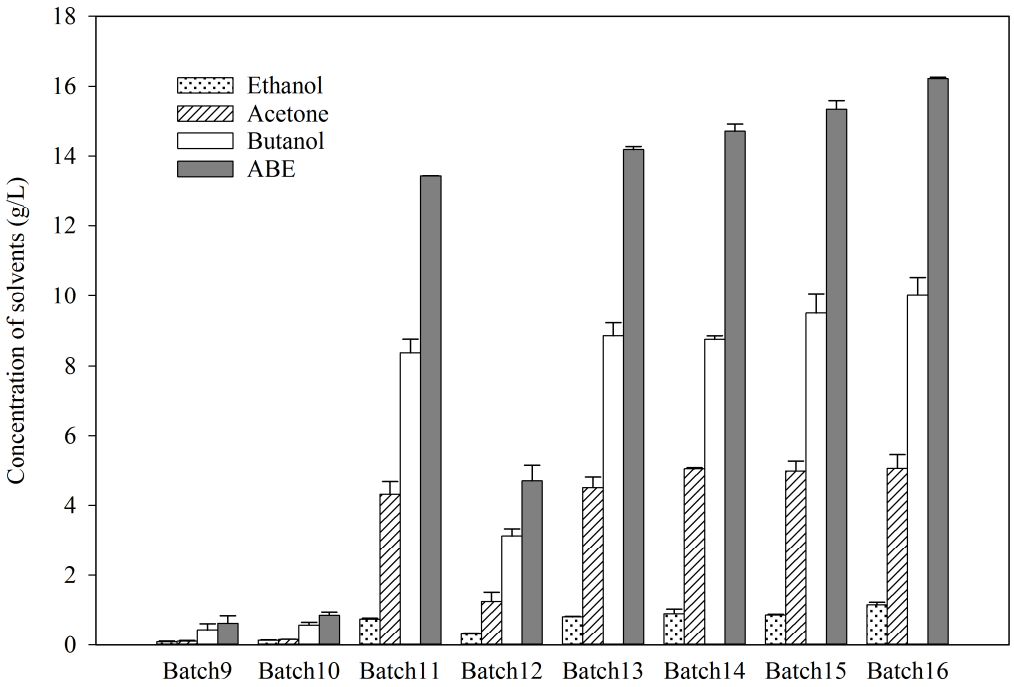
Fig. 3. Fermentation of hemicellulosic hydrolysate of *S. schwerinii* by supplementation of starchy slurry to replace the P2 solutions.

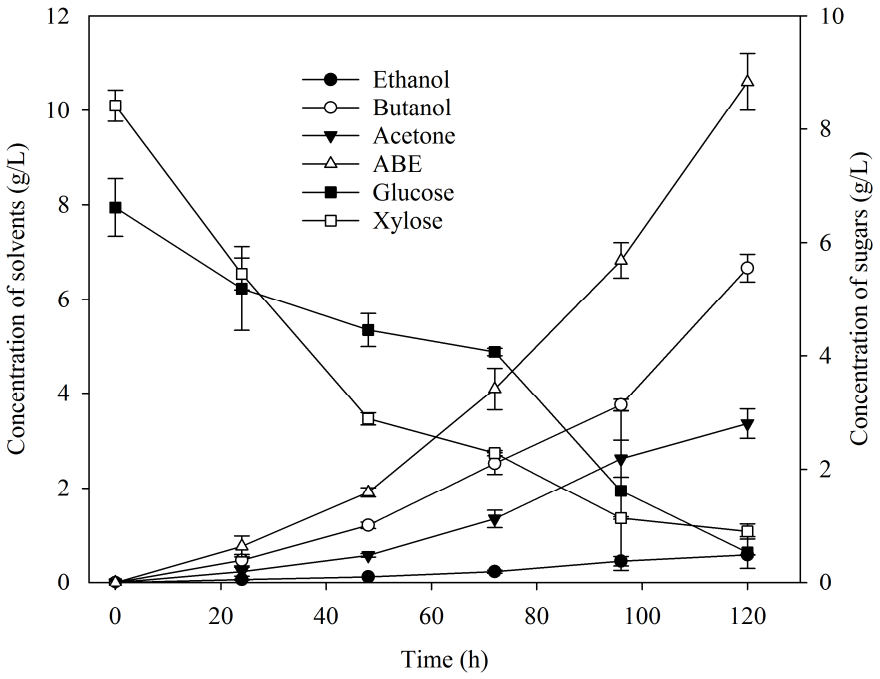
Fig. 4. Hydrolysis of cellulosic solid by different dosages of CEL in 50 mM sodium citrate buffer at pH 5.0 and at 50°C. (A) Kinetic hydrolysis yields; (B) The hydrolysis yields obtained by addition of PEG 4000 after 48 h. CEL: Celluclast 1.5L and Novozyme 188.

