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Metabolism of cellobiose and cellulose by *Bacteroides cellulosolvens**

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SUMMARY

The metabolism of *Bacteroides cellulosolvens* was studied on cellobiose and cellulose as energy and carbon sources. The growth rate was faster on cellobiose; however, growth on cellulose resulted in consumption of 55% more hexose equivalents, and in production of 49% more biomass, and 30% more metabolites (ethanol, acetate, and lactate). On each substrate *B. cellulosolvens* exhibited two distinct ranges of molar growth yields (Y_H, g cells/mol hexose). At low substrate concentrations (less than 30 mmol) hexose Y_H values were 25.5 for cellulose and 28.5 for cellobiose, while at hexose levels greater than 30 mmol Y_H values were 13.5 and 15, respectively. Shifts in metabolism towards greater lactic acid production resulted in decreased ATP production; however, this did not cause early growth cessation, as these shifts occurred after the drop in Y_H.

INTRODUCTION

Research on the upgrading of lignocellulosic biomass to fuels and solvents has largely centered on the development of two-stage processes. *Trichoderma* cellulases are first used to saccharify the lignocellulosic material; the released sugars may then be fermented to ethanol by *Saccharomyces cerevisiae* or *Zymomonas mobilis* [11], to acetone and butanol by *Clostridium acetobutylicum*, or to 2,3-butanediol by *Klebsiella pneumonia* [24].

A one-step process, using a naturally occurring anaerobic coculture, has been studied in this laboratory. The coculture consists of a cellulolytic microbe, *Bacteroides cellulosolvens* [20], and *Clostridium saccharolyticum* [19], a saccharolytic microbe capable of converting both hexose and pentose sugars to ethanol [16].

These two microbes exhibit a mutualistic symbiotic relationship where the cellulolytic microbe supplies the saccharolytic microbe with nutrients, and in turn the saccharolytic microbe removes a secondary metabolite toxic to the primary microbe [15]. This symbiotic relationship ensures the advantage of coculture stability. Also, unlike two-step processes, the coculture can convert biomass to ethanol at one incubation temperature.

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The conditions that promote maximum ethanol production by *C. saccharolyticum* have been studied [17,18], and a mutant improved in both ethanol production and tolerance has been produced [21]. Research is now focussed on improving the fermentation of cellulose by *B. cellulosolvens* [13], and investigating the possible causes of early growth cessation. In this communication the metabolism of cellobiose and cellulose by this microorganism is reported.

MATERIALS AND METHODS

Microorganism, medium, and test conditions

B. cellulosolvens (NRCC 2944, ATCC 35603) was maintained in a synthetic medium containing cellobiose (1% w/v) as carbon source [13].

All tests were conducted in 60-ml serum vials containing 10 ml of medium. Cheese cloth was used as the cellulosic substrate rather than commercial powdered forms because of the relative ease of residual cellulose collection. The other test substrate, cellobiose (Sigma Chemical Co., St. Louis, MO), was sterilized separately and added aseptically to the medium by hypodermic syringe. To ensure that pH did not affect metabolic studies, extra buffering capacity was achieved by the addition of 50 g/liter of Ca^{2+}-charged cation-exchange resin (Amberlite IRC-50, Sigma Chemical Co., St. Louis, MO) to the medium [25].

The ability of *B. cellulosolvens* to metabolize cellobiose and cellulose was tested in triplicate on two separate occasions. Tests were conducted at 35°C, with rotary shaking, using cellobiose concentrations of 0.5–20 g/liter and cellulose concentrations of 1–50 g/liter. Cellobiose fermentations were carried out for 4 days, and cellulose fermentations for 10 days.

Analytical techniques

The contents of the vials were centrifuged and the supernatant liquid was used for metabolite and soluble sugar determinations, and the pellet was used for residual cellulose estimation as previously described [14], except that the supernatant fraction after NaOH treatment was saved for protein determination. Protein concentration was converted to biomass dry weight by reference to a standard curve relating protein concentration to the dry weight of washed *B. cellulosolvens* cells grown in cellobiose broth. The amount of hydrolyzed cellulose present in the culture broth as unused reducing sugars was assayed by the dinitrosalicylic acid method [12]. This amount was subtracted from the amount of cellulose degraded to determine the actual number of glucose equivalents consumed by the microbe.

