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BIOLOGICAL PRETREATMENT OF HIGH ENERGY SORGHUM*

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하이에너지수수의 생물학적 전처리*

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ABSTRACT

Degradation of structural carbohydrates has been observed in samples of sweet sorghum inoculated with either *Clostridium cellulolyticum* or *Bacteroides succinogenes*. However, conditions under which these cellulolytic organisms can compete effectively with lactic acid bacteria have not yet been determined. Degradation of cellulose by *B. succinogenes* was found not to be inhibited by either glucose or succinate.

INTRODUCTION

Development of anaerobic digestion processes for large-scale production of methane from biomass has been a major focus of several research groups during the last decade. Although a variety of approaches to anaerobic conversion have been investigated, all suffer from high production costs. These high costs result from a combination of low rates of conversion, low yields of methane, and high costs of crop production, harvesting, storage and transportation [1]. One means of reducing production costs would be to pretreat the biomass to make it more susceptible to digestion.

An effective biomass pretreatment process should begin the process of breaking down the structural components, i. e., the lignocellulose fraction. Since biomass crops generally are seasonal, storage times of six to nine months may be required prior to utilization in a biological conversion system. This provides an opportunity to use a slow, low-cost pretreatment process which could be effected during the storage period.

Ensiling has been shown to be the preferred method of storage for high moisture biomass because of lower storage losses compared to other methods [2, 3]. Ensiling is a natural fermentation process in which lactic acid bacteria convert sugars available in the biomass to lactic acid. The process occurs under anaerobic conditions and causes pH to drop rapidly into a range which inhibits other microorganisms from growing. Unfortunately, from a biomass processing viewpoint, lactic acid bacteria are not capable of hydrolyzing structural carbohydrates present in the biomass, and the low pH of the system prevents the activity of any cellulolytic organisms which may be present as part of the natural microflora on the biomass.

The overall goal of our research is to develop a biological pretreatment process in which significant degradation of the lignocellulose components of biomass is brought about during the storage period. Pursuant to this goal, we have been investigating methods of forcing the ensiling process to undergo a different type of fermentation, e. g., a clostridial fermentation. In addition, we have been studying growth of various anaerobic,

cellulolytic bacteria on sweet (high energy) sorghum and their utilization of the lignocellulose components.

We have previously reported that increasing the moisture content and altering the water soluble carbohydrates (WSC) content of the sorghum were not sufficient to cause a clostridial fermentation to occur [4]. Although samples with the lowest WSC content underwent a lactic acid fermentation, conditions did not appear to be stable. The pH of those samples did not drop as low during the first few days and started to increase after about two months In addition, there were some differences in the fermentation products observed.

We have also previously reported that *Bacteroides succinogenes*, a cellulolytic rumen bacteria, could grow on sorghum substrates and utilize a portion of the structural carbohydrates [5]. The primary product of metabolism was succinic acid, and it was found that B. succinogenes grew equally well on fresh sorghum, sorghum that had been roller milled and washed to extract soluble sugars and sorghum that had been ensiled. However, the organism did not appear to grow as well on sorghum substrates as on a pure cellulose substrate.

The purpose of this paper is to report our current progess toward developing a biological pretreatment process. Work has continued in the investigation of altering the ensiling fermentation and in the study of growth and product formation kinetics for pure cultures utilizing the structural components of sweet sorghum.

MATERIALS AND METHODS

A culture of *Clostridium cellulolyticum*, obtained from the American Type Culture Collection, was used to investigate the potential for forcing the ensiling process into a different type of fermentation. Pure culture kinetic studies were conducted using a culture of *Bacteroides succinogenes* S85, kindly provided by Dr. Bryant A. White, Departement of Animal Scienece, University of Illinois.

