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Optimization of Butanol Production from Mixed Sugars and Sweet Sorghum Bagasse Hydrolysate Using *Clostridium beijerinckii* TISTR 1461

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Abstract: This study investigated the capability of *Clostridium beijerinckii* TISTR 1461 to utilize mixed sugars (glucose and xylose) in synthetic media and sweet sorghum bagasse (SSB) hydrolysate for butanol production. Synthetic media containing 60 g/L of glucose and xylose at various ratios were used for butanol production. *C. beijerinckii* TISTR 1461 preferentially utilized glucose over xylose for butanol production. The highest butanol concentration (P_B, 10.25–10.60 g/L), butanol yield ($Y_{B/S}$, 0.27–0.28 g/g), butanol productivity (Q_B, 0.22 g/L·h), and sugar consumption (*SC*, 61–63%) were achieved when the glucose content was at least 75% of the total sugars. When an SSB hydrolysate (produced via enzymatic hydrolysis) containing 60.83 g/L of total sugars (glucose:xylose ratio = 88:12, *w*/*w*) was used as a substrate for butanol production, the SSB hydrolysate supplemented with 1 g/L of yeast extract and buffers significantly yielded higher P_B (15.10 g/L), $Y_{B/S}$ (0.31 g/g), Q_B (0.31 g/L·h), and *SC* (82%) values compared to the synthetic media. These results indicate that sweet sorghum bagasse hydrolysates containing glucose and xylose mixtures show promise as cost-effective substrates for sustainable butanol fermentation, demonstrating the potential of agricultural residues in biofuel production.

Keywords: biobutanol; *Clostridium beijerinckii*; glucose and xylose; mixed sugars; sweet sorghum bagasse

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1. Introduction

As global concerns over climate change and the environmental impact of fossil fuels intensify, there has been a growing emphasis on developing renewable and sustainable alternatives. Butanol has garnered attention as a promising biofuel due to several advantageous properties: it blends easily with gasoline, has a high energy content, maintains a high blending vapor pressure, and exhibits high water tolerance [1–3]. Beyond its potential as a fuel, butanol also serves as a crucial chemical in various industrial processes, making it a valuable commodity.

One of the main obstacles to butanol production is its high substrate costs. Starchy feedstocks (potatoes, corn, cassava, etc.), sugary feedstocks (sugarcane, sugar beets, and molasses), and lignocellulosic materials are the three main substrate classes used for butanol production [4]. Lignocellulosic materials, potential low-cost carbon sources for biobased butanol production, are agricultural residues that cannot be used as food [5–8]. Sweet sorghum bagasse (SSB), a residue of the extraction of free sugars from sorghum stalks, is a

lignocellulosic material that can be used as a substrate for butanol production. However, the compact structure of bagasse must be converted into a fermentable hydrolysate using proper pretreatment and hydrolysis processes [9–12]. Generally, delignification is performed as the primary pretreatment of lignocellulosic material. Many researchers reported that NaOH is an effective chemical in extracting lignin from lignocellulosic material [13–15]. Subsequently, enzymatic methods are used to hydrolyze the pretreated lignocellulosic materials to obtain fermentable sugars for bioproduct fermentation [16,17].

Biobutanol can be produced by Clostridia strains, including *Clostridium acetobutyricum*, *C. sporogenes*, *C. carboxidivorans*, *C. saccharoperbutylacetonicum*, *C. saccharobutyricum*, and *C. beijerinckii*, via acetone–butanol–ethanol (ABE) fermentation under strictly anaerobic conditions. ABE fermentation has two characteristic phases: acidogenesis and solventogenesis. During acidogenesis, acetic acid, butyric acid, and ATP are produced in the exponential growth phase. When the acid levels greatly increase and the pH dramatically decreases, bacterial cells in the stationary phase convert the acids into solvents (acetone, butanol, and ethanol) [18–21].

This research aims to address the limitations in previous studies regarding high substrate costs and the understanding of several factors in butanol production: the effects of mixed-sugar ratios in lignocellulosic hydrolysates, nitrogen source requirements, and pH control and buffering effects. Therefore, this study evaluates the capability of *C. beijerinckii* TISTR 1461 to utilize mixed sugars, i.e., glucose and xylose, at different ratios in synthetic media as well as in SSB hydrolysates for butanol production. The findings can be applied to other lignocellulosic residues, and the study includes a comparison of butanol production between synthetic media and SSB hydrolysates.

2. Materials and Methods

2.1. Inoculum Preparation

A spore suspension (~1 × 10^6 spores/mL) of *C. beijerinckii* TISTR 1461 from the Thailand Institute of Scientific and Technology Research Center (TISTR) was kept at 4 °C in a refrigerator (SF-C997 GYN, Sanyo Commercial Solution, Mueang Chachoengsao, Thailand). The spores were heat shocked at 80 °C for 1 min to activate them and then immediately cooled in ice water for 1 min to prevent cell damage [22]. The cultures were grown for 10 to 14 h at 37 °C in a sterile cooked meat medium (CMM, Oxoid, Basingstoke, UK) containing 1 g of CMM and 0.08 g of glucose (BDH, Leuvn, Belgium) under anaerobic conditions created using oxygen-free nitrogen (OFN) gas, as described by Daengbussadee et al. [23]. Sterile tryptone–glucose–yeast extract (TGY) medium (Oxoid, Basingstoke, UK) was flushed with OFN gas to create anaerobic conditions. Thereafter, 5% (*v*/*v*) of the culture in CMM medium was inoculated into TGY medium and cultivated at 37 °C for 4 to 6 h to obtain a 0.5 OD (optical density at 600 nm) in the log phase of growth (~0.9 g dry cell weight/L) before use as an inoculum for the ABE fermentation [24].

