Bioresource Technology 102 (2011) 10065–10071

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

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

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ARTICLE INFO

Article history:
Received 30 May 2011
Received in revised form 4 August 2011
Accepted 15 August 2011
Available online 25 August 2011

Keywords:
Consolidated bioprocessing
Clostridium sp.
Fermentation
Co-culture
Lignocellulosic ethanol

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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doi:10.1016/j.biortech.2011.08.067

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

Table 1a
Effect of initial ethanol addition (0–4 g/L) and substrate types on ethanol production (g/L) at pH = 9 for *C. thermocellum* mono-culture, *C. thermolacticum* mono-culture and co-culture fermentations. The ethanol and acetate productions are the average value of triplicates runs under each chosen condition. SD is the standard deviation of the replicates of each condition.

	Initial ethanol (g/L)	*C. thermocellum*		*C. thermolacticum*		Co-culture	
		Ethanol (g/L)	SD	Ethanol (g/L)	SD	Ethanol (g/L)	SD
Glucose	0	0.476	0.004	0.028	0.001	0.285	0.005
	0.5	0.839	0.029	0.038	0.001	0.194	0.004
	1	1.229	0.005	0.043	0.004	0.965	0.003
	2	2.000	0.019	0.071	0.003	1.189	0.001
	4	2.638	0.050	0.116	0.004	1.358	0.001
Cellobiose	0	0.399	0.001	0.036	0.003	0.258	0.003
	0.5	0.738	0.010	0.046	0.002	0.503	0.001
	1	1.128	0.030	0.058	0.006	1.427	0.008
	2	1.615	0.037	0.072	0.004	1.750	0.002
	4	1.673	0.033	0.112	0.003	3.163	0.001
MCC	0	0.016	0.009	0.004	0.001	0.430	0.001
	0.5	0.144	0.004	0.016	0.001	0.582	0.001
	1	0.304	0.006	0.032	0.001	1.109	0.006
	2	0.680	0.004	0.066	0.008	1.935	0.005
	4	1.339	0.006	0.104	0.002	3.811	0.032
Xylose	0	0.000	0.000	0.026	0.001	0.226	0.003
	0.5	0.000	0.000	0.033	0.001	0.200	0.007
	1	0.000	0.000	0.037	0.001	0.217	0.001
	2	0.000	0.000	0.064	0.004	0.754	0.010
	4	0.000	0.000	0.097	0.083	4.539	0.011
Hemicellulose	0	0.000	0.000	0.001	0.000	0.007	0.000
	0.5	0.000	0.000	0.002	0.000	0.008	0.001
	1	0.000	0.000	0.001	0.001	0.006	0.001
	2	0.000	0.000	0.002	0.001	0.021	0.001
	4	0.000	0.000	0.000	0.000	0.053	0.001

(Raman et al., 2009; Pellerina et al., 1991), and *C. thermolacticum* is able to utilize the degraded substrates, which are less favorable for *C. thermocellum* (Talabardon et al., 2000a). The improvement of MCC degradation clearly demonstrates this synergistic effect.

3.2. Impact of ethanol concentration on fermentation

Tables 1a and 1b compare the product formation during saccharide fermentations applying *C. thermocellum*, *C. thermolacticum* or *C. thermocellum*/*C. thermolacticum* co-cultures. Similar to mono-culture fermentations, ethanol and acetate were found to be the major end-products in *C. thermocellum*/*C. thermolacticum* co-culture fermentation.

The impact of the ethanol production and accumulation within the medium on total solvent production was studied by adding various levels of ethanol at the beginning of the fermentation. The addition of 0 g/L of ethanol represents the beginning stage of the fermentation when no ethanol was produced. By adding ascending amounts of ethanol from 0.5 to 4 g/L at the start, this study simulated the ethanol accumulation progress as a sequence of the continuous fermentation. Compared to mono-culture fermentations, *C. thermolacticum*/*C. thermocellum* co-culture fermentation lead to higher ethanol production when the original ethanol concentration at the start of the fermentation is around 4 g/L for all substrates used under all pH values (Tables 1a and 1b, Fig. 1). However, the increase of ethanol yield caused by applying a co-culture was observed not to correlate to ethanol/acetate ratio improvement.