Ensiling Studies

The sorghum used for this work was harvested from plots of Grassl at the Texas A & M University Experimental Farm, chopped and stored in the freezer until used. Samples of 40g each were placed in 500mℓ serum bottles and 360mℓ of distilled water was added to each bottle. Bottles were prepared using the Hungate technique to obtain anaerobic conditions.

Various treatments were investigated to determine their effect on type of fermentation obtained. The treatments included sterilization in the autoclave for 40 min, adjustment of initial pH to 7.5 with NaOH, no pH adjustment initially, incubation at 37℃ and incubation at room temperature. A control sample was prepared using unsterilized sorghum which was left uninoculated. Table 1 lists the various treatments evaluated.

Table 1. Sample treatments for ensiling studies.

Sample	Treatment
A	Unsterilized, no inoculum, no pH adjustment, room temp.
B	Unsterilized, initial pH 7.5, 37℃
C	Unsterilized, no pH adjustment, room temp.
D	Sterilized, initial pH 7.5, 37℃
E	Sterilized, no pH adjustment, room temp.

Afer preparation, samples were inoculated with 5 day old cultures grown on the maintenance medium (cellobiose carbon source). Inoculum volume was 6mℓ per sample. Following inoculation, samples were incubated at the indicated temperature. To determine effects of the different treatments of fermentation, liquid samples were withdrawn periodically from each serum bottle using a syringe. These samples were analyzed immediately for pH then frozen for later analyses of acid and sugar contents by HPLC.

Pure Culture Kinetics Studies

Substrate for this work was prepared by drying gresh and grinding through a 40 mesh screen using Wiley mill. The ground sorghum was then

extracted with neutral detergent to remove sugars and other soluble components leaving only the structural components.

Batch fermentations were conducted with *B succinogenes* using a mineral salts medium supplemented with required nutritional factors and the prepared sorghum as carbon source. Filter paper, glucose and cellobiose were used as control substrates. Initial substrate concentrations were 0.06, 0.1, 0.2, and 0.5% as cellulose, glucose or cellobiose. Since the sorghum substrate contained approximately 62% noncellulosic materials, initial substrate concentrations were increased accordingly to give the desired levels of cellulose in the medium. In addition, 0.2% succinate was added to some fermentations and 0.5 or 1.0% glucose to others to determine if product or glucose inhibited utilization of cellulose. Table 2 summarizes the different substrates evaluated.

Table 2. Substrates used for *B. succinogenes* fermentations.

Batches	Substrate
1-4	Glucose
5-8	Glucose+0.2% Succinate
9-12	Cellobiose
13-16	Cellobiose+0.2% Succinate
17-20	Cellobiose+0.5% Glucose
21-24	Cellobiose+1.0% Glucose
25-28	Sorghum
29-32	Sorghum+0.2% Succinate
33-36	Sorghum+1.0% Glucose
37-40	Filter Paper

Fermentations were conducted in 1 L flasks with 900mℓ medium per flask. Each flask was inoculated with one-day cultures grown on cellobiose medium using 45mℓ per flask to give an initial cell density of about 33mg/ℓ. After inoculation, flasks were incubated at 37℃.

Samples were collected periodically from each flask to determine the time course of the fermentation. To obtain homogeneous samples of flask contents, the flasks were placed on a magnetic stirrer to suspend the solids and the sample was then withdrawn. Samples have been analyzed for pH, total solids, and neutral detergent fiber.

RESULTS AND DISCUSSION

Ensiling Studies

The pH profile for each sample is shown in Figure 1. The pH in each of the unsterilized samples (A, B, C) dropped rapidly to approximately 3.7 within the first two days and then remained constant. This final pH was independent of initial pH value, and the time required to reach the final value was also independent of the initial value. The behavior was typical of a lactic acid fermentation.

