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Full Length Article

Acetone-butanol-ethanol (ABE) fermentation of soluble and hydrolyzed sugars in apple pomace by *Clostridium beijerinckii* P260

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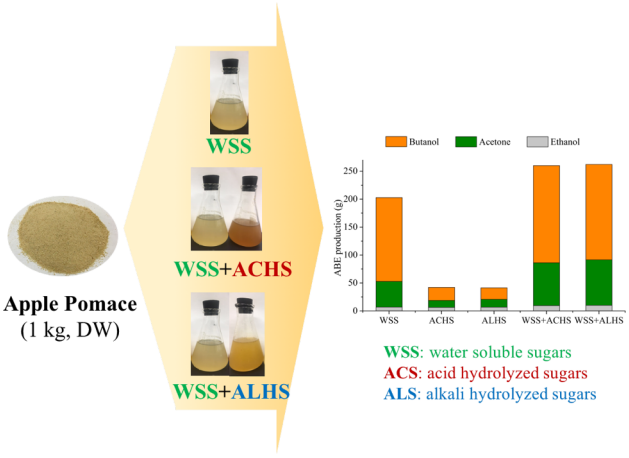
GRAPHICAL ABSTRACT



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ABSTRACT

The decreasing supply of fossil fuels and increasing environmental concern of food waste disposal have raised interests in food waste conversation to biofuels such as butanol. Apple pomace, a food processing waste rich in carbohydrates, is a good feedstock for butanol production. The goal of this study is to present and evaluate a process to thoroughly convert apple pomace water soluble sugars (WSS) and hydrolyzed sugars from structural carbohydrates to acetone-butanol-ethanol (ABE) by fermentation. WSS was extracted from apple pomace by hot water. The solid residue was pretreated with acid or alkali followed by enzymatic hydrolysis to obtain acid hydrolyzed sugars (ACHS) or alkali hydrolyzed sugars (ALHS). Finally, WSS, ACHS, ALHS, WSS + ACHS, and WSS + ALHS were used as substrates to produce ABE by *Clostridium beijerinckii* P260, respectively. Acid and alkali pretreated apple pomace showed significantly ($p < 0.05$) higher glucose yield after cellulase hydrolysis compared with that of unpretreated apple pomace. Addition of pectinase increased hydrolyzed glucose yield by 27.9%, 26.9%, and 33.0% for acid pretreated sample, alkali pretreated sample, and unpretreated sample, respectively. Fermentation results revealed that inhibitors generated during pretreatment could negatively affect

Abbreviations: ABE, acetone-butanol-ethanol; ACHS, acid hydrolyzed sugars; ALHS, alkali hydrolyzed sugars; ANOVA, analysis of variance; DAD, diode array detector; DW, dry weight; HPLC, high performance liquid chromatograph; NREL, national renewable energy laboratory; RID, refractive index detector; SD, standard deviation; WSS, water soluble sugars

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the ABE fermentation rate and titers; however, this negative effect could be alleviated by mixing the hydrolyzed sugars with water soluble sugars. A total of 202.8, 42.1, 41.4, 260.1, and 262.2 g of ABE was produced from each kg of dry apple pomace using WSS, ACHS, ALHS, WSS + ACHS, and WSS + ALHS as the substrates, respectively, based on the mass balance.

1. Introduction

Apple (the Rosaceae family, genus *Malus*), as one of the most widely cultivated fruits, has an increasing production over the past ten years, reaching more than 70 million metric tons production in 2015 [1,2]. About 30% of apples are industrially processed to produce juice, cider, or puree which generate large amounts of pulp, skin, and seed wastes, called pomace. Apple pomace occupies around 25–30% of the original fruits in dry mass [3]. As the industrial byproduct, it contains valuable compounds such as soluble sugars, structural carbohydrates (e.g., cellulose and hemicellulose), minerals, and vitamins. However, due to its low protein content, apple pomace has low nutritional value as animal feed, and most apple pomace ends up in landfills [1]. The high acidity and seed anti-germination activity of apple pomace pose a potential threat to the soil. In addition, the high soluble sugars in apple pomace can be fermented in rumen causing alcoholaemia which intoxicates the animals [4]. Since apple pomace is abundantly available and contains compounds that can be valorized, it calls for a shift from simple pomace waste pollution control to a more holistic approach, that is, apple pomace as the valuable resource can be sustainably processed to value-added chemicals and biofuels [5].

Recently, apple pomace has been used to produce organic acids, antioxidants, enzymes, ethanol, biogas, and butanol [4,6]. Among the aforementioned biomolecules, butanol is a particularly promising product due to its several advantages: 1) butanol occupies a current market over $6 billion each year, which is expected to reach $18 billion by 2020 due to the increasing need for bio-based chemicals [7]; 2) butanol serves as an excellent sustainable biofuel alternative to ethanol. The energy content in butanol is 30% higher than ethanol and is closer to gasoline; moreover, it has lower vapor pressure and is less flammable, making it compatible with gasoline in various proportions [8]; 3) butanol is an important industrial intermediate chemical which can be used to generate other products such as acrylic esters, butyl acetate, and glycol ethers [9].

Currently, butanol is industrially produced from petroleum or fermentation of corn and cassava. Due to the rising food price, food wastes such as wheat straw, rice straw, barley straw, corn stover, and corn cob have been studied as substrates for butanol production through acetone-butanol-ethanol (ABE) fermentation by *Clostridium beijerinckii,* or *C. acetobutylicum*, etc [10]. Our previous research also showed the advantages of using food waste to produce biofuels [11]. Apple pomace, as agricultural solid waste, is abundantly available and rich in soluble sugars and structural carbohydrates, making it a good candidate for butanol production. The relevant research using apple pomace as the feedstock for ABE fermentation is limited. A published study used the soluble sugars in apple pomace to produce butanol [12]. Recently, researchers applied different pretreatments (autohydrolysis, acids, alkali, organic solvent, and surfactant) followed by enzymatic hydrolysis to break down the structural carbohydrates of apple pomace into hydrolyzed sugars, which were further fermented to butanol [13]. Although separate studies on soluble sugars or structural carbohydrates utilization exist, no research has been conducted to evaluate the technical feasibility of comprehensive utilization of all (both soluble and insoluble) carbohydrates in apple pomace to obtain butanol. The thorough utilization of carbohydrates in apple pomace will not only maximize the butanol production but also minimize the residual waste after processing.

