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# Effects of Hemicellulose and Lignin on Enzymatic Hydrolysis of Cellulose from Dairy Manure

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## Abstract

This study focused on the effect of hemicellulose and lignin on enzymatic hydrolysis of dairy manure and hydrolysis process optimization to improve sugar yield. It was found that hemicellulose and lignin in dairy manure, similar to their role in other lignocellulosic material, were major resistive factors to enzymatic hydrolysis and that the removal of either of them, or for best performance, both of them, improved the enzymatic hydrolysis of manure cellulose. This result combined with scanning electron microscope (SEM) pictures further proved that the accessibility of cellulose to cellulase was the most important feature to the hydrolysis. Quantitatively, fed-batch enzymatic hydrolysis of fiber without lignin and hemicellulose had a high glucose yield of 52% with respect to the glucose concentration of 17 g/L at a total enzyme loading of 1300 FPU/L and reaction time of 160 h, which was better than corresponding batch enzymatic hydrolysis.

**Index Entries:** Cellulose; fed-batch enzymatic hydrolysis; glucose; hemicellulose; yield.

## Introduction

Nearly 160 million dry tons of manure is produced annually in the United States. Approximately 55 million dry tons of animal manures are collected for disposal, with about 75% of the total coming from dairy and feed-lot cattle *(1)*. Animal manures are rich in carbohydrate and protein, which could be further converted into biobased chemicals, materials, and energy. Dairy manure, representing the largest percentage, contains about 12% hemicellulose and 22% cellulose *(2)* which represents a large potential

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source of carbohydrates that are capable of producing monosugars such as glucose, xylose, arabinose, and galactose through various hydrolysis processes.

Enzymatic hydrolysis has attracted increasing attention as an alternative to acid hydrolysis for converting lignocellulosic materials to sugars, because the process has highly specific yet mild reaction conditions (pH around 5 and temperature less than 50°C) as well as a lack of corrosion problems *(3)*. Cellulase is widely used to carry out enzyme hydrolysis of lignocellulosic material such as wood and wheat straw. Cellulase consists of endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase, and β-glucosidase. The endoglucanase attacks cellulose to create free chain ends, the exoglucanase degrades the molecule by removing cellobiose from the free chain end, and the β-glucosidase produces glucose by breaking down the cellobiose *(4)*. It is well known that in lignocellulosic materials cellulose is physically associated with hemicellulose and physically and chemically associated with lignin *(5)*. The resulting matrix prevents cellulose from being attacked by cellulase *(5,6)*. There are numerous studies on the effect of this hemicellulose and lignin matrix on enzymatic hydrolysis of cellulose *(7–11)*. None of these efforts, however, has investigated the hydrolysis of cellulose from animal manure, which might be one of the most difficultly digestible cellulosic materials, because most of the easily accessible fiber from forage has already been degraded by cellulolytic enzymes in the animal's digestive system.

There are a number of pretreatment methods, in particular chlorite and dilute acid, which are capable of removing hemicellulose and lignin *(12,13)*. Sodium chlorite has been reported as one of the most effective reagents for the removal of lignin from lignocellulosic materials and is widely used by the paper industry to do deligninfication and bleaching. Dilute-acid treatment has been shown in our previous work to be effective in completely removing all of the hemicellulose in manure with the least loss of cellulose *(2)*. Thus in this study, chlorite treatment and dilute acid treatment were applied for the removal of lignin and hemicellulose, respectively.

The objective of this work was to study the effect of hemicellulose and lignin in dairy manure on enzymatic hydrolysis of cellulose, and further optimize the enzymatic hydrolysis process to obtain the best performance. The three treated manure samples investigated in the study were: hemicellulose-free manure fiber from dilute-acid treatment, lignin-free manure fiber from sodium chlorite treatment, and manure fiber without lignin and hemicellulose from a combination of dilute-acid and chlorite treatments.

## Materials and Methods

### *Materials*

Fresh dairy manure was obtained from the Dairy Center of Washington State University. The manure had 15.3% dry matter (DM) with

Table 1
Characteristics of Sample Manures[a]

| | Manure fiber |
|---|---|
| Dry matter, % | 14.20 ± 0.05 |
| NDF, % dry basis | 61.32 ± 1.02 |
| ADF, % dry basis | 47.45 ± 0.56 |
| ADL, % dry basis | 16.03 ± 0.17 |
| Cellulose (=ADF-ADL), % dry basis | 31.42 ± 0.50 |
| Hemicellulose(=NDF-ADF), % dry basis | 13.87 ± 0.60 |
| N, % dry basis | 1.23 ± 0.06 |
| C, % dry basis | 41.52 ± 1.89 |

[a]Data are the average of triplicates with standard deviations ($n = 3$) at $\alpha = 0.05$.

a total carbon content of 46.9 g/100 g DM and total nitrogen content of 2.6 g/100 g DM. Ten kilograms of original manure was mixed with 5 kg of water and blended for 1 min to achieve size reduction, and then 10 kg of the mixture was washed three separate times with 5 kg of water. Solid–liquid separation using a centrifuge at 988 g for 10 min isolated most of the soluble nitrogen and impurities within the liquid fraction. The solid part was collected and dried as manure fiber for hydrolysis. The data for the manure fiber are presented in Table 1.