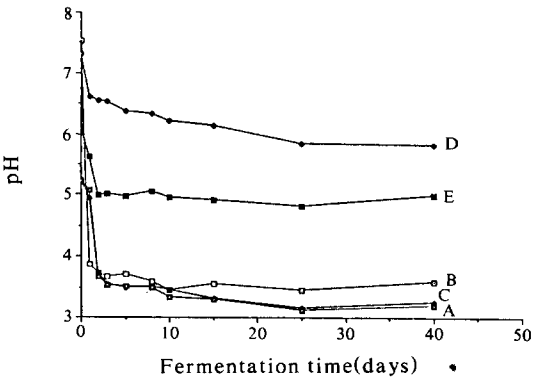

Figure 1. pH profiles during fermentation of sweet sorghum. Sample treatments are defined in Table 1.

The sterilized sorghum samples (D, E in Figure 1) each showed a decline in pH of approximately one unit, from pH 7.5 to 6.5 for the sample with pH initially adjusted to 7.5 (D) and from 6.1 to 5.0 for the sample that did not have an initial pH adjustment (E). The drop in pH is not as great as for the unsterilized samples, indicating a different fermentation pattern.

Lactic acid production in the sample is shown in Figure 2. The unsterilized samples showed a rapid increase in lactic acid, again typical of lactic acid fermentation. The sterilized samples produced very little lactic acid, and their visual appearance indicated that not much growth of *C. cellulolyticum* had occurred. However, the drop in pH indicates that there was some microbial

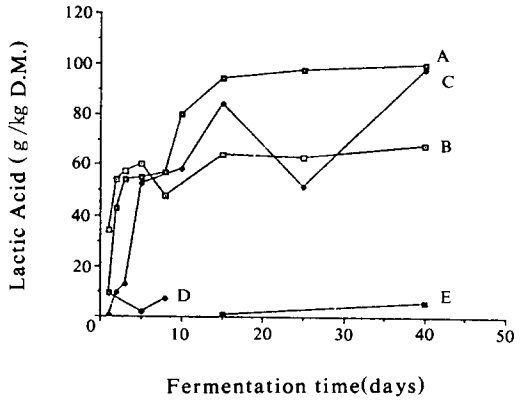

Figure 2. Lactic acid production during fermentation of sweet sorghum. Sample treatments are given in Table 1.

activity in those samples.

Another indication that there was microbial activity in the sterilized sample (D, E) is given by the increase in cellobiose in the medium as shown in Figure 3. The unsterilized samples (A, B, C), on the other hand, showed decreasing or very low levels of cellobiose. These data indicate that some cellulolytic activity was present in the sterilized samples. Although it is not clear whether that activity was the result of microbial growth in the samples or was from enzyme present in the inoculum, it seems more likely that it was produced by microbial growth.

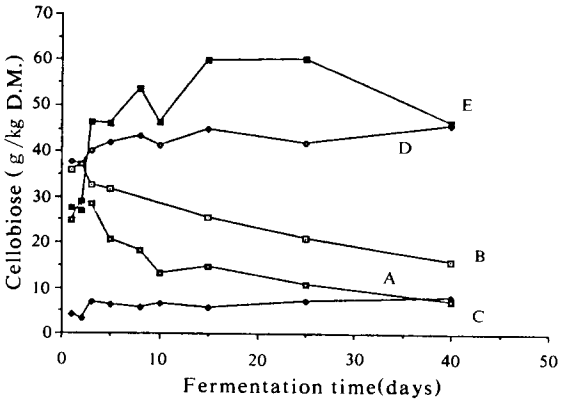

Figure 3. Cellobiose concentrations during fermentation of sweet sorghum. Sample treatments are given in Table 1.

Pure Culture Kinetic Studies

Growth curves for batch fermentations of glucose and cellobiose media are shown in Figure 4-6. The specific growth rate of $0.26h^{-1}$ on cellobiose was slightly lower than the rate of $0.29h^{-1}$ on glucose. Specific growth rates were affected by substrate concentration within the range of 0.06% to 0.5%. However, at 1.0% glucose the specific growth rate was slightly decreased, indicating that substrate inhibition was occurring at that concentration. Addition of 0.2% succinate to the medium reduced the lag time at the start of the fermentation for both glucose and cellobiose substrates but did not appear to affect the sorghum fermentation. Therefore, the fermentation does not appear to be inhibited by product (succinate) at this concentration.