While the ethanol production by *C. thermolacticum* mono-culture and co-culture are significantly enhanced through exposure to initial ethanol levels up to 4 g/L on all substrates and at all pH values (Table 1a, Fig. 1), for *C. thermocellum* the positive effect of initial ethanol appeared to be dependent on pH and substrate. If cellobiose is added as the sole carbon source, the positive correlation between initial ethanol addition and final ethanol concentration is terminated when the ethanol concentration approaches 2 g/L. As the ethanol concentration exceeds 2 g/L no additional positive effect on final ethanol yield is observed. For glucose and MCC fermentation, the ethanol production continues to increase as the initial ethanol concentration rises. On the other hand, the acetate production decline stopped at 2 g/L of initial ethanol level, and bounces back up slightly at 4 g/L initial ethanol level. In general, a higher initial ethanol level favors the ethanol production for both cultures and the co-culture is accompanied by a lower acetate yield (Table 1b). At the optimized condition, the ethanol production in MCC co-culture fermentation increases from 1.0 g/L at an initial ethanol level of 0 g/L to 3.8 g/L at an initial ethanol level of 4 g/L, an improvement of more than 3 g/L. Moreover, for ethanol yields in xylose co-culture fermentations, the increase reached over 4 g/L by increasing the initial ethanol concentration from 0 to 4 g/L (Table 1a). The initial ethanol might contribute to changed fermentation in two ways: changing the metabolism pathway or changing the cell membrane fluidity (Herrero et al., 1982).

It is proven that the presence of ethanol will change the fatty acid composition of the cell membrane of *Clostridium* sp., which leads to higher cell membrane fluidity (Herrero et al., 1982). Under extreme ethanol concentrations, which is over 8–16 g/L in this study, this fluidity change will lead to the blockage of the glycolysis, and results in low solvent production (Herrero et al., 1985).

It is also conceivable that metabolic pathway regulation caused by the raising of the initial ethanol level can lead to higher ethanol production. In the co-culture fermentations, the enhancement of ethanol yield is accompanied with a decreased acetate yield. This observation suggests that the initial existence of ethanol might contribute to the shift of the co-culture fermentation pathway, shifting the hetero-fermentation towards the ethanol production, while reducing acetate production.

The other most likely possible explanation is the interaction of the ethanol concentration and optimum culture temperature. In this study, the *C. thermocellum* is the major ethanol producer, the growth

Table 1b
Effect of initial ethanol addition (0–4 g/L) and substrate types on acetate production (g/L) at pH = 9 for *C. thermocellum* mono-culture, *C. thermolacticum* mono-culture and co-culture fermentations. The ethanol and acetate productions are the average value of triplicates runs under each chosen condition. SD is the standard deviation of the replicates of each condition.

	Initial ethanol (g/L)	*C. thermocellum*		*C. thermolacticum*		Co-culture	
		Acetate (g/L)	SD	Acetate (g/L)	SD	Acetate (g/L)	SD
Glucose	0	0.689	0.007	0.028	0.001	0.533	0.001
	0.5	0.672	0.001	0.026	0.001	0.666	0.006
	1	0.583	0.003	0.025	0.001	0.557	0.006
	2	0.472	0.002	0.024	0.001	0.437	0.002
	4	0.514	0.009	0.022	0.001	0.357	0.005
Cellobiose	0	0.433	0.005	0.027	0.002	0.649	0.003
	0.5	0.403	0.006	0.029	0.001	0.643	0.003
	1	0.307	0.006	0.028	0.002	0.616	0.008
	2	0.424	0.003	0.026	0.002	0.526	0.002
	4	0.468	0.001	0.027	0.001	0.491	0.008
MCC	0	0.040	0.001	0.009	0.001	1.117	0.001
	0.5	0.043	0.002	0.007	0.002	1.026	0.002
	1	0.042	0.001	0.006	0.002	0.976	0.001
	2	0.041	0.002	0.005	0.002	0.651	0.001
	4	0.041	0.001	0.007	0.002	0.648	0.002
Xylose	0	0.000	0.000	0.029	0.002	0.453	0.001
	0.5	0.000	0.000	0.028	0.001	0.397	0.001
	1	0.000	0.000	0.027	0.002	0.374	0.001
	2	0.000	0.000	0.027	0.002	0.373	0.004
	4	0.000	0.000	0.028	0.002	0.332	0.001
Hemicellulose	0	0.000	0.000	0.065	0.009	0.010	0.001
	0.5	0.000	0.000	0.064	0.006	0.011	0.001
	1	0.000	0.000	0.055	0.008	0.010	0.001
	2	0.000	0.000	0.054	0.003	0.010	0.001
	4	0.000	0.000	0.000	0.002	0.009	0.001

