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Direct Production of Butanol and Ethanol from Cane Sugar Factory Wastewater and Cellulosic Ethanol Pilot Plant Wastewater by Clostridium Beijerinckii CG1

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Abstract

The ability of Clostridium beijerinckii CG1 to utilize sugar from cane sugar factory wastewater (CSFW) and cellulosic ethanol pilot plant wastewater (CEPW) as a renewable carbon source and complete fermentation medium to produce acetone-butanol-ethanol (ABE) was investigated. CSFW and CEPW were used as the unsupplemented culture media (50 mL) for ABE production by C. beijerinckii CG1 fermentation in 100-mL serum bottles. No acetone was produced from fermentation of either CEPW or CSFW, in contrast to the small amount (20 g L\(^-1\)) produced from glucose in the last 24 h of a 120 h fermentation. The average ethanol, butanol and butyric acid production from CEPW (butanol 0.6 g L\(^-1\), ethanol 0.4 g L\(^-1\) and butyric acid 2.5 g L\(^-1\)) was higher than that from CSFW (butanol 0.3 g L\(^-1\), ethanol 0.3 g L\(^-1\) and butyric acid 0.9 g L\(^-1\)), but still less than that from glucose (butanol 1.87 g L\(^-1\), ethanol 0.86 g L\(^-1\) and butyric acid 6.47 g L\(^-1\)). However, the productivity rate in this 50-mL fermentation was three-fold higher with CSFW (0.06 g L\(^-1\) h\(^-1\)) than with CEPW (0.02 g L\(^-1\) h\(^-1\)). These results suggest that CSFW and CEPW can be applied as appropriate substrates to produce solvents by C. beijerinckii CG1 at a reasonable concentration without the need for additional exogenous nutrient supplements or pretreatment. The idea then being to consolidate the bioprocess for low-cost biofuel production.

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1. Introduction

Bioalcohols, principally butanol and ethanol, are potential renewable energy sources for now and in the future that can be produced efficiently by the acetone-butanol-ethanol (ABE) fermentation process. Currently, bioalcohol production cannot compete economically with petroleum energy because of the substrate cost. In order to develop a cost effective bioprocess to produce bioalcohol, recent research has largely focused on new substrates or raw materials that are renewable, readily available, relatively cheap, produce a reasonable yield and do not compete with food or other product uses (i.e. are waste products).

Industrial wastewater requires treatment to remove the pollutants prior to discharge into the environment. The wastewater treatment system is important but the cost of the treatment system in each process plant is varied, and along with the method of wastewater treatment, it depends on the wastewater properties. [1] Wastewater that contains a high level of organic substances requires a very specific treatment processes because of its diverse components, such as a mixture of carbohydrates, fats or even polymers and salts. For this reason, it is often inappropriate to use only one (typically chemical) treatment processes but rather requires physical, chemical and biological treatment processes to efficiently eliminate most of the pollutants. [2] Biological treatment processes are, or are based upon, traditional methods that combine aerobic and anaerobic treatment systems and have a high efficiency at reducing the biological oxygen demand (BOD) and chemical oxygen demand (COD), which are critical values used to determine the wastewater hazard to the environment. [3]

Carbohydrate-rich wastewater is one of many kinds of wastewater from food processing industries [4], such as from the cassava starch, vermicelli, cane sugar and deproteinized whey processing wastewaters. They may contain relatively high and diverse levels of monosaccharides and disaccharides (e.g., glucose, mannose, xylose and arabinose), as well as more complex polymers [5]. In this study, the concept was to combine and consolidate the biological wastewater treatment processes with ABE fermentation without the need for any pretreatment or enzyme digestion processes of the substrate (wastewater) before the ABE fermentation. One of the important factors is the selection of an appropriate microorganism, which must have a good potential to convert the carbohydrate mixture to added value chemicals, in this case ethanol, butanol and butyric acid, efficiently enough so as to obtain a yield and fermentation rate that are economically viable [6, 7]. Clostridium beijerinckii was selected for this research because of its high ABE production level in a short time period, and its ability to use various carbon sources [8] and to produce endospores for poor environment resistance [9].

