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Butanol Production from Cellulosic Substrates by Sequential Co-culture of *Clostridium thermocellum* and *C. acetobutylicum*

Yu, E.K.C., M.K.-H. Chan and J.N. Saddler
Biotechnology Department, Forintek Canada Corp.
800 Montreal Road, Ottawa, Ontario, Canada K1G 3Z5

ABSTRACT

A sequential co-culture approach was investigated for the conversion of lignocellulosic substrates to fuels and chemicals. Growth of *Clostridium acetobutylicum* on solka floc (or a mixture of solka floc and aspenwood xylan), in co-culture with *C. thermocellum*, resulted in the efficient utilization of all the hydrolysis products derived from the lignocellulosic substrates. This co-culture approach resulted in a 1.7-2.6 fold increase in the total fermentation products formed. The majority of the fermentation products were acids and not solvents, however the solventogenesis step could be induced by the addition of butyric acid to the fermentation medium.

INTRODUCTION

During the past few years we have studied a variety of methods for converting lignocellulosic residues to liquid fuels and chemicals. Our earlier work has shown that *Clostridium acetobutylicum* can utilize most of the hexose (including cellobiose) and pentose sugars present in biomass hydrolyzates and convert them into almost quantitative yields of n-butanol (Mes-Hartree and Saddler, 1982; Yu and Saddler, 1983). This organism is able to utilize a range of lignocellulosic substrates which have been hydrolyzed either by acid (Yu *et al*, 1984a; 1984b) or cellulase enzymes (Saddler *et al*, 1983; Yu *et al*, 1984b). Since the enzymatic hydrolysis approach requires the costly steps of separate enzyme production and handling, we hope to simplify the overall process by adopting a co-culture system using the cellulolytic anaerobe *C. thermocellum*. This anaerobe produces active cellulolytic and xylanolytic enzymes; it can directly convert cellulose to ethanol and it has a tendency to accumulate glucose and pentose sugars in the culture filtrate when it is grown on lignocellulosic substrates (Saddler and Chan, 1982; 1984). By co-culturing *C. thermocellum* with *C. acetobutylicum* we hoped that all of the hemicellulose and cellulose-derived sugars would be directly converted to butanol and ethanol using a single fermentation process.

MATERIALS AND METHODS

Microorganisms and media: *Clostridium acetobutylicum* (ATCC 824) was grown anaerobically under a nitrogen headspace at 37°C without shaking (Yu and Saddler, 1983), using anaerobic techniques as described by Miller and Wolin (1974). *C. thermocellum* was obtained from the National Research Council of Canada (NRCC 2688). Cultures were grown anaerobically under a nitrogen headspace gas in DSM medium at 60°C without shaking (Saddler and Chan, 1984).

Sequential co-culture approach: C. thermocellum was grown on solka floc or a mixture of solka floc and aspenwood xylan in DSM medium at 60°C for 3 days. Cultures of C. acetobutylicum were used (5%, v/v, inoculum) to inoculate medium containing cultures of C. thermocellum and the mixed cultures were then incubated without shaking at 37°C for various times. Controls of C. thermocellum incubated at 60°C for 3 days on corresponding substrates were grown under identical conditions to the mixed culture systems. When any extra ingredients were added to the co-culture system, the ingredients were prepared as concentrated solutions under anaerobic conditions, autoclaved, and then transferred anaerobically to the culture vials. All cultures were analyzed at regular intervals for enzyme activities, reducing sugars, glucose, and volatile fatty acids.

Analytical methods: Culture supernatant fluids were analyzed for volatile fermentation products using previously defined methods (Yu and Saddler, 1983). Reducing sugars were analyzed with dinitrosalicylic acid reagent (Miller, 1959). Glucose was assayed colorimetrically by the glucostat enzyme assay (Raabo and Terkildsen, 1969). Endoglucanase activity was determined by a previously described, modified carboxymethylcellulase assay methods (Saddler and Chan, 1984), using citrate-phosphate buffer (pH 6.0) and incubation at 60°C . Similar modifications were incorporated in the assay of xylanase activity, using aspenwood xylan as the substrate (Saddler et al, 1983).

RESULTS AND DISCUSSION

Initial studies were carried out to ensure that the growth conditions for C. thermocellum and C. acetobutylicum were compatible. When C. thermocellum was grown on solka floc, extracellular cellulase and xylanase enzymes were released into the culture medium (Fig. 1a). The cellulase enzyme(s) could readily hydrolyze solka floc to reducing sugars and glucose (Fig. 1b), but the hydrolyzed substrates were poorly utilized as reflected by the accumulation of sugars in the culture medium (Fig. 1b) and the low levels of ethanol and acetic acid formed (Fig. 1c). The sequential co-culture was initiated by the addition of C. acetobutylicum to C. thermocellum cultures, after the incubation temperature of the C. thermocellum cultures was lowered from 60°C to 37°C as a compromised temperature for the co-culture system. Both the endoglucanase and xylanase enzyme activities were realtively stable in the culture medium for the duration of the co-culture incubation time (Fig. 1a), indicating that there would always be active enzymes present for the continued hydrolysis of the hemicellulose and cellulose substrates to fermentable sugars. The addition of C. acetobutylicum to the C. thermocellum cultures also led to the rapid utilization of the sugars accumulated in the medium (Fig. 1b) and the formation of additional fermentation products (Fig. 1c). The end result was that the total fermentation products obtained in the co-culture were twice those obtained in the corresponding monoculture grown under identical conditions. This clearly demonstrated the advantage of using the co-culture approach to enhance product formation from cellulosic substrates.

