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Improved ethanol production from various carbohydrates through anaerobic thermophilic co-culture

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ABSTRACT

Saccharification is one of the most critical steps in producing lignocellulose-based bio-ethanol through consolidated bioprocessing (CBP). However, extreme pH and ethanol concentration are commonly considered as potential inhibitors for the application of *Clostridium* sp. in CBP. The fermentations of several saccharides derived from lignocellulosics were investigated with a co-culture consisting of *Clostridium themocellum* and *Clostridium thermolacticum*. Alkali environments proved to be more favorable for ethanol production. Fermentation inhibition was observed at high ethanol concentrations and extreme pH. However, low levels of initial ethanol addition resulted in an unexpected stimulatory impact on the final ethanol productions for all cultures under selected conditions. The co-culture was able to actively ferment glucose, xylose, cellulose and micro-crystallized cellulose (MCC). The ethanol yield observed in the co-culture was higher (up to twofold) than in mono-cultures, especially in MCC fermentation. The highest ethanol yield (as a percentage of the theoretical maximum) observed was 75% (w/w) for MCC and 90% (w/w) for xylose.

1. Introduction

Affordable bio-fuel has become an important topic due to an imbalance between supply and demand for existing energy sources as well as the problems associated with recent fuel refinery processes (Kumar et al., 2009). Lignocellulosic biomass offers a great potential as a bio-fuel resource. This is mainly because lignocellulosics are an abundant raw material and bypass the issue of utilization of food material for fuel production.

Consolidated bioprocess (CBP) fermentation is a promising solution offering the potential for higher efficiency and lower production cost compared to other current conversion processes (Van Zyl et al., 2007). However, as a relatively new technology, technical difficulties are unavoidable, especially since during the CBP fermentation cellulase is generated inside the reaction vessel, making this process more complex than simply adding enzymes.

Proper microbes with specific traits combined with suitable process modifications (Perlack et al., 2005) can help deal with the extreme conditions and inhibitors present in CBP fermentation. Co-cultures have been widely studied (Kleijntjens et al., 1986). Although production of biofuels in co-culture systems usually is higher than in their separate mono-cultures, the overall ethanol production even in co-culture systems remains low. For wild strains, less than 70% theoretical ethanol yield was produced per glucose equivalent (Balusu et al., 2005), and less than 66% was reported to be produced per xylose equivalent (Lin and Tanaka, 2006). In this study, attempts were made to evaluate the potential advantages of integrating *Clostridium themocellum* and *Clostridium thermolacticum* into a CBP fermentation process with respect to ethanol and acetate formation, as compared to their mono-cultures. The ability of this co-culture and their mono-cultures to saccharify major biomass polymers and their fragments under a wide pH range (5–10) was examined. This innovative co-culture containing *C. themocellum* and *C. thermolacticum* were selected because of their remarkable de-polymerization ability for polysaccharides (Willaert and Baron, 1996). Both microbes can hydrolyze a wide range of saccharides and tolerate relatively high temperatures up to 60 °C (Fardeau et al., 2001; Weimer and Zeikus, 1977). *C. themocellum* ATCC 27405 which contains cellulosomes is proficient in converting both crystalline and amorphous cellulose efficiently into ethanol, acetate and hydrogen (Weimer and Zeikus, 1977). *C. thermolacticum* ATCC 43739 can produce a variety of de-polymerization enzymes but is especially apt in degrading pentoses (Fardeau et al., 2001).

A secondary purpose of this research is the optimization of this co-culture fermentation for ethanol production, and potentially reducing side reactions towards unwanted products such as acetate. Although much research has been devoted to investigating the effect of pH and ethanol concentrations during *C. themocellum* or *C. thermolacticum* mono-culture fermentation processes as independent variables, less attention has been paid to the interactions of these fermentation parameters in any of these mono-culture

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fermentations. In addition, the influences of pH values and ethanol concentration in previous studies are inconsistent (Talabardon et al., 2000a, 2000b) requiring further explorations.

In summary, this study is attempting to optimize a *C. themocellum*/*C. thermolacticum* co-culture, by overcoming the inhibitor effect of high pH and high ethanol concentrations, allowing for an effective merging of pretreatment and fermentation processes. CBP fermentation will result in reduced equipment and processing cost, bringing the technology closer to economically feasible biofuels from non-food based biomass.

2. Methods

2.1. Microbial species and media

Both the *C. thermocellum* (ATCC 27405) and *C. thermolacticum* (ATCC 43739) used for this study were obtained from the American Type Culture Collection (ATCC). Media was prepared as described by He et al. (2009). The basic media was used for both strains unless otherwise noted. All media were prepared using standard anaerobic techniques under controlled conditions (80%N_2/20%CO_2). The redox potential decrease in media was tested using resazurine which changes from reddish pink to light yellow if reduced.

Cultures were maintained in standard media containing either a paper strip (Whatman No. 1) or sucrose (Sigma–Aldrich). The cultures were stored in the refrigerator and were subcultured once every month. The purity of the microbes was regularly checked under the microscope.