Cellobiose consumption was determined by measuring initial and final cellobiose concentrations by the dinitrosalicylic acid method. Ethanol and acetic acid were assayed by gas chromatography by the method of Ackman [1] for GLC of volatile fatty acids. Lactic acid was determined enzymatically by the method of Olson [23]. Moles of ATP produced were calculated according to the method of Giallo et al. [8], where ATP = two acetate plus one lactate plus one ethanol, and 0.25 mol of ATP per mol of hexose was added, assuming that 50% of the cellobiose was phosphorylated by cellobiose phosphorylase action.

RESULTS

B. cellulosolvens consumed 55% more hexose equivalents, and produced 49% more biomass and 30% more metabolites (ethanol, acetate, and lactate) when grown in medium containing 20 g/liter cellulose, than it did in medium containing the same concentration of cellobiose (Table 1).

When biomass yields were plotted as a function of hexose consumption (cellobiose or cellulose) two

Table 1

Comparison of *B cellulosolvens* metabolism of cellobiose and cellulose

Substrate (20 g/l)	Hexose consumed (mmol/l)	Biomass (g/l)	Total soluble metabolites[a] (mmol)
Cellobiose	66.6	0.94	88
Cellulose	103.4	1.40	114

[a] Ethanol + acetate + lactate.

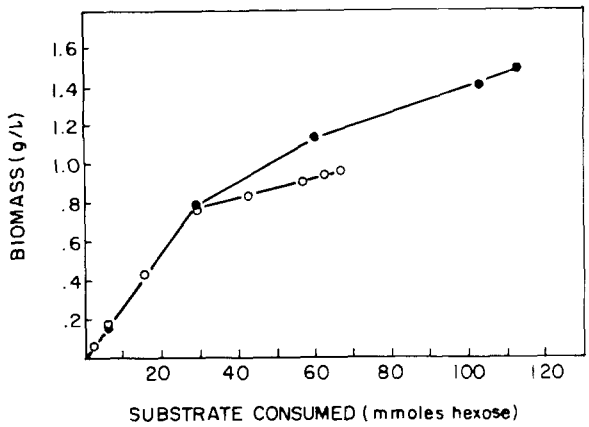

Fig. 1. *B. cellulosolvens* biomass yield on cellulose (●) and cellobiose (○).

distinct linear relationships were noted (Fig. 1). At hexose concentrations up to 30 mmol an increase in the amount of hexose consumed resulted in a proportional increase in biomass production. The breaking point in this relationship occurred at 30 mmol of hexose. Above this point the slope of the line changed, which suggested a slowing down of cell growth but continued conversion of carbon source to metabolites.

The major products of cellulose and cellobiose fermentation were acetate, ethanol, lactate, H_2, and CO_2. Fig. 2 shows the amounts of soluble metabolites accumulated in relation to hexose consumed. When the substrate was cellobiose there was a constant increase in acetate levels up to 43 mmol of consumed hexose. At this point acetate production began to level off and a strong shift to lactate production occurred. Ethanol production was essentially linear over the hexose range of 3–66 mmol. With cellulose as the substrate there was a constant increase in acetate level until 60 mmol hexose were used. There was then a shift in metabolite production to increased ethanol production at the expense of acetate. There was also an increase in lactate production; however, this shift to lactate was more gradual than that observed with cellobiose as substrate.