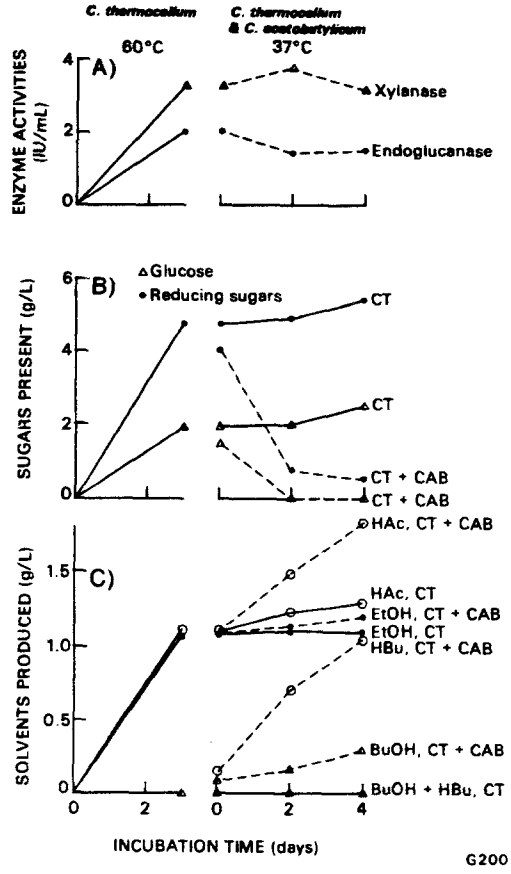

Fig. 1 Enzyme activities, sugar utilization, and product formation during co-culture of C. thermocellum and C. acetobutylicum grown on solka floc (1%, w/v). CT (——), mono-culture of C. thermocellum (control); CT + CAB (---), co-culture of C. thermocellum and C. acetobutylicum. EtOH, ethanol; HAc, acetic acid; BuOH, butanol; HBu, butyric acid.

We next investigated the efficiency of the co-culture approach to mixed substrate utilization. By growing C. thermocellum and C. acetobutylicum on a mixture of solka floc and aspenwood xylan (Table 1), the total product levels obtained from the co-culture system were 2.6 times greater than those obtained with the corresponding mono-culture system grown under identical conditions. These results demonstrated that the combined cellulose and hemicellulose components of biomass could be converted to products using this approach.

Table 1. Solvent production by C. acetobutylicum grown in co-culture with C. thermocellum [a]

Substrate & Organism	Incubation Temperature & Time (days)	Sugars present (g/L) Reducing sugars	Glucose	Solvents produced (g/L) Ethanol	Acetic Acid	Acetone	Butyric Acid	Butanol
Solka floc (1%) & Xylan (2)								
C. thermocellum	60°C 3	9.9	N.D. [b]	0.9	1.8	0.2	0.3	0.1
C. thermocellum	37°C 2	8.3	N.D.	1.0	1.8	0.2	0.3	0.3
	4	10.4	N.D.	0.9	2.0	0.1	0.1	0.2
C. thermocellum	37°C 2	2.5	N.D.	1.0	2.8	0.2	3.7	0.3
& C. acetobutylicum	4		N.D.	1.0	3.1	0.2	3.9	0.3
Solka floc (3%)								
C. thermocellum	60°C 3	8.9	1.0	0.9	1.9	0.1	0.2	0.1
C. thermocellum	37°C 2	10.6	7.7	1.0	1.9	0.1	0.2	0.1
	4	11.1	8.6	1.0	1.7	0.1	0.2	0.1
C. thermocellum	37°C 2	1.3	0.0	1.0	2.9	0.1	3.0	0.1
& C. acetobutylicum	4	1.0	0.0	1.0	2.8	0.1	3.1	0.3

[a] All media for sequential co-culture studies were first inoculated with C. thermocellum and incubated without shaking at 60°C for 3 days. One set was then inoculated with C. acetobutylicum and incubated at 37°C for 2-4 days without shaking. An identical set without inoculation of C. acetobutylicum was used as control for the mono-culture under same experiment conditions

[b] N.D. Not determined

When the co-culture was grown under the earlier described conditions the end-products were mainly acetic and butyric acids rather than ethanol, acetone, and butanol. This finding suggested that the fermentation process had stopped at the acid-forming phase and prior to the solvent-formation phase of growth of C. acetobutylicum, according to the biphasic fermentation pattern earlier proposed by Davies and Stephenson (1974). Similar results were reported recently with a co-culture of C. acetobutylicum and a mesophilic cellulolytic Clostridium species (Fond et al, 1983; 1984; Petitdemange et al, 1983). The failure of solventogenesis to occur was attributed to the slow glucose feeding rate as a direct result of the rate-limiting enzymatic hydrolysis of the lignocellulosic substrates. Other workers (Monot et al, 1983) have also demonstrated that low glucose concentration in the fermentation medium resulted in acid formation.