2.2. Substrate

Sterilized anaerobic fermentation substrate solutions containing glucose (Sigma–Aldrich), xylose (Sigma–Aldrich), cellobiose (Sigma–Aldrich), microcrystal cellulose (Sigma–Aldrich), or hemicellulose (Kaufert Lab, University of Minnesota, Saint Paul, MN) were added after autoclaving the medium.

2.3. Growth of organisms

A low initial substrate/medium ratio (1% w/v) was used and a 5 mL homogeneous microbe culture with a cell weight of 5 g/L was transferred into 100 mL anaerobic bottles containing 50 mL of medium prepared under anaerobic conditions unless otherwise noted. For the co-culture, the inoculation was performed by adding 2.5 mL 5 g/L *C. thermocellum* and 2.5 mL 5 g/L *C. thermolacticum* per 50 mL prepared medium. Cultures incubated for 48 h at 57 °C without shaking were analyzed for the end-products (ethanol and acetate). Fermentations on each substrate without adjusting the pH and without adding exogenous ethanol were used as controls and mixtures with only medium and substrates were used as blanks. Triplicates were conducted for each treatment.

2.4. Analytical methods

The cell density was monitored by measuring the turbidity of the culture at 575 nm with a spectrophotometer (Sequoia-Turner Model 340) using a 13 mm path length test tube. One unit of OD575 was found to be equivalent to 1.1 g dry cell/L of *C. thermolacticum* and 0.5 g dry cell/L *C. thermocellum*.

End products (ethanol and acetate yield) in the water phase were determined using a Waters high performance liquid chromatograph unit (HPLC) with a Bio-Rad Aminex HPX-87H column with a de-ashing BIO-RAD Micro-guard refill cartridge filter. The column temperature was set to 30 °C and the detector temperature was set to 50 °C. Waters 2414 refractive index detector and Waters 2478 dual λ absorbance detector are used. Distilled water was used as the mobile phase delivered at the flow rate of 0.5 mL/min, with a sample injection volume of 5 μL. Calibration curves were generated using pure ethanol and acetate (Sigma–Aldrich) and the culture medium. Percentages of yields for final products were calculated in relation to original saccharides addition.

3. Results and discussion

In order to analyze the effect of major fermentation parameters on the CBP fermentation, the conversions of selected materials such as cellulose, hemicellulose and their mono- or di-saccharide intermediates are investigated. The major saccharides include glucose, cellobiose, and xylose. The effect of minor saccharides such as arabinose mannose and galactose, however, are not considered in this study. Both strains produce ethanol, acetate and hydrogen. But depending on conditions, the product distribution varies. Preliminary studies were performed with both mono-cultures at different substrate concentrations, initial ethanol concentrations, and pH values. 1% w/v substrate loading proved to be the most suitable for fermentation for both strains, which agrees with earlier studies (Kundu et al., 1983). Initial ethanol equivalent of 8 g/L is the highest ethanol concentration both strains can tolerate. Any sample exposed to an ethanol level over 8 g/L, showed no strain growth because of the irreversible change of cell membrane lipids through ethanol, as discussed by Baskaran et al. (1995). It is also observed that a pH between 5 and 10 leads to the production of ethanol and acetate, any pH below or above appears to be unsuitable for the viability of the cells. The influences of pH, ethanol concentration and substrate types were further compared between *C. thermocellum*/*C. thermolacticum* mono-cultures and co-culture fermentation.

3.1. Benefit of co-culture fermentation

This study clearly demonstrated the advantage of applying a co-culture in CBP fermentations to improve the ethanol production for selected substrates. It demonstrated the potential of using simultaneous *C. thermocellum*/*C. thermolacticum* co-culture to converting components of lignocellulosics directly into ethanol and acetate at initial ethanol levels of up to 4 g/L. Although this co-culture is unable to shift the heterofermentative pathway into a homofermentative pathway or at least decrease the acetate production, ethanol production is significantly increased compared to the mono-cultures. Almost all the ethanol yields obtained from co-culture fermentation are above any of its mono-culture fermentation yields, often almost tripled as compared to the mono-cultures under identical conditions on all the substrate. The only exceptions are the experiments at pH = 7 to pH = 9 for the substrate glucose. The by-product acetate generation, which occurs in smaller concentration of mostly below 1 g/L, turns out to lie in the same narrow range irrespective of the substrate or pH value used. In addition, although acetate is not desirable as a final product, it has been shown that the formation of acetate can stimulate ethanol production during fermentation indirectly contributing to final ethanol yield (He et al., 2009). Thus, small amounts of acetate production are not considered to have a negative effect on ethanol production in this study. The absence of the correlation between ethanol productivity improvement and increased E/A ratios suggests that the changes caused by co-culture application are caused by both mass action effects and membrane fluidity change. In addition, this co-culture exhibited a shorter lag period (48 h) than any of their mono-culture before the initiation of the selected sugar fermentations.

We hypothesize that our approach is effective because *C. thermocellum* can produce active cellulotyic and xylanolytic enzymes