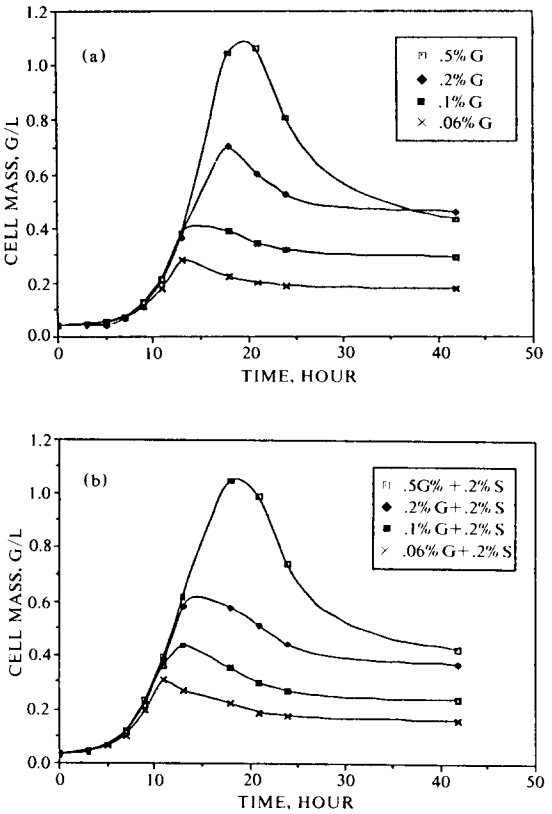

Figure 4. Growth of *Bacteroides succinogenes* on varying levels of glucose(a) and glucose plus 0.2% succinate (b).

The growth curves for glucose and cellobiose also show that after cells reached the stationary phase, 10 to 20 hr after inoculation depending on substrate concentration, cell lysis occurred. The amount of lysis was greater in the fermentations with higher substrate concentrations.

Growth curves for solid substrates such as sorghum and filter paper cannot be obtained directly from dry weight measurements as can be done for soluble substrates. However, substrate utilization can be used as an indication of the amount of growth and this can be measured as the decrease in neutral detergent fiber (NDF). Figures 7-10 show NDF concentrations for each of the sorghum and filter paper fermentations. In general, these figures indicate a steady decrease

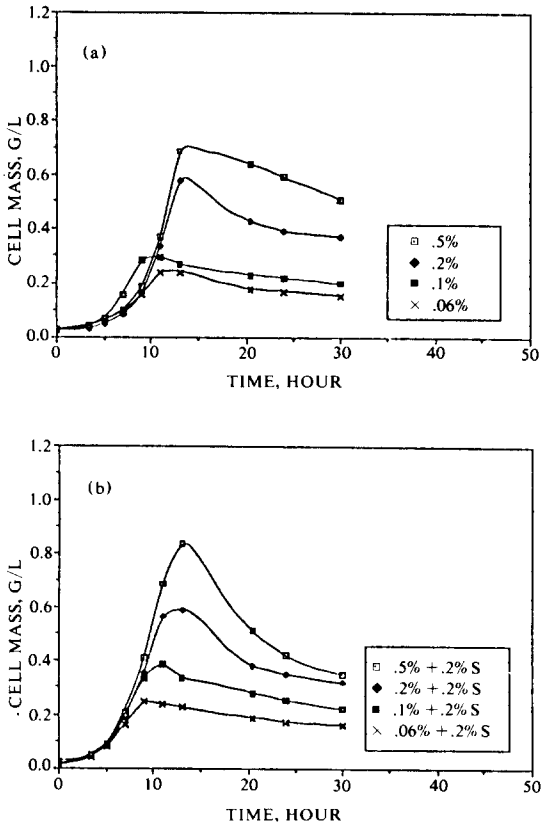

Figure 5. Growth of *Bacteroides succinogenes* on varying levels of cellobiose(a) and cellobiose plus 0.2 % succinate (b).