Therefore, the objective of this study is to develop a process to make a full utilization of the carbohydrates (both soluble and structural sugars) in apple pomace to maximize butanol production. Apple pomace was extracted by hot water to obtain soluble sugars at first. The solid residue, which is rich in lignocellulose, was then pretreated with dilute acid or alkali solution followed by enzymatic hydrolysis to get hydrolyzed sugars. Pretreatment and enzymatic hydrolysis parameters were optimized in these steps. Finally, soluble sugars and acid/alkali hydrolyzed sugars were combined as the substrate for butanol production by *C. beijerinckii* P260.

2. Materials and methods

2.1. Chemicals and materials

Sulfuric acid, sodium hydroxide, acetone, butanol, ethanol, acetic acid, and butyric acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Glucose, xylose, arabinose, fructose, sucrose, galactose, furfural, and HMF were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cooked meat medium was obtained from Difco™ (Becton, Dickinson and Company, Sparks, MD, USA). Yeast extract was purchased from Bacto-Dickinson & Co. (Sparks, MD, USA). *C. beijerincki* P260 spores were maintained in sterile distilled water at 4 °C until utilization. The enzymes including cellulase (Cellic CTec2, 132 FPU/mL) and pectinase (Pectinex® Ultra SPL, 4186 EU/mL) were supplied by

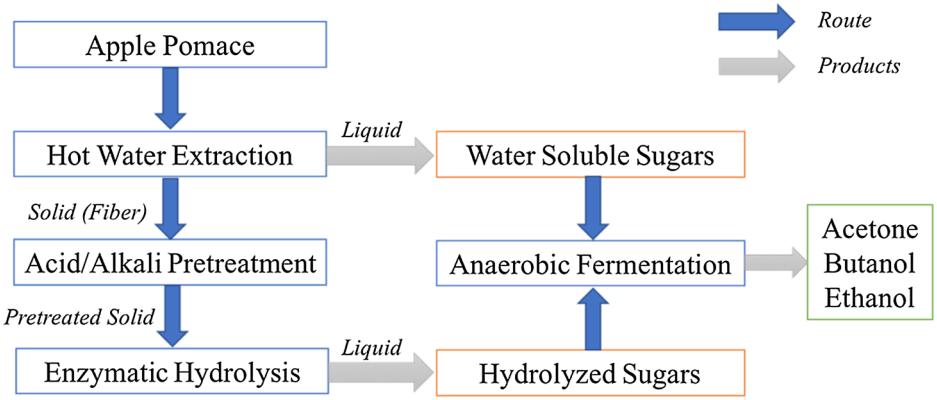

Fig. 1. The flow diagram of the proposed process to comprehensively utilize both water soluble and hydrolyzed sugars from apple pomace to produce acetone-butanol-ethanol (ABE).

Novozymes North America Inc. (Franklinton, NC, USA).

Apple pomace (Golden Delicious, 3 kg) was obtained from the pilot-scale food processing plant of Virginia Tech (Blacksburg, VA 24061) in October 2016. After filter press to extract juice, the leftover pomace was collected and stored at −20 °C until utilization. Apple pomace was dried (moisture content 7.3%) and milled with a laboratory hammer mill to a particle size of less than 0.85 mm, and then stored at −20 °C until further utilization.

2.2. Soluble sugars extraction

Fig. 1 illustrates the flow diagram of the proposed process. The hot water extraction of apple pomace was applied to obtain residual soluble sugars in apple pomace. In details, apple pomace powder (15 g) was subjected to deionized water (300 mL) extraction in the autoclave at 121 °C for 30 min [14,15]. The autoclaved mixture was centrifuged (16,639 × *g*, 10 min, 4 °C) to separate solid and supernatant. The supernatant was determined for sugar composition and concentration using high performance liquid chromatograph (HPLC) as described later. The solid was collected and subjected to sequential pretreatment and enzymatic hydrolysis to obtain hydrolyzed sugars. The sugar extraction was conducted in triplicate.

2.3. Acid and alkali pretreatments

The apple pomace solid residue (with 4.0% of pectin, dry weight, DW) was pretreated by dilute sulfuric acid or sodium hydroxide. Varied concentrations of sulfuric acid (1 and 2%, v/v) or sodium hydroxide (1 and 2%, w/v) with a solid to liquid ratio of 1:20 for different pretreatment times (30 and 60 min) at 121 °C were evaluated to identify the best pretreatment conditions. After pretreatment, the solid was separated from the liquid by centrifugation (16,639 × *g*, 10 min, 4 °C). The solid residue was then washed with deionized water five times to remove most of residual sulfuric acid or sodium hydroxide and potential inhibitors generated during pretreatment. Each pretreatment was performed in triplicate.

2.4. Enzymatic hydrolysis

Enzymatic saccharification of unpretreated apple pomace solid residue (moisture content of 86.6%), acid pretreated apple pomace solid residue (moisture content of 88.9%), and alkali pretreated apple pomace solid residue (moisture content of 89.7%) was performed with 13 FPU/g dry biomass of cellulase addition. Two solid loadings including 2.5 and 5% were selected herein due to the high viscosity of the pretreated samples. The pH of the samples was adjusted to 5.0 using 0.1 M sodium citrate buffer. The saccharification was conducted in a shaking water bath (50 °C) at 120 rpm for 72 h. After selecting one best acid pretreatment and one best alkali pretreatment, 183 EU/g dry biomass of pectinase was added to test the synergistic action of cellulase and pectinase in releasing sugars from apple pomace. Pectin (approximate 0.1% in apple pomace after both acid and alkali pretreatments) matrix may act as a barrier for the enzymatic hydrolysis of cellulose. Thus, the degradation of pectin by pectinase may help sugar release from cellulose. The selection of cellulase and pectinase loadings was based on the manufacturer's guidelines and previous studies [3,16]. During the enzymatic hydrolysis, samples (1 mL) were collected at different times (0, 6, 12, 24, 48, 72 h), and then centrifuged (16,639 × *g*, 10 min, 4 °C) to collect supernatant for further sugar analysis. Enzymatic hydrolysis was performed in triplicate.

