

## Influence of Urea on Butanol Production from Sugarcane Juice by *Clostridium beijerinckii* TISTR 1461

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### Abstract

Nitrogen is the key factor influencing for butanol production. To improve the butanol production efficiency, the urea concentration was investigated for butanol production from sugarcane juice by *Clostridium beijerinckii* TISTR 1461. The addition of nutrients from P2 medium (excluding glucose) resulted in a butanol (9.29 g l<sup>-1</sup>) and acetone-butanol-ethanol (ABE) (12.27 g l<sup>-1</sup>) concentrations higher than the fermentation without supplemented with those in the sugarcane juice (butanol; 3.31 and ABE; 5.43 g l<sup>-1</sup>). The result suggested that some nutrients in P2 medium might be necessary for butanol production. Due to the high cost of yeast extract, urea was used as the nitrogen source in subsequent experiments. The juice supplemented with P2 medium (excepted glucose and yeast extract) and varied urea concentrations ranged from 0.27-1.35 g l<sup>-1</sup> were used as butanol production media. The results showed that the butanol and ABE concentrations were increased with increasing the urea concentration from 0.27-0.81 g l<sup>-1</sup>. The highest butanol (9.41 g l<sup>-1</sup>) and ABE (11.78 g l<sup>-1</sup>) concentrations were observed at 0.81 g l<sup>-1</sup> of urea concentrations and showed 0.30 of butanol yield and 0.20 g l<sup>-1</sup> h<sup>-1</sup> of productivity. The results concluded that urea promotes butanol production efficiency and suitable for instead of yeast extract.

**Keywords:** *Clostridium beijerinckii* TISTR 1461, Butanol production, Sugarcane juice, Urea

## Introduction

Now, increasing concerns over global warming have renewed interest in biobutanol as an alternative renewable liquid fuel [1]. Butanol as a fuel offers many superior advantages to ethanol. Its vapor pressure is lower and fuel value higher than ethanol. Moreover, it is less impacted by water contamination, which makes it easier to distribute and use [2]. Butanol can be produced by fermentation process called the acetone–butanol–ethanol (ABE) fermentation [3]. The primary microorganisms involved in this fermentation are spore-forming bacteria, i.e., *Clostridium* spp. A typical feature of clostridia solvent production is its biphasic nature. The first phase is an acidogenic phase, in which the acid-forming pathways are activated producing acetate, butyrate, hydrogen and carbon dioxide as major products. This phase usually occurs during the exponential-growth phase of cell division. The second phase is a solventogenic phase in which acetic and butyric acids are assimilated and used in the production of acetone and butanol, respectively [4]. Biobutanol can be produced from a variety of renewable sources. The nitrogen concentration is a key factor for growth of *Clostridium* spp. and butanol production [5]. Yeast extract is usually used as a nitrogen source in laboratory scale of butanol production [6], but it is relatively expensive when used as the nitrogen source for biofuel production. Therefore, urea was used in this research instead of yeast extract as a low cost nitrogen source for butanol production. In this research, we focused on sugarcane as substrates for butanol production since sugarcane is renewable agricultural raw material widely available in Thailand.

This research aimed to consider the feasibility of using urea instead of yeast extract for nitrogen source and investigated the influence of urea concentration for butanol production from sugarcane juice by *C. beijerinckii* TISTR 1461.

## Materials and methods

### Microorganism and inoculum preparation

*C. beijerinckii* TISTR 1461 [3] was purchased from the Thailand Institute of Scientific and Technological Research (TISTR). A spore suspension was heat shocked at 80 °C for 1 min and cooled in ice-water [5]. The 5% (v/v) of spore suspension was inoculated into cooked meat medium (CMM) and incubated at 37 °C for 10-12 h under anaerobic condition to obtain vegetative cells [5]. The 5% (v/v) of vegetative cells were transferred into tryptone-glucose-yeast extract (TGY) medium and incubated at 37 °C for 4-6 h under anaerobic condition before use as an inoculum for Acetone-Butanol-ethanol (ABE) production [3].

### Culture medium

CMM and TGY media were used for inoculum preparation. CMM was composed of CMM powder, 10 and glucose, 0.8 g l<sup>-1</sup> (modified from [6]). TGY medium was composed of typtone, 5; glucose, 1; yeast extract, 5 and K<sub>2</sub>HPO<sub>4</sub>, 5 g l<sup>-1</sup> [6]. Both CMM and TGY media were autoclaved at 121 °C for 15 min and purged with sterile oxygen free nitrogen (OFN) gas to create strictly anaerobic conditions before inoculation.