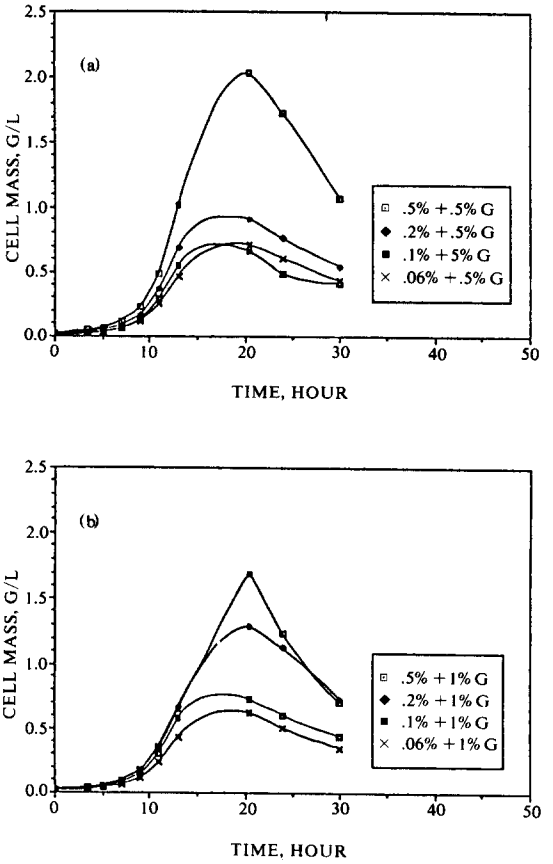

Figure 6. Growth of *Bacteroides succinogenes* on varying levels of cellobiose plus 0.5% glucose(a) and cellobiose plus 1.0% glucose (b).

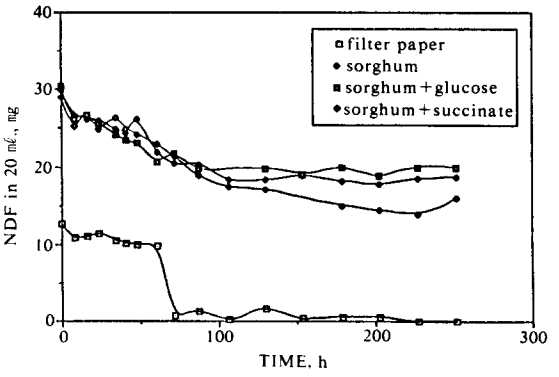

Figure 7. Neutral detergent fiber profile for *B. succinogenes* fermentations using 0.06% initial substrate concentrations.

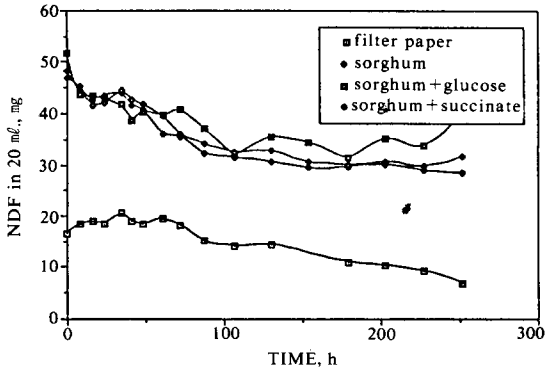

Figure 8. Neutral detergent fiber profile for *B. succinogenes* fermentations using 0.1% initial substrate concentrations.