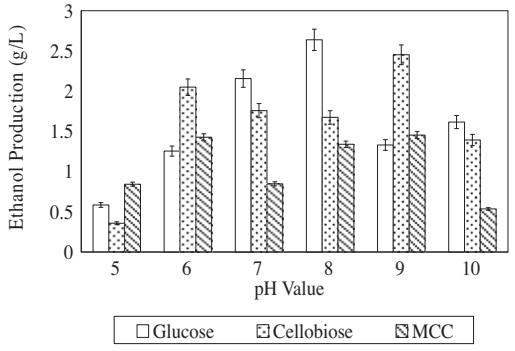

Fig. 1a. Effect of pH on ethanol production at 4 g/L initial ethanol level for *C. thermocellum* fermentation.

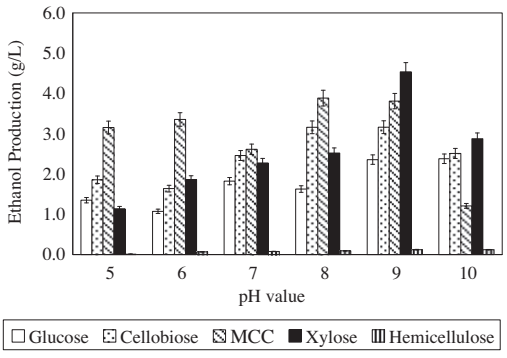

Fig. 1c. Effect of pH on ethanol production at 4 g/L initial ethanol level for co-culture fermentation.

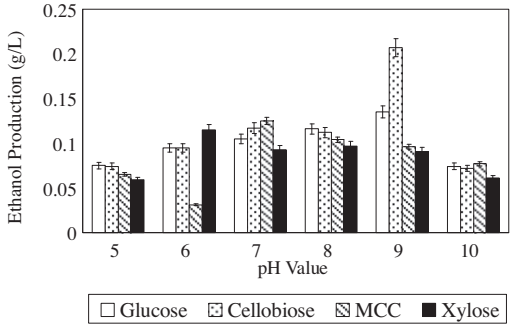

Fig. 1b. Effect of pH on ethanol production at 4 g/L initial ethanol level for *C. thermolacticum* fermentation.

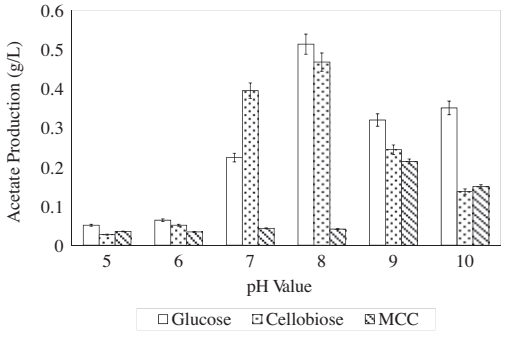

Fig. 1d. Effect of pH on acetate production at 4 g/L initial ethanol level for *C. thermocellum* fermentation.