2.5. Acetone-butanol-ethanol (ABE) fermentation

C. beijerinckii P260 spores (100 μL) were heat shocked at 80 °C for 2 min for activation. The heat shocked spore solution (20 μL) was then transferred to the cooked meat medium (3.5 g cooked meat, 0.6 g glucose, and 35 mL distilled water). The medium was previously autoclaved at 121 °C for 15 min followed by cooling down to room temperature. After spore inoculation, the medium was incubated at an anaerobic environment (35 °C) for 16–18 h, and the culture was used as the first-stage inoculum. Following that, 7–8 mL of the first-stage culture was transferred to the P2 medium. The medium containing carbon source and yeast extract was autoclaved at 121 °C for 15 min followed by cooling to 35 °C, and then, filter-sterilized P2 stock solutions [(buffer: KH_2PO_4, 50 g/L; K_2HPO_4, 50 g/L; ammonium acetate, 220 g/L), (vitamin: para-amino-benzoic acid, 0.1 g/L; thiamin, 0.1 g/L; biotin, 0.001 g/L), and (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)] were aseptically added (1 mL/each) [17]. The P2 medium culture was allowed to grow for 7–8 h at 35 °C anaerobically, after which it was ready to be inoculated into the ABE fermentation medium.

Five media including water soluble sugar (WSS), acid hydrolyzed sugars (ACHS), alkali hydrolyzed sugars (ALHS), WSS combined with ACHS, and WSS combined with ALHS were used as ABE fermentation substrates, respectively. ABE fermentation with pure glucose as a substrate was also conducted for a control purpose. The ABE fermentation was conducted in 50 mL centrifuge tubes (20 mL substrates). Before inoculation, all substrates were amended with 2 g/L yeast extract and sterilized at 121 °C for 15 min followed by adding 1 mL of each filter-sterilized stock solution including vitamins, buffer, and minerals [17]. Then, 1.5 mL of actively growing cells developed in P2 medium were added to different substrates. The fermentation was conducted anaerobically at 35 °C. ABE fermentation was performed in duplicate. ABE yield and productivity were calculated to evaluate the fermentation efficiency:

$$\text{ABE yield} = \frac{\text{g/L of Total ABE}}{\text{g/L of Total sugar utilized}} \quad (1)$$

$$\text{ABE productivity(g/L/h)} = \frac{\text{g/L of Total ABE}}{\text{h of Fermentation time}} \quad (2)$$

2.6. Analytical methods

For the soluble sugars determination, apple pomace (1 g) was extracted by 85% ethanol (50 mL) for 30 min with constant shaking in a water bath at 50 °C. The extraction procedure was repeated twice. The extracted liquid was combined and vacuum evaporated at 50 °C to remove ethanol, and the residue was re-suspended in ultrapure water (obtained from Thermo Scientific™ Barnstead™ MicroPure™ Water Purification System, 18.2 MΩ cm, Fisher Scientific, Fair Lawn, NJ, USA) for sugars determination [18]. Structural carbohydrates including glucan, xylan, arabinan, galactan, and mannan, lignin, and extractives of apple pomace were analyzed according to the National Renewable Energy Laboratory (NREL) procedures [19–22]. Pectin was determined by the hot acid extraction method [23]. To be specific, 5 g of samples were mixed with 250 mL distilled water (adjusting pH to 2.5 with citric acid), and the mixture was incubated in the water bath (95 °C) for 30 min. After that, the mixture was filtered (Whatman #1 filter paper) and the filtrate was cooled overnight at 4 °C. Then, 125 mL of 96% ethanol was added to the cold filtrate. After stirring for 10 min, the liquid was left overnight to precipitate pectin. Finally, the precipitate was filtered (Whatman #1 filter paper) and dried in the oven (55 °C) for 24 h. The pectin content was determined gravimetrically.

Sugars (glucose, sucrose, xylose, fructose, galactose, arabinose, and mannose) were determined by Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID). Samples were filtered through 0.2 μm syringe filter (Waters Corporation, Milford, MA, USA) before analyzing by HPLC. A Bio-Rad Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA, USA) was used with ultrapure water as the mobile phase (flow rate of 0.6 mL/min) at 80 °C. The total running time was 30 min, and the injection

Table 1
Chemical composition of apple pomace after hot water extraction.

Components	Content (%, DW)
Extractives	29.7 ± 1.6
Structural carbohydrates	
Glucan	34.9 ± 1.1
Xylan	7.2 ± 0.5
Arabinan	4.7 ± 0.5
Galactan	5.9 ± 0.5
Lignin	
Acid soluble lignin	2.4 ± 0.2
Acid insoluble lignin	12.4 ± 0.1

Data are expressed as mean ± SD.

volume was 20 μL.

Total phenolic compounds were measured by the Folin-Ciocalteu method [24]. To be specific, liquid sample (0.5 mL) was mixed with Folin-Ciocalteau reagent (0.2 N, 2.5 mL) and saturated sodium carbonate (7.5%, w/v, 2 mL). The mixture was incubated at room temperature for 2 h and then measured the absorbance at 765 nm by the Genesys™ 10S UV/VIS spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA). Gallic acid was used as the standard.

Fermentation products including acetone, butanol, ethanol, acetic acid, and butyric acid and degradation products including acetic acid, HMF, and furfural were measured by Agilent 1200 HPLC system (Agilent Technologies) with a 1260 RID and a 1200 diode array detector (DAD). Samples were centrifuged at 15,871 × *g* for 5 min, and then filtered through 0.2 μm syringe filter (Waters Corporation) prior to analyses. A Bio-Rad Aminex HPX-87H column (50 °C) were used for fermentation products separation. Sulfuric acid (5 mM) solution was used as mobile phase. Acetic acid, ethanol, and butanol were detected with the RID, and other chemicals were detected by the DAD with different wavelengths (butyric acid, 210 nm; acetone, 265 nm; HMF and furfural, 280 nm). The flow rate was set at 0.6 mL/min with the injection volume of 5 μL.

2.7. Statistical analysis

Comparisons among treatments were analyzed by analysis of variance (ANOVA) according to Tukey's test using SPSS software (Version 19.0, SPSS Inc., Chicago, IL, USA). Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Water soluble sugars extraction

The composition of apple pomace is affected by numerous factors such as variety, ripen stage, physical and chemical properties of the apples, juice extraction technologies, various enzyme utilization during extraction, and with or without press aid such as rice hulls [25]. The variability in the composition of apple pomace will affect the process design. For example, the contents of soluble sugars such as sucrose (from 1.4 to 11.2%, DW) [26,27], fructose (from 13.6 to 35.0%, DW) [12,28], and glucose (from 6.1 to 50.8%, DW) in different apple pomaces may determine if recovery of those soluble sugars is needed [28,29]. In addition, the variation in soluble and insoluble fibers in apple pomace may affect the selection of pretreatment methods and enzymes to obtain hydrolyzed sugars. A detailed discussion about selection pretreatment methods and enzymes is addressed in later sections.