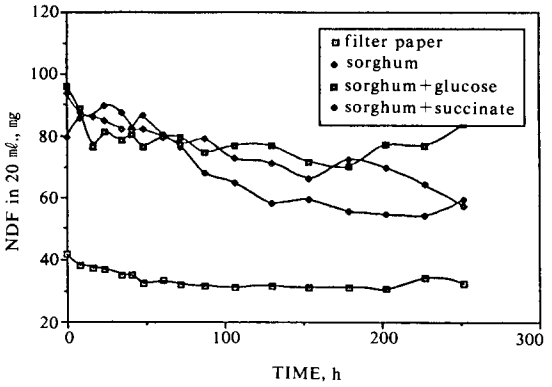

Figure 9. Neutral detergent fiber profile for *B. succinogenes* fermentations using 0.2% initial substrate concentrations.

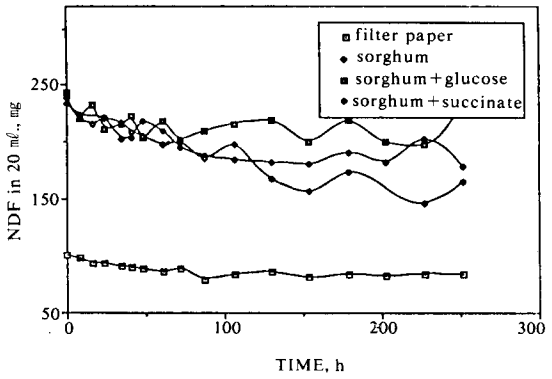

Figure 10. Neutral detergent fiber profile for *B. succinogenes* fermentations using 0.5% initial substrate concentrations.

in NDF for each of the substrates at all concentrations. Although several of the curves showed an increase in NDF toward the end of the fermentation, these trends may have resulted from nonuniform sample collection.

The results presented in Figure 7-10 indicate the same trend in NDF for the sorghum fermentation regardless of the addition of either succinate or glucose. Therefore, conversion of NDF in sorghum is not inhibited by product (succinate) or glucose at the concentrations studied.

Since it is known that *B. succinogenes* can hydrolyze hemicellulose but cannot utilize the pentoses which are the major product of hydrolysis, decreases in NDF in the solid substrate fermentations cannot be related directly to cell growth. Analyses for Kjeldahl nitrogen and composition of residual materials must be completed to determine actual cell mass production and conversion of the cellulose fraction.

CONCLUSIONS

The *C. cellulolyticum* appears to have expressed some cellulolytic activity in the sorghum samples as indicated by the increase in cellobiose during the sterilized sample fermentations. However, it does not appear to have been able to compete effectively with the naturally occurring lactic acid bacteria in the unsterilized samples at the inoculation level used.

Results from the *B. succinogenes* fermentations indicate that it can degrade structural components of sorghum and that this degradation is not subject to inhibition by either glucose or succinate.

요 약

식물체 biomass 에너지 생산, 즉 메탄과 에탄올에서 부딪치는 제일 큰 문제는 식물체의 structural components, 즉 lignocellulose가 자연상태에서 발생하는 lactic acid bacteria에 의해 분해되지 않는데 있다. 이에 structural components의 주성분인 lignocellulose를 분해시키기 위해 여러가지 처리를 가

한 High Energy Sorghum에 *C. cellulolyticum*과 *B. succinogenes*를 주입하여 그 효과를 이 논문에서 규명하였다. 살균한 High Energy Sorghum에 C. cellulolyticum을 주입한 결과 cellobiose가 structural components에 생산되었다. 그러나, 살균처리를 안한 샘플에서는 *C. cellulolyticum*가 lactic acid bacteria와 처리범위 내에서는 효과적으로 경쟁하지 못한것으로 사료된다. 또, *B. succinogenes*도 High Energy Sorgum의 structural components를 어느 정도 분해시켰다. *B. succinogenes*에 의한 cellulose의 degradation에서 glucose와 succinate에 의해 inhibition 효과는 없었다.

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