The enzyme, celluclast 1.5 L, purchased from Sigma (Sigma, St. Louis, MO) was used for the study of enzymatic hydrolysis. Celluclast 1.5 L contained 145.5 FPU/g solution of cellulase (filter paper activity unit, FPU). One unit of FPU is defined by the Commission on Biotechnology, IUPAC, as the enzyme amount that releases 1 µmol of glucose equivalents from Whatman No. 1 filter paper in 1 min *(14)*.

### *Pretreatment*

Hemicellulose-free manure fiber was obtained by applying optimized dilute-acid treatment from a previous study on manure fiber *(2)*. The treatment was operated at 1% sulfuric acid, 135ºC, and 5% substrate concentration for 2 h. After dilute-acid treatment, the samples were filtered through Whatman No. 5 filter paper and washed by water until the pH of solution was around 4. After the washed solid part was dried at 100ºC overnight, the hemicellulose-free manure fiber sample was ready to be hydrolyzed.

Chlorite treatment was used to extract lignin from the manure fiber *(12)*. The substrate concentration was 5%. The chemical mass ratio was 0.3 g sodium chlorite and 0.1 mL glacial acetic acid per grams of manure fiber. The sample was treated at 70ºC for 1 h. The ensuing liquid–solid sep-

aration step was carried out using a No. 120 standard screen with the solid part being washed by 1000 mL of water. After being dried at 100ºC, the lignin-free manure fiber sample was obtained.

Manure fiber without hemicellulose and lignin was obtained by applying both the dilute-acid treatment and the chlorite treatment. The acid treatment was used first, followed by chlorite treatment. Each individual step was the same as described before.

### *The Effect of Hemicellulose and Lignin on Enzymatic Hydrolysis*

Two and one-half grams of dry sample from each different type of manure fiber was mixed with 50 mL of cellulase solution. Two levels of enzyme loading (650 FPU/L and 1000 FPU/L) were studied. The pH of enzymatic hydrolysis was set at 4.8 and the reaction temperature was 50°C *(15)*. Samples were taken at 3 h intervals for the first 10 h and at 24 h intervals for the rest of the 96 h.

### *Enzymatic Hydrolysis of Ensuring Treated Manure Fiber*

The same ratio of dry sample to enzyme solution was used in this part of the study. The effect of enzyme loading was studied at four levels of 650 FPU/L, 800 FPU/L, 1000 FPU/L, and 1300 FPU/L. The pH and reaction temperature were the same as those in the previous section. Total reaction time was 144 h.

### *Fed-Batch Enzymatic Hydrolysis of Ensuring Treated Manure Fiber*

The enzymatic hydrolysis of treated manure fiber started at a level of 650 FPU/L with another 650 FPU/L enzyme solution added into the solution at 24 h. In evaluating the performance, the fed-batch enzymatic hydrolysis was compared with two separate batch hydrolyses with 650 FPU/L enzyme and 1300 FPU/L enzyme, respectively.

### *Statistical Analysis*

An analysis of variance (ANOVA) of sugar yields was tested for each individual experiment mentioned above using the Statistical Analysis System (SAS 8.0) program.

### *Analytical Methods*

Enzyme activities during hydrolysis were measured according to the recommendation for dilute enzyme solution by the IUPAC committee *(14)*. One-half milliliter of enzyme solution was added into 1 mL of 0.05 *M* sodium-citrate buffer (pH 4.8) contained in a strip of 50 mg Whatman No.1 filter paper. The solution was incubated in a shaker at 50ºC for 1 h. The enzyme unit was defined as the total amount of glucose release per minute from 0.5 ml enzyme solution.