### Butanol production medium

Sugarcane juice (cv. Suphanburi 50, containing total sugar of ~165 g l<sup>-1</sup>) was obtained from a local market in Mahasarakham province, Thailand. Then, it was diluted with distilled water to obtain 60 g l<sup>-1</sup> of total sugar without and with nutrient from P2 medium supplementation (excepted glucose). The P2 medium was composed of total sugar, 60 and yeast extract, 1 g l<sup>-1</sup> and stock solutions A, B and C [6]. Stock solution A consisted of K<sub>2</sub>HPO<sub>4</sub>, 50; KH<sub>2</sub>PO<sub>4</sub>, 50 and ammonium acetate, 220 g l<sup>-1</sup>. Stock solution B consisted of para-amino-benzoic acid, 0.1; thiamine, 0.1 and biotin, 0.001 g l<sup>-1</sup>. Stock solution C consisted of MgSO<sub>4</sub>·7H<sub>2</sub>O, 20; MnSO<sub>4</sub>·H<sub>2</sub>O, 1.0; FeSO<sub>4</sub>·7H<sub>2</sub>O, 1.0 and NaCl, 1.0 g l<sup>-1</sup>. Stock solutions A and C were autoclaved at 121 °C for 15 min, whereas stock B was

sterilized using a sterile 0.2  $\mu\text{m}$  cellulose acetate membrane. Then, 1% (v/v) of each stock solution was aseptically added into the sterile P2 medium. To investigate the influence of urea concentration for butanol production from sugarcane juice by *C. beijerinckii* TISTR 1461. The urea concentration was varied as 0.27-1.35  $\text{g l}^{-1}$  (0.27  $\text{g l}^{-1}$  of urea containing a nitrogen content equivalent to that in 1  $\text{g l}^{-1}$  of yeast extract [5]) on butanol fermentation. The media were transferred into 1-L air-locked bottles and autoclaved at 121  $^{\circ}\text{C}$  for 15 min. Before inoculation, the pH of the medium was adjusted to 6.5 by addition of sterile 8 N NaOH [5].

### Analytical methods

The fermentation broth was centrifuged at 10,000 rpm for 15 min to remove particles. Acetone, butanol ( $P_B$ ), ethanol, acetic and butyric acids were determined using gas chromatography with stainless steel column packed with Parapack Q, 80/100 mesh (Resteck, USA). The injector and detector were operated at 220 and 230  $^{\circ}\text{C}$ , respectively. Nitrogen gas was used as a carrier gas at a pressure of 150 kPa. The column temperature was 10 min at 160  $^{\circ}\text{C}$ , 15  $^{\circ}\text{C min}^{-1}$  to 180  $^{\circ}\text{C}$  and 20 min at 180  $^{\circ}\text{C}$ , and iso-butanol was used as an internal standard (modified from [7]). Total sugar concentration was measured using a phenol-sulphuric acid method. The butanol yield ( $Y_{B/S}$ ) and butanol productivity ( $Q_B$ ) were calculated as follows:  $Y_{B/S} = P_B/TS$  and  $Q_B = P_B/t$ , where  $TS$  is the total sugars utilized ( $\text{g l}^{-1}$ ), and  $t$  is the fermentation time (h) giving the highest butanol concentration. Total ABE concentration (PABE) was also calculated.