Several approaches were therefore used to try to increase the level of fermentable sugars in the medium. The use of higher substrate concentrations (3%, as compared to 1%, solka floc) resulted in increased accumulation of reducing sugars and glucose in the C. thermocellum fermentation medium (Table 1). However, the utilization of the sugars by the mixed culture still resulted in acid formation with little or no solvent being detected. When the incubation temperature of the co-culture system was raised to 46°C, in an attempt to enhance enzymatic hydrolysis, the release of reducing sugars was only marginally higher (6-8%) at 46°C as compared to the levels obtained at 37°C (data not shown). Increasing the glucose level in the medium by the addition

of 5 g/L, or even 10 g/L, of glucose also failed to enhance the production of solvents (Fig. 2a). This again suggested that glucose levels or glucose feed rates alone did not determine the final outcome of the fermentation process in the present co-culture system.

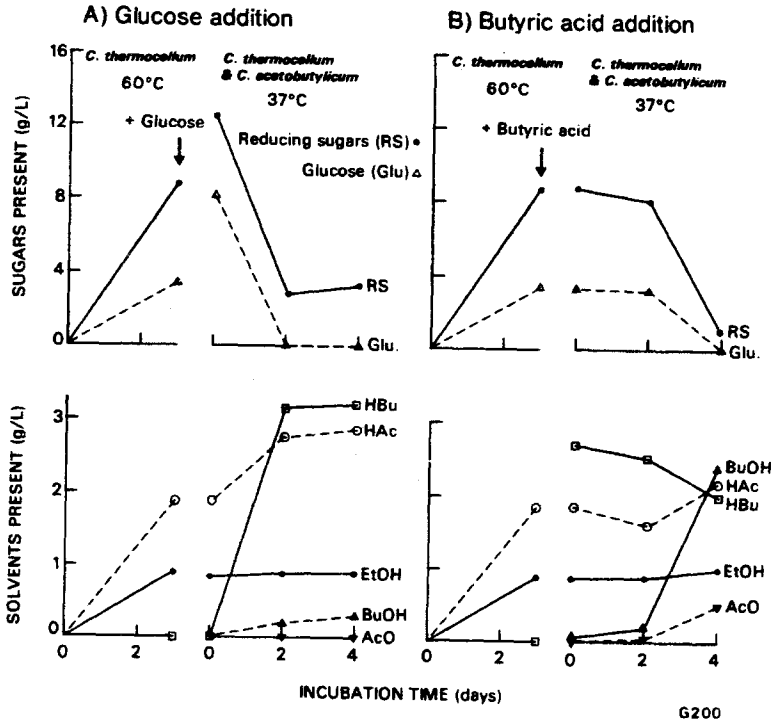

Fig. 2 Fermentation profiles of co-culture of C. thermocellum and C. acetobutylicum grown on solka floc (3%, w/v) in the presence of (A) added glucose (5 g/L), and (B) added butyric acid (3 g/L). EtOH, ethanol; HAc, acetic acid; AcO, acetone; BuOH, butanol, HBu, butyric acid.

Recent studies (Monot et al, 1983) have shown that solvent formation by C. acetobutylicum occurred only when the concentration of undissociated butyric acid in the medium reached a critical level. These findings were in agreement with our earlier work which showed that enhanced solvent production from xylose was obtained with C. acetobutylicum when the medium was supplemented with butyric acid (Yu and Saddler, 1983). The feasibility of using added butyric acid to induce solvent formation was therefore tested in the co-culture

system (Fig. 2b). Although the addition of butyric acid led to an initial inhibition of the utilization of sugars in the fermentation medium, the inhibition was subsequently overcome, as illustrated by the rapid disappearance of sugars and formation of solvents. The induction of solvent formation in the present study was not due to a lowering of the pH of the medium as the pH of the culture at the onset and termination of the co-culture work were not significantly different from the control set where no addition of acid was carried out (data not shown). This conclusion was further substantiated when the lower pH attained in the co-culture system, which was carried out in the absence of pH buffer (Sodium β-glyerol phosphate), failed to induce solvent formation (data not shown). The results therefore indicated that the level of butyric acid was responsible for the observed induction of solvent production.

The present sequential co-culture approach, with added butyric acid, led to the net production of butanol and acetone in addition to the ethanol produced by C. thermocellum. The net solvent yield was around 0.3 g per g of reducing sugar utilized, or around 80% of the theoretical efficiency (Leung and Wang, 1981). When the overall bioconversion efficiency (i.e., hydrolysis and fermentation) was taken into consideration, conversion yields of up to 38% of the theoretical could be achieved from mixed cellulose and hemicellulose substrates (Table 1). The results therefore demonstrated the potential of using the sequential co-culture approach as a simple means of converting lignocellulosic substrates directly to fuels and chemicals. Work is currently in progress to use this approach for the production of butanol from wood and agricultural residues after steam-explosion treatment.

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