The shift in metabolite production resulted in a decrease in the moles of ATP produced per mole of hexose consumed for both cellulose and cellobiose

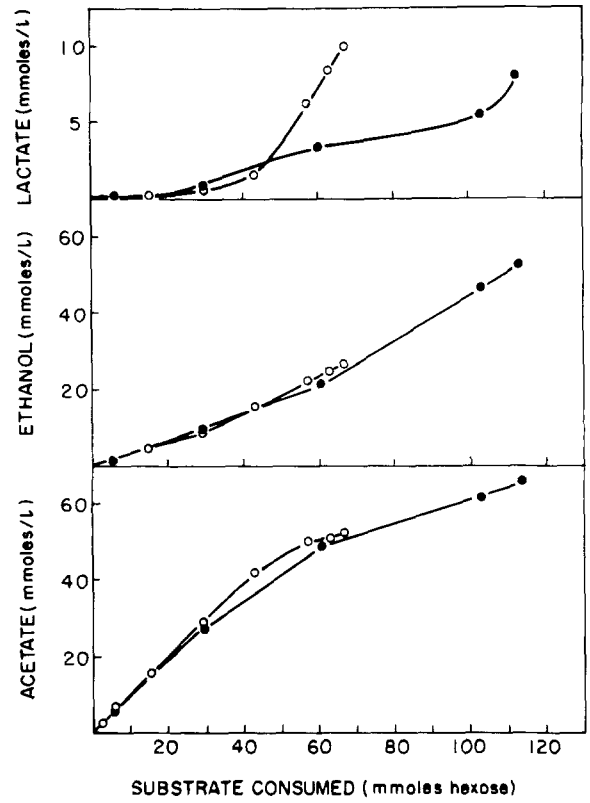

Fig. 2. The production of acetate, ethanol, and lactate by *B. cellulosolvens* grown on cellulose (●) and cellobiose (○).

fermentations (Tables 2 and 3). Molar growth yields (Y_H, in grams of cells per mole of hexose consumed) were slightly greater on cellobiose. At low substrate levels Y_H was approximately 25.5 for cel-

Table 2

Growth yields of *B. cellulosolvens* grown on cellulose

Initial cellulose (g/l)	Hexose consumed (mol)	ATP (mol/mol hexose)	Y_H[a]	Y_{ATP}[b]
1	0.006	2.56	25.6	10.0
5	0.03	2.42	25.8	10.6
10	0.06	2.26	18.7	8.3
20	0.103	1.95	13.5	6.9
35	0.113	1.94	13.2	6.8
50	0.113	1.91	13.7	7.2

[a] Y_H – molar growth yield (grams of cells/mole of hexose).
[b] Y_{ATP} – growth yield (grams of cells/mole ATP produced).

Table 3

Growth yields of *B. cellulosolvens* grown on cellobiose

Initial cellobiose (g/l)	Hexose consumed (mol)	ATP (mol/mol hexose)	Y_H[a]	Y_{ATP}[b]
0.5	0.003	2.74	28.6	10.4
1.0	0.006	2.72	28.6	10.5
2.5	0.015	2.60	28.2	10.8
5.0	0.029	2.56	26.0	10.2
7.5	0.043	2.59	19.4	7.5
10	0.057	2.49	15.7	6.3
15	0.063	2.36	14.9	6.3
20	0.066	2.35	14.2	6.0

[a,b] Y_H and Y_{ATP} as defined in Table 2.

lulose and 28.5 for cellobiose, while at higher substrate levels the Y_H values dropped to approximately 13.5 and 15, respectively. The drop in Y_H was not proportional to the drop in ATP yield (Tables 2 and 3). For both cellulose and cellobiose fermentations the Y_{ATP} (cell yield (g) per mol ATP) dropped from about 10.5 at low substrate concentrations to 6.5 at high substrate levels, showing that less biomass was being produced per mole of available ATP. Accordingly, the shift in metabolite production which caused decreased ATP production was not responsible for cessation of *B. cellulosolvens* growth. This conclusion is substantiated by the data in Fig. 1 and 2, which show that *B. cellulosolvens* reached a breaking point in its growth yield prior to shifts in metabolite production. Meaningful growth yield data are best obtained under conditions where growth ceases because of substrate depletion. This ensures that differences in growth stoppages are not due to dissimilar accumulation of inhibitory metabolites. In this study it was found that cellobiose concentrations below 57 mM, and cellulose concentrations below 95 mM, were growth limiting. Accordingly, both the breaking point in biomass yield and the drop in Y_H and Y_{ATP} occurred in media where the substrate concentration was growth limiting.

