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Production of Cellulase/β-Glucosidase by the Mixed Fungi Culture of *Trichoderma reesei* and *Aspergillus phoenicis* on Dairy Manure

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Abstract

A cellulase production process was developed by growing the fungi *Trichoderma reesei* and *Aspergillus phoenicis* on dairy manure. *T. reesei* produced a high total cellulase titer (1.7 filter paper units [FPU]/mL, filter paper activity) in medium containing 10 g/L of manure (dry basis [w/w]), 2 g/L KH_2PO_4, 2 mL/L of Tween-80, and 2mg/L of $CoCl_2$. However, β-glucosidase activity in the *T. reesei*–enzyme system was very low. *T. reesei* was then cocultured with *A. phoenicis* to enhance the β-glucosidase level. The mixed culture resulted in a relatively high level of total cellulase (1.54 FPU/mL) and β-glucosidase (0.64 IU/mL). The ratio of β-glucosidase activity to filter paper activity was 0.41, suitable for hydrolyzing manure cellulose. The crude enzyme broth from the mixed culture was used for hydrolyzing the manure cellulose, and the produced glucose was significantly ($p < 0.01$) higher than levels obtained by using the commercial enzyme or the enzyme broth of the pure culture *T. reesei*.

Index Entries: Dairy manure; cellulase; β-glucosidase; *Trichoderma reesei*; *Aspergillus phoenicis.*

Introduction

During the past decade, the US animal industry has undergone a substantial structural change that has led to a rapid reduction in the number of animal operations and an increase in herd size on the remaining farms. These large, concentrated animal operations are a cause of great concern regarding the environmentally acceptable disposal and effective utilization of animal manure produced at these facilities. In particular, the current

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manure management practice of direct land application is coming under increased environmental and regulatory scrutiny, because the limited amount of land available for manure disposal may result in surface and groundwater contamination and air quality issues.

Animal manure contains a variety of nutrients including undigested crude fiber; protein; nitrogen; phosphorus; and minerals such as K, Ca, Mg, Fe, Cu, Mn, and Zn *(1,2)*. These nutrients represent a large potential bioresource for producing bio-based chemicals, materials, and energy. To date, however, limited efforts have been focused on utilizing the lignocellulosic components (the fiber) within the manure. Manure lignocellulosics can be hydrolyzed into saccharides, which can be further fermented into ethanol and other value-added products *(3)*. An enzyme-based process converting dairy manure cellulose into glucose has been developed previously. However, the process was considered economically infeasible because of the high cost of the commercial enzymes used *(4,5)*.

As a potentially less expensive alternative, cellulolytic enzymes could be produced by a number of bacteria and fungi that can use cellulose as a primary carbon source. Many cellulosic materials such as wood *(6,7)*, wastepaper *(8)*, bagasse *(9,10)*, wheat straw *(11,12)*, corncob *(13)*, wheat bran *(14)*, and fruit pomace *(15,16)* have been studied as potential substrates for microbial production of cellulase. There is, however, a lack of investigation on the cellulase production from manure cellulosics.

The aim of the present work was to study the potential of using dairy manure for cellulolytic enzyme production. The information would be useful for the development of a cost-effective process for cellulase production and subsequent enzymatic hydrolysis of manure cellulose.

Materials and Method

Collection and Characterization of Manure

Dairy manure was collected from the Dairy Center at Washington State University in Pullman, WA, and stored for later use in a freezer. The manure was mixed with water (2:1 [w/w]) and homogenized by an Osterizer® blender. The homogenized samples were analyzed for total carbon, total nitrogen, ammonium, phosphorus, potassium, calcium, magnesium, sodium, sulfur, and trace elements (iron, manganese, zinc, cobalt, copper) content. Dry matter (DM) and lignocellulosic content including neutral-detergent fiber (NDF), acid-detergent fiber (ADF), and acid-detergent lignin (ADL) were also analyzed.

Microorganism, Medium, and Culture Conditions

Trichoderma reesei RUT-C30 (ATCC 56765) and *Aspergillus phoenicis* QM 329 (ATCC 52007) were maintained (at 4