Table 2
Fiber Characteristics of Differently Treated Manure[a]

| | Manure fiber (control) | Hemicelllulose-free manure fiber | Lignin-free manure fiber | Manure fiber without hemicellulose or lignin |
|---|---|---|---|---|
| Cellulose, % dry basis | 31.42 ± 0.50 | 42.76 ± 0.18 | 48.11 ± 0.43 | 65.95 ± 0.17 |
| Hemicellulose, % dry basis | 13.87 ± 0.60 | approx 0 | 17.80 ± 0.63 | approx 0 |
| Lignin, % dry basis | 16.03 ± 0.17 | 28.85 ± 0.74 | 3.65 ± 0.48 | 3.06 ± 0.26 |

[a]Data are the average of triplicates with standard deviations ($n = 3$) at $\alpha = 0.05$.

Fiber data including neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were analyzed using the reflux apparatus *(16)*. NDF was used to estimate the total cellulosic materials (cellulose, hemicellulose, lignin, and insoluble ash) while ADF was used to estimate the concentration of lignin and cellulose. Hemicellulose was determined by the difference (%NDF−%ADF). The content of each monosugar in the hydrolyzed solution was determined using an ion chromatograph (IC) from Dionex *(2)*. Carbon and nitrogen contents in solid samples were measured by automated combustion techniques. The LECO CNS-2000 was used to measure the total carbon and total nitrogen of manure samples. The structure changes during the acid hydrolysis were qualitatively studied using a scanning electron microscope (SEM) from Hitachi *(2)*.

## Results and Discussion

### *The Effect of Hemicellulose and Lignin on Enzymatic Hydrolysis of Manure Fiber*

Dilute-acid and chlorite treatments not only removed the corresponding hemicellulose and lignin, but also simultaneously significantly increased the cellulose content (Table 2). After treatments, hemicellulose and cellulose in lignin-free manure fiber reached 18% and 48%, respectively, and hemicellulose-free manure fiber had 43% of cellulose and 29% of lignin. Meanwhile, the combination of dilute acid and chlorite treatments removed almost all of the hemicellulose and lignin, making the manure fiber without hemicellulose and lignin have the highest cellulose content at 66%.

The enzymatic hydrolysis of differently treated manure fiber demonstrates that removing either hemicellulose or lignin, especially both, was beneficial for improving the glucose yield of the hydrolysis