DISCUSSION

Many cellulolytic anaerobes display growth rates on cellulose greater than that of *Trichoderma reesei* [10,22]. Although specific rates of cellulose hydrolysis are high, total cell biomass and cellulase yield are low. Various explanations for the early growth stoppage of cellulolytic anaerobes have been suggested, such as low pH, toxic accumulation of major metabolites, inhibition of cellulolytic enzymes by soluble sugars, and decreased growth on crystalline cellulose after rapid hydrolysis of the amorphous regions [2,4,9]. However, these main causes of growth cessation were not applicable to *B. cellulosolvens* [14,15] or to *Clostridium cellulolyticum* [7]. Under conditions of pH control and excess nutrients *B. cellulosolvens* stopped growing before inhibitory levels of H_2, CO_2, acetate, ethanol or lactate accumulated [14]. It grew well on highly polymerized forms of cellulose, and soluble sugars did not accumulate in the culture medium until after the microbe stopped growing. The only explanation for early growth stoppage has been evidence that *B. cellulosolvens* is sensitive to low level accumulation of an unidentified secondary metabolite [15].

The metabolism of cellulose and cellobiose by *B. cellulosolvens* is similar in many ways to that reported for *C. cellulolyticum* [7,8]. The growth rates of *B. cellulosolvens* and *C. cellulolyticum* were both slower on cellulose than cellobiose, but the final amounts of hexose equivalents consumed were greater on cellulose (103 versus 67 mmol for *B. cellulosolvens*, and 25 versus 15 mmol for *C. cellulolyticum*). Both microbes showed two distinct ranges of molar growth yields; high yield at low or limiting substrate concentrations, and a lower yield at high substrate levels. The breaking point between these two Y_H ranges occurred at 5 mmol of consumed hexose for *C. cellulolyticum*, as compared to 30 mmol for *B. cellulosolvens*. The occurrence of the Y_H breaking point for *C. cellulolyticum* at a much lower hexose level, and the fact that this microbe consumes less total cellobiose or cellulose than *B. cellulosolvens* indicates that *C. cellulolyticum* is more susceptible to early growth cessation.

Giallo et al. [7] in their study of cellobiose and cellulose metabolism by *C. cellulolyticum* stated that the decrease in molar growth yields correlated with higher production of lactic acid. From their data it can be seen that when *C. cellulolyticum* was grown on cellobiose a shift in metabolism towards increased lactic acid production began at the Y_H breaking point. However, this was not the case with growth on cellulose, where *C. cellulolyticum*, like *B. cellulosolvens* (Figs. 1 and 2), showed an increase in lactic acid production after the Y_H breaking point.

A shift in metabolism from heterolactic to homolactic fermentation has also been correlated with decreased growth yields in species of *Streptococcus* [3,6]. Although ATP production is lower during the homolactic fermentation this was not the cause of decreased growth yields, for it was shown that the total ATP pool actually increased during this period [6]. Forrest [5] suggested that this situation could arise where growth was limited by some environmental factor other than energy source. Under this condition there would be decreased or arrested growth, but continued ATP production by the metabolically active cells.

In the present study, *B. cellulosolvens* growth yield was correlated with decreased ATP production; however, the decrease in Y_H was greater than the decrease in ATP yield. This indicates that growth was affected by some other factor which caused an uncoupling of growth and catabolism. Decreased ATP production, therefore, was not the cause of early growth cessation, but may very well have been a consequence of it. Previously, the only factor found to limit the growth of *B. cellulosolvens* was toxicity of a secondary metabolite [15]. Such sensitivity is a reflection of the conditions imposed by pure culture. When *B. cellulosolvens* was grown with its natural symbiont *C. saccharolyticum*, the inhibitory secondary metabolite was removed from the culture. This resulted in prolonged growth and increased cellulose digestion [15]. These findings have relevance in the understanding of microbial interactions in natural environments, and in the use of naturally occurring mixed cultures for applied processes such as the one-step conversion of cellulose to fuels and solvents.

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