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Cellulosic Bioethanol from *Clostridium Thermocellum* Fermentation

Kwanchanok Viravaidya [1, 3*], Waranya Tangjerdjaras [1] and Chakrit Tachaapaikoon [2 +]

[1] Chemical Engineering Practice School (ChEPS), Department of Chemical Engineering, King Mongkut's University of Technology, Thonburi, Bangkok, Thailand

[2] Pilot Plant Development and Training Institute, King Mongkut's University of Technology, Thonburi, Bangkok, Thailand

[3] Biological Engineering Program, Faculty of Engineering, King Mongkut's University of Technology, Thonburi, Bangkok, Thailand

Abstract. Due to oil price hike, efforts have been made to find renewable sources of energy that can be locally generated. In Thailand, more emphasis has been put on the production of ethanol from crops, since Thailand has abundant supplies of agricultural products. These materials, however, have relatively high market values, making bioethanol production not economically feasible. The interest has been shifted to the conversion of cellulose to ethanol since cellulose is considered as waste in most agricultural processing. The production of ethanol from cellulose, often called cellulosic ethanol, can be achieved with several methods including the fermentation of cellulose using *C. Thermocellum* (ATCC 27405). In this study, the effects of important factors, such as initial substrate concentrations and types of cellulosic materials, on the production of cellulosic ethanol were investigated. The cellulosic materials used in this study were classified into three physical forms: microcrystalline (Avicel), microgranular and amorphous. It was found that both cell growth rate and ethanol production inversely depended on the initial substrate concentrations. The lower substrate concentration resulted in the higher concentration of both glucose and ethanol. In addition, an increase in the crystallinity in substrate's structure caused decreases of both cell growth and ethanol yield. The maximum specific growth rate, glucose yield, and ethanol concentration, which were 2.96 day^{-1}, 0.22 g/g of substrate and 0.83 g/L, respectively, could be achieved with 5.0% (v/v) *C. Thermocellum*, using 1% (w/v) amorphous cellulose as a substrate.

Keywords: cellulosic ethanol, *C. Thermocellum* and avicel

1. Introduction

Thailand is currently facing an energy problem due to oil price hike. Efforts have been made to find renewable sources of energy or new sources of energy that can be domestically generated. Ethanol has been an alternative fuel that received a great deal of attentions recently, as it can be blended with gasoline to make gasohol fuel which is sold in all gas stations in Thailand. Ethanol can be produced using fermentation of sugar or hydration of ethylene. In Thailand, more emphasis has been put on the production of ethanol from crops, since Thailand has abundant of agricultural products. Several raw materials such as starch, sugar beet, and cassava have been used in the production of bioethanol. These materials, however, have relatively high market values, making bioethanol production not economically feasible for long-term. The interest has been shifted to the conversion of cellulose to ethanol since cellulose is considered as waste in most agricultural processing. The production of ethanol from cellulose, often called cellulosic ethanol, can be achieved by fermentation of cellulose with cellulase. Cellulase, produced by bacteria, fungi, and yeast, is a class of enzyme that catalyzes cellulolysis.

+ Corresponding author. Tel.: + 662-470-9222, ext. 205; fax: +662-872-9118.
E-mail address: kwanchanok.vir@kmutt.ac.th

In this study, we are interested in using *Clostridium Thermocellum* in the fermentation of cellulose to produce ethanol. These thermophilic bacteria can produce active cellulase complex with high specificity for cellulose hydrolysis and, at the same time, ferment ethanol in a single step [1]. The effects of initial substrate concentrations and cellulose structure on the cell growth and the production of ethanol were investigated.

2. Materials and Methods

2.1. Materials

Avicel, a purified microcrystalline cellulose with particle size of 30 micron, microgranular cellulose, amorphous cellulose and all chemicals were purchased from Sigma Aldrich (USA), unless otherwise noted.

2.2. Cell culture, medium composition and preparation

C. Thermocellum (ATCC 27405) was maintained in BM 7.0 medium, composed of KH_2PO_4, K_2HPO_4, urea, yeast extract and mineral solution. All of these components were added to DI water and stirred until well-mixed. The pH of the medium was adjusted to 7.0 prior to the addition of L-cysteine and resazurin. More water was added to the solution to make up for 95 ml final volume. 0.5 g of Avicel (0.5% w/v) was added to the medium as a carbon source for the cells. Due to an anaerobic process, air was removed by incubating the solution in an oven at 70^{o}C for 2.5 hours. Then, the bottle was flushed with nitrogen in an anaerobic chamber for 5 minutes or until the color of the solution turned to yellow. After that, the bottle was tightly capped prior to autoclaving at 121^{o}C for 20 minutes. The bottle was left at room temperature to cool down. Afterwards, 5% (v/v) of *C. Thermcellum* in stock solution was transferred to the freshly prepared BM 7.0 medium using a syringe and incubated at 60 ^{o}C in an incubator shaker for 2 days before the experiments.