2.2. Butanol Production from Mixed-Sugar Synthetic Media

2.2.1. Synthetic Mixed-Sugar Media or Modified P2 Media

A modified standard synthetic medium (P2 medium) for butanol production consisting of glucose and xylose (Sigma-Aldrich, St. Louis, MO, USA) at different ratios (60 g/L of total sugars) was prepared (Table 1). Apart from the sugars, the modified P2 medium comprised 10 mL/L of stock solutions A, B, and C and 1 g/L of yeast extract (Oxoid, Basingstoke, UK). Ammonium acetate (BDH, Leuvn, Belgium), 220 g/L; K_2HPO_4 (BDH, Leuvn, Belgium), 50 g/L; and KH_2PO_4 (BDH, Leuvn, Belgium), 50 g/L, were the components of stock solution A. The ingredients of stock solution B were para-amino-benzoic acid (Fluka, Steinheim, Sweden), 0.1 g/L; thiamin (Labchem, New South Wales, Australia), 0.1 g/L; and

biotin (Sigma-Aldrich, MO, USA), 1 mg/L. Stock solution C contained $MgSO_4 \cdot 7H_2O$ (BDH, Poole, UK), 20 g/L; $MnSO_4 \cdot H_2O$ (BDH, Poole, UK), 1 g/L; $FeSO_4 \cdot 7H_2O$ (Riedel-de Haën, Seelze, Germany), 1 g/L; and NaCl (Ajax, Auckland, New Zealand), 1 g/L [25,26]. The P2 media (except stock solution B) were sterilized in an autoclave (SX-700, TOMY, Nerima City, Japan) at 110 °C for 28 min [22,27]. Stock solution B was sterilized by passing it through a 0.2 µm cellulose acetate membrane. Then, 1% (*v*/*v*) of stock solution B was aseptically added into the sterile modified P2 medium.

Table 1. Ratios of glucose and xylose in modified P2 media (60 g/L of total sugars) for butanol production.

Glucose:Xylose (%, *w*/*w*)	Concentration (g/L)	
	Glucose	Xylose
100:0	60	0
75:25	45	15
50:50	30	30
25:75	15	45
0:100	0	60

2.2.2. ABE Fermentation from Mixed-Sugar Synthetic Media

The pH of sterile modified P2 media containing different ratios of glucose and xylose (Table 1) was adjusted to 6.50. The media were sparged with OFN gas to create anaerobic conditions. Then, a butanol producer, active *C. beijerinckii* TISTR 1461 cells in TGY medium (from Section 2.1), was inoculated (5%, *v*/*v*) into the modified P2 media to initiate the ABE fermentation. The fermentation was carried out in 1 L screw-capped bottles (700 mL working volume) at 37 °C with an agitation rate of 150 rpm.

2.3. Butanol Production from SSB Hydrolysate

2.3.1. Preparation of Sweet Sorghum Bagasse Particles

Sweet sorghum (*cv*. KKU 40) was obtained from the Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University, Thailand. After juice extraction from sweet sorghum stalks, the obtained sweet sorghum bagasse (SSB) was chopped, ground, and milled to obtain small particles (4 mesh) and dried at 60 °C to a constant weight in an oven (FD240, Binder, Tuttlingen, Germany). The particles were kept at 4 °C until use.

2.3.2. Sweet Sorghum Bagasse Pretreatments

1. Delignification

Ten-percent NaOH (*w*/*v*) was mixed with the SSB particles at a liquid/solid ratio (LSR) of 20 mL liquid/g dry weight of SSB particles (modified from [14]). The mixture was autoclaved at 121 °C for 20 min and cooled at room temperature. The solid fraction in the mixture was filtered through a cheesecloth and washed with distilled water until the pH of the wash water became neutral. Thereafter, the solid particles were dried at 60 °C to a constant weight (modified from [28–30]).

2. Enzymatic hydrolysis

Dried pretreated SSB after delignification was enzymatically hydrolyzed with 8% (*w*/*v*) solid loading in 0.05 M citrate buffer at pH 5.0, and 8% (*v*/*v*) cocktail enzyme [cellulase (iKnowZyme, Reach Biotechnology Co., Ltd., Thanyaburi, Thailand): xylanase (iKnowZyme, Reach Biotechnology Co., Ltd., Thanyaburi, Thailand) in a 1:1 (*v*/*v*) ratio] was added to activate enzymatic hydrolysis. The cellulase and xylanase activities were

59.53 FPU/mL and 420 U/mL, respectively. The mixtures were incubated at 50 °C and 150 rpm for 72 h (modified from [11,31,32]). After that, the reaction was stopped by heating in a water bath at 100 °C for 10 min. Finally, the solid fraction was separated via centrifugation (Thermo Scientific™ Sorvall LYNX 4000, Waltham, MA, USA) at room temperature with an RCF of 8602 g. Thereafter, the sugar content (glucose and xylose), 5-hydroxymethylfurfural (HMF), furfural, and lignin in the supernatant or SSB hydrolysate were analyzed before use as a substrate for butanol production.