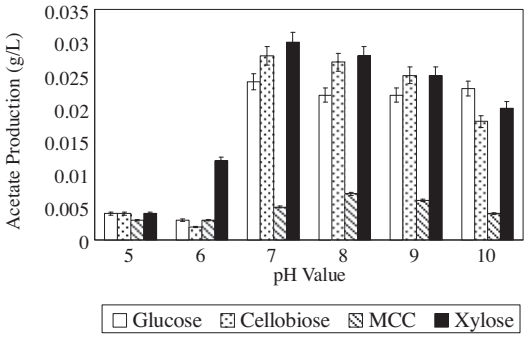

Fig. 1e. Effect of pH on acetate production at 4 g/L initial ethanol level for *C. thermolacticum* fermentation.

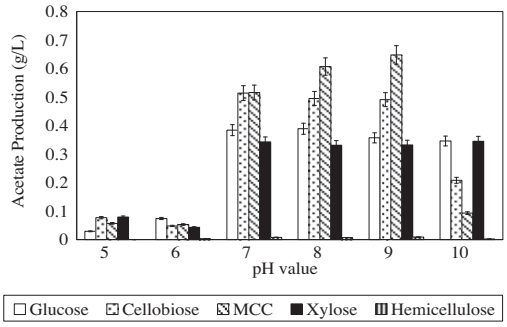

Fig. 1f. Effect of pH on acetate production at 4 g/L initial ethanol level for co-culture fermentation.

of *C. thermocellum* is extremely important. Previous studies suggest that the optimum temperature for the *C. thermocellum cultivation* will decrease with increasing ethanol or acetate concentrations within the medium (Kundu et al., 1983). A culture with 5–10 g/L ethanol and acetate was proven to have an optimum temperature at 58–55 °C, instead of 60 °C at 0 g/L ethanol level (Kundu et al., 1983). Increasing the initial ethanol level from 0 to 4 g/L, will lower the optimum cultivation temperature from 60 °C to around 56–57 °C which is close to the temperature chosen in this study. As a result, the lower the initial ethanol, the more the cultivation temperature (57 °C) deviated from the optimum conditions, resulting in lower solvent productions. A study focusing on optimizing the cultivation temperatures for co-culture at different ethanol levels is underway.

No obvious effect was detected beyond this initial ethanol level within the tolerance range. Further rising of the original ethanol concentration from 4 to 8 g/L does not show additional improvement in solvent production for neither mono-cultures nor co-culture. Higher ethanol concentrations will lead to the termination of the fermentation. Increase of the initial ethanol concentration over 8 g/L stopped the fermentation activity completely for *C. thermocellum*, and the co-culture. Initial ethanol concentrations over 16 g/L will terminate the activity of *C. thermolacticum*.

3.3. Substrate selectivity

As shown in Tables 1a and 1b, the selection of substrate has significant impact on ethanol and acetate yield for co-culture fermentation as well as mono-culture fermentations. Ethanol yield for *C. thermocellum* fermentation was non-detectible for hemicellulose and xylose conversions. This finding agrees with previous results demonstrating that *C. thermocellum* is unable to use xylose or xylan as sole carbon sources (Prates et al., 2001). Cellobiose and glucose were metabolized preferentially for both ethanol and acetate production. The efficiency of fermenting MCC is lower than fermenting simple sugars, including cellobiose and glucose especially under alkaline conditions (Figs. 1c and 1f). Different from *C. thermocellum*, *C. thermolacticum* can directly convert all the selected substrates (crystalline cellulose, glucose, xylose and cellobiose) except hemicellulose to ethanol and acetate. However, the proportions of these two major products are dramatically different between these two strains. For the conditions where the co-culture did not show any advantage, for example glucose conversion at pH = 9, the end-products obtained from *C. thermolacticum* fermentations were lower than *C. thermocellum* fermentation. Only exception was the fermentation of xylose. Starting ethanol concentration equal to 0 g/L at pH = 6 is the only condition at which *C. thermolacticum* produces more product than *C. thermocellum*. These lower yields in *C. thermolacticum* fermentation can be explained by the media used in this study, which is optimized for *C. thermocellum* growth. The solvent production capacity of *C. thermolacticum* fermentation might not be fully utilized.