2.3. Ethanol fermentation

For the ethanol fermentation studies, appropriate amount of avicel (1%, 5% and 10% w/v) was added to BM 7.0 medium. The solution with the final volume of 500 ml was incubated in the oven at 70^{o}C for 1 hour and autoclaved as previously described. To initiate the fermentation process, 5% v/v of *C. Thermocellum* was transferred to the medium containing Avicel and placed in an incubator shaker at 60^{o}C and 180 rpm. At each predetermined time, 3 ml of a sample was aliquoted from each fermentation bottle for cell count and the determination of cell dry weight, sugar and ethanol contents. The samples were stored at -4^{o}C until the analyses to stop enzymatic processes involved during the fermentation. YSI sugar analyzer was used to determine the concentrations of glucose and ethanol. Each experiment was performed in triplicate (n = 3) and repeated at least twice.

3. Results and Discussion

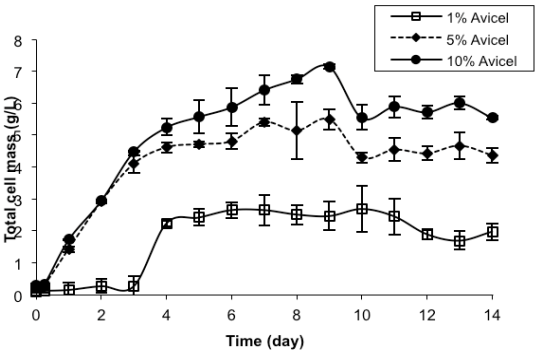
3.1 Effect of initial concentrations of avicel

In this study, Avicel concentrations were varied between 1%, 5% and 10% w/v to determine an appropriate concentration of the raw material for further studies. The growth of *C. Thermocellum* was reported as total cell mass as shown in Fig. 1A. The result shows that the lag phase was approximately 6 hours for *C. Thermocellum* in all substrate concentrations. It seems that the cells were still in the stationary phase after 2 weeks of fermentation, possibly because some substrates still remained in the fermentor. As expected, higher concentration of the substrate resulted in higher total cell mass (Table 1). However, 1% (w/v) Avicel gave the highest specific growth rate (1.5 day^{-1}), indicating faster cell production rate.

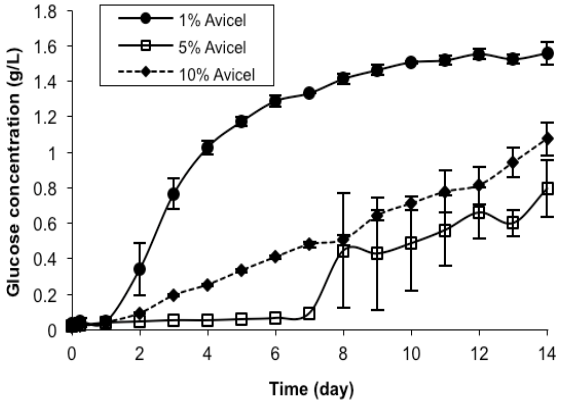
On the other hand, the highest glucose and ethanol concentrations were obtained from the fermentation of 1% w/v Avicel, while the fermentation of both 5% and 10% w/v Avicel yielded similar amounts of glucose and ethanol (Fig. 1B, 1C and Table 1). Because much more Avicel was used, the yields of glucose and ethanol from 5% and 10% w/v Avicel fermentation were quite low. This implies that the cells had reached its production limit because increasing initial substrate concentration did not increase the concentrations of the products.

Although the total cell mass depended on the initial substrate concentration, the maximum specific growth rates, glucose and ethanol contents of 5% and 10% w/v Avicel were similar as shown in Table 1. It is likely that these substrate concentrations reached the saturation where increasing the substrate concentration

would not have any effect on either the cell mass or the end products. Moreover, a significant amount of Avicel in both concentrations still remained in the fermentor even after the cells had reached the death phase (data not presented). This result implies that the cell growth did not stop because of carbon limitation which was in agreement with earlier reports on the influence of initial cellulose concentration on the carbon flow distribution during batch fermentation [2]. Thus, under carbon excess condition, growth of cell was limited by other factors such as product inhibition. Although 1% w/v Avicel yielded the lowest total cell mass, highest specific growth rate, glucose and ethanol concentrations were achieved at this Avicel concentration. It is possible that more carbon source or substrate was consumed to produce cell mass instead of producing the enzyme to convert glucose to ethanol. Therefore, 1% w/v Avicel was used in the next study.