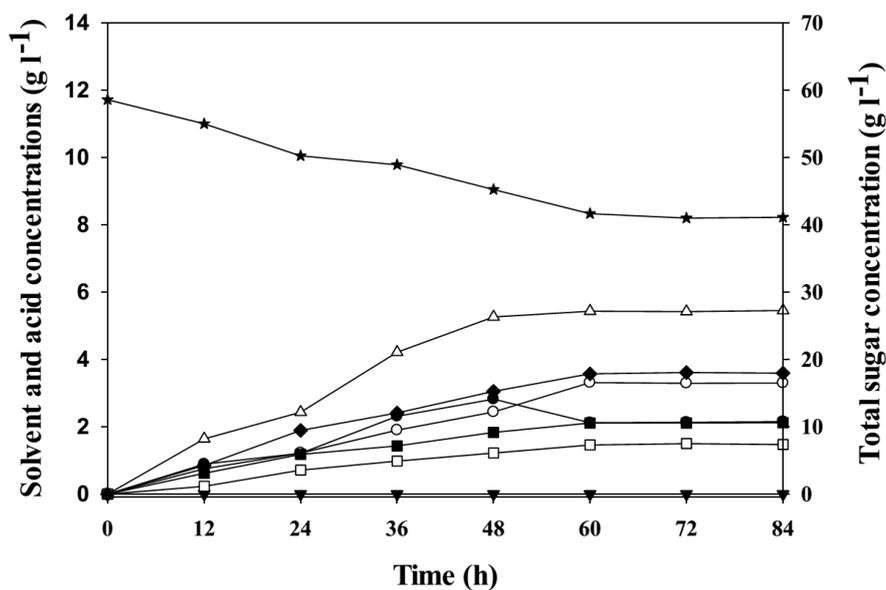
## Results and discussion

### Effect of nutrient P2 medium supplements on butanol production from sugarcane juice

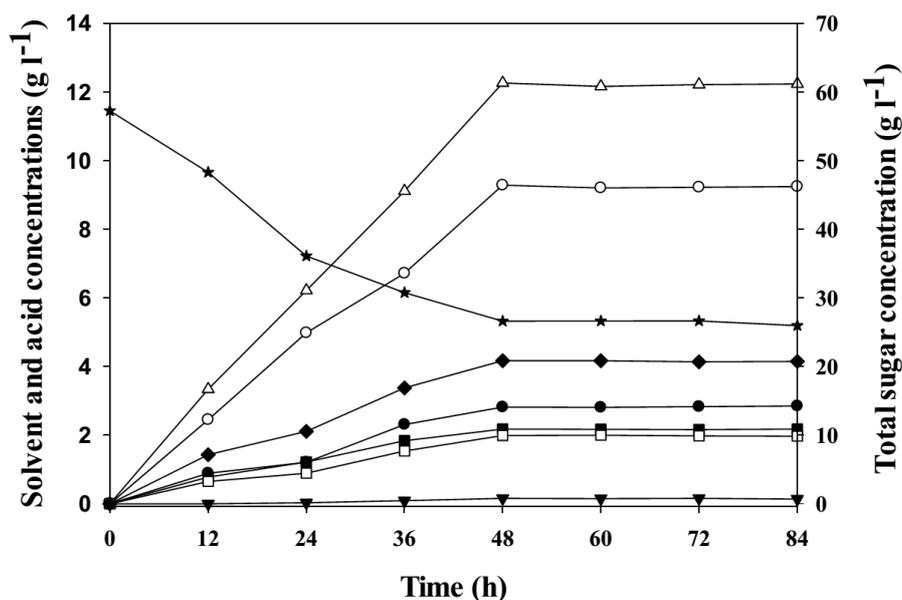
With no nutrient supplementation in the sugarcane juice, the  $P_B$  and  $P_{ABE}$  concentrations were increased with increasing fermentation time (Figure 1). Under this condition, the maximum  $P_B$  and  $P_{ABE}$  concentrations were 3.31 and 5.43  $\text{g l}^{-1}$  at 60 h corresponding to  $Y_{B/S}$  and  $Q_B$  were 0.20 and 0.06  $\text{g l}^{-1} \text{h}^{-1}$  whereas, ethanol was not detected. Total sugar

concentration decreased from 58.57 to 41.64 g l<sup>-1</sup> at 60 h and the sugar utilized was 16.93 g l<sup>-1</sup> (~29%). The acetic and butyric concentrations were increased immediately during 12 h, and they were 2.11 and 1.46 g l<sup>-1</sup>, respectively at 60 h (Figure 1).