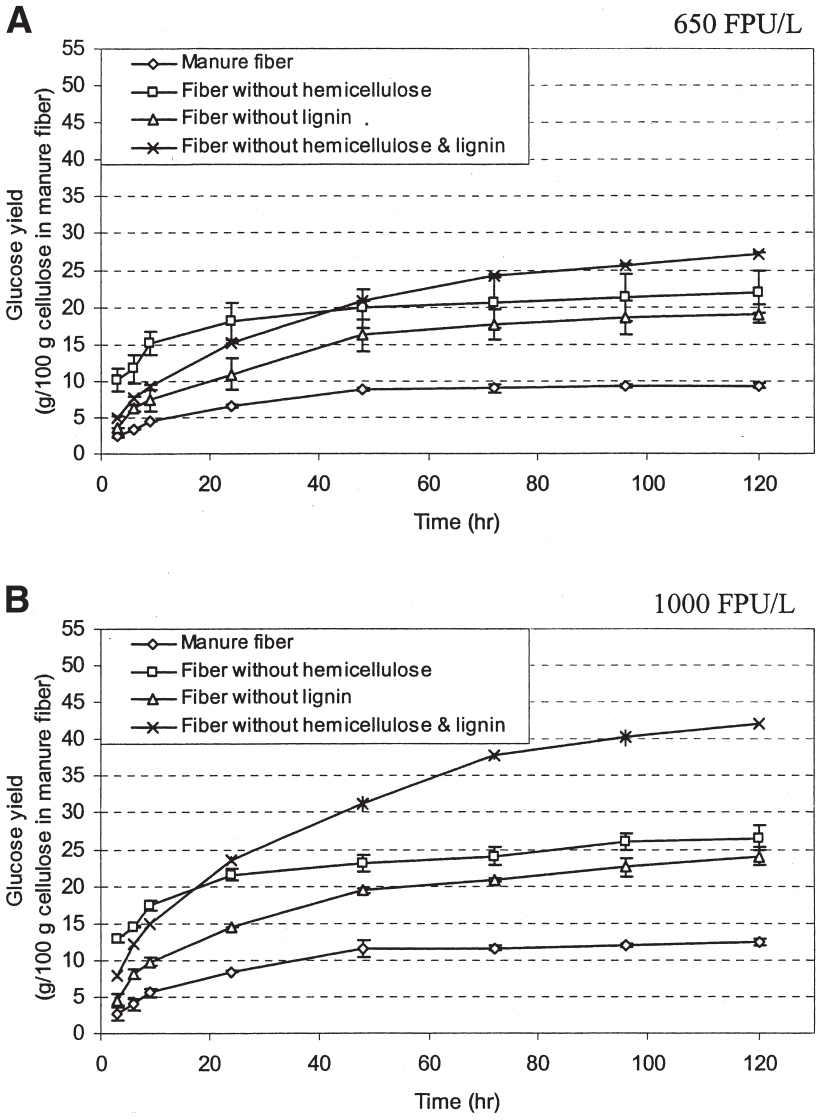


**Fig. 1.** Comparison of enzymatic hydrolysis of differently treated manure fibers. Data are presented as the mean of triplicates and the error bars show the standard deviation. **(A)** 650 FPU/L; **(B)** 1000 FPU/L.

(Fig. 1). Compared to manure fiber, all three of the treated manure fibers showed much higher glucose yields from both of the enzyme loading levels of 650 and 1000 FPU/L. At the low enzyme loading level of 650 FPU/L, hydrolysis of hemicellulose-free manure fiber performed better than the other two treated fibers during the first 48 h. After that, glucose yields from hydrolysis of manure fiber without hemicellulose and lignin kept increasing while that from hemicellulose-free fiber started slowing

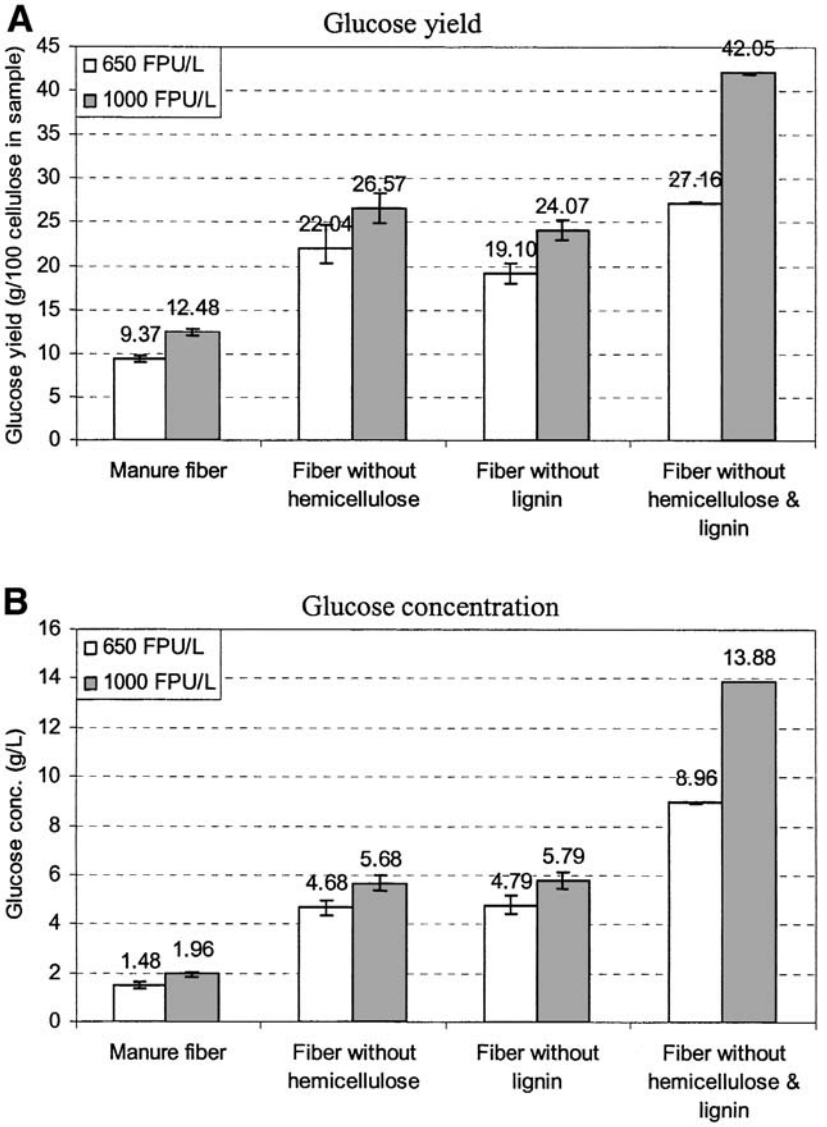


**Fig. 2.** Glucose concentrations and yields from hydrolysis of differently treated manure fibers. Results were from the point of 120h. Data are presented as the mean of triplicate and the error bars show the stanard deviation. **(A)** Glucose yield; **(B)** Glucose concentration.