The innovative co-culture is extremely efficiently in fermenting separate components of lignocellulosics for ethanol production. The substrates include cellulose, cellobiose, and xylose, which can be explained by selective substrate utilization ability observed in both strains. This improvement was most significant for ethanol yield at pH = 9 with high initial ethanol level (4 g/L). Under this condition, the ethanol yield from xylose was increased from 0.1 g/L for *C. thermolacticum* and 0 g/L for *C. thermocellum* to 4.5 g/L using a co-culture, which almost approached 90% of the theoretical xylose fermentation efficiency (Table 1a). This agrees with former studies showing the existence of cellulosic enzymes and xylanase in both strains (Raman et al., 2009; Pellerina et al., 1991). The co-culture is extremely efficient in converting MCC to ethanol. Ethanol yields from MCC fermentation were dramatically improved through the use of a co-culture, from 1.4 g/L for *C. thermocellum* and 0.1 g/L for *C. thermolacticum* to a maximum of 3.8 g/L. This represents 1.5 mol of ethanol per mol of sugar utilized for co-culture fermentation (around 75% of the theoretical efficiency; Kim and Weigand, 1992), almost three times the ethanol yield of the combined mono-cultures. For hemicellulose and it fragments, an increase in the production of ethanol and acetate was observed with the application of the co-culture. No effective enzymes capable of fermenting high molecule weight hemicellulose were reported in pervious studies for these two strains, only xylanase that can utilize degraded or partially degraded high molecular hemicellulose during fermentation (Raman et al., 2009; Pellerina et al., 1991). In our case, the co-culture should only be able to utilize di- or mono-saccharides, including xylose. Nevertheless, tiny amounts of polymeric hemicellulose are also consumed. Hemicellulose fermentation appeared to be slower and less effective than conversion of other fermentable sugars. In this case, by applying the co-culture of *C. thermolacticum* and *C. thermocellum* to lignocellulosic fermentation, optimization of the conditions should focus on the degradation conditions for cellulosic material and the hemicellulose fragments, mainly xylose.

The polymerized hexoses appear to improve the fermentation end-product yields for the co-culture, whereas it depresses the fermentation for the mono-cultures. For fermentation applying a single strain, the substrate with simpler structure will be preferred. The cellobiose and glucose, which have simpler molecular structure, are more accessible than MCC since they don't have access restricting crystalline areas. This accessibility led to a higher microbial conversion which resulted in higher solvent productions compared to MCC. This result is consistent with earlier research suggesting that solubility can stimulate the fermentation (Weimer and Zeikus, 1977).

Comparatively, it is clear that higher polymerization or lower solubility is able to initiate higher ethanol production during co-culture fermentation. For the co-culture increased utilization was observed for both cellobiose and MCC. MCC is proven to be the best substrate for ethanol production in the co-culture application in this study. However, decreased utilization of glucose was detected in co-culture as compared to mono-culture, and this observation is consistent under all chosen conditions. This is probably because for poly- or even di-saccharides, two strains are able to utilize different substrate instead of competing for one specific substrate. While *C. thermocellum* is degrading di- or poly-saccharides into monosaccharides (glucose) (Shoham et al., 1999), *C. thermolacticum* and to some extent *C. thermocellum* ferment the glucose into end-products (Collet et al., 2006; Shoham et al., 1999). However, for the fermentation with only glucose, since both strains are able to utilize glucose, enzyme substrate competition is present between these strains, which mean the total final yield in glucose fermentation is not improved.

3.4. Impact of pH value on co-culture fermentation

Determining the optimum pH range for both mono-culture fermentation systems is critical because of the interaction between pH value and substrate influence on microbial performance. However, the reported capability to digest the five chosen substrates for *C. thermocellum* and *C. thermolacticum* varies in previous reports due to the lack of uniform cultivation conditions (Fardeau et al., 2001; Wang et al., 1983). In this work, the wild type *C. thermocellum* strains ATCC 27405 and *C. thermolacticum* strain ATCC 43739 were grown in a standard medium as discussed by He and Sanford (He et al., 2009) on chosen substrates for 10 d. The influence of pH value on end product yield is shown in Fig. 1 Fermentation yields for both strains were sensitive to pH changes. The sensitivity towards pH change was enhanced with the increase of initial ethanol concentration. When the starting ethanol concentration reaches 4 g/L, pH influence have the maximum input on ethanol production from all substrates conversion and for all strains. No growth was found for the fermentation performed at a pH below 5 or above 10.