Ethanol and butanol production from waste materials has been studied in various systems, such as with the sugar from organic waste (domestic organic waste; DOW) to produce butanol using C. acetobutylicum ATCC824. However, the dry and wet garbage had to first be pretreated to attain a high enough simple (fermentable) sugar concentration, typically reaching a post-treatment sugar concentration of 27.7–39.3% (w w⁻¹). The resulting fermentation by C. acetobutylicum ATCC824 did not require the addition of other nutrients and yielded 3 g L⁻¹ butanol at 0.03 g L⁻¹ h⁻¹ from 100 g of organic waste [10]. However, the required pretreatment (including enzymatic treatment) to convert the wet garbage to fermentable sugar will adversely affect the cost and time logistics of the production process at a commercial scale.

Thailand is a largely agricultural country producing a large mass of lignocellulosic and other carbohydrate-rich waste and so has the potential for non-food fermentation [11]. To date, the use of lignocellulosic (agricultural) wastes as fermentation substrates to produce ethanol and butanol by ABE fermentation have been studied the most, and include dried distiller's grains and wastewater from the production of ethanol [12], bran (rice bran) [4], corn cob [8], cassava and sugar cane bagasse [13].

Both cane sugar factory wastewater (CSFW) and cellulosic ethanol pilot plant wastewater (CEPW) are types of carbohydrate-rich wastewater. The goal of this research was to evaluate the potential of CSFW
and CEPW as a substrate in the ABE fermentation process from a consolidated bioprocess designed especially for *C. beijerinckii* CG1.

2. Materials and Methodology

2.1. Wastewater collection and analysis

The CSFW and CEPW samples were collected from the Singburi sugar factory in Singburi province, Thailand, and the cellulosic ethanol pilot plant in Ayutthaya province, Thailand, respectively. Samples were stored upon collection in an ice bucket for transportation and then frozen at -20 °C until use.

2.2. Microorganism storage, preculture and culture

The *C. beijerinckii* CG1 strain was selected from the culture collection of the Biofuels by Biocatalysts Research Unit, Chulalongkorn University, Thailand, and was stored in a 4:1 (v/v) ratio of medium with 20 g L⁻¹ glucose (MMS) [7]: glycerol at -20 °C. To initiate cultures, *C. beijerinckii* CG1 cells were heat shocked at 80 °C for 10 min and then the heat shocked spores were inoculated into MMS at 10% (v/v) and incubated at 37 °C for 48 h in an anaerobic chamber under a 18:1:1 (v/v/v) ratio N₂: CO₂: H₂ atmosphere. When the culture reached an optical density of 1.2 (absorbance at 600 nm (A₆₀₀) in a 1 cm light path) it was inoculated into fresh MMS medium at a 10% (v/v) inoculum level and incubated at 37 °C for 24–48 h.

2.3. Preparation of CSFW and CEPW medium

The frozen CSFW and CEPW were thawed at 25 °C and aliquoted at 50 mL per 100-mL serum bottle and then flushed with N₂ (99.995%) for 15 min. The serum bottles were sealed with a Neoprene stopper and sterilized (100 °C, 1.5 mPa) in an autoclave for 20 min.

2.4. Acetone-ethanol-butanol (ABE) fermentation and product detection

The CSFW and CEPW media, plus the MMS medium for direct comparison, were inoculated with *C. beijerinckii* CG1 at 10% (v/v) and cultured anaerobically at 37 °C for 120 h. Samples (1 mL) were removed at 0, 24, 48, 72, 96 and 120 h after culture initiation and examined for bacterial growth by measuring the A₆₀₀, and for acetone, ethanol, butanol and butyric acid concentrations by gas chromatography (GC; Shimadzu, Japan) using a DB-WAX column (Agilent Technologies, USA). The residual reducing sugar concentration was analyzed by the modified dinitrosalicylic method [14]

3. Results and discussion

3.1. Characteristics of the CSFW and CEPW

The CEPW was a black-brown color in appearance with a pH (at 25 °C) of 4.4, while the CSFW was yellow in color with a pH (at 25 °C) of 5.3. The total reducing sugar level of CSFW and CEPW was approximately 0 g L⁻¹ and 6 g L⁻¹, respectively.

