Report No. 06

Degrading Sludge With A Cellulase Complex

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ABSTRACT

The ability of indigenous and commercial cellulase complexes to degrade native and pretreated sludge was evaluated. Indigenous enzyme was produced in our laboratory, where as commercial cellulase was purchased from a chemical supplier. Pretreatments included detergent solutions, sodium hydroxide, hydrochloric acid, sulfuric acid, sulfur trioxide, alkaline peroxide and sonification. Glucose production was used to infer degradability.

Compared controls (no glucose produced), hydrochloric acid and sodium hydroxide treatment resulted in most degradation. Other treatments were much less effective. The commercial cellulase degraded standards (cellulose) much more rapidly and extensively than indigenous. Although commercial cellulase and either sodium hydroxide or hydrochloric acid treatment increased degradability of sludge considerably above the controls, improvement was much less than expected. Physical appearance of sludges was altered greatly by pretreatment but the implications of this are unclear.

PURPOSE AND OBJECTIVE

Disposal of sludges produced during pretreatment of washwater from dairy processing plants is becoming an economical burden to the dairy industry. Washwater from dairy plants typically is high in BOD (i.e. 3000-5000 mg/l). Extensive volatilization (pretreatment) is necessary to reduce BOD to the 300-500 mg/l concentration required before the effluent can be discharged or sent to city treatment facilities (Bough et al., 1988. Carawan, 1988). Whether trickle-filter or activated sludge systems are used for pre-treatment, tremendous amounts of sludge are generated. A typical dairy plant processing 1 million pounds of milk per day will produce about 3 million pounds of wash water, which, when pretreated, yields about 4000 pounds of sludge (dry weight). Sludge disposal has typically been either by land application or in land fills; both of these options are becoming very restricted and new alternatives are needed.

In another project we have investigated the potential feed value of dairy wash water sludge (termed Biomass in that program) for swine, sheep, cows and poultry. Data from that research indicates: (1) that dairy sludges do not contain EPA priority pollutants (inorganic or organic) (2) that animals will consume diets containing significant quantities of sludge; (3) that longterm feeding of diets containing sludge does not cause health, reproductive nor terratalogical problems; and (4) that, although sludge contains significant amounts of nutrients, availability of these nutrients appears limited (Brown et al., 1985; Clevenger et al., 1987., Fellner et al., 1985., May et al., 1989).

Because of limited availability of nutrients (particularily protein) in sludge, we undertook the following study to try to understand why availability was low and if it could be improved using certain physical treatments in conjunction with enzymes.

METHODS

The investigation consisted of two parts: part I, determining enzyme kinetics and part II, effects of pre-treatments upon cellulase activity.

PART I KINETICS

A. Enzyme Preparation

Cellulase was prepared by culturing conidia from Trichoderma reesei Simmons culture QM 9419 (Natick Laboratory, Natick, MA) in salt liquid medium as described by Bughrara and Sleper (1986).

B. Optimum Amount of Enzyme

Filter paper, Sigmacell (commercially available cellulose) and Biomass were used as three substrates to determine the optimum amount of enzyme for maximum cellulase hydrolysis. The assays were set up as follows:

1. Filter Paper

Nine amounts of enzyme (.1, .15, .20, .40, .60, .80, 1.0, 2.0, 3.0 ml) were added to .05 gm (1 x 6 cm strips) of Whatman No. 1 filter paper. One ml of .05 M acetate buffer, pH 4.85, was added to all samples and the total volume was brought to 5.0 ml with distilled water.

2. Biomass

Six amounts of enzyme (1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 ml) were added to .05 gm of dried dairy biomass, which was available from another project. All samples were treated similarly to those in the filter paper assay.

3. Sigmacell

Eight different amounts of enzyme (.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.0, and 4.0 ml) were added to .05 gm of Sigmasell. All samples were treated similar to those in the above two assays.

All of the assays were carried in a water bath at 40C for 24 hr (filter paper and biomass) and for 12 hr (sigmacell). At the end of the incubation period, samples were immersed in ice to stop the reaction. The assays were replicated 10 times.

C. Enzyme Activity

The filter paper assay was used for cellulase activity determination (Reese and Mandels, 1971). Glucose was measured colorimetrically using the coupled reactions of glucose oxidase-peroxidase (Raabo and Terkildsen, 1960 : Sigma Diagnostic Kit No. 510, Sigma Chemical Co., St. Louis, MO).