2.3.3. ABE Fermentation from SSB Hydrolysate

Four butanol production media from SSB hydrolysates were prepared as follows: SSB hydrolysate with no supplement, called SSBH; SSBH supplemented with 1.0 g/L of yeast extract, called SSBH-YE; SSBH supplemented with 1.0 g/L of yeast extract and buffer in P2 medium (stock solution A), called SSBH-YE-Buf; and SSBH supplemented with all nutrients in a P2 medium called SSBH-Nut in P2. Additionally, modified P2 medium supplemented with mixed sugars of glucose and xylose at the ratio detected in the SSB hydrolysate (Section 2.3.2) was prepared and used as the control treatment.

For the ABE fermentation, the pH of sterile media was adjusted to 6.50. The media were sparged with OFN gas to create anaerobic conditions. An active butanol producer, *C. beijerinckii* TISTR 1461, was then immediately inoculated into the media. The fermentation was performed in 1 L screw-capped bottles (700 mL working volume) at 37 °C with agitation at 150 rpm.

2.4. Analytical Methods

During ABE fermentation, samples were taken from the 1 L screw-capped bottles at 12 h intervals. To maintain anaerobic conditions in the screw-capped bottles, OFN gas was flushed through a PTFE filter (0.45 µm) into the headspace to increase the pressure. The samples were then withdrawn through a sampling port using an aseptic technique. The pH of the fermentation broth was measured using a pH meter (Mettler-Toledo, FiveEasy™ Plus FEP20, Greifensee, Switzerland). Fermentation products, such as acetone, butanol, ethanol, acetic acid, and butyric acid, were analyzed via gas chromatography (Shimadzu, GC-2014, Kyoto City, Japan) using a Porapak Q column (80/100 mesh, 3 m × 3 mm, GL sciences, Shinjuku City, Japan) (modified from [23]). The total sugar concentration was determined using a phenol–sulfuric acid method [33]. For inoculum preparation, the cell growth (optical density, OD) and biomass concentrations [dry cell weight, DCW (g/L)] were spectrophotometrically measured at 600 nm (OD_{600}) [34,35] and oven dried at 90 °C to a constant weight, respectively. Quantitative analyses of xylose, glucose, formic acid, 5-hydroxymethylfurfural (HMF), and furfural were conducted using high-performance liquid chromatography (HPLC) with a refractive index detector (Waters, Milford, MA, USA). The stationary and mobile phases were an Aminex HPX-87H column (300 mm × 7.8 mm ID, Bio-Rad, Hercules, CA, USA) and 5 mM H_2SO_4, respectively [29].

The lignin content in the SSB hydrolysate was determined using the Folin reagent method according to *Standard Methods for the Examination of Water and Wastewater* [36]. The absorbance of the reaction was measured using a spectrophotometer at 649 nm. The free amino nitrogen (FAN) concentration in the fermentation broth was measured using a ninhydrin assay [37].

The kinetic parameters of butanol yield ($Y_{B/S}$), volumetric butanol productivity (Q_B), and sugar consumption (SC) were calculated using the following equations:

$$Y_{B/S}\ (\mathrm{g/g}) = \frac{P_B}{TS} \quad (1)$$

$$Q_B\ (\mathrm{g/L{\cdot}h}) = \frac{P_B}{t} \quad (2)$$

$$SC\ (\%) = \frac{(ITS - RTS)}{ITS} \times 100 \quad (3)$$

P_B is the butanol concentration produced (g/L), TS is the total sugar concentration utilized (g/L), t is the fermentation time giving the highest P_B (h), ITS is the initial total sugar concentration (g/L), and RTS is the residual total sugar concentration (g/L).

2.5. Statistical Analyses

Means and standard deviations (SD) are reported for each treatment. The experiments were conducted in triplicate. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to determine significant differences in the mean values at an $\alpha = 0.05$ level.

3. Results and Discussion

3.1. Butanol Production from Mixed-Sugar Synthetic Media

The butanol production profiles by *C. beijerinckii* TISTR 1461 from synthetic mixed-sugar media (modified from P2 medium) supplemented with glucose and xylose (60 g/L of total sugars) at various ratios (100:0, 75:25, 50:50, 25:75, and 0:100) are shown in Figure 1. All fermentation profiles were similar except in the medium containing only xylose (0:100). The pH values decreased sharply with slight decreases in the sugar concentration (~2 to 12 g/L) in the first 12 h of fermentation (Figure 1a,b). This indicated that acids were produced (Figure 1c). The results implied that *C. beijerinckii* TISTR 1461 cells were in their acidogenesis phase. High acetic acid and butyric acid levels indicated that acetate kinase and butyrate kinase were, respectively, active, coupled with ATP generated in post-pyruvate metabolism. ATP was also reused in pre-pyruvate metabolism to generate ADP. These systems operated during the acidogenesis phase of ABE fermentation. Then, the acids were converted into solvents (acetone, butanol, and ethanol). These results imply that acetoacetate decarboxylase, butanol dehydrogenase, and ethanol dehydrogenase were, respectively, active in the solventogenesis phase [38–41]. Butanol and ethanol were detected, suggesting that NADH from post-pyruvate metabolism was consumed, while NAD^+ was produced. NAD^+ was also regenerated for use in pre-pyruvate metabolism for NADH production. These phenomena occurred to balance redox reactions in the fermentation system.