3.2. Growth of *C. beijerinckii* CG1 in CSFW and CEPW media
The sterilized CSFW, CEPW and MMS media (pH adjusted to 6.5) were used to culture a 10% (v/v) inoculum of \textit{C. beijerinckii} CG1. From the $A_{600}$ values, the bacteria reached a maximum growth (stationary phase) within 72 h in each medium to an $A_{600}$ value of between ~1.03 (CEPW) to ~1.17 (CSFW and MMS) (Fig. 1). The media pH decreased during the fermentation from an initial pH 6.3 (0 h) down to pH 4.6 (CEPW) or pH 4.3 (CSFW and MMS) indicating substantial acid production.

Fig. 1 Average (a, c, e) cell growth and media pH and (b, d, f) ABE production level from the fermentation of (a, b) CEPW, (c, d) CSFW and (e, f) MSS by \textit{C. beijerinckii} CG1.
3.3. **ABE production**

Using CSFW and CEPW at 100% (v v\(^{-1}\)) without any pretreatment or addition of other substrates, the ABE production capacity by *C. beijerinckii* CG1 was compared to that with 20 g L\(^{-1}\) glucose (MMS media) as the carbon source (Fig. 1). No acetone was produced in the CSFW or CFPW media over the 120 h ferment, and only ~0.3 g L\(^{-1}\) in the MMS media from 96 to 120 h of fermentation. A two-fold higher amount of butanol was produced by 120 h in the CFPW (0.6 g L\(^{-1}\)) than in the CSFW medium (0.3 g L\(^{-1}\)), but these were both much lower than that in MMS (2.3 g L\(^{-1}\)). Likewise for ethanol after 120 h fermentation, a 1.33-fold higher level was produced from the CFPW (0.4 g L\(^{-1}\)) than the CSFW (0.3 g L\(^{-1}\)), but in MMS 1.06 g L\(^{-1}\) was obtained. Thus, the ABE ratio differed between the different media, being 0:3:1 in CSFW, 0:3:1, 0:1:1 in CFPW, and 0:2.5:1 in MMS, whilst the efficiency, in terms of the productivity rate, was 0.06, 0.02 and 0.11 g L\(^{-1}\) h\(^{-1}\) for CSFW, CEPW, and MMS, respectively.

For comparison, rice bran and rice bran oil as substrates to produce butanol by *C. saccharoperbutylacetonicum* N1–4 fermentation gave a broadly similar productivity rate of 0.06 g L\(^{-1}\) h\(^{-1}\) [15]. Although hydrolyzed wheat straw fermentation by *C. beijerinckii* P260 gave a higher butanol productivity rate of 0.29 g L\(^{-1}\) h\(^{-1}\) [16], this was attained only after the expensive pretreatment (hydrolysis) of the wheat straw to yield a final sugar concentration of 60.2 g L\(^{-1}\). Thus, CSFW and CEPW have suitable fermentation efficiencies, like rice bran, to use as a direct substrate for ABE production without pretreatment.

Consistent with the pH decrease, butyric acid was produced in the ferments of all three media, again being higher (~2.8-fold) in the CFPW (2.5 g L\(^{-1}\)) than in the CSFW (0.9 g L\(^{-1}\)) media, compared to 5.9 g L\(^{-1}\) in the MMS medium.

4. **Conclusion**

In this work CSFW and CEPW were used as substrates to produce a high butanol and ethanol concentration by anaerobic fermentation with *C. beijerinckii* CG1 without the addition of any other nutrient supplements or any pretreatment process. Thus, these two renewable substrates have the potential to be used as feedstock for bioprocesses and have the advantage of being waste products and so not competing for food or other applications. The absence of any requirement for pretreatment and their waste product nature should make CSFW and CEPW economically attractive as fermentation feedstock for ethanol and butanol production. Further development to optimize the fermentation condition and to upscale the fermentor size for commercial fermentation would make conversion to ethanol and butanol even more attractive.

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