In the present study, the composition analysis showed that the major soluble sugars extracted by 85% ethanol in apple pomace included fructose (27.2%, DW), sucrose (16.3%, DW), and glucose (8.0%, DW); minor amounts of xylose (0.2%, DW), galactose (0.3%, DW), and arabinose (0.1%, DW) were also detected. The high content of soluble sugars remained in apple pomace might be due to the incomplete pressing of ground apples when producing juice [3].

Due to the high amount of soluble sugars existed in the apple

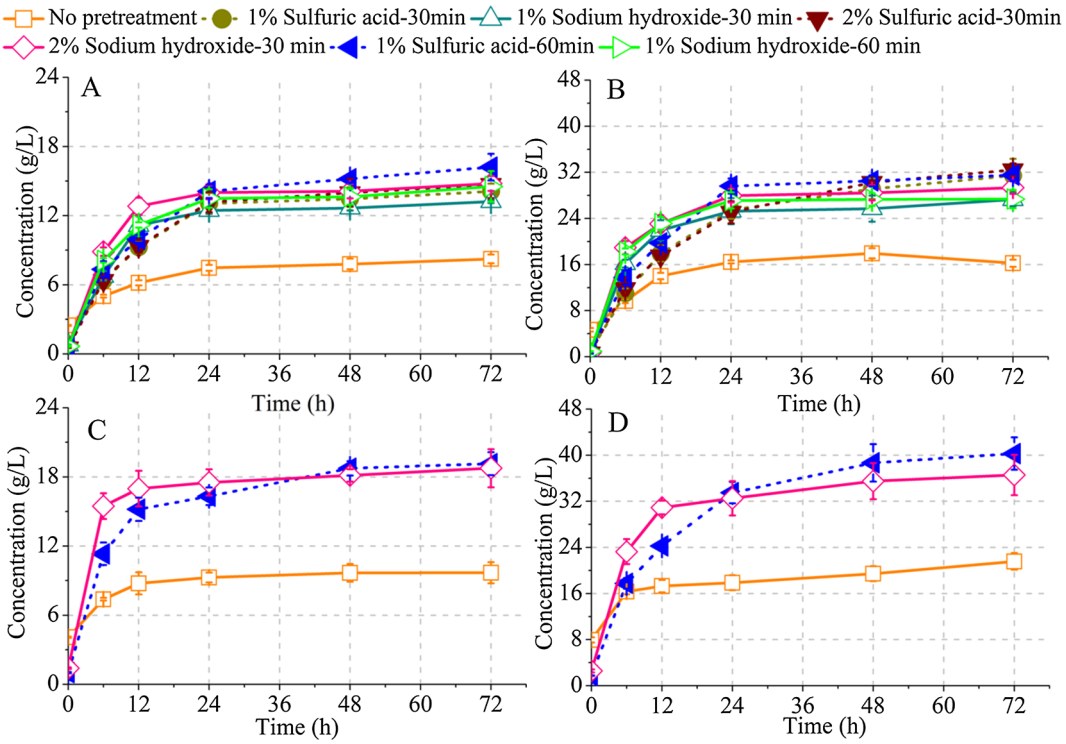

Fig. 2. Glucose produced from different acid/alkali pretreatments followed by enzymatic hydrolysis of apple pomace solid residue (after water extraction). A: Cellulase + 2.5% solid loading during enzymatic hydrolysis; B: cellulase + 5% solid loading during enzymatic hydrolysis; C: cellulase + pectinase + 2.5% solid loading during enzymatic hydrolysis; D: cellulase + pectinase + 5% solid loading during enzymatic hydrolysis. The error bars represent SD.

pomace, the first step we applied herein was using hot water to extract those sugars for further fermentation utilization. As a result, the extracted solution contained a total of 28.3 g/L of sugars. Among them, fructose (14.7 g/L) was the major sugar, followed by sucrose (8.2 g/L) and glucose (5.1 g/L). Small amounts of other sugars (less than 0.3 g/L) were also extracted. The soluble sugar enriched liquid was used as the substrate for subsequent ABE fermentation.

3.2. Hydrolyzed sugars production

After hot water extraction, there was 29.7% of extractives (mainly ethanol soluble fats and waxes, soluble dietary fiber, and some minerals) left in the solid residue. The extractives might be recovered for nonpolar compounds extraction. In the present study, the extractives were removed by acid or alkali pretreatment and following washing steps. Besides extractives, the solid residue was rich in cell wall materials such as lignin and structural carbohydrates (Table 1). A total of 14.8% (DW) lignin was found in the solid residue. As to the structural carbohydrates, glucan was the major one (34.9%, DW), the total contents of other structural carbohydrates (hemicellulose components including xylan, arabinan, and galactan) were 17.8% (DW).

Lignin, some crosslinks between lignin and structural carbohydrates such as xylan, and some fibers such as pectin serve as physical barriers to reduce the accessibility for enzymatic saccharification of the structural carbohydrates [30]. Therefore, a pretreatment process was performed to disrupt the recalcitrant structure of biomass and make the structural carbohydrates more accessible by enzymes. In the present study, acid (sulfuric acid) and alkali (sodium hydroxide) pretreatments, the two of the most effective pretreatments to reduce biomass recalcitrance, with different concentrations (1 and 2%) and pretreatment times (30 and 60 min) were tested. In addition, structural carbohydrates could not be directly fermented by *C. beijerinckii* P260. Therefore, after pretreatment, enzymatic hydrolysis is needed to break the structural carbohydrates to fermentable (hydrolyzed) sugars. In this study, cellulase with or without pectinase addition was applied in the enzymatic hydrolysis step, during which two solid loadings (2.5 and 5%, w/v) were conducted.

The aim of evaluating these parameters was to identify the best operating conditions to get the maximum hydrolyzed sugars for the subsequent ABE fermentation. Glucose was selected as the optimization target. Other hydrolyzed sugars such as xylose, arabinose, and galactose were not reported herein due to their negligible concentrations in hydrolysates. As could be seen in Fig. 2A, both the acid and alkali pretreatments of apple pomace improved the glucose production at 2.5% solid loading compared with the unpretreated sample. After 72 h of cellulase hydrolysis, the maximum 16.2 and 14.8 g/L of glucose were obtained from acid (1% sulfuric acid for 60 min) and alkali (2% sodium hydroxide for 30 min) pretreatments, respectively, while the glucose production from unpretreated apple pomace solid residue was significantly ($p < 0.05$) lower, only 8.2 g/L. When hydrolysis was conducted at 5% solid loading, the maximum glucose concentration doubled to 32.4 and 29.3 g/L for acid (2% sulfuric acid for 30 min) and alkali (2% sodium hydroxide for 30 min) pretreatments, respectively (Fig. 2B). The glucose concentration in the unpretreated pomace sample (at 5% solid loading) was 16.2 g/L.

