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Ethanol from Cellulosic Biomass [and Discussion]

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Ethanol from cellulosic biomass

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The major objective of this project is to achieve the direct microbiological conversion of cellulosic biomass to liquid fuel, ethanol. Within the scope of this objective, it is also the intent to maximize the conversion efficiency of ethanol production from biomass. This can be achieved through the effective utilization of both the cellulosic (six-carbon sugar) and hemicellulosic (five-carbon sugar) fractions in biomass. The degradation of cellulosic biomass is achieved through the use of a thermophilic and anaerobic bacterium, *Clostridium thermocellum*. This microorganism is unique in that it is able to hydrolyse both the cellulosic and hemicellulosic fractions of biomass but, unfortunately, it is not able to metabolize the pentoses. Therefore, to achieve total utilization of biomass, a second thermophilic and anaerobic microorganism, *Clostridium thermosaccharolyticum*, has been under study owing to its ability to convert pentoses to ethanol. Mutation, selection and adaption programmes have yielded ethanol tolerant strains of both organisms.

A fermentation process using mutant strains of the anaerobic, thermophilic bacteria *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* has been investigated for the direct production of ethanol from agricultural cellulosics. Through strain improvements for increased ethanol tolerance and catabolite selectivity, alcohol yields of 85% of the theoretical maximum have been obtained from solka floc with mixed culture. The method of isolation and the performance of these improved strains on both refined cellulosics and a realistic biomass, corn [maize] stover, is presented in detail.

Introduction

Renewable resources from the agricultural and forestry sectors represent a significant quantity of potential raw material for the conversion to useful products. In a country such as the U.S.A., agricultural residues have been considered by many investigators as raw materials particularly for the production of a liquid fuel, ethanol.

One reason in selecting agricultural residues is their abundance. Taking, for example, one of the major agricultural food crops, corn [maize], it was reported by the U.S. Department of Commerce (1978) that 6.4×10^9 bushels (225×10^9 l) of corn were harvested in 1978. It is also reported by Tsao *et al.* (1978) that for every pound of corn kernels harvested, there is one pound of residue produced in the form of cobs, stalks, husks, leaves and roots. This residue is often known as 'corn stover'. From these statistics it was estimated by Avgerinos & Wang (1980) that the theoretical amount of ethanol that can be produced from corn stover can be as much as 14×10^9 U.S. gallons (130×10^9 l) which is nearly 10% of the total petrol consumption of the U.S.A.

The conventional approaches in the utilization of biomasses are through the enzymic or chemical hydrolysis of cellulose and then through the microbiological conversions of the hydrolysis products to the desired end-products. These methods generally require several

different processes. For example, in the enzymatic saccharification of cellulose leading ultimately to the production of a liquid fuel such as ethanol, one can expect the following required processes: enzyme production; fermentation and recovery, cellulose hydrolysis; construction of an enzyme reactor, ethanol production; fermentation. Although significant advances and progress have been achieved in the above listed processes, one should not ignore other concepts to obtain a more simplified system.

It was hypothesized in our laboratory that there must be attractive alternatives in the degradation of biomass for the production of useful chemicals. However, we also recognised that new concepts are often viewed with scepticism and apprehension owing to a lack of basic information. It is through research and development that such information can be obtained and, it is hoped, help to erase the apprehension and scepticism. We present in this paper some new concepts in the production of chemicals from cellulosic biomass by microbiological means.

In searching for novel bioconversion processes for ethanol production, the M.I.T. group has been exploring a different concept. It was rationalized that a single-step conversion of cellulosic biomass to ethanol could offer potential economic advantages over those using multiple processes. Within this single-step conversion scheme, it was also the intent to use both the cellulosic (six-carbon sugars) and hemicellulosic (five-carbon sugars) fractions in biomass to produce ethanol. To achieve these objectives, we have focused our attention on an anaerobic and thermophilic (optimum temperature 60 °C) bacterium, *Clostridium thermocellum*, which is able to hydrolyse cellulose and hemicellulose in biomass. This microorganism is also able to metabolize the six-carbon sugars to produce ethanol and other organic acids. However, it is not able to metabolize five-carbon sugars such as xylose. A second anaerobic and thermophilic (optimum temperature 60 °C) bacterium, *Clostridium thermosaccharolyticum*, is therefore being examined for the conversion of the hemicellulosic fraction to ethanol. The overall goal is to use a mixed-culture fermentation for the direct conversion of biomass to ethanol.