Figure 1b,d show sugar consumption and butanol production during ABE fermentation. The % sugar consumption (SC) and butanol concentration (P_B) from the medium containing only glucose (100:0) were not significantly different from those of the medium containing 75% glucose (75:25) (Table 2). The highest P_B (10.25–10.60 g/L), SC (61.76–63.03%), butanol yield ($Y_{B/S}$, 0.27–0.28 g/g), and butanol productivity (Q_B, 0.22 g/L·h) were obtained when the mixed sugars contained at least 75% glucose. A lower P_B and P_{ABE} were obtained when the glucose contents in the medium were under 75% or the xylose contents were greater than 25% (Table 2 and Figure 1d,e). The corresponding % SC was lower when the xylose content was higher, implying that *C. beijerinckii* TISTR 1461 preferentially metabolized glucose. Birgen et al. [42] showed that the glucose and xylose ratio influences growth and butanol synthesis via carbon catabolite suppression. Butanol production was the lowest in the medium containing only xylose. Under this condition, P_B, $Y_{B/S}$, and Q_B were only 1.65 g/L, 0.22 g/g, and 0.05 g/L·h, respectively. The results show that the enzymatic activity of *C. beijerinckii* TISTR 1461 in a xylose metabolic pathway was low. Little is known about xylose catabolism in *Clostridium* spp. Liu et al. [43] stated that the phosphoketolase and pentose phosphate pathways were active in

C. acetobutylicum when xylose was used in the culture medium. Zhao et al. [44] reported that *C. beijerinckii* and *C. acetobutylicum* metabolized xylose through the pentose phosphate and phosphoketolase pathways. Domingues et al. [45] reported that some wild types of *Clostridium* metabolized xylose through three different pathways: xylose isomerase, oxidoreductase, and non-phosphorylative pathways (including the Weinberg and Dahms pathways).

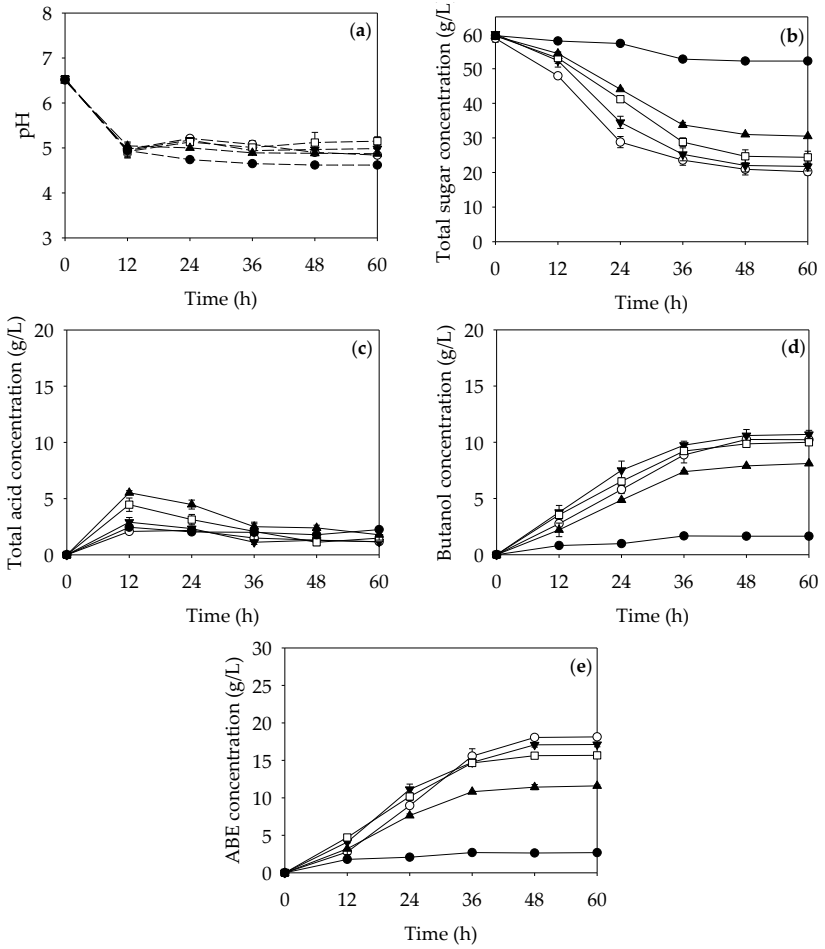


Figure 1. Profiles of pH (**a**), sugar (**b**), acids (**c**), butanol (**d**), and ABE (**e**) during ABE fermentation by *C. beijerinckii* TISTR 1461 in modified P2 media containing mixed sugars (60 g/L of total sugars) of glucose and xylose at different ratios: glucose:xylose = 100:0 (○), 75:25 (▼), 50:50 (□), 25:75 (▲), and 0:100 (●).

Table 2. ABE fermentation results by *C. beijerinckii* TISTR 1461 from modified P2 media containing mixed sugars (60 g/L of total sugars) of glucose and xylose at different ratios.