One of the factors that affecting pretreatment efficiency is the composition of the apple pomace. Acid pretreatment is mainly aimed to break down hemicellulose such as xylan in biomass and make cellulose more accessible to enzymes during enzymatic hydrolysis [30]. This pretreatment method has been studied on different biomass and is more favorable in the industrial application [30]. However, some degradation products such as furfural, HMF, acetic acid, and phenolic compounds may be generated during the pretreatment process, which may affect the metabolism of microorganism in the fermentation [31]. Alkali pretreatment is effective on removing lignin and acetyl groups to increase the cellulose digestibility during enzymatic hydrolysis [30]. Compared with acid pretreatment, less structural carbohydrates such as cellulose and hemicellulose are solubilized during alkali pretreatment

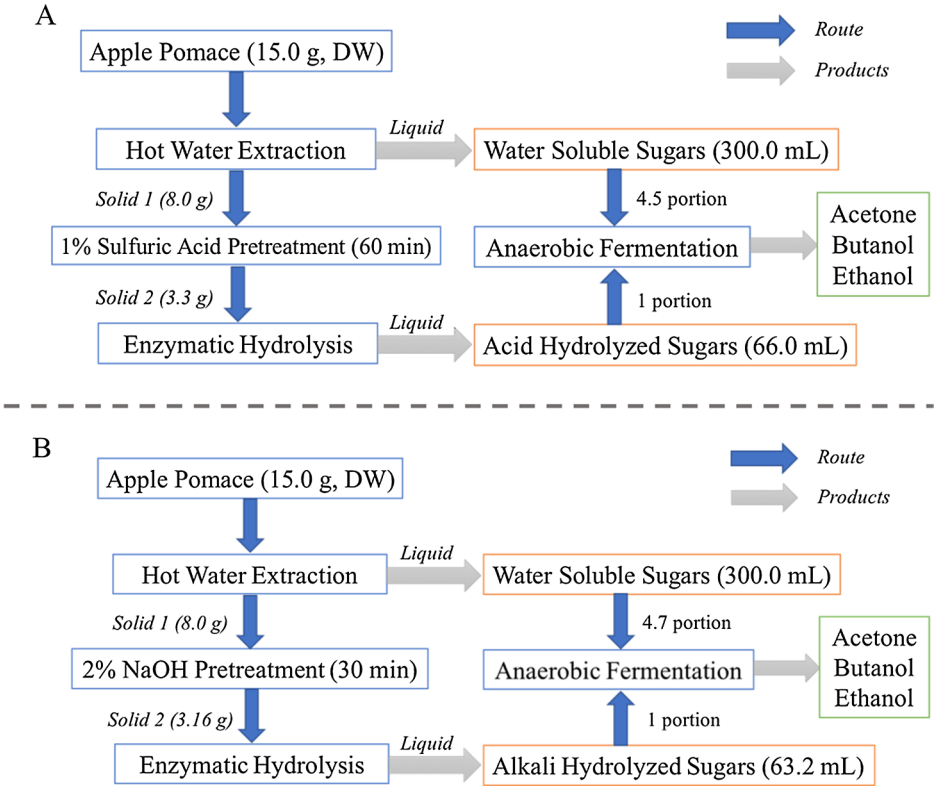

Fig. 3. Water soluble sugars (WSS) combined with acid or alkali hydrolyzed sugars (ACHS or ALHS) for acetone-butanol-ethanol (ABE) fermentation. A: WSS + ACHS; B: WSS + ALHS.

[31]. However, the efficiency of alkali pretreatment is highly dependent on the lignin content of the biomass [30]. Therefore, the selection of pretreatment method should be based on the composition due to the content variation of the lignin (from 6.4 to 20.4%, DW) and hemicellulose (from 4.1 to 24.4%, DW) in apple pomaces [4,25,27]. As in the present study, the apple pomace residue contained 14.8% (DW) of lignin and 17.8% (DW) of hemicellulose. Although no significant ($p > 0.05$) difference was found within different acid or alkali pretreatments, the acid pretreatment groups had a higher glucose production compared with the alkali pretreatment groups on average. A similar result was found in a previous study that alkali (sodium hydroxide and potassium hydroxide) was not as effective as acids (sulfuric acid, hydrogen chloride, and nitric acid) to pretreat apple pomace for the production of hydrolyzed sugars [13].

We further investigated the synergistic cooperation of cellulase and pectinase for the glucose production during hydrolysis. In this experiment, acid (1% sulfuric acid for 60 min) and alkali (2% sodium hydroxide for 30 min) pretreatments were selected due to their optimal performance as shown in Fig. 2A and B. As could be seen in Fig. 2C, by addition of pectinase, the glucose yield increased by 17.5%, 18.1%, and 26.9% for unpretreated, sulfuric acid pretreated, and sodium hydroxide pretreated samples at 2.5% solid loading, respectively. At 5% of solid loading, the maximum of 40.3 and 36.6 g/L glucose could be obtained after sulfuric acid and sodium hydroxide pretreatments followed by enzymatic (cellulase and pectinase) hydrolysis, respectively (Fig. 2D). The glucose yield increased by 33.0% for the unpretreated sample, 27.9% for the sulfuric acid pretreated sample, and 26.9% for the sodium hydroxide pretreated sample. Studies have shown that the cell wall of apple pomace contained a pectin matrix, which could block cellulose, leading to inefficient enzymatic digestibility [16]. Therefore, breaking down pectin network using pectinase could facilitate enzymatic attachment to cellulose, consequently releasing more glucose.