Materials and methods

The microorganisms used in the studies were *Clostridium thermocellum* ATCC 27405 (American Type Culture Collection, Rockville, Maryland, U.S.A.) and *Clostridium thermosaccharolyticum* isolated and identified in our laboratory. Medium composition, equipment and the associated analytical procedures have been presented previously by Gordon *et al.* (1978), Cooney *et al.* (1979), Wang *et al.* (1979) and Avgerinos *et al.* (1981), and will not be repeated. However, we wish to present our adaption and isolation procedure for the selection of ethanol tolerant strains of *C. thermocellum* and *C. thermosaccharolyticum*.

The organisms were grown in media containing a soluble carbon source such as cellobiose (for *C. thermocellum*) and xylose (for *C. thermosaccharolyticum*) and also, initially, ethanol at 10 g l^{-1}. Serial transfers of the culture were then performed and the ethanol concentration was increased stepwise at 2 g l^{-1} each time. At each concentration of ethanol, the cellulolytic activity of *C. thermocellum* was also tested by examining its ability to grow on cellulose. The total number of transfers with progressively higher ethanol concentrations varied between 40 and 65. After these numbers of transfers, an isolate that possesses cellulolytic activity as well as the ability to grow in the presence of high ethanol concentration was selected for subsequent studies.

Other mutation and selection procedures relevant to the results to be presented are also

given below. Mutation of the cultures was accomplished with ethyl-methanesulphonate (EMS) or *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NMNG). Selection of reduced lactate producers of *C. thermocellum* was achieved by plating mutagenized cultures of solka floc plates (2% agar). After 5 days of incubation in a glove box the colonies were replica-plated and lactate production detected by the method given below. The plates were sprayed with a reagent containing

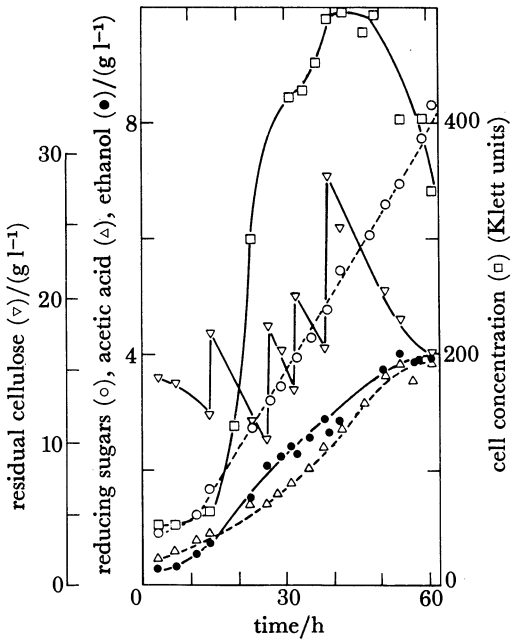

FIGURE 1. Growth and product formation by *Clostridium thermocellum* ATCC 17405 at pH 7, with batch feeding of cellulose (solka floc).

nicotinamide adenine dinucleotide (NAD^+) (8 mg ml^{-1}), phenazine methosulphate (MPS) (0.01 mg ml^{-1}), tetranitroblue tetrazolium (TNBT) (0.5 mg ml^{-1}) and lactate dehydrogenase (LDH) (0.5 units ml^{-1}). After 2 h of incubation at 37 °C, a purple precipitate of tetrazolium appeared around lactate-producing colonies as a result of the reactions

$$\text{L}(+)\text{lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH;$$
$$NADH + \text{PMS (oxidized)} \rightleftharpoons NAD^+ + \text{PMS (reduced)};$$
$$\text{PMS (reduced)} + \text{TNBT} \rightleftharpoons \text{PMS (oxidized)} + \text{TNBT-formazan (purple)}.$$

Selection for decreased acid producing colonies of *C. thermosaccharolyticum* was accomplished by plating mutagenized cultures on xylose plates with the addition of ethanol (2% by volume) and methyl red (0.04 g l^{-1}) as a pH indicator. Colonies remaining yellow after 72 h were selected for further examination.