down. The same trend happened at the higher enzyme loading level of 1000 FPU/L although the glucose yield without hemicellulose and lignin passed the others in just 24 h. The comparison of sugar concentrations and sugar yields of these two enzyme-loading levels at the end of the reaction is presented in Fig. 2. The highest values of glucose concentration and yield of 14 g/L and 42%, respectively, were from the hydrolysis of fiber without hemicellulose and lignin, although all three pretreat-

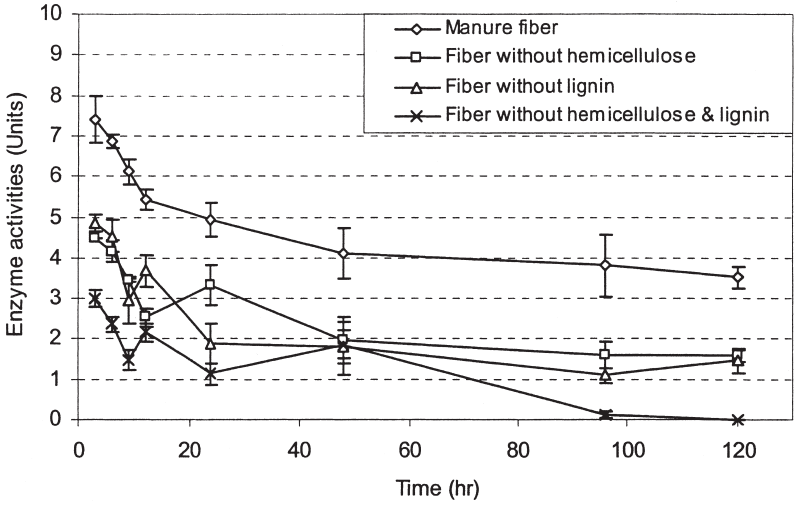


**Fig. 3.** The effect of hemicellulose, lignin on cellulase during the hydrolysis. The initial enzyme activity was 650 FPU/L. All of enzyme activities in the figure are from the liquid part of samples. Data are presented as the mean of triplicate and the error bars show the standard deviation.

ments showed improvements based on the control. Additionally, glucose production for all three pretreatments increased as enzyme loading increased and at a rate that was higher than was observed in the control. In particular, fiber without hemicellulose and lignin showed the largest increase of around 15%, which was almost three times higher than the increases of the other two treated samples.

The enzyme activity changes of each individual sample were then studied in an attempt to better understand the influences of hemicellulose and lignin on the enzymes during the hydrolysis (Fig. 3). Because enzymatic hydrolysis of manure fiber is a type of heterogeneous system, the functional enzyme should be that absorbed on the surface of the fiber. This means, the more enzyme activity in the liquid, the less enzyme absorbed by the fiber. Figure 3 shows that enzyme in liquid from hydrolysis of fiber without hemicellulose and lignin had the lowest enzyme activity. This fact, combined with the observation that cellulase had a slight decay during the retention time of 160 h (data not shown) in the buffer solution, led to the conclusion that hydrolysis of fiber without hemicellulose and lignin had more enzyme absorbed by the cellulose than the other treated manure fibers. This result was consistent with the observations of glucose yields in Fig. 1 which showed that the more enzymes that were absorbed by fiber, the more glucose that was produced. This also indicated that either hemicellulose or lignin might partially inhibit the enzyme physically or chemically, preventing cellulose from being attacked. Figure 3 also shows that the aqueous enzyme activities within lignin-free fiber and hemicellulose-free fiber had no significant difference ($p > 0.05$) during most of the reaction

times (except at 12 h and 24 h). This means that almost the same amount of enzyme was absorbed by both samples. However, as previously discussed, hemicellulose-free fiber had higher glucose yields than lignin-free fiber (Fig. 1). This indicated that the functional enzyme was more readily absorbed in hemicellulose-free fiber than in lignin-free fiber. The reason might be that although lignin-free fiber and hemicellulose-free fiber had similar surface areas to absorb the enzyme, hemicellulose-free fiber might have been slightly more accessible to the enzyme because of the changes made to the structure during the high-temperature acid pretreatment used to obtain the pretreated sample. This difference in pretreatment and in particular the temperature at which they were exposed might explain the observation that both samples absorbed the same amount of enzyme, but produced slightly but significantly ($p < 0.05$) different amounts of glucose.