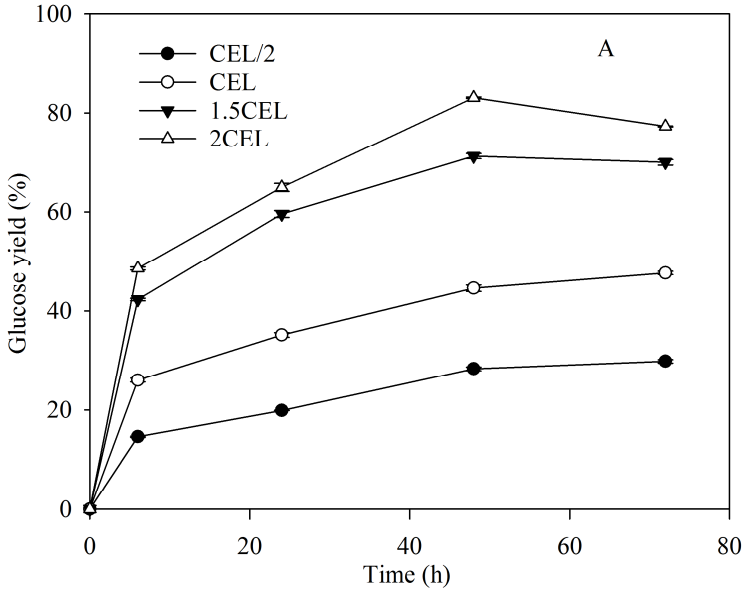
Fig. 5. Fermentation of cellulosic hydrolysate of *S. schwerinii* by supplementation of starchy slurry to replace the P2 solutions. Star: Starchy slurry.

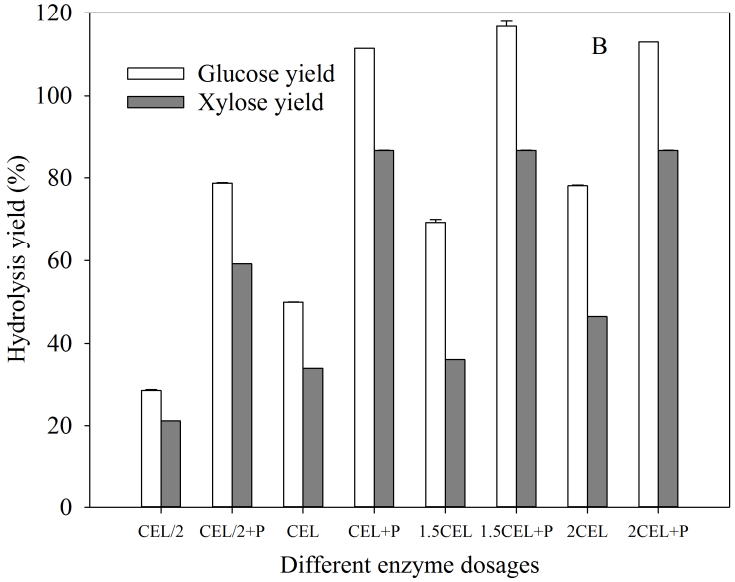
Fig. 6. Mass balance of the ABE production from *S. schwerinii* and barley grain slurry. Star: Starchy slurry.