3.3. ABE production

Five types of sugar including WSS, ACHS, ALHS, WSS + ACHS, and WSS + ALHS obtained before were used as the substrates for the ABE fermentation, respectively. The liquid to liquid ratios of WSS to ACHS (4.5:1) and WSS to ALHS (4.7:1) were calculated based on the mass balance of the designed process (Fig. 3). To be specific, after hot water extraction, 300 mL WSS and 8 g fiber enriched solid residue could be obtained from 15 g dry apple pomace. The solid residue was then pretreated by sulfuric acid or sodium hydroxide to get 3.3 g or 3.16 g pretreated solid residue, respectively. After that, the acid and alkali pretreated solid residues were enzymatically hydrolyzed, obtaining 66.0 mL of ACHS and 63.2 mL of ALHS, respectively. Therefore, for 1

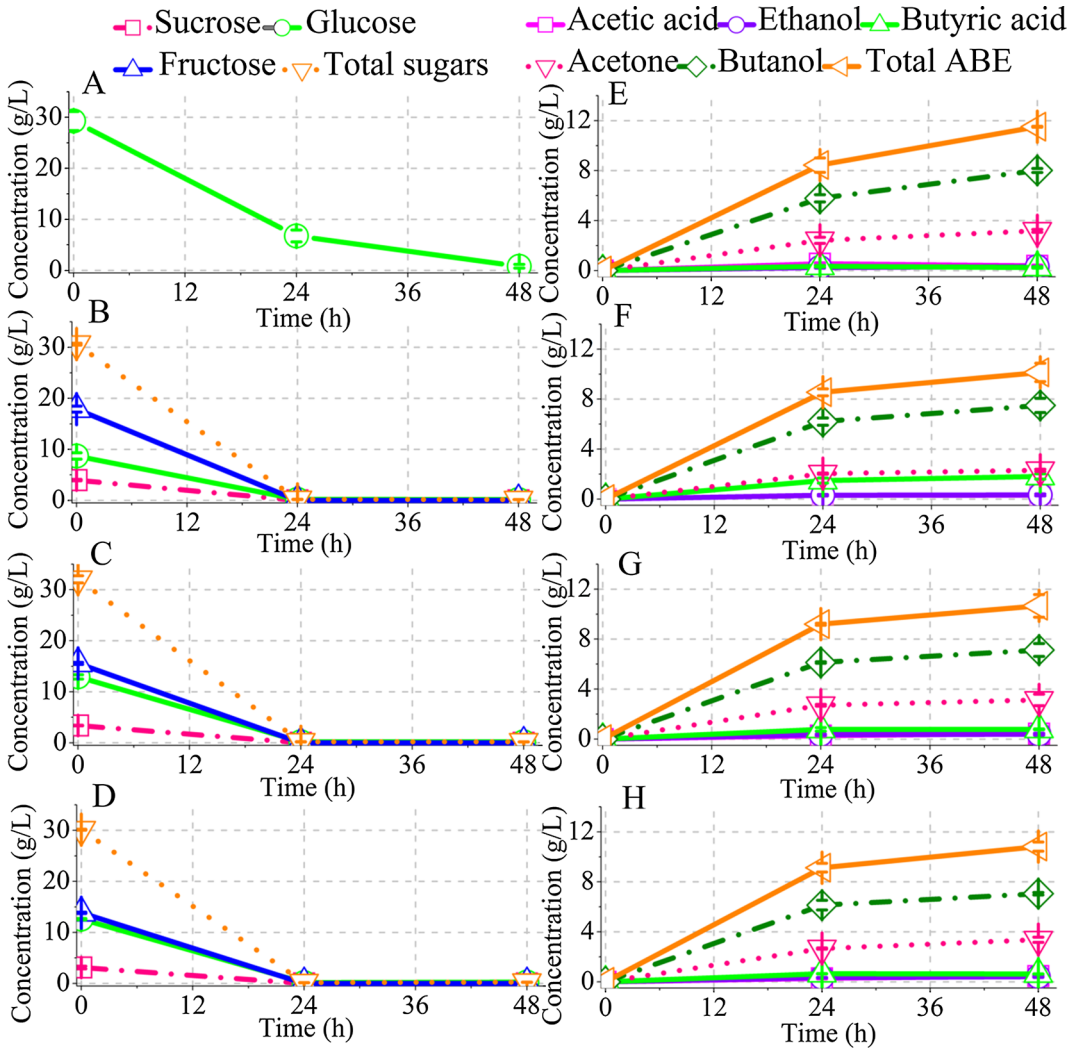

Fig. 4. Soluble and hydrolyzed sugars utilization and fermentation products generated by *Clostridium beijerinckii* P260. A: Sugars utilization in control; B: sugars utilization in apple pomace water soluble sugars (WSS), C: sugars utilization in WSS combined with acid hydrolyzed sugars (ACHS); D: sugars utilization in WSS combined with alkali hydrolyzed sugars (ALHS); E: fermentation products generated in control; F: fermentation products generated in WSS; G: fermentation products generated in WSS + ACHS; H: fermentation products generated in WSS + ALHS. The error bars represent SD.

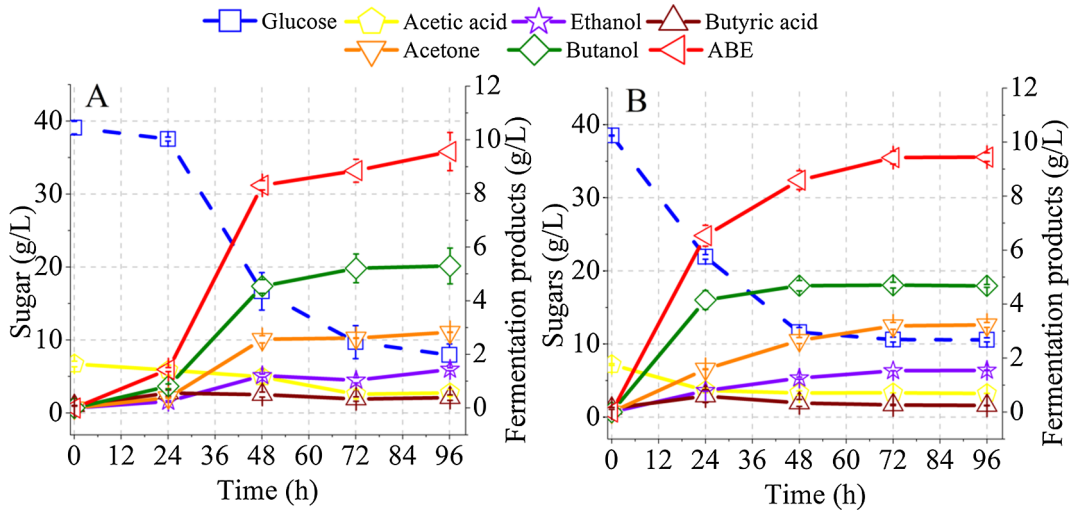

Fig. 5. Acid and alkali hydrolyzed sugars utilization and fermentation products generated by *Clostridium beijerinckii* P260. A: Fermentation using acid hydrolyzed sugars (ACHS) as the substrate; B: fermentation using alkali hydrolyzed sugars (ALHS) as the substrate (fermentation completed at 72 h).