Structural changes of differently treated manure fibers were observed using a scanning electronic microscope (SEM) in order to better determine how lignin and hemicellulose influence the hydrolysis, physically or chemically (Fig. 4). The differences in fiber structure between manure fiber and treated manure fiber are presented in Figs. 4A–D. The pictures qualitatively show the removal or washing off of a considerable amount of substance from the backbone of the pretreated fiber. However, it is apparent that the main structure of lignin-free and hemicellulose-free fibers was not broken down (Fig. 4B,C). The pictures with the characteristics of the fibers (Table 2) elucidated that either the lignin in hemicellulose-free fiber or the hemicellulose in lignin-free fiber was still attached to the cellulose, which formed the shield to protect those cellulose inside of the fiber. The structure of manure fiber without hemicellulose and lignin was clearly different from the other two treated fibers with most of the cellulose exposed, which made the fiber more easily attacked by the enzyme. After enzymatic hydrolysis, hydrolyzed lignin-free and hemicellulose-free fibers (Fig. 4F,G) clearly show that the textured valleys were much narrower than before hydrolysis, which may mean part of the cellulose on the outside of the main structure was washed off. However, most of the cellulose inside of the structure was still not touched by the enzyme, so the efficiency of hydrolysis was still low. Hydrolyzed manure fiber without hemicellulose and lignin in Fig. 4H demonstrated that the main structure of fiber was completely destroyed, explaining why the glucose yield and concentration reached much higher levels for this pretreatment. These phenomena combined with other results, such as glucose yields and enzyme activity, further prove the effects of lignin and hemicellulose on enzymatic hydrolysis of dairy manure are mainly physical.

### *Effects of Enzyme Loading on Enzymatic Hydrolysis of Manure Fiber Without Hemicellulose and Lignin*

Based on the above findings, manure fiber without hemicellulose and lignin was chosen as the substance to study the effect of enzyme loading