Glucose:Xylose (%, *w*/*w*)	P_B (g/L)	P_{ABE} (g/L)	*SC* (%)	*t* (h)	$Y_{B/S}$ (g/g)	Q_B (g/L·h)
100:0	10.25 ± 0.16 [a]	18.06 ± 0.01 [a]	63.03 ± 1.96 [a]	48	0.27 ± 0.01 [a]	0.22 ± 0.01 [a]
75:25	10.60 ± 0.44 [a]	17.08 ± 0.31 [b]	61.76 ± 1.68 [a]	48	0.28 ± 0.00 [a]	0.22 ± 0.01 [a]
50:50	9.78 ± 0.12 [b]	15.63 ± 0.16 [c]	56.94 ± 0.22 [b]	48	0.28 ± 0.00 [a]	0.21 ± 0.00 [a]
25:75	7.90 ± 0.04 [c]	11.43 ± 0.34 [d]	47.85 ± 0.36 [c]	48	0.28 ± 0.01 [a]	0.16 ± 0.01 [b]
0:100	1.65 ± 0.01 [d]	2.64 ± 0.11 [e]	12.34 ± 0.45 [d]	36	0.22 ± 0.01 [b]	0.05 ± 0.01 [c]

[a, b, c, d, e]: Means followed by the same letter within the same column are not significantly different using Duncan's multiple range test at $\alpha = 0.05$. All experiments were performed in triplicate, and the results are expressed as mean values ± SDs. P_B = butanol concentration, P_{ABE} = acetone–butanol–ethanol concentration, *SC* = sugar consumption, $Y_{B/S}$ = butanol yield, and Q_B = volumetric butanol productivity.

At the end of fermentation, fermentable sugars were not completely utilized. Some sugars remained in the broth, even in the medium containing only glucose (100:0) (Figure 1b). This may be due to butanol toxicity or catabolite repression [46–49]. When the final glucose and xylose contents were determined, the most glucose consumed, 42.20 g/L, was in the medium containing only glucose (no xylose addition). Glucose consumption was lower in the media containing higher xylose contents (Figure 2). *C. beijerinckii* consumed more xylose when the xylose in the medium was increased from 25% to 75%. Xylose consumption in a medium containing only xylose (100%) was not different from the medium containing 75% xylose, indicating that the highest capability of *Clostridium* to consume xylose was ~17 g/L.

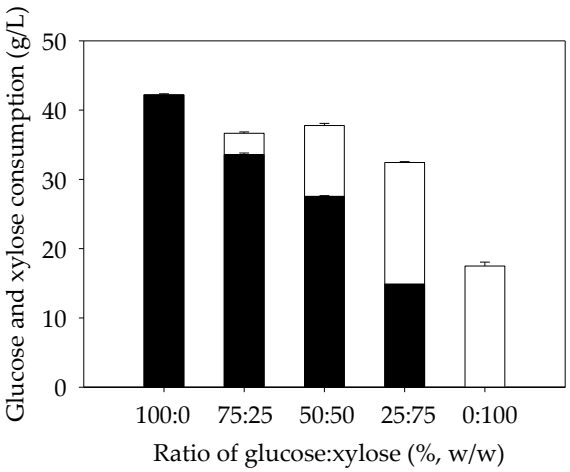


Figure 2. Consumption of glucose (black bars) and xylose (white bars) in ABE fermentations from the modified P2 media containing mixed sugars (60 g/L of total sugars) of glucose and xylose at different ratios: glucose:xylose = 100:0, 75:25, 50:50, 25:75, and 0:100 (*w*/*w*).

Our results indicate that xylose content in the medium did not affect the $Y_{B/S}$ values (0.27–0.28 g/g) as long as there was glucose in the medium at any ratio, suggesting that the butanol metabolic pathway was unchanged. However, when using xylose as a sole carbon source, $Y_{B/S}$ was lower, at 0.22 g/g, implying that by-product pathways were operating.

According to the results (Table 2), a modified P2 medium supplemented with mixed sugars of at least 75% glucose with a xylose content not higher than 25% was suitable for use as a medium for butanol production.

3.2. Sugar Composition of SSB Hydrolysate

The sugar composition of the SSB hydrolysate was determined before use as a substrate for butanol production. After enzymatic hydrolysis, the SSB hydrolysate comprised 60.83 g/L of total sugars, 53.53 g/L of glucose, and 7.30 g/L of xylose corresponding to a glucose:xylose ratio of 88:12. Formic acid and 5-hydroxymethylfurfural (HMF) were detected at less than 0.5 g/L. These results may be due to a dehydration process under thermal decomposition of hexose sugar (glucose) gained from the cellulose fraction of lignocellulose biomass when the enzymatic reactions were stopped in a water bath [50–53]. Furfural was not detected in the SSB hydrolysate, suggesting that the dehydrated five-carbon sugar (xylose) obtained from the hemicellulose fraction of lignocellulosic biomass was not formed via enzymatic hydrolysis. Additionally, the lignin in the SSB hydrolysate was below the detectable level (0.05 mg/L). The total sugar concentration in the SSB hydrolysate was not different from the sugar concentration in the standard butanol production medium or P2 medium (60 g/L of glucose). According to the results of Section 3.1, using the modified P2 medium containing mixtures of glucose and

xylose, a suitable glucose content of a medium for butanol production should be at least 75% of the total sugars (or a xylose content less than 25%). The SSB hydrolysate in the study contained 88% glucose and 12% xylose. Very low concentrations of inhibitors (formic acid, HMF, furfural, and lignin) were detected in the hydrolysate. Therefore, the SSB hydrolysate (SSBH) was used directly as a substrate for butanol production by *C. beijerinckii* TISTR 1461.