D. Estimation of Kinetic Parameters

Sigmacell was used as the substrate to characterize the K_m and V_{max}. Two assays were conducted, one to determine the initial velocity as a function of enzyme concentration and a second to determine the effect of substrate concentration on initial

velocity for a fixed enzyme concentration. The data were arranged in a linear form based on the Lineweaver-Burk reciprocal plot (Segal, 1976).

PART II PRETREATMENT AND HYDROLYSES

A. Pretreatment

Nine pretreatments were evaluated:

1. Control
2. Untreated Sludge
3. Neutral detergent extraction (ND)
4. Acid detergent extraction (AD)
5. Hydrochloric acid (HCL)
6. Sulfuric acid (H_2SO_4)
7. Alkaline peroxide (AP)
8. Sulfurtrioxide (SO_3)
9. Sonification (Sonif)

The rational for these treatments was as follows:
ND, AD, HCL, H_2SO_4, AP and SO_3 are known to increase the availability of low quality feed stocks for animals or industrial fermentations. Sonification will hydrolyze (physically disrupt) microbial all walls. Our intention was that one or more of these methods would increase degradability of sludge as measured by glucose appearance in the filtrate.

B. Sample Preparation and Pretreatment

Samples of dairy sludge (biomass) were obtained from two dairy plants. Subsamples were dried and ground (1.0 mm screen).

Triplicate samples (.15 gm) were placed in test tubes. Two milliliters of different chemical pretreatments (described later) were added to the tubes and samples were vortexed. Another 1.0 ml was added to wash the sides of the tubes. Samples were incubated at 80C for 45 minutes in a water bath. After incubation samples were removed and centrifuged at 3000 rpm for 10 min. Supernatants were carefully aspirated using pasteur pipets and several ml of dH_2O were added to each tube to wash the sample free of its pretreatment chemical. Samples used test effects of sonification were exposed to sonic waves for .5, 1.0, 2.0, 3.0 minutes, and 4500 hours.

C. Hydrolyses

For enzymatic hydrolyses, the above samples then were incubated with 3.0 ml of cellulase for 0, 3, 6, 9, 12, 15, 24 and 30 hr in a water bath at 40C. To all samples was added 1.0 ml of a .05 M acetate buffer (pH 4.85). Each batch included a control, which consisted of .05 gm of Sigmacell as the substrate. Blanks

for each hour interval had 3.0 ml of dH_2O instead of the enzyme. Although the water bath was equipped with a side-to-side rotatory plate, tubes were also intermittently vortexed. At the respective hours, samples were removed and immersed in ice to inhibit any further enzyme reaction. All samples were stored in a refrigerator till further analysis. Glucose was assayed within 24 hours. Samples that were icteric were deproteinized prior to glucose determination as per the procedure outlined in the diagnostic kit.

RESULTS AND DISCUSSION

PART I KINETICS

A. Enzyme Preparation

A batch (5 liters) of cellulase was prepared and frozen for later use. This batch will be referred to as the indigenous preparation. The enzyme was active for several months as shown by repeated tests prior to hydrolysis experiments.

Our first objective was to determine the amount of enzyme needed to obtain maximum hydrolysis without exceeding practical limits. The initial velocity assays for both filter paper and sludge indicated that maximum glucose was released using 3.0 ml of enzyme (Fig.1). For the sludge assay increasing the volume above 3.0 ml was chosen for subsequent hydrolysis experiments. The first order region of the filter paper assay (Fig. 1-inset) was used to determine the kinetic parameters of the enzyme (discussed below).

B. Activity and Kinetics

Protein in the crude cell free cellulase extract was .63 mg/ml. Two hundred microliters of this extract in a standard total reaction volume of 5 ml catalyzed the formation of 6.43 mg/dl of glucose in 24 hr under optimum assay conditions (Fig.1-inset). The following calculations were made to determine the activity:

$$\underline{\text{Velocity expressed in nmoles x ml}^{-1} \text{ x min}^{-1}}$$

$$6.43 \text{ mg/dl} = .357 \text{ mmol/L}$$

$$= (3.57 \times 10^5 \text{ nmoles/24 hr})/5 \text{ ml}$$

$$= 49.58 \text{ nmoles x ml}^{-1} \text{ x min}^{-1}$$

Enzyme concentration in assay mixture

$49.58 \text{ nmoles} \times \text{ml}^{-1} \times \text{min}^{-1}$

$= .0496 \text{ umoles} \times \text{ml}^{-1} \times \text{min}^{-1}$

$[E]_t = .05$ units/ml of assay mixture

Enzyme concentration in extract

The actual assay volume of 5 ml contained .25 units (.05 x 5 ml). These .25 units came from 200 ul of extract. Therefore:

$[E]_t = .25 \text{ units}/.2 \text{ ml} = 1.25$ units/ml extract

Specific activity

S.A. = (1.25 units/ml) /.63 mg protein/ml

= 1.98 units/mg protein

Kinetics (V_{max} and K_m)

To determine the K_m and V_{max} of cellulase, Sigmacell was used as the substrate and initial velocity was determined at various enzyme and substrate concentrations (Fig. 2 and 3). From the initial velocity data of Sigmacell (Fig. 2) an enzyme volume of .5 ml (from the first order region) was used in subsequent experiments to determine the V_{max} and K_m (Fig. 3). The initial velocity versus substrate concentration curve (Fig. 3) is described by the Henri-Michaelis-Menten equation (Siegel, 1976). Although V_{max} and K_m can be calculated from the data, it is evident from the plot that the exact value for both parameters is not discernable. The best way to obtain V_{max} and K_m is to plot the data by the method of Lineweaver-Burk Reciprocal plot (Fig. 4). This plot is based on the rearrangement of the Henri-Michaelis-Menton equation into a linear (y=mx + b) form:

$$v = \frac{V_{max}\,[S]}{K_m + [S]}$$

$$1/v = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{Vmax}$$

From the double reciprocal plot (Fig. 4) V_{max} and K_m were calculated as follows:

$$1/V_{max} = .599 \text{ mg} \times 10^{4} \times \text{min}^{-1}$$
$$V_{max} = 1.67 \times 10^{-4} \text{ mg} \times \text{ml}^{-1} \times \text{min}^{-1}$$

and,

$$-1/K_m = -.933 \text{ mg} \times 10^{-2}$$
$$K_m = 1.07 \times 10^{2} \text{ mg}$$

Part II. Pretreatments and Hydrolysis

Hydrolysis experiments on dairy biomass were conducted using indigenous cellulase following pretreatments using indigenous cellulase. For comparisons a commercial cellulase (Sigma Cellulase C0898, type IV) was also used with Sigmacell as the substrate. Data (table 1) were plotted (Fig. 5) to represent the hydrolysis curve. The Sigma cellulase had a greater intercept and steeper curve than the indigenous enzyme. The two curves can be viewed as (a) High Enzyme to Substrate Ratio, E_a = 2.13 units/ml (Sigma cellulase) and, (b) Low Enzyme to Substrate Ration, E_a = 1.25 units/ml (indigenous cellulase). This plot also points certain distinctive features of cellulose hydrolysis. At the end of 30 hr glucose reached a maximum of 25.6 mg/dl for the indigenous cellulase. There did not appear to be any end product inhibition. However, glucose concentration for Sigma cellulase was 28.7 mg/dl by the end of 3 hr and peaked at 78.9 mg/dl by 15 hr. Following 15 hr glucose concentration decreased slightly before plateauing between 24 and 30 hr. Glucose might be causing end product enhibition in this case. There might also be variation in the accessibility of the substrate. Wald et al. (1984) showed that glucose yield is a function of initial enzyme loading at 8 hr and 47 hr. Glucose yield increased until its concentration reached a point, beyond which it remained constant. Glucose reaches a maximum because the substrate becomes saturated with enzyme. Rate of hydrolysis is proportional to the amount of enzyme adsorbed; the rate also reaches a maximum. Wald et al. (1984) increased enzyme:substrate ratio beyond this point but there were no significant increases in glucose yields. This suggests that the reaction was not first order in enzyme

concentration and Wald et al. (1984) deduced that an adsorption process was the controlling factor.

A third explanation for lack of activity may relate to intramolecular structure of the substrate (sludge), which is complex and not well understood. Therefore, it cannot be stated on a priori grounds that further modification will result in increased conversions to glucose by hydrolysis with cellulase. Also, the indigenous preparation was evidently a full-complement cellulase system consisting of perhaps endoglucanases, cellobiohydrolyses, exoglucanases and beta-glucosidases. Hence, measuring activity on this system is dependent on all components working in a synergistic manner. End product inhibition could be mistaken for a low activity of any one of these enzyme components.