Table 2
Degradation products in pretreated liquid and enzymatic hydrolysis liquid.

Degradation products (g/L)	Acid pretreatment (1% sulfuric acid, 60 min)		Alkali pretreatment (2% sodium hydroxide, 30 min)	
	After pretreatment	After hydrolysis	After pretreatment	After hydrolysis
Acetic acid	0.4 ± 0.06	1.8 ± 0.1	0.6 ± 0.03	1.3 ± 0.08
HMF	0.8 ± 0.007	0.02 ± 0.0004	0.005 ± 0.0001	0.003 ± 0.0001
Furfural	0.04 ± 0.005	0.002 ± 0.0001	0.002 ± 0.0002	0.002 ± 0.00001
Total phenolic compounds	1.5 ± 0.05	0.4 ± 0.02	1.8 ± 0.1	0.3 ± 0.02

Data are expressed as mean ± SD.

portion of ACHS or ALHS obtained from apple pomace, 4.5 or 4.7 portions of WSS could be generated from apple pomace simultaneously. Therefore, to make a full utilization of apple pomace sugars, we combined 4.5 portions of WSS with 1 portion of ACHS, and 4.7 portions of WSS with 1 portion of ALHS as the ABE fermentation medium, respectively. A control (glucose) with a similar amount of total sugars as WSS, WSS + ACHS, and WSS + ALHS was also conducted to test the performance of *C. beijerinckii* P260.

The results of sugars (fructose, glucose, and sucrose) utilization and fermentation products (acetone, butanol, ethanol, acetic acid, and butyric acid) generation using control, WSS, WSS + ACHS, and WSS + ALHS are shown in Fig. 4. Prior to the initiation of ABE fermentation, 29.3, 30.6, 32.1, and 30.1 g/L total sugars were present in the control, WSS, WSS + ACHS, and WSS + ALHS substrates, respectively. These sugars were consumed at the end of 48 h fermentation (Fig. 4A–D); therefore, we stopped fermentation at 48 h. Compared with control, sugars in apple pomace substrates were depleted faster. At 24 h, sugars in WSS, WSS + ACHS, WSS + ALHS were almost used up, while about 6.7 g/L glucose still remained in the control substrate. One of the possible reason is that some nutrients such as vitamins or minerals in apple pomace may stimulate the sugar consumption by *C. beijerinckii* P260 [32]. From 24 to 48 h, a slight increase of ABE production was found in WSS, WSS + ACHS, and WSS + ALHS (Fig. 4F–H), which was attributed to the utilization of residual monomer sugars such as glucose, sucrose, xylose, galactose, and fructose (about 0.1–0.2 g/L) and might also be due to the utilization of some water extractable polysaccharides such as starch [33,34]. At last, a total of 11.5, 10.1, 10.7, and 10.8 g/L ABE were produced from control, WSS, WSS + ACHS, and WSS + ALHS, respectively. The concentrations of butanol in control, WSS, WSS + ACHS, and WSS + ALHS were 8.0, 7.5, 7.1, and 7.1 g/L, respectively (Fig. 4E–H). The mechanism behind the lower ABE yield in apple pomace substrates compared with control is currently unclear. However, from the application perspective, applying apple pomace waste material as the fermentation substrate could solve the apple pomace waste management problem to a certain extent, and in the meantime, benefit the environment.

ABE fermentation was also conducted using 100% ACHS or ALHS as a substrate, respectively. Only the utilization of glucose is shown in Fig. 5 since the concentration of the sum of other sugars including sucrose, xylose, galactose, and fructose is less than 2.5 g/L. The results showed that fermentation using ACHS or ALHS resulted in a longer fermentation time, and thus a lower fermentation rate, compared with fermentation using WSS, WSS + ACHS, and WSS + ALHS as substrates. When ALHS was used as a fermentation medium, the fermentation was not completed until 72 h with an ABE productivity of 0.13 g/L/h. When ACHS was used as the fermentation medium, the ABE productivity was even lower than the ALHS fermentation. Only 1.6 g/L of glucose was consumed during the first 24 h, and the fermentation was not completed until 96 h, with an ABE productivity of 0.10 g/L/h. This low fermentation rate might be due to the generated inhibitors that inhibit fermentation [31]. As can be seen in Table 2, the contents of degradation products (inhibitors) including HMF and furfural are significantly ($p < 0.05$) higher in liquids after acid pretreatment and hydrolysis than those in liquids after alkali pretreatment and hydrolysis. A previous study found that the apple pomace hydrolysate obtained after nitric acid pretreatment and enzymatic hydrolysis was not fermentable and the possible reason was the high content of inhibitors generated in the acidic treatment [13]. At last, a total of 9.6 and 9.4 g/L of ABE were generated by consumption of 30.4 and 28.0 g/L of total sugars from ACHS and ALHS, respectively. Overall, the result indicates that the generated inhibitors from the pretreatments could negatively affect the ABE fermentation rate and titers; however, this negative effect could be alleviated by mixing the hydrolyzed sugars with water

Table 3
Comparison of ABE production from apple pomace substrates.

Strain	Pretreatment	Enzymatic hydrolysis	Fermentation substrate	Sugar utilized (g/L)	Total ABE produced (g/L)	ABE productivity (g/L/h)	Solvent yield (g/g)	Ref.
C. beijerinckii P260	Sulfuric acid (1%, 60 min, 121 °C)	Cellic CTec 2 and pectinase	Soluble sugars and hydrolyzed sugars	31.9	10.7	0.22	0.34	This study
C. beijerinckii P260	Sodium hydroxide (2%, 30 min, 121 °C)	Cellic CTec 2 and pectinase	Soluble sugars and hydrolyzed sugars	29.9	10.8	0.23	0.36	This study
C. beijerinckii P260	–	–	Soluble sugars	30.5	10.1	0.21	0.33	This study
C. beijerinckii P260	Sulfuric acid (1%, 60 min, 121 °C)	Cellic CTec 2 and pectinase	Hydrolyzed sugars	30.4	9.6	0.10	0.32	This study
C. beijerinckii P260	Sodium hydroxide (2%, 30 min, 121 °C)	Cellic CTec 2 and pectinase	Hydrolyzed sugars	28.0	9.4	0.13	0.34	This study
C. beijerinckii CECT 508	Autohydrolysis (12 min, 142.4 °C)	Cellic CTec 2	Hydrolyzed sugars	38.9	8.3	0.09	0.23	[13]
C. beijerinckii CECT 508	HNO_3 (1.83%, 7.3 min, 124.2 °C)	Cellic CTec 2	Hydrolyzed sugars	3.1	0.1	0.001	0.03	[13]
C. beijerinckii CECT 508	Acetone (10%, 5 min, 112.1 °C)	Cellic CTec 2	Hydrolyzed sugars	37.4	15.1	0.16	0.42	[13]
C. beijerinckii CECT 508	PEG 6000 (1.96%, 5 min, 100.2 °C)	Cellic CTec 2	Hydrolyzed sugars	38.2	12.9	0.13	0.40	[13]
C. acetobutylicum NRRL B 596	–	–	Soluble sugars	33.6	11.4 (A + B)*	0.16 (A + B)	0.34 (A + B)	[12]
C. butylicum NRRL B 592	–	–	Soluble sugars	34.0	13.1 (A + B)	0.18 (A + B)	0.38 (A + B)	[12]
C. butylicum NRRL B 593	–	–	Soluble sugars	32.0	12.8 (A + B)	0.18 (A + B)	0.40 (A + B)	[12]