(A)



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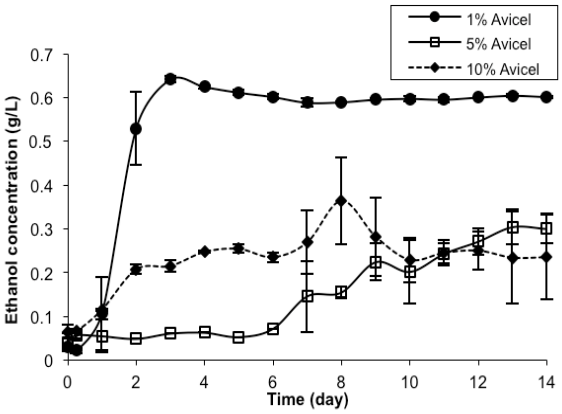


(C)

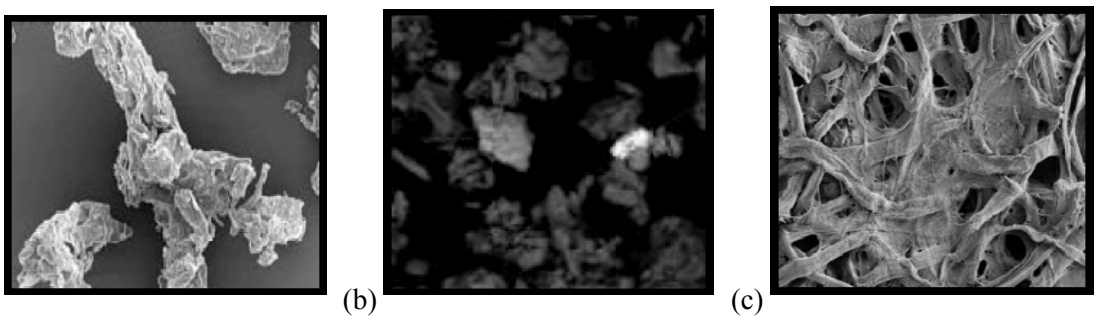
Fig. 1: (A) Cell growth of *C. Thermocellum*, (B) glucose and (C) ethanol concentrations from the fermentation of 1%, 5% and 10% w/v Avicel.

Table 1: Summary of total cell mass, specific growth rate, glucose and ethanol concentrations, and glucose and ethanol yields from the fermentation of 1%, 5% and 10% w/v Avicel.

			Concentration (g L^{-1})		Yield (g g substrate^{-1})	
Substrate structure	Total Cell Mass (g L^{-1})	μ_{max} (day^{-1})	Glucose	Ethanol	Glucose	Ethanol
1% w/v Avicel	3.65	1.50	1.52	0.60	0.152	0.06
5% w/v Avicel	4.43	1.28	1.41	0.30	0.028	0.006
10% w/v Avicel	5.73	1.22	1.41	0.32	0.014	0.003

3.2 Effect of the cellulose structures

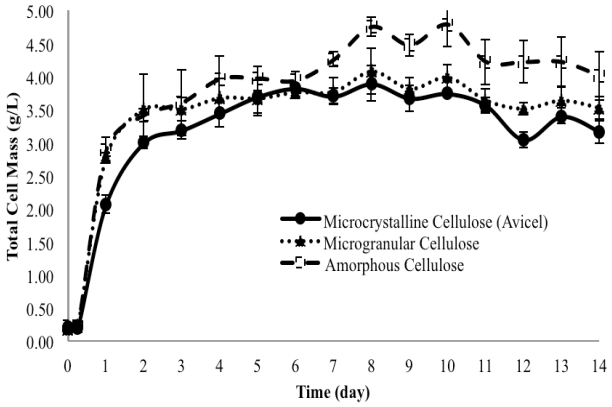
Three types of substrates, namely microcrystalline (Avicel), microgranular and amorphous cellulose, were used in this study (Fig. 2). Avicel has the highest crystallinity in its structure, while amorphous cellulose appears to form long fiber. The highest total cell mass and growth rate of 4.22 g L^{-1} and 2.96 day^{-1}, respectively, were achieved when amorphous cellulose was used as a substrate (Fig. 3A and Table 2). It is clear that the crystallinity in the structure of cellulose had an effect on the cell growth. The cell growth rate decreased as the substrate's crystallinity increased.