For the mono-cultures, pH = 8 and pH = 9 are the pH values providing the shortest lag period during fermentation for both strains, which is usually around 72 h. The optimum pH values for both strains dependent on the substrate. For cellulose and its fragments, including glucose, cellobiose and MCC, the largest ethanol production yields are found at pH = 8 or 9. In contrast to this, for pentose fermentation, the biggest ethanol productions were seen at pH = 6 to pH = 9.

Alkaline conditions, especially pH equals to 9, is preferred for *C. thermocellum*/*C. thermolacticum* co-culture fermentation if the goal is high solvents production (Fig. 1c), confirming results obtained in our mono-cultures fermentations (Figs. 1a and 1b). When the pH was adjusted to 9, the co-culture fermentation was the most efficient ethanol producer, except for glucose. Almost all of the ethanol yields obtained from co-culture fermentation at this pH were higher than the sum of their mono-culture fermentations. At higher pH values (pH > 9) the overall ethanol production is reduced and the advantage of a co-culture process disappeared for the mono sugars substrates. For MCC conversion, the co-culture was observed to be more effective than mono-cultures, even at less than optimum pH. No significant yield change was detected in hemicellulose fermentation under high pH co-culture condition. A decline of ethanol formation was observed as pH decreased below 9. At lower pH, ethanol produced on selected substrates was significantly reduced.

Similarly, the acetate production during fermentation by co-culture is higher when the strains were grown on cellulose, cellobiose, glucose, and xylose under neutral or alkaline conditions (Fig. 1f). Both pH = 8 and pH = 9 were considered as the optimum pH for acetate production since no significant yield difference was detected within this pH range. PH = 7 resulted in a 0.1 g/L decrease in acetate production for MCC fermentation as compared to pH = 9. Acetate production on none of the other substrates was observed to be affected by pH drop. However, once the pH dropped below 7, the acetate formed by co-culture fermentation dropped sharply from an average of 0.5 g/L to less than 0.1 g/L. In addition, under acidic conditions, co-culture application did not show significant advantages over mono-culture fermentations on cellobiose, glucose, hemicellulose and xylose (Figs. 1d and 1e). The only exception is the MCC fermentation. A higher acetate yield always existed in co-culture fermentation of MCC, regardless of the pH. For acetate production, instead of *C. thermocellum* as observed for ethanol production, *C. thermolacticum* usually showed a better yield whenever co-culture did not provide any advantage.

In the relationship of pH and solvent production, mass action effect plays the most important role. Both *C. thermocellum* and *C. thermolacticum* have a similar metabolic pathway. During hexose fermentation, both strains convert the substrates into pyruvate, and then into acetyl-coA and lactate. The acetyl-coA is fermented to a reductive product (ethanol) or an oxidative product (acetate) through the Embden-Meyerhof pathway (EMP) (Collet et al., 2006; Bothun et al., 2004). For xylose conversion, the pentose phosphate pathway and EMP are also important (Jeffries, 1981). In the Embden-Meyerhof pathway, during the formation of -acetyl-CoA from pyruvate, pyruvate:ferredoxin oxidoreductase (E.C. 1.2.7.1) is the key enzyme to catalyzes the pyruvate oxidative decaboxylation (Collet et al., 2006). The optimum pH for this enzyme is reported to be around 7 (Meinecke et al., 1989). An activity decline of pyruvate:ferredoxin oxidoreductase will occur as the pH decreases below neutral condition (Bothun et al., 2004). Instead of producing acetyl-CoA, glycolytic intermediates will form. Thus the yield of ethanol and acetate converted from acetyl-CoA dropped sharply under acidic condition. No obvious change in yield was detected by increase pH from 7 to 9 for *C. thermolacticum* and from 8 to 9 for *C. thermocellum* in this research.