However,  $P_B$  and  $P_{ABE}$  values were lower than that (9.29 and 12.27 g l<sup>-1</sup>) using the sugarcane juice supplemented with nutrients from P2 medium as the butanol medium. When sugarcane juice was supplemented with the nutrients of the P2 medium (excluding glucose), the  $P_B$  and  $P_{ABE}$  concentrations of this media were significantly different. Under this condition, the  $Y_{B/S}$  and  $Q_B$  were 0.30 and 0.19 g l<sup>-1</sup> h<sup>-1</sup>, whereas, ethanol was 0.16 g l<sup>-1</sup> and also reduced the fermentation time from 60 to 48 h (Figure 2). Total sugar concentration decreased from 57.13 to 26.26 g l<sup>-1</sup> at 48 h and the sugar utilized was 30.68 g l<sup>-1</sup> (~54%). The acetic, butyric were 2.18 and 1.99 g l<sup>-1</sup>, respectively at 48 h (Figure 2). These results implied that some nutrients in P2 medium might be necessary for butanol production from sugarcane juice [5]. Therefore, the sugarcane juice supplemented with the nutrients of the P2 medium (excluding glucose) was selected as a butanol production medium in the subsequent experiments.



**Fig.1** ABE batch fermentation results from sugarcane juice without P2 medium by *C. beijerinckii* TISTR 1461 (●; acetone, ○; butanol, ▼; ethanol, Δ; ABE, ■; acetic acid, □; butyric acid, ◆; total acid and ★; Total sugar)



**Fig. 2** ABE batch fermentation results from sugarcane juice with P2 medium by *C. beijerinckii* TISTR 1461 (●; acetone, ○; butanol, ▼; ethanol, Δ; ABE, ■; acetic acid, □; butyric acid, ◆; total acid and ★; Total sugar)

### Effect of initial sugar concentrations for butanol production

When the urea used as the nitrogen source and it was varied the initial urea concentrations between 0.27-1.35 g l<sup>-1</sup>, the results showed in Table 1. Under the condition of 0.27 g l<sup>-1</sup> of urea concentration, the pH was 5.21 at 48 h and the sugar utilized was 28.06 g l<sup>-1</sup> (~45%). The  $P_B$  and  $P_{ABE}$  concentrations were 6.02 and 7.25 g l<sup>-1</sup> at 48 h resulting to  $Y_{B/S}$  and  $Q_B$  were 0.21 and 0.13 g l<sup>-1</sup> h<sup>-1</sup> (Table 1). However, ethanol was not detected under these conditions, indicating that acetaldehyde dehydrogenase and ethanol dehydrogenase were not viable [8]. Acetic and butyric acids were 1.09 and 1.41 g l<sup>-1</sup>. For the condition of 0.54 g l<sup>-1</sup> of urea concentration, the pH and sugar utilized were 5.26 and 29.55 g l<sup>-1</sup> (~49%). The  $P_B$  and  $P_{ABE}$  concentrations were 7.32 and 9.14 g l<sup>-1</sup> at 48 h corresponding to  $Y_{B/S}$  and  $Q_B$  were 0.25 and 0.15 g l<sup>-1</sup> h<sup>-1</sup> (Table 1). Under the condition of 0.81 g l<sup>-1</sup> of urea concentration, the pH was 5.34 at 48 h and the sugar utilized was 31.05 g l<sup>-1</sup> (~52%). The  $P_B$  and  $P_{ABE}$  concentrations were 9.41 and 11.78 g l<sup>-1</sup> at 48 h resulting to  $Y_{B/S}$  and  $Q_B$  were 0.30 and 0.20 g l<sup>-1</sup> h<sup>-1</sup> (Table 1). Under the condition of 1.08 g l<sup>-1</sup> of urea concentration, the pH was 5.36 at 48 h and the sugar utilized was 30.09 g l<sup>-1</sup> (~51%). The  $P_B$  and  $P_{ABE}$  concentrations were 9.32 and 11.71 g l<sup>-1</sup> at 48 h resulting to  $Y_{B/S}$  and  $Q_B$

were 0.30 and 0.19 g l<sup>-1</sup> h<sup>-1</sup> (Table 1). Whereas, the condition of 1.35 g l<sup>-1</sup> of urea concentration, the pH and sugar utilized were 5.30 and 31.11 g l<sup>-1</sup> (~51%). The  $P_B$  and  $P_{ABE}$  concentrations were 9.22 and 11.37 g l<sup>-1</sup> at 48 h resulting to  $Y_{B/S}$  and  $Q_B$  were 0.30 and 0.19 g l<sup>-1</sup> h<sup>-1</sup> (Table 1). In addition, the ethanol concentration was observed in the range of 0.18-0.33 g l<sup>-1</sup> under the 0.54-1.35 g l<sup>-1</sup> of urea.