3.3. Butanol Production from SSB Hydrolysate

The P2 medium (a standard butanol production medium) supplemented with glucose and xylose at the same ratio detected in SSBH (glucose:xylose = 88:12) was prepared and used as a control treatment. This was done to evaluate the feasibility of using SSBH as a substrate for butanol production. Changes in the pH, total sugars, total acids, butanol, and ABE concentrations during fermentation by *C. beijerinckii* TISTR 1461 from SSBH and the P2 medium (containing 88% glucose and 12% xylose) are shown in Figure 3. The P_B value of SSBH with no supplementation was very low (1.18 g/L) compared to that of the P2 medium containing the same mixed-sugar ratio (10.77 g/L). This is likely due to insufficient nutrients and/or no buffering agents in the SSBH. Therefore, the effects of nitrogen supplementation (yeast extract, YE), minerals (in P2 medium), and buffers (K_2HPO_4, KH_2PO_4, and ammonium acetate) on butanol production from the SSBH by the *Clostridium* were investigated. When SSBH supplemented with 1.0 g/L of yeast extract (SSBH-YE), SSBH supplemented with 1.0 g/L of yeast extract and buffers in P2 medium (SSBH-YE-Buf), and SSBH supplemented with all nutrients in P2 medium (SSBH-Nut in P2) were used for butanol production, the fermentation profiles of SSBH-YE were similar to those of SSBH. The pH sharply decreased in 12 h from 6.50 to 4.82 and continuously decreased until the end of fermentation, with a final value of 4.49 (Figure 3a). The pH values indicated high acid production (Figure 3c). Sugar consumption, butanol, and ABE were very low (Figure 3b,d,e). Low ABE production in SSBH and SSBH-YE is likely due to a lack of buffering in the medium, so the acids produced (acetic and butyric) were not converted into solvents (ABE). In SSBH-YE-Buf and SSBH-Nut in P2, the pH profiles in the presence of buffers were higher than those with no buffer addition (SSBH and SSBH-YE), suggesting that buffers (ammonium acetate, K_2HPO_4, and KH_2PO_4) are essential to controlling the pH during ABE fermentation (Figure 3a). The *SC*, P_B, and P_{ABE} values markedly increased under buffer addition (Figure 3b,d,e). Additionally, a lower total acid concentration (P_{acid}, 0.55–2.68 g/L) (Table 3) remaining in the fermentation broth was observed under buffer addition (Figure 3c), whereas a higher P_{acid} (5.40–7.99 g/L) was detected with no buffering. The buffers could activate butyrate-CoA-transferase, butyraldehyde dehydrogenase, and butanol dehydrogenase in the butanol pathway, along with acetoacetyl-CoA-acetate, acetyl-CoA-acetyltransferase, and acetoacetate decarboxylase in the acetone pathway. The results indicated that the nitrogen source in SSBH was insufficient for butanol production. Yeast extract addition as a nitrogen source is essential and can boost ABE fermentation in the presence of buffers. The highest kinetic parameter values of P_B (15.10 g/L), P_{ABE} (23.66 g/L), *SC* (82%), $Y_{B/S}$ (0.31 g/g), and Q_B (0.31 g/L·h) were obtained using SSBH-YE-Buf. Surprisingly, these values were higher than those using a synthetic P2 medium. However, the $Y_{B/S}$ values (0.30–0.32 g/g) were not significantly different with buffer addition. Hence, an SSBH medium supplemented with yeast extract and a buffer (SSBH-YE-Buf) is a promising substrate for butanol production by *C. beijerinckii* TISTR 1461.