However, although acidic conditions reduced the solvent production, neutral condition (pH = 7) is not the optimum pH for *C. thermocellum* and this co-culture, which indicates that other factors are involved to improve the co-culture activity. Higher acetate production compared to *C. thermolacticum* would be a possible reason leading to a higher optimum pH range. According to our observations, the accumulation of acetate within the medium will bring the pH down to 6 within the fermentation period which leads to lower yields. Thus, by applying higher initial pH, it will take longer for these microbes to drop the pH below 7.

In sum, the co-culture produced the largest amount of acetate during fermentation under high initial ethanol levels, and the optimum pH of 9. For *C. thermocellum*, which leads to slightly lower acetate production compared to co-culture, pH = 8 is efficient to provide a high pyruvate:ferredoxin oxidoreductase activity. This result suggests that in order to archive higher solvent productions, initial mild alkaline condition, prompt removal of acetate from the system, or pH-control is essential.

3.5. Interaction among factors on formation of end-products

Based on ethanol and acetate yield after 10 d fermentation, statistical analysis indicated that the solvent yields were significantly related to the growth conditions, including the interactions among application of co-culture, initial ethanol level, pH value and substrate type. Interactions between each two factors, and among these four factors were found to be statistically significant ($P < 0.0001$) at the 95% confidence level. These results

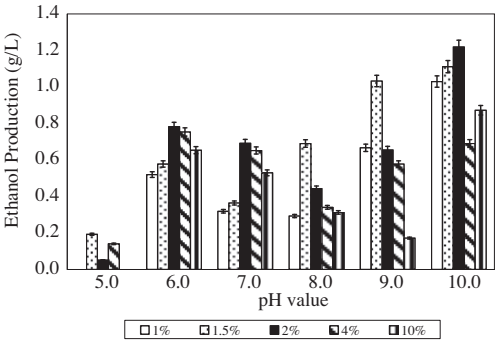

Fig. 2. pH effects on ethanol yield for aspen co-culture fermentation with wood loading rate from 1% to 10%.

demonstrated that adjustment of pH, initial ethanol concentration, substrates type and application of co-culture are all interrelated. Thus, the optimization of the co-culture fermentation should not only take the three main effects into account, the interactions among them cannot be ignored.

3.6. Fermentation of untreated aspen powder

The feasibility of fermenting untreated biomass by applying this co-culture was examined. Aspen powder, due to its complicated lignocellulosic structure, was expected to require longer fermentation times. At the optimized condition, the highest ethanol production of 1.28 g/L was observed. This is significantly lower than ethanol yields observed with the pure sugar media (Fig. 2). In addition, this maximum ethanol production was detected at pH = 10 instead of pH = 9 in pure saccharide fermentations. Higher wood loading rate did not have significantly positive effect on ethanol production. By changing the substrate amount from 1% to 1.5%, only a slight increase of ethanol production was observed. Continuously increasing the aspen concentration from 1.5% to 2%, the ethanol yield only changed from 1.1 to 1.2 g/L. Further increasing the wood loading rate from 2% to 10%, however, led to a decrease of ethanol production for co-culture fermentations.

4. Conclusion

This study clearly shows the technical feasibility of using lignocellulosics for ethanol production with our innovative co-culture because of shorter lag time and higher solvent production. Comparatively higher starting pH is necessary to obtain maximum ethanol yield due to the acetyl groups found in native aspen. The ethanol yield obtained from aspen fermentation is relatively low compared to pure cellulosics due to the hindered accessibility of wood or lack of fermentable substrates at the early fermentation stage. Therefore, adding sugars to initiate the microbial growth, or modifying the substrate or adding hemicellulose degradator to provide higher accessibility might be necessary.

Acknowledgements

The authors acknowledge the support of the University of Minnesota and the Buckman Fellowship fund for supporting this research.

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