Physical Effects of Chemical Pretreatments

Acidic pretreatment has been known to increase degradability of cellulose. Improvement in the hydrolosis rate of cellulose has been related to the modification of the lignin-carbohydrate linkage (Fan et al., 1981a). Following pretreatment, Fan et al. (1981b) and Taso et al. (1979) described their substrates as appearing light in weight and brown in color. Data obtained in the present study were not consistant with all of the findings reported above but certain distinct observations were apparent, regarding the pretreated substrate. Since the original color of biomass was brown, it was difficult to determine whether the color was affected by pretreatments. However, there were definite physical alterations in biomass and changes in the color of the supernatants. The NaOH and NDS-pretreated substrates were normal (brown) in color with the NaOH appearing somewhat granulated. The NDS substrate appeared lighter and not very compacted. The ADS pretreated substrate was similar in color to NDS but finer in texture and well compacted. Some granules in ADS substrate appeared white in color. The supernatant color af ADS was similar to that of NDS. The colors Hcl, AP and H_2SO_3 but not nearly as granulated. The H_2O_2 substrate was also white with a cloudy appearance. Sonification changed the substrate into a rubbery plastic texture.

All of the above comparisons were made with a control, which was sludge in dH_2O. The control substrate appeared light brown and fine. Supernatant was light brown to white in color and had an opaque appearance. These observations infer that pretreating biomass altered its physical appearance.

Others have observed similar results and though the amount of glucose released from simple acid pretreatment might be low there is little disagreement on the reduction of the degree of polymerization indicating extensive structural modification (Bungay, 1981; Fan et al., 1981).

Compared to H_2SO_4, NaOH only slightly hydrolyzed the polysaccharides, resulting in a low ration of reducing sugar to total sugars (Beldman et al., 1987). Solubilization of nonsugar components was substantial in the sample treated with NaOH. With low acid concentrations, large amounts of monomers (arabinose) were released and at higher concentrations (.5 N H_2SO_4) several oligomers were produced and almost no glucose was released. This suggests that at higher H_2SO_4 concentration cellulose was not hydrolyzed. In the soluble fraction of the alkali treated material, only larger oligomers and no monomers were found.

Enzymatic Hydrolysis of Chemically Pretreated Samples

The results obtained from enzymatic hydrolysis of the pretreated samples are presented in tables 2 and 3. There were no differences in total glucose released from Sigmacell due to the pretreatments (Table 2). However, when sludge was pretreated, there were some differences in release of glucose at the end of
30 hrs. Compared to untreated sludge, all pretreatments resulted in some glucose release even though the amounts were very small. The maximum glucose released was for the Hcl pretreatment (.47 mg %) followed by NaOH and ADS (.23 mg % for both) and AP (.21 mg %) and marginal increases with SO_3, H_2O_2 and NDS (.09, .09 and .03 mg % respectively).

Compared to the NaOH pretreatment, HCl resulted in the release of twice as much glucose. Beldman et al. (1987) characterized the nature of the sugars which differed considerably; sugars released from acid hydrolysis were almost completely in monomeric form (glucose, xylose and arabinose). The NaOH pretreated samples showed large amounts of oligomers. The reason why these oligomers were not further hydrolyzed to smaller products is not clear. Beldman et al. (1987) speculate that another enzyme (lacking in the cellulase complex), such as an arabinofuranosidase, might be required to further degrade these oligomers.

CONCLUSION

Kinetic constants for cellulase were calculated from initial reaction velocities. Effects of several chemical pretreatments on hydrolysis of biomass by cellulases were investigated. Pretreatments resulted in varied degrees of glucose released. Maximum glucose increase was with the HCL and NaOH pretreatments. Other chemical pretreatments resulted in moderate to low amounts of glucose being released. Sonification gave no glucose release. The mechanisms responsible for enzymatic hydrolysis of cellulose are not clearly understood. Results from our experiments were generally similar to other studies. However, it is important to keep in mind that explanations for cellulolytic hydrolysis in one study may not be true for another study. The cellulase enzyme

complex is a mixture of several enzyme components, each of which might exhibit an independent activity level. This obviously will affect the hydrolysis of substrates differently. But, more importantly, the nature of the substrate is perhaps the controlling factor. The affect of crystallinity and structure of sludge on denying the enzyme easy access to the substrate and retarding enzymic hydrolysis has been discussed. As mentioned earlier, the molecular structure of biomass is not clearly known as yet and it is possible that a combination of enzymes along with cellulase (pectinase, chitinase, etc.) might be required for increased release of hydrolytic products. It is entirely possible that dairy sludge is generally refractory to pretreatments and enzymes that are normally regarded as effective on typical cellulosic substrates. The kinetics determined for cellulase degradation of sludge in this study did not appear to correlate well with kinetics we obtained for situ or in vitro degradation. The reason is not evident but probably suggests that other enzymatic activity, i.e. proteolysis, may be more important than cellulolysis.

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