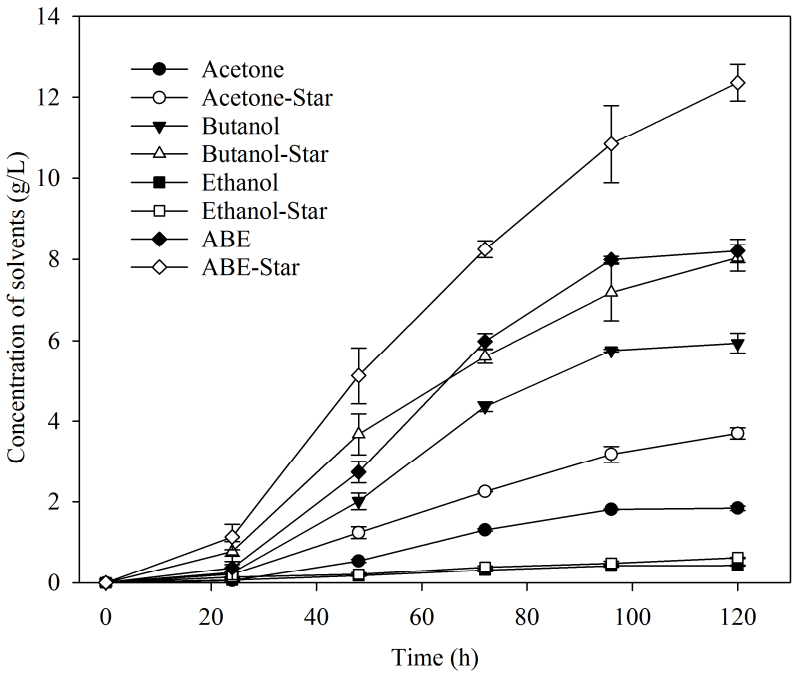












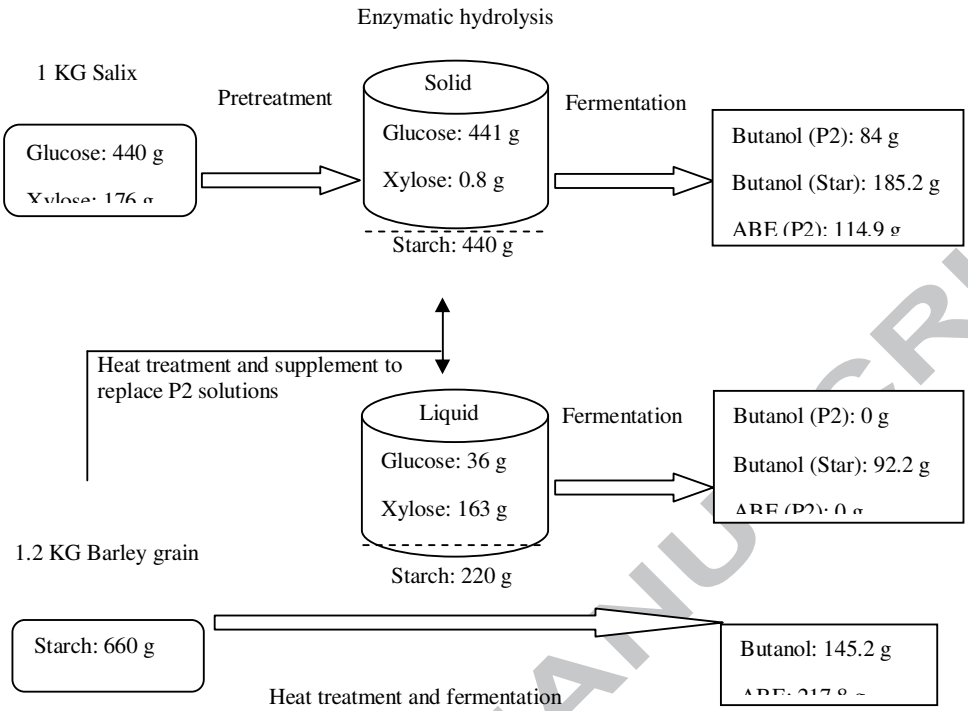


Table 1. Fermentations of glucose by supplementation of starchy slurry to replace P2 solutions.

Batches	Substrates	Original sugars (g/L)		Residual sugars (g/L)		Sugar consumption (%)	Butanol yields (g/g)	ABE yields (g/g)
		Glucose	Starch	Glucose	Starch			
1	Glu+P2	50	0	9.1±3.7	0	82	0.23	0.31
2	Glu	50	0	45.4±4.5	0	9	0.23	0.28
3	Glu+P2+Star(10)	40	10	6.3±5.5	0.3±0	87	0.23	0.36
4	Glu+Star(10)	40	10	25.4±1.5	0.0±0	49	0.21	0.29
5	Glu+P2+Star(20)	30	20	6.1±5.2	1.1±0	86	0.23	0.34
6	Glu+Star(20)	30	20	9.4±1.6	0.3±0	78	0.23	0.33
7	Glu+P2+Star(30)	20	30	6.2±5.1	1.8±0	84	0.24	0.36
8	Glu+Star(30)	20	30	8.7±1.6	2.5±0.1	79	0.25	0.39

Glu: Glucose; Star: Starchy slurry; The numbers in bracket show the volumes of supplemented starchy slurry (mL).

Table 2. Fermentations of xylose by supplementation of starchy slurry to replace P2 solutions.