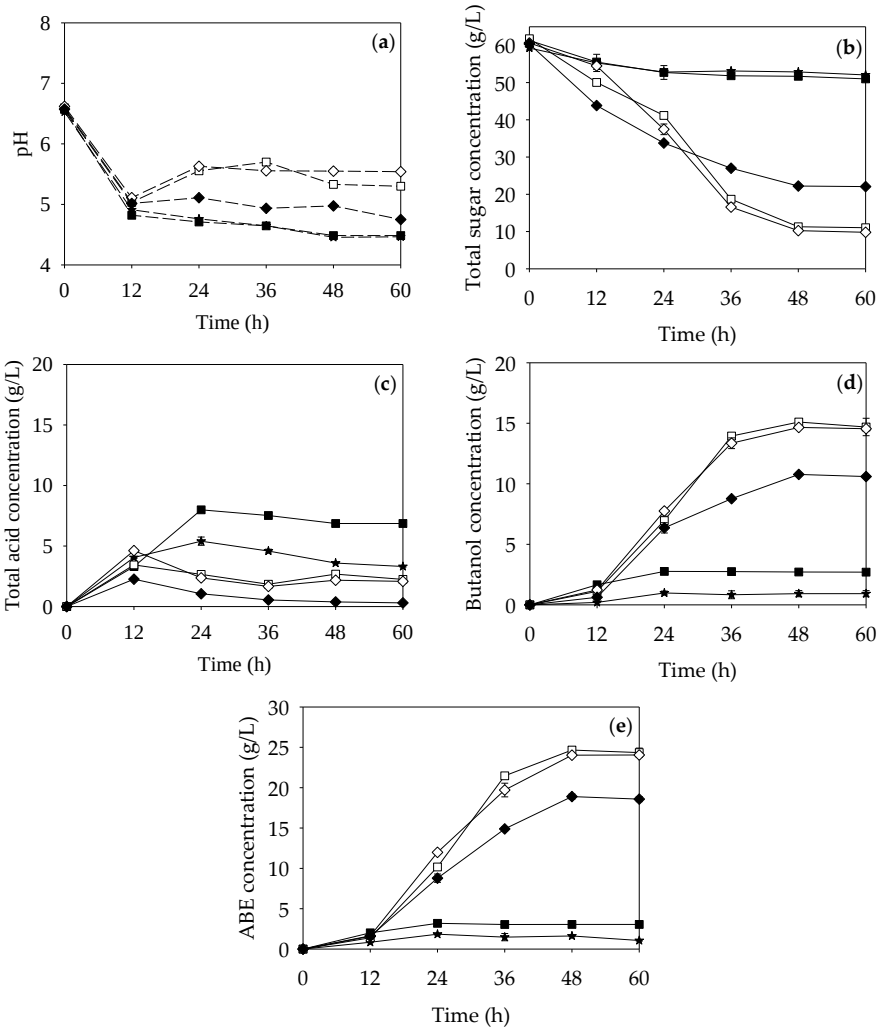


Figure 3. Profiles of pH (**a**), total sugar (**b**), total acid (**c**), butanol (**d**), and ABE (**e**) concentrations during ABE fermentation by *C. beijerinckii* TISTR 1461 from SSBH with no supplementation (★), SSBH-YE (■), SSBH-YE-Buf (□), SSBH-Nut in P2 (◇), and P2 medium (⧫).

Table 3. ABE fermentation results by *C. beijerinckii* TISTR 1461 from SSB hydrolysates containing different supplements and modified P2 medium.

Butanol Production Media	P_B (g/L)	P_{ABE} (g/L)	P_{acid} (g/L)	*SC* (%)	*t* (h)	$Y_{B/S}$ (g/g)	Q_B (g/L·h)
SSBH	1.18 ± 0.13 ^{e}	2.04 ± 0.08 ^{d}	5.40 ± 0.34 ^{b}	10.89 ± 1.22 ^{e}	24	0.17 ± 0.02 ^{c}	0.05 ± 0.01 ^{d}
SSBH-YE	2.76 ± 0.02 ^{d}	3.19 ± 0.15 ^{c}	7.99 ± 0.16 ^{a}	15.28 ± 0.10 ^{d}	24	0.29 ± 0.00 ^{b}	0.12 ± 0.00 ^{c}
SSBH-YE-Buf	15.10 ± 0.21 ^{a}	23.66 ± 0.37 ^{a}	2.68 ± 0.05 ^{c}	81.66 ± 0.22 ^{b}	48	0.31 ± 0.01 ^{a}	0.31 ± 0.00 ^{a}
SSBH-Nut in P2	14.66 ± 0.14 ^{b}	24.02 ± 0.17 ^{a}	2.18 ± 0.00 ^{d}	83.30 ± 0.13 ^{a}	48	0.29 ± 0.01 ^{a}	0.31 ± 0.01 ^{a}
* Modified P2 medium	10.77 ± 0.12 ^{c}	18.89 ± 0.23 ^{b}	0.55 ± 0.04 ^{e}	63.20 ± 0.71 ^{c}	48	0.28 ± 0.01 ^{a}	0.22 ± 0.01 ^{b}

$^{a, b, c, d, e}$: Means followed by the same letter within the same column are not significantly different using Duncan's multiple range test at $\alpha = 0.05$. All experiments were performed in triplicate, and the results are expressed as mean values ± SDs. P_B = butanol concentration, P_{ABE} = acetone–butanol–ethanol concentration, *SC* = sugar consumption, $Y_{B/S}$ = butanol yield, and Q_B = volumetric butanol productivity. SSBH: SSB hydrolysate containing 60 g/L of total sugars (88% glucose and 12% xylose). SSBH-YE: SSBH supplemented with 1.0 g/L of yeast extract. SSBH-YE-Buf: SSBH supplemented with 1.0 g/L of yeast extract and buffer in P2 medium. SSBH-Nut in P2: SSBH supplemented with all nutrients in P2 medium. * Modified P2 medium: a synthetic medium (modified from [25,26]) containing 60 g/L of total sugars (88% glucose and 12% xylose), 1 g/L of yeast extract and solutions A, B, and C in P2 medium (from Section 2.2.1).

The free amino nitrogen (FAN) concentrations in all media before and after ABE fermentation were determined. The initial FAN level in SSBH was ~70 mg/L. Interestingly, the FAN concentration consumed was about 27–29 mg/L in SSBH and SSBH-YE (Figure 4),

implying that *C. beijerinckii* consumed little FAN in an unbuffered medium. The initial FAN concentration in P2 medium was significantly lower than that in SSBH-YE-Buf, and SSBH-Nut in P2 (Figure 4). FAN consumption in P2 medium was less than in SSBH-YE-Buf and SSBH-Nut in P2. The P_B, P_{ABE}, and *SC* levels of the P2 medium were lower than those of the SSBH-YE-Buf and SSBH-Nut in P2 (Figure 3 and Table 3). This might be the reason that FAN consumption was high in SSBH-YE-Buf and SSBH-Nut in P2, corresponding to greater P_B, P_{ABE}, and *SC* values (Figure 4). This is likely due to the positive effect of buffering in ABE fermentations. Therefore, SSBH-YE-Buf, a simplified medium, was the most suitable for butanol production.