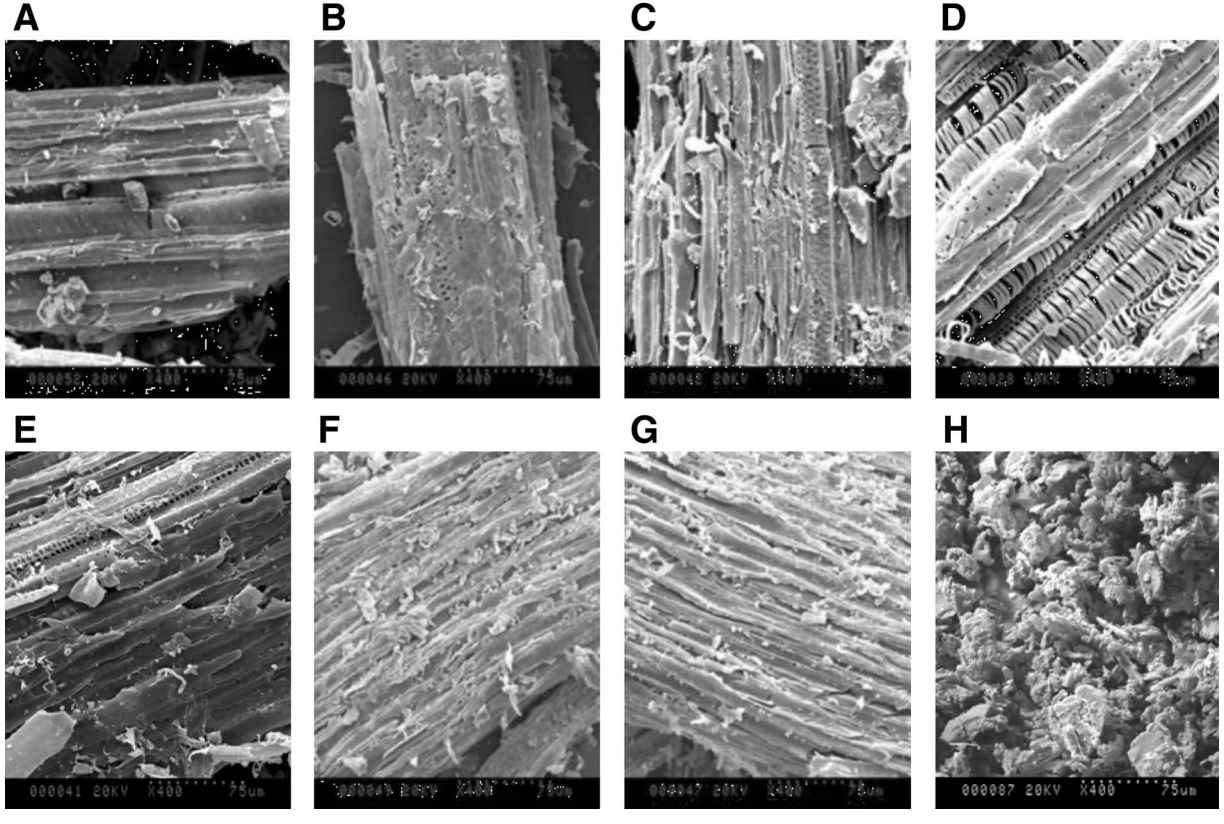


**Fig. 4.** SEM of hydrolysis of different types of manure samples (400×) from hydrolysis of enzyme loading of 1000 FPU/L. **(A)** Manure fiber; **(B)** manure fiber without hemicellulose; **(C)** manure fiber without lignin; **(D)** manure fiber without hemi and lignin; **(E)** manure fiber after enzymatic hydrolysis; **(F)** manure fiber without hemicellulose after enzymatic hydrolysis; **(G)** manure fiber without lignin after enzymatic hydrolysis; **(H)** manure fiber without hemicellulose and lignin after enzymatic hydrolysis.

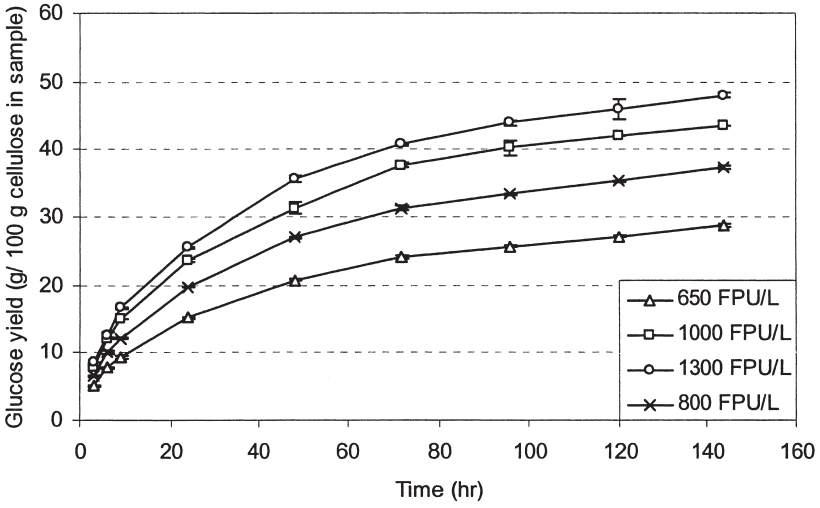


**Fig. 5.** Enzymatic hydrolysis of manure fiber without hemicellulose and lignin Data are presented as the mean of triplicate and the error bars show the standard deviation.

on enzymatic hydrolysis. Figure 5 shows that glucose yields kept increasing with an increase in enzyme loading. The highest glucose yield of 48% was obtained at an enzyme loading of 1300 FPU/L and reaction time of 144 h (at a glucose concentration of 16 g/L). This means that the more enzyme, the better the hydrolysis. However, the increase of the yield was not linearly proportional to the increase in initial enzyme loading because it leveled off from 650 to 1300 FPU/L. The results concluded that the efficiency of the enzyme during the hydrolysis decreased following the increase of enzyme loading. The reason might be that there was much more enzyme than available binding sites on cellulose at the beginning of the reaction. The excess enzyme might merely have been absorbed on the surface of the cellulose with inactive binding sites or stuck on a small pole of crystal structure like the trunk in the middle of Fig. 4D, which did not work on degrading the cellulose. If it is true, the process of batch reaction has to be modified in terms of improving the efficiency.

## *Fed-Batch Enzymatic Hydrolysis of Manure Fiber Without Hemicellulose and Lignin*

Fed-batch reaction as a production technique is widely used in the fermentation industry *(17,18)*. The advantages of this technique are to increase the productivity and reduce the total reaction time. In this particular case, it could be an effective way to improve the efficiency of enzymatic hydrolysis of manure fiber. The hypothesis was that the first enzyme loading is not only to convert hydrolysis-ready cellulose to glucose but also to enzymatically break down the crystal structure of cellulose that