Batches	Substrates	Original sugars (g/L)		Residual sugars (g/L)			Sugar consumption (%)	Butanol yields (g/g)	ABE yields (g/g)
		Xylose	Starch	Xylose	Glucose	Starch			
9	Xyl+P2	50	0	35.6±5.3	0.0±0	0	29	0.04	0.06
10	Xyl-P2	50	0	37.5±4.5	0.0±0	0	25	0.04	0.05
11	Xyl+P2+Star(10)	40	10	6.1±2.7	0.1±0	0±0	88	0.19	0.31
12	Xyl+Star(10)	40	10	24.9±1.1	3.3±0.9	0±0	44	0.14	0.22
13	Xyl+P2+Star(20)	30	20	4.4±0.5	0.3±0.2	0.1±0	90	0.20	0.32
14	Xyl+Star(20)	30	20	8.7±1.3	0.7±0.2	0.1±0	81	0.22	0.36
15	Xyl+P2+Star(30)	20	30	7.8±4.0	0.3±0.2	0.1±0	84	0.23	0.37
16	Xyl+Star(30)	20	30	6.3±0.7	0.9±0.5	0.1±0	85	0.23	0.38

Xyl: Xylose; Star: Starchy slurry; The numbers in bracket show the volumes of supplemented starchy slurry (mL).

Table 3. The percentage of the compositions of raw material, pretreated solid (cellulosic solid) and liquid (hemicellulosic hydrolysate) in 100 g raw materials.

Materials	Glucose (%)	Xylose (%)	Arabinose (%)	Galactose (%)	Manose (%)	Rhamanose (%)	Lignin (%)
Raw material	44.0	17.6	0.3	0.7	1.6	0.40	21.3
Pretreated solid	41.1	0.8	0.0	0.0	0.2	0	22.7
Pretreated liquid	3.6	16.3	-	-	-	-	

Table 4. Fermentation of hemicellulosic and cellulosic hydrolysates of *S.schwerinii* by supplementation of starchy slurry to replace P2 solutions.

Substrates	Original sugars (g/L)			Residual sugars (g/L)			Butanol yields (g/g)	ABE yields (g/g)
	Glucose	Xylose	Starch	Glucose	Xylose	Starch		
Hemi-hydr+P2	3.6	16.3	0	2.8±0.3	14.6±0.4	0	0	0
Hemi-hydr +Star	2.2	9.8	20	0.5±0.5	0.9±0.1	0.2±0.1	0.22	0.35
Cellu-hydr+P2	36	0.6	0	3.6±0.1	1.1±0.1	0	0.19	0.26
Cellu-hydr+Star	20	0.4	20	1.2±0.1	0.4±0.1	1.3±0.2	0.21	0.33

Hemi-hydr: hemicellulosic hydrolysate; Cellu-hydro: Cellulosic hydrolysate; Star: Starchy slurry.

Table 5 Comparison of ABE fermentations from lignocellulosic hydrolysates.

Substrates	Strains	Hydrolysis methods	Inhibitors removal	Nutrients	ABE (g/L)	Yields (g/g)	References
Wheat bran	*C. beijerinckii* ATCC 55025	Dilute sulfuric acid	Overliming with $Ca(OH)_2$	P2	11.8	0.32	Liu et al. 2010
Wheat straw	*C. beijerinckii* P260	Alkaline peroxide and enzyme	Electrodialys	P2	22.2	0.42	Qureshi et al. 2008
Corn stalk	*C. acetobutylicum* ABE 1301	Dilute alkaline and enzyme	No	P2	7.7-12.2	0.30-0.32	Cai et al., 2016
Spent liquor of spruce	*C. acetobutylicum* DSM 792	SO_2-ethanol-water pulping	Evaporation, steam stripping, liming,oxidation	P2	8.8	0.20	Survase et al. 2011
Corncorb	*C. acetobutylicum* ATCC 824	Alkaline and enzyme	Washing	P2	11.2	-	Zhang et al. 2013
Eucalyptus	*C. saccharoperbutylacetonicum* N1-4	Steam explosion and enzyme	No	No	13.1	0.41	Zheng et al. 2015
Willow	*Clostridium beijerinckii*	Dilute sulfuric acid	No	P2	9.4	-	Han et al. 2013
Salix (hemicellulosic hydrolysate)	*C. acetobutylicum* DSM 1731	Dilute sulfuric acid	No	No	10.6	0.35	This work
Salix (Cellulosic hydrolysate)	*C. acetobutylicum* DSM 1731	Dilute sulfuric acid and enzyme	Washing	No	12.4	0.33	This work

Highlights

- The normal supplements of ABE fermentation medium were replaced with starchy slurry.
- The starchy slurry as supplement ensured the essential nutrients for ABE fermentation.
- Supplement of the starchy slurry enhanced the ABE production from lignocelluloses.
- The starchy slurry was helpful for the utilization of xylose by *C. acetobutylicum.*