RESULTS AND DISCUSSION

The growth of the parent strain *Clostridium thermocellum* ATCC 27405 on solka floc is shown in figure 1. Because high concentrations of cellulose cannot be easily agitated in a fermentor, solka floc had to be fed in batches. This can be seen in figure 1 as the 'sawtooth' noted as residual cellulose. Cell growth accompanies the utilization of cellulose up to an optical density of 500 Klett units (330 Klett = 1 g l^{-1} cell concentration). A decline in the cell concentration was

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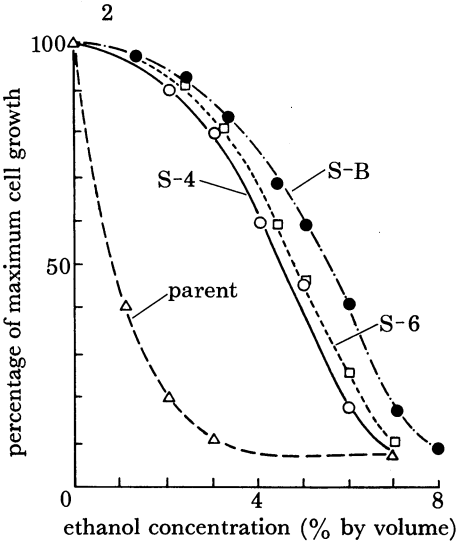
noted; and this was due to the absorption of cells onto the cellulose. Reducing sugars are produced as a result of cellulose degradation up to about 8 g l^{-1}. Previous quantitative and qualitative analyses by high-performance liquid chromatography have shown that the reducing sugars are mainly glucose, cellobiose, xylobiose and xylose. The catabolic products ethanol and acetic acid were accumulated at equal concentrations of 4 g l^{-1}. These results show that *C. thermocellum* is able to degrade cellulose and produce soluble sugars, ethanol and acetic acid. Analysis of the biochemical pathway in the catabolism of cellulose by *C. thermocellum* indicates that the natural products are ethanol and acetic acid in equimolar quantities (Wang 1978). Therefore, if a liquid fuel, ethanol, is the primary product of interest, the accumulation of acetic acid must be eliminated. Furthermore, the concentration of the end-products must also be increased.



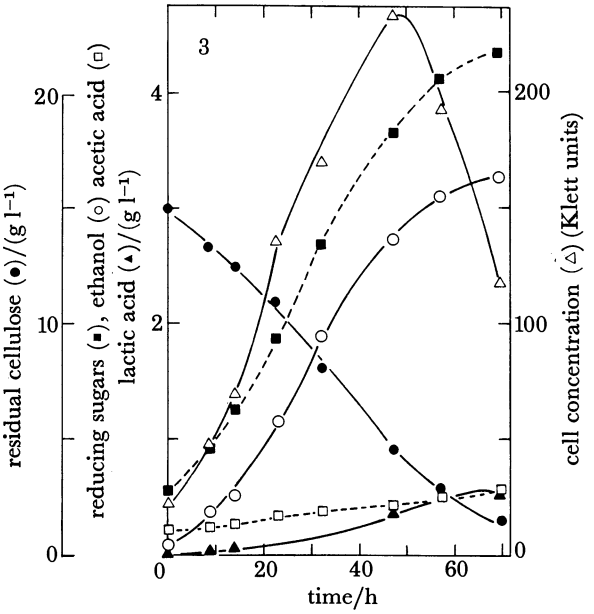


FIGURE 2. Comparision of ethanol tolerance for different strains of *Clostridium thermocellum* (ATCC 27405 → S-4 → S-6 → S-B).

FIGURE 3. Fermentation profile of cellulose (solka floc) by *Clostridium thermocellum* (S-4).

To increase the concentration of ethanol by *C. thermocellum*, the organism's tolerance to this product was also examined. Adaptation and selection programmes were performed to increase *C. thermocellum*'s growth tolerance to ethanol. The results from these studies are shown in figure 2. The growth of the parental strain of *C. thermocellum* is strongly inhibited by low concentrations of ethanol. For example, at an ethanol concentration of 1% by volume, growth inhibition to 40% of maximum resulted. From our selection procedure, three isolates, designated as S-4, S-6 and S-B, were obtained. All of these isolates can be seen from figure 2 to have a higher tolerance to ethanol than the parent (ATCC 27405). The properties of these isolates will now be presented.