The results revealed that when increased the initial urea concentrations from 0.27-0.81 g l<sup>-1</sup> increased the sugar utilized,  $P_B$  and  $P_{ABE}$  concentrations. The maximum sugar utilized,  $P_B$  and  $P_{ABE}$  concentrations were obtained with the values of 31.15, 9.41 and 11.78 g l<sup>-1</sup>, respectively under the 0.81 g l<sup>-1</sup> of initial urea concentration and resulting to 0.30 and 0.20 g l<sup>-1</sup> h<sup>-1</sup> of  $Y_{B/S}$  and  $Q_B$  (Table 1). However, the  $P_B$  and  $P_{ABE}$  were slightly different under the condition of 1.08 and 1.35 g l<sup>-1</sup> (9.22-9.32 g l<sup>-1</sup> and 11.37-11.71 g l<sup>-1</sup>) (Table 1). The results indicated that the *C. beijerinckii* TISTR 1461 was not stimulated by higher nitrogen concentrations (urea > 0.81 g l<sup>-1</sup>). Li et al. [9] demonstrated that butanol production dropped when the yeast extract concentration was increased beyond 3 g l<sup>-1</sup>. Higher nitrogen concentrations might distort the C/N ratio. Tran et al. [10] reported that the C/N ratio played an important role in carbon utilization. At high C/N ratios, utilization of carbon was less effective, and high levels of ABE production were obtained at a low C/N ratio.

Butanol production from sugarcane juice supplemented with P2 medium urea (excepted glucose) and 0.81 g l<sup>-1</sup> of urea by *C. beijerinckii* TISTR 1461 (9.41 g l<sup>-1</sup>) was higher than that of *C. acetobutylicum* ATCC824 (7.00 g l<sup>-1</sup>) [1] and *C. beijerinckii* JCM 1390 (7.56 g l<sup>-1</sup>) [7]. The butanol production level in our study was close to those of *C. acetobutylicum* ATCC 824 (8.20 g l<sup>-1</sup>) [11] and *C. saccharoperbutylacetonicum* N1-4 (8.69 g l<sup>-1</sup>) [2]. The result showed that the sugarcane juice is a suitable substrate for butanol production by *C. beijerinckii* TISTR 1461.

**Table 1.** Fermentation results of ABE fermentations from sugarcane juice at various urea concentrations

Fermentation results	without P2 medium	with P2 medium	Urea concentrations (g l <sup>-1</sup> )				
			0.27	0.54	0.81	1.08	1.35
Acetone (g l <sup>-1</sup> )	2.12	2.82	1.23	1.61	2.04	2.11	1.97
Butanol (g l <sup>-1</sup> )	3.31	9.29	6.02	7.32	9.41	9.32	9.22
Ethanol (g l <sup>-1</sup> )	0	0.16	0	0.21	0.33	0.28	0.18
Total ABE (g l <sup>-1</sup> )	5.43	12.27	7.25	9.14	11.78	11.71	11.37
Acetic acid (g l <sup>-1</sup> )	2.11	2.18	1.09	1.32	1.85	2.01	2.15
Butyric acid (g l <sup>-1</sup> )	1.46	1.99	1.41	1.22	0.78	1.02	1.26
Total Acids (g l <sup>-1</sup> )	3.57	4.17	2.5	2.54	2.63	3.03	3.41
Sugar utilized (g l <sup>-1</sup> )	16.93	30.68	28.06	29.55	31.15	30.9	31.11
Time (h)	60	48	48	48	48	48	48
<i>Y<sub>p/s</sub></i> butanol (g g <sup>-1</sup> )	0.20	0.30	0.21	0.25	0.30	0.30	0.30
<i>Q<sub>p</sub></i> butanol (g l <sup>-1</sup> h <sup>-1</sup> )	0.06	0.19	0.13	0.15	0.20	0.19	0.19

## Conclusions

The addition of nutrients from P2 medium resulted in a butanol and ABE concentrations higher than the fermentation without supplemented with those in the sugarcane juice. The result suggested that some nutrients in P2 medium might be necessary for butanol production. In addition, the butanol and ABE concentrations were increased with increasing the urea concentration from 0.27-0.81 g l<sup>-1</sup>. The results concluded that the urea promoted butanol production efficiency and suitable for instead of yeast extract.

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