* A + B means the results are calculated based on acetone and butanol.

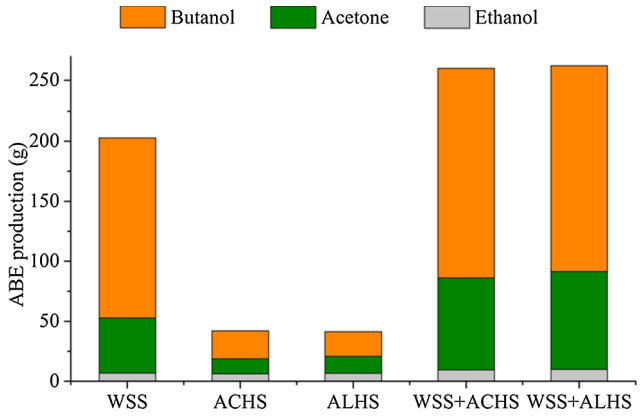

Fig. 6. Overall ABE production from each kg of dry apple pomace based on the mass balance.

soluble sugars. This clearly shows the advantage of the proposed process of using both water soluble and hydrolyzed sugars by combining them together as the fermentation substrate.

To see if the results of current ABE fermentation from apple pomace are comparable with previous studies, a detailed comparison is shown in Table 3. As can be seen from this table, ABE production, solvent yield, and ABE productivity are similar to those reported in other references using apple pomace soluble sugars or structural carbohydrates as the substrates.

Finally, the overall ABE production from apple pomace was calculated based on the mass balance. Fig. 6 shows the overall ABE production from the fermentation of each kg of dry apple pomace using different substrates including WSS, ACHS, ALHS, WSS + ACHS, and WSS + ALHS. By fermenting WSS, about 202.8 g ABE including 46.0 g acetone, 150.0 g butanol, and 6.8 g ethanol could be generated. By fermenting only ACHS or ALHS, about 42. 1 or 41.4 g ABE including 12.5 or 14.0 g acetone, 23.3 or 20.7 g butanol, and 6.3 or 6.7 g ethanol could be generated, respectively. However, by the combination of about 20% of acid or alkali hydrolyzed sugars with WSS, significantly ($p < 0.05$) higher amount of ABE could be generated. For the WSS + ACHS substrate, a total of 260.1 g ABE (76.6 g acetone, 174.0 g butanol, and 9.5 g ethanol) was obtained from each kg of dry apple pomace. As to WSS + ALHS, a total of 262.2 g ABE (81.6 g acetone, 170.7 g butanol, and 9.9 g ethanol) can be generated from each kg of dry apple pomace.

From the economic perspective, the comprehensive utilization of both water soluble and hydrolyzed sugars from apple pomace provides several benefits. First, the proposed process will lead to an increase of ABE yield from 203 kg (WSS) to 262 kg (WSS + ALHS) from each tonne of apple pomace (Fig. 6), Considering the current market price of butanol ($1.2/kg), acetone ($0.9/kg), and ethanol ($0.5/kg) [35], the potential economic value of the increased yield of ABE is about $58 for processing each tonne of apple pomace (DW), which could contribute significantly to the economic performance of the process, e.g., internal rate of return. Second, by mixing water soluble sugars with the hydrolyzed sugars, we found that the negative effect of inhibitors in the hydrolyzed sugar substrate was mitigated in ABE fermentation. This could potentially reduce the operating and capital costs of the butanol production by eliminating the expensive detoxification step. Usually the overliming process is used to detoxify the pretreated biomass; however, this process results in a significant amount of sugar lost (~13%) due to side reactions occurring at high pH and has a high capital cost [36]. Third, the conversion of water soluble and hydrolyzed sugars to butanol could share the same equipment (e.g., fermenters, distillation columns), which would further reduce the capital cost per unit of butanol production. On the other hand, compared with only using water soluble sugars, more unit operations such as pretreatments and enzymatic

hydrolysis are needed for using both the water soluble and hydrolyzed sugars. Therefore, a detailed techno-economic analysis is warranted in the future to evaluate the economic feasibility of this designed process.

In the present study, we find that there is still a potential to further increase the production of total ABE. One way is to increase the solid to liquid ratio in the soluble sugar extraction, and thus can increase the sugar concentration in the fermentation medium. In addition, in-situ solvent recovery technologies such as vacuum stripping and pervaporation membranes can be integrated to the fermentation system to selectively recover ABE from the fermentation broth [11,37,38].

4. Conclusion

In this study, an integrated process aiming at a comprehensive utilization of both apple pomace soluble sugars and acid or alkali hydrolyzed sugars to produce ABE by anaerobic fermentation was proposed. All substrates including WSS, ACHS, ALHS, WSS + ACHS, and WSS + ALHS were fermentable by *C. beijerinckii*. The fermentation using ACHS as the substrate had the lowest productivity which was probably due to the highest degradation products such as HMF and furfural that inhibited fermentation. The fermentations using WSS, WSS + ACHS, or WSS + ALHS resulted in higher productivities and final ABE concentrations than the fermentations using ACHS or ALHS. Based on the mass balance, 42.1, 41.4, 202.8, 260.1, and 262.2 g of ABE were produced from each kg of dry apple pomace using ACHS, ALHS, WSS, WSS + ACHS, and WSS + ALHS as the fermentation substrates, respectively. This result showed the superiority of the combination of water soluble sugars and hydrolyzed sugars in apple pomace for ABE production. The designed process in this study can be adapted and applied to other food processing byproducts or wastes, such as white grape pomace, citrus wastes, and pineapple peels.

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