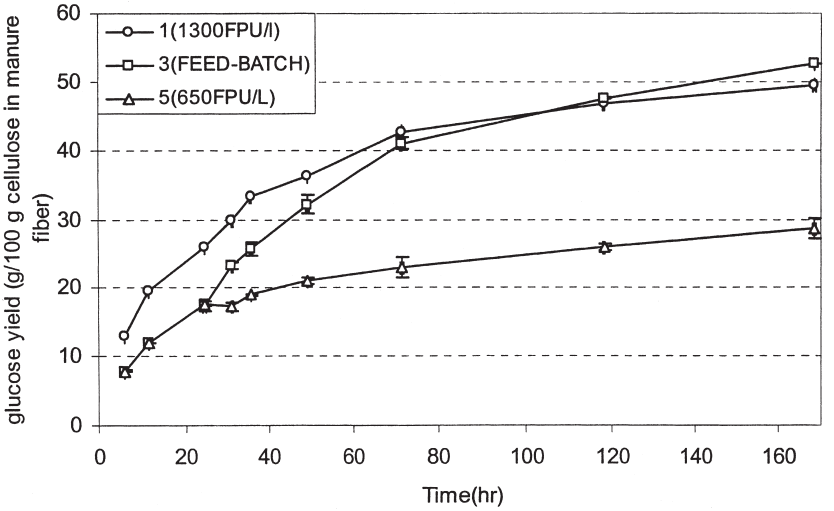


**Fig. 6.** Fed-batch enzymatic hydrolysis. Data are presented as the mean of triplicate and the error bars show the standard deviation.

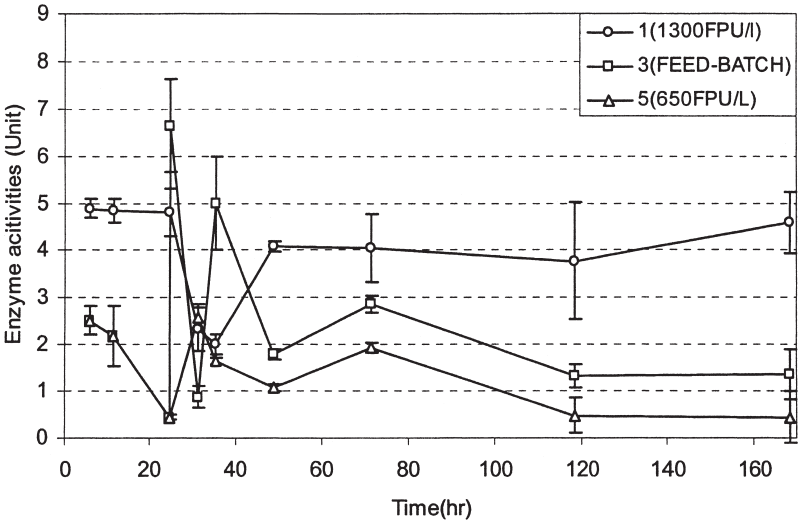


**Fig. 7.** Enzyme activity changes of fed-batch enzymatic hydrolysis. Data are presented as the mean of triplicate and the error bars show the standard deviation.

remains after pretreatment in order to diminish the negative aspects of the inactive adsorption and heterogeneous structure of the substrate.

Application of this hypothesis resulted in a fed-batch enzymatic hydrolysis that significantly ($p < 0.05$) improved the final glucose yield compared to the control with a high enzyme loading level of 1300 FPU/L (Fig. 6). The highest yield of 52% (with respect to a glucose concentration of 17 g/L) was obtained from fed-batch hydrolysis with a reaction time of 168 h. The analysis of enzyme activity showed that the fed-batch system had more enzyme absorbed than the high enzyme level control (Fig. 7).

This means the negative factors of inactive adsorption and structure blockage were partially removed by fed-batch enzymatic hydrolysis resulting in enhanced performance for the enzymatic hydrolysis.

## Conclusion

Hemicellulose and lignin in dairy manure, like other lignocellosic materials, have similar functions as major resistors to enzymatic hydrolysis. The removal of either component partially improved the enzymatic hydrolysis of manure cellulose, while removal of both resulted in even greater hydrolysis performance, producing a glucose yield of 42% with respect to the concentration of 14 g/L at a reaction time of 120 h and enzyme loading of 1000 FPU/L. This result, along with the observations of enzyme activity changes during the hydrolysis and SEM pictures, further prove that the accessibility of cellulose to cellulase was the most important structural feature influencing the enzymatic hydrolysis. Finding an environmentally friendly treatment method to substitute for the standard methods of chlorite and alkaline treatments will play a key role for commercial utilization of cellulose in lignocellulosic materials. Furthermore, besides the importance of structural features, process optimization also can effectively help improve enzyme performance. Fed-batch enzymatic hydrolysis of fiber without hemicellulose and lignin at a total enzyme loading of 1300 FPU/L produced 17 g/L glucose in 160 h with respect to a glucose yield of 52%, which was higher than those from the corresponding batch hydrolyses. This means that fed-batch enzymatic hydrolysis is a promising process for hydrolyzing lignocellulosic materials and further study on optimizing fed-batch enzymatic hydrolysis should be conducted.

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