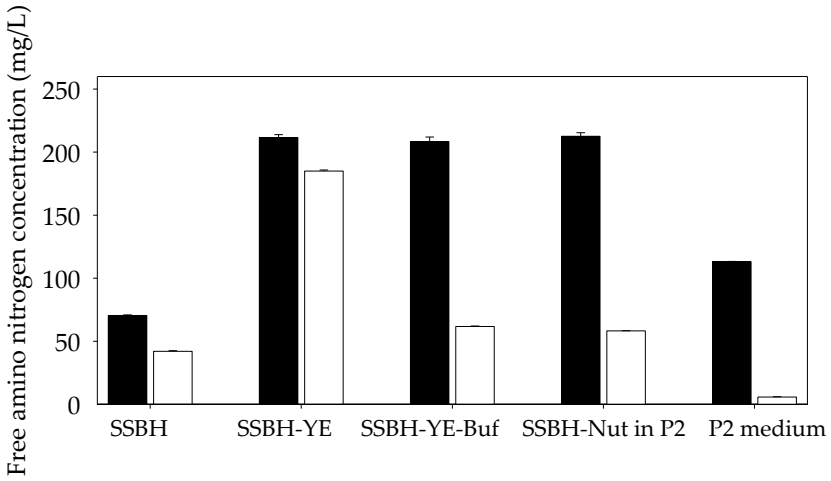


Figure 4. FAN concentrations in the broth during ABE fermentation from SSBH, SSBH-YE, SSBH-YE-Buf, SSBH-Nut in P2, and P2 medium by *C. beijerinckii* TISTR 1461 at the beginning (black bars) and end of fermentation (white bars).

Our results were supported by Liu et al. [54], Welch et al. [55], Dürre et al. [56], and Chandgude et al. [57], who reported that the amino acid L-cysteine acts as a reducing agent, which reduces NAD + back to NADH by donating electrons. Elevated NADH levels might have contributed to enhancing the activities of crucial enzymes in the butanol production pathway, such as butanol dehydrogenase (BDH) and butyraldehyde dehydrogenase (BADH), which are NADH dependent. Additionally, Wang et al. [58] reported that increased levels of certain amino acids (threonine, glycine, alanine, phenylalanine, tyrosine, tryptophan, aspartate, and glutamate) might affect butanol tolerance in *C. acetobutylicum*, resulting in higher butanol production.

Table 4 compares butanol production from various lignocellulosic biomasses from other research works. The P_B values produced by different microorganisms using various substrates ranged from 6.70 to 16.11 g/L. P_B and Q_B values in the current study using SSBH-YE-Buf as a butanol production medium were relatively high compared to earlier studies. However, the Q_B value was lower than that reported by Dziemianowicz et al. [59]. This may be due to differences in the initial sugar concentration, bacterial strains, and nutrient supplementation.

Table 4. Butanol production by *Clostridium* spp. from various lignocellulosic biomasses.

Substrate	Strain	P_B (g/L)	$Y_{B/S}$ (g/g)	Reference
Sweet sorghum bagasse hydrolysate (SSBH) (60 g/L, containing glucose:xylose = 88:12)	*C. beijerinckii* TISTR 1461	15.10	0.31	Current study
Modified P2 medium (60 g/L, containing glucose:xylose = 88:12)	*C. beijerinckii* TISTR 1461	10.77	0.28	Current study
Rye straw (43 g/L)	*C. acetobutylicum* DSM 1731	16.11	0.40	[59]
Wheat straw hydrolysate (60 g/L)	*C. beijerinckii* BA101	10.11	0.22	[60]
Sugarcane bagasse hydrolysate and molasses (60 g/L)	*C. saccharoperbutylacetonicum* DSM 14923	10.00	0.31	[61]
Sugarcane bagasse hydrolysate (60 g/L)	*C. tyrobutyricum* Δ*ack-adhE2*	12.00	0.20	[62]
Sugarcane bagasse hydrolysate and molasses (30 g/L)	*C. saccharoperbutylacetonicum* DSM 14923	7.79	0.28	[7]
Rice bran (40 g/L)	*C. acetobutylicum* YM1	8.27	0.21	[63]
Salix hydrolysate (20 g/L)	*C. acetobutylicum* DSM 1731	6.70	0.22	[64]
Soybean hull hydrolysate (65 g/L)	*C. tyrobutyricum* ATCC 25755	15.70	0.24	[65]

P_B = butanol concentration; $Y_{B/S}$ = butanol yield.

4. Conclusions

This research provides insights for optimizing mixed-sugar utilization in butanol fermentations. *C. beijerinckii* TISTR 1461 produces high butanol yields when glucose comprises at least 75% of the total sugars in mixed-sugar media. This bacterium preferentially utilizes glucose over xylose for butanol production. The highest xylose consumption capability was approximately 17 g/L. Sweet sorghum bagasse (SSB) hydrolysate (approximately 61 g/L total sugars), consisting of glucose and xylose in an 88:12 ratio and supplemented with yeast extract and buffers, outperforms a synthetic mixed-sugar medium for butanol production. The process achieved the highest butanol concentration of 15.10 g/L, with a butanol yield of 0.31 g/g, butanol productivity of 0.31 g/L·h, sugar consumption of 82%, and total ABE production of 23.66 g/L. Buffer addition is crucial for controlling the pH and improving the butanol yield from an SSB hydrolysate. The research demonstrates the potential of using lignocellulosic biomass, specifically sweet sorghum bagasse, as a low-cost substrate for butanol production. Further studies should focus on process development, such as investigating in situ product removal techniques to reduce butanol toxicity and optimizing the buffer composition and concentration for better pH control, to improve butanol production from a lignocellulosic hydrolysate.

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