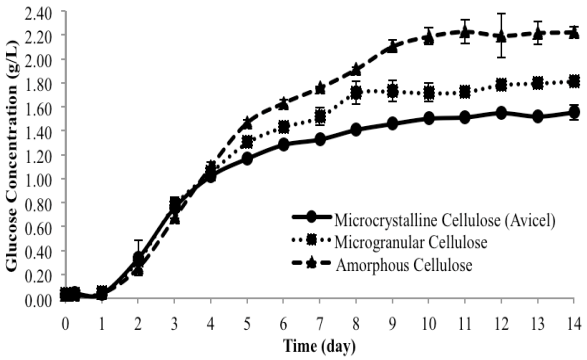
(a) (b) (c)

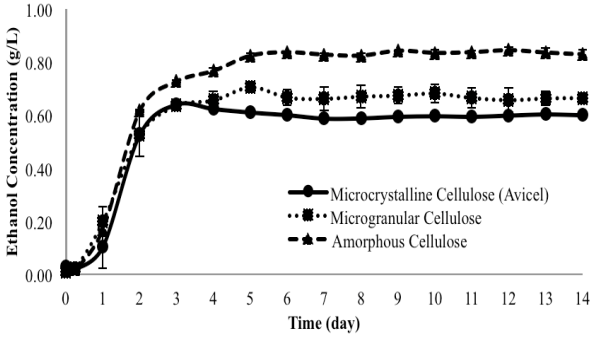
Fig. 2: SEM images of (a) microcrystalline or Avicel, (b) microgranular and (c) amorphous cellulose [3]

As expected, the fermentation of amorphous cellulose yielded the highest glucose (2.22 g L^{-1}) and ethanol (0.6 g L^{-1}) contents, as shown in Fig. 3B, 3C and Table 1. In cellulose molecules, each monomer is linked by β-1,4 glycosidic bond and hydrogen bond, forming a crystalline structure which neither a molecule of water nor enzyme can penetrate them. The crystalline cellulose only allows *exogluconase*, a subgroup of cellulase enzyme, to attack the terminal glucosidic bond while, amorphous cellulose allows both *exo-* and *endogluconase*, another subgroup of cellulase enzyme, to break the terminal and internal glucosidic bond [4]. Therefore, the hydrolysis rate of amorphous cellulose is much faster than those of microgranular and microcrystalline cellulose. As a result, the total cell mass, cell growth rate, glucose and ethanol concentrations were higher when amorphous cellulose was used as a substrate.



(A)





(B) (C)

Fig. 3: (A) Cell growth of *C. Thermocellum*, (B) glucose and (C) ethanol concentrations from the fermentation of microcrystalline, microgranular and amorphous cellulose.

Table 2: Summary of total cell mass, specific growth rate, glucose and ethanol concentrations, and glucose and ethanol yields from the fermentation of microcrystalline, microgranular and amorphous cellulose.

			Concentration* (g L^{-1})		Yield* (g g substrate^{-1})	
Substrate structure	Total Cell Mass* (g L^{-1})	μ_{max} (day^{-1})	Glucose	Ethanol	Glucose	Ethanol
Microcrystalline	3.65	1.50	1.52	0.60	0.15	0.06
Microgranular	3.68	2.85	1.79	0.66	0.18	0.07
Amorphous	4.22	2.96	2.22	0.83	0.22	0.08

Interestingly, there was abundant glucose remaining in the system, while the concentration of ethanol was leveled off as early as 3 days, implying that the glucose-to-ethanol conversion was terminated at very early in the fermentation process. It is possible that glucose may act as an enzyme inhibitor preventing the production ethanol. The previous report has demonstrated that glucose with a small amount of alcohol can provide a synergistic effect which considerably limited the rate of fermentation [5]. To overcome this problem, a co-culture fermentation system is suggested. Another microbial strain or yeast that can efficiently utilize glucose to form ethanol should be added to the system. Consequently, the accumulation of glucose can be reduced to the level that enzyme inhibition does not occur, while increasing the concentration of ethanol, at the same time.

4. Conclusion

In this study, the effects of initial substrate concentrations and cellulose structures on the cell growth and ethanol production were investigated. It was found that the total cell mass strongly depended on the initial substrate concentration. However, the specific growth rate, glucose and ethanol contents decreased as the initial substrate concentrations increased. Among the three concentrations (1%, 5% and 10% w/v of Avicel) tested, 1% w/v Avicel gave the highest glucose and ethanol yields. In the second part, the study of the effect of cellulose structure on ethanol fermentation was carried out using three types of cellulose: microcrystalline, microgranular and amorphous. High crystallinity in the cellulose structure resulted in lower total cell mass, specific growth rate and both glucose and ethanol concentrations. It was more difficult for the enzymes to penetrate through the tightly packed structure of microcrystalline cellulose, leading to a slower hydrolysis process and lower ethanol production.

5. Acknowledgements

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6. References

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