The fermentation behaviours of the new ethanol-tolerant strain, S-4, are shown in figure 3. Here again, the cellulose used was solka floc. From an initial cellulose concentration of 15 g l^{-1}, almost total degradation occurred. Reducing sugars were accumulated to a final concentration of 4.5 g l^{-1}. The most interesting and surprising behaviour of this new strain is the high ratio of ethanol formation in comparison to acetic acid. From figure 3, it can be seen that about

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3.2 g l^{-1} of ethanol was formed but less than 0.5 g l^{-1} of acetic acid accumulated. It is hypothesized that this new strain, S-4, not only is able to tolerate higher concentrations of ethanol, but also has an altered metabolism. This is possible if one considers the catabolic pathway of this organism with respect to ethanol and acetate formation. For example, the conventional catabolic reactions leading to ethanol and acetic acid can be represented as

$$C_6H_2O_6 + H_2O + 3ADP + 3P_i \longrightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2 + 3ATP. \quad (1)$$

In this case, one would expect equimolar ratios of ethanol and acetic acid as well as H_2 and CO_2. On the other hand, when carbon and oxidation–reduction balances were performed by using the results in figure 3, the reaction shown in (1) was found to be no longer valid. It is postulated that the new strain, S-4, produces very little hydrogen gas and nearly all of the reducing power generated is used to produce NADH and thus increase the ratio of ethanol to acetic acid formation.

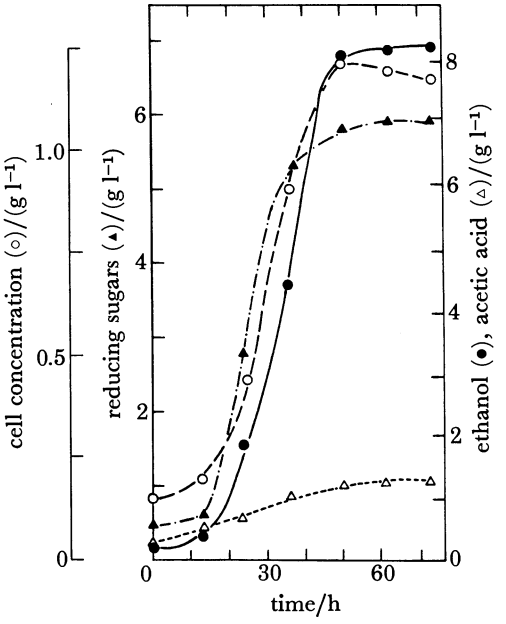

FIGURE 4. Fermentation profile of *Clostridium thermocellum* (strain S-6) on solka floc. Initial and final concentrations of solka floc, 35 and 0 g l^{-1} respectively. Yield, 44%.

Further adaptation and selection of strain S-4 to increase its ethanol tolerance resulted in the isolation of strain S-6. The fermentation characteristics of this strain are shown in figure 4. In this experiment, 35 g l^{-1} of cellulose (solka floc) was used. At the end of the fermentation no residual cellulose was found, thus showing 100% cellulose degradation. From an initial 35 g l^{-1} cellulose, the fermentation products were: cells, 1.2 g l^{-1}; ethanol, 8 g l^{-1}; acetic acid, 1.0 g l^{-1}; reducing sugars, 6 g l^{-1}. These results again show that this new strain (S-6) still maintains the favourably high ethanol to acetic acid production ratio, as well as having a high efficiency of cellulose degradation. The conversion yield of products from cellulose is also excellent. For example, the overall yield, including all catabolite and degradative products, was calculated to be 0.44 g of product per gram of cellulose. Since the conversion efficiency from feedstock is of primary importance, these results show an excellent potential in the use of *C. thermocellum* for biomass conversion.

In addition to increasing the ethanol tolerance of these isolates this selection procedure also

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resulted in a favourable increase in the ratio of ethanol to acetate appearing in the products. Growth of the wild-type ATCC 27405 on solka floc or cellobiose typically yielded a ratio of 1:1 in contrast to ratios of 4:1 and 5:1 for S-4 and S-6. However, as growth of these strains slowed at the end of the fermentation, large quantities of lactate (more than 2 g l^{-1}) were shown to accumulate in a non-growth associated manner, thus reducing the overall alcohol

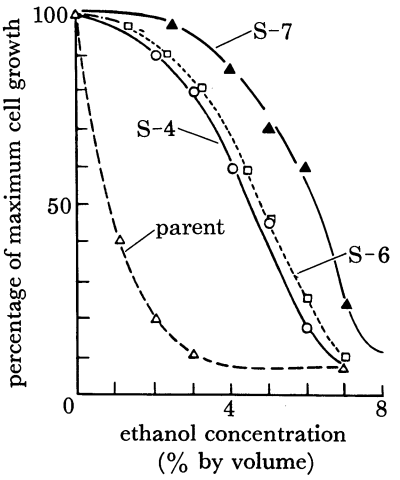

FIGURE 5. Growth of *Clostridium thermocellum* isolates in the presence of ethanol.

yield. Since the homofermentative production of ethanol by this strain appeared to be feasible on theoretical grounds, we concentrated our efforts on isolating an LDH-negative mutant of the improved strain S-6. Through mutation with NMNG and selection of reduced lactate producers with tetrazolium reagent, the isolate S-7 (figure 5), shown to produce less than 0.1 g l^{-1} lactate was obtained. During growth on 40 g l^{-1} solka floc (figure 6) under pH-controlled conditions, this strain shows virtually no lactate production, and while ethanol is still produced in a high ratio to acetate of approximately 5:1. The bulk of the solka floc degradation, however, resulted in the production of 24 g l^{-1} reducing sugars giving a low yield of ethanol based on substrate degraded. The fermentation of 40 g l^{-1} corn stover (figure 7) was examined under similar conditions and also resulted in little lactate production, although ethanol and acetate were produced in a lower ratio of 2:1. After 80 h of fermentation, 60% of the initial dry mass was lost, resulting in an average rate of degradation of this substrate of 0.3 g l^{-1} h^{-1}, compared with 0.8 g l^{-1} h^{-1} for solka floc. Although a lower average rate of degradation of this lignaceous substrate than with solka floc was observed, over 80% of the available carbohydrate of the stover was degraded.

The ability to convert the cellulosic (six-carbon sugars) fraction of biomass to useful products represents the solution of only one-half of the overall problem. This is because most natural biomass such as corn stover contains about an equal amount of hemicellulose (five-carbon sugars) and cellulose. If the hemicellulose cannot be utilized effectively, the overall conversion efficiency would be quite unfavourable with respect to process economics. An equally important segment of our project has therefore been the conversion of xylose to ethanol. We have shown in our laboratory that the cellulase enzyme complex from *Clostridium thermocellum* is as effective in the hydrolysis of hemicellulose in biomass to xylose as it is in that of cellulose. Unfortunately, *C. thermocellum* cannot metabolize pentoses. Therefore, to utilize the five-carbon sugars effectively, we have been examining the capability of another anaerobic thermophile. This organism was

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isolated and identified in our laboratory to be *Clostridium thermosaccharolyticum*. The unique attributes of this organism are its xylose catabolism and its ability to form a mixed culture with *C. thermocellum*. The behaviours of *C. thermosaccharolyticum* in pure and mixed culture are presented below.

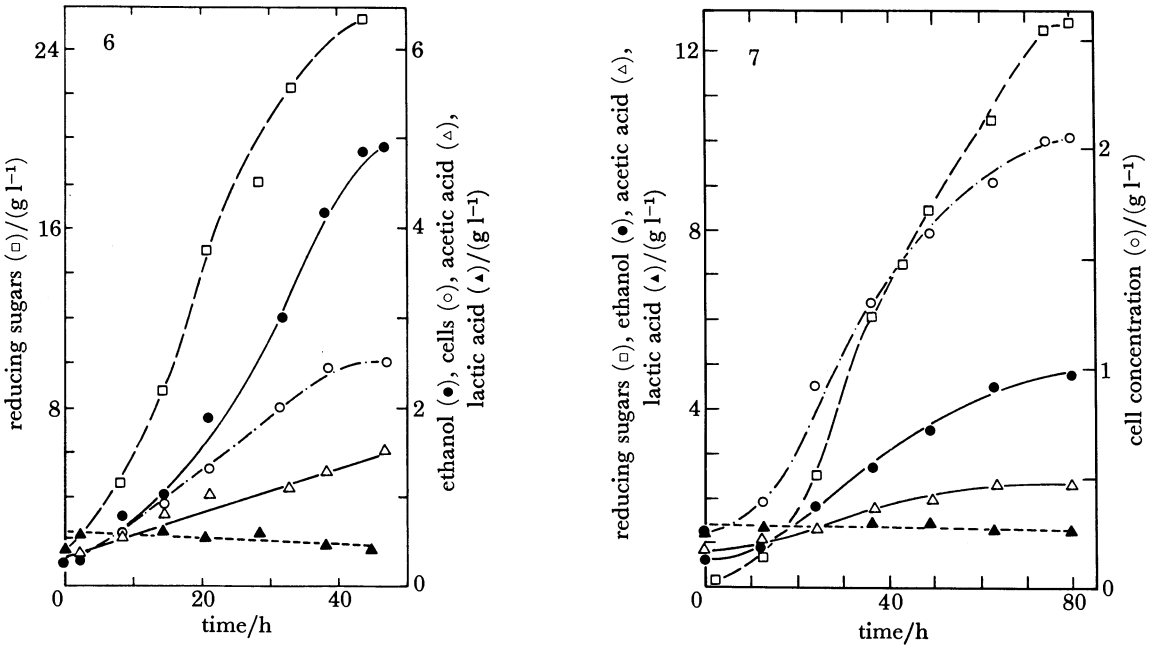


FIGURE 6. Fermentation profile of lactic acid negative mutant of *Clostridium thermocellum* (S-7) on solka floc.
FIGURE 7. Fermentation profile of *Clostridium thermocellum* (S-7) on corn stover.

Our immediate efforts with *C. thermosaccharolyticum* were aimed at characterizing its growth tolerance and selecting for strains with an increased tolerance towards ethanol. By using the adaptation technique, a more tolerant strain of this organism was selected; its behaviour compared with that of the parent is shown in figure 8. The parental strain (HG-2) is more resistant to ethanol than wild-type *Clostridium thermocellum*. For example, for the parent (HG-2), a 50% growth inhibition occurred at an ethanol concentration of about 3% by volume. The new strain, HG-3, was found to be more resistant to ethanol, as figure 8 shows. For example, 50% growth inhibition for strain HG-3 has now been increased to an ethanol concentration of 4% by volume. These results are encouraging since they demonstrate that the selection procedure established for *C. thermocellum* can be readily adapted to other microorganisms. We are confident that further increased tolerance to ethanol is still attainable through this adaptation and serial transfer technique.

The behaviour of *Clostridium thermosaccharolyticum* strain HG-3 as a pure culture with xylose as the carbon source is shown in figure 9. Cell growth is excellent, attaining a maximum of 2.4 g l^{-1}. Xylose was fed in batches because our earlier studies have shown that high concentrations of xylose are not desirable. In this fed-batch fermentation, the xylose concentration was maintained at between 5 and 10 g l^{-1}. The catabolic products were ethanol (14.5 g l^{-1}), lactic acid (16 g l^{-1}) and acetic acid (10 g l^{-1}). It should be mentioned that the ratio of ethanol

to acetic acid produced by the new ethanol-tolerant strain (HG-3) is increased similarly to that observed for *C. thermocellum*. For example, the parental strain (HG-2) produced equal concentrations of ethanol and acetate, whereas the new strain (HG-3) has an ethanol:acetate ratio of 3:2. A total of 57.7 g of xylose was consumed in the fermentation, as shown in figure 9. This represents a total product (ethanol, acetic acid, lactic acid) conversion efficiency of 58.8%. This is encouraging because it shows that *C. thermosaccharolyticum* is already quite efficient in catabolizing xylose.

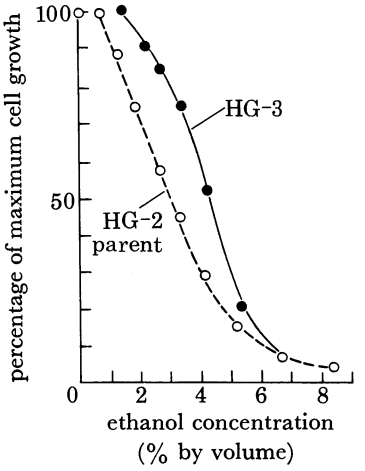
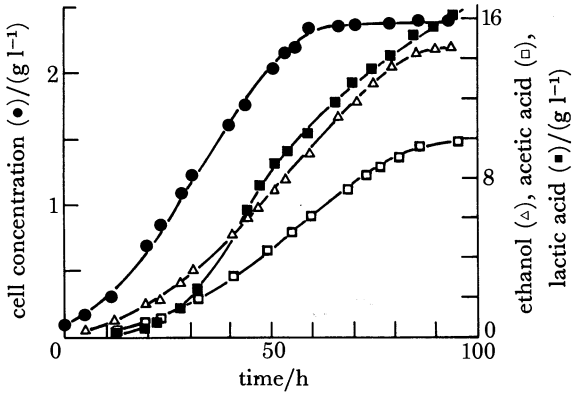


FIGURE 8. Comparison of ethanol tolerance of *Clostridium thermosaccharolyticum* (parent: HG-2 → HG-3).



xylose consumed, 67.8 l^{-1}
ethanol produced, 14.5 g l^{-1}
lactic acid produced, 15.3 g l^{-1}
acetic acid produced, 10.0 g l^{-1}
overall yield, 58.7%

FIGURE 9. Fermentation profile of *Clostridium thermosaccharolyticum* (strain HG-3) through batch feeding of xylose.

To increase the ethanol yield of this organism further, a strategy of mutation with EMS and selection for low acid producing strains with methyl red pH indicator dye on xylose plates was conducted. Of approximately 4000 colonies plated, 160 produced less acid as indicated by the dye. Fourteen of the largest colonies were picked to yield HG-4 which produced ethanol and acetate in a ratio of 4:1 (see figure 10). To ascertain the maximum capability of this strain for alcohol production, a fed-batch fermentation of xylose was conducted in an 8 l fermentor controlled at pH 6.7. The xylose concentration was monitored and maintained between 3 and 9 g l^{-1}. During 60 h of fermentation, 75 g l^{-1} xylose was fed, producing 27 g l^{-1} ethanol, 6.5 g l^{-1} acetate, and 3.0 g l^{-1} lactate. This level of alcohol production currently obtained represents 70% of the theoretical maximum yield of 0.5 g ethanol g^{-1} xylose consumed (see table 1).

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TABLE 1. ETHANOL YIELD OF *CLOSTRIDIUM THERMOSACCHAROLYTICUM* ISOLATES ON XYLOSE

(Ethanol yield is defined as grams of ethanol produced per gram of xylose consumed.)

strain	ethanol yield
HG-2	0.17
HG-3	0.23
HG-4	0.35

The compatability of these two high-yielding alcohol-tolerant strains was examined during fed-batch fermentations of solka floc in anaerobic flasks with pH control at 6.7 (see figure 11). At time zero S-7 was inoculated and HG-4 was added after 24 h of fermentation. A total of 80 g l^{-1} solka floc was fed, resulting in the production of 25.3 g l^{-1} ethanol. The mixed culture degraded 60 g l^{-1} of the substrate fed and was successful in rapidly consuming the reducing sugars hydrolysed by S-7. The resulting ethanol yield of this fermentation of 0.43 g ethanol g^{-1} solka floc consumed represents 58% of the theoretical yield based on substrate consumed.

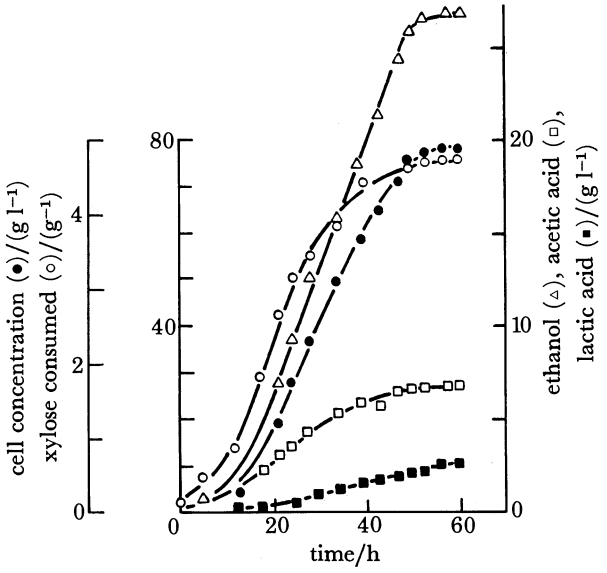

FIGURE 10. Fermentation profile of *Clostridium thermosaccharolyticum* (strain HG-4) through batch feeding of xylose.

Encouraged by these results, the fed-batch fermentation of corn stover by these strains was examined in a similar manner (see figure 12). During the course of this fermentation, 100 g l^{-1} corn stover was fed. The mixed culture consumed the bulk of the sugars produced and 37% of the stover was degraded. However, the fermentation of the real substrate, in contrast to solka floc, resulted in the production of 9.7 g l^{-1} ethanol and 6.7 g l^{-1} acetate. This increased level of acetate production reduced the overall alcohol yield to 0·26 g ethanol g^{-1} substrate consumed.

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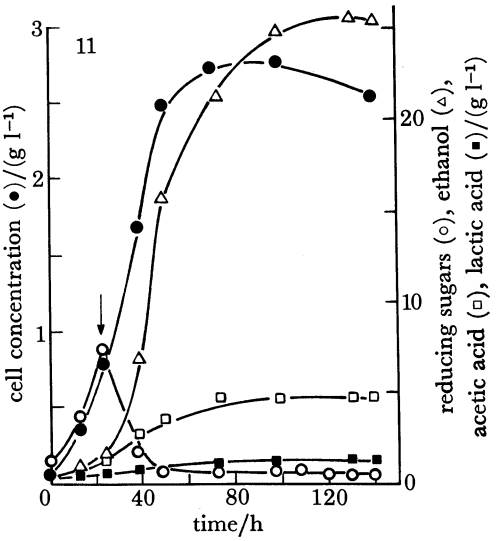

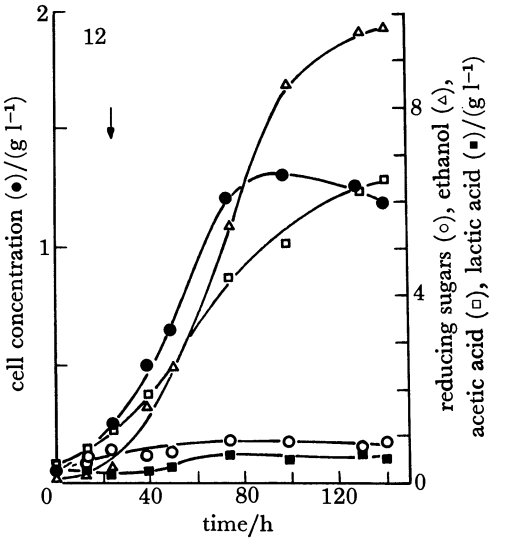

FIGURE 11. Mixed-culture fermentation of *Clostridium thermocellum* (S-7) and *Clostridium thermosaccharolyticum* (HG-4) on solka floc. The time of inoculation with HG-4 is indicated by the arrow.

FIGURE 12. Mixed-culture fermentation of *Clostridium thermocellum* (S-7) and *Clostridium thermosaccharolyticum* (HG-4) on corn stover. The time of inoculation with HG-4 is indicated by the arrow.

CONCLUSIONS

The isolation of more ethanol-resistant and higher-yielding mutants of S-7 of *C. thermocellum*, and of HG-4 of *C. thermosaccharolyticum*, has enabled us to demonstrate the direct conversion of the model cellulosic solka floc to ethanol at 85% of the theoretical yield. The isolation of HG-4 represents a means for converting pentoses to alcohol in high concentrations and yields. Mixed-culture fermentation of the non-chemically pretreated corn stover by these organisms results in 52% degradation of the available carbohydrates and consumption of all the soluble reducing sugars produced. However, a decrease in the ratio of ethanol to acetic acid in the products during corn stover fermentation results in a decrease in the alcohol yield to 50% of the theoretical maximum attainable. Possible explanations of this behaviour with real biomass include the potential toxicity of the lignaceous components, which are not present during fermentation of refined cellulosics such as solka floc. In addition, increased levels of acetic acid may result from the enzymic cleavage of acetylated hemicellulose residues in corn stover before their assimilation by the cells. Both of these possibilities are currently under investigation. Nevertheless, this process concept for the single-step production of ethanol represents an attractive alternative approach to alcohol production from cellulosics, which may benefit from further strain improvements in the future.

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Discussion

F. E. Young (*University of Rochester, New York, U.S.A.*). I compliment Professor Wang on his excellent approach to this complex problem. I agree that the anaerobic organisms offer much to biotechnology. These organisms make up most of the microbial world, are extensively involved in organic acid production and are the producers of some extremely important hydrolyses. We have been devoting efforts to developing a cloning system in *Bacteroides fragilis*. It would be helpful if Professor Wang or others were to develop a cloning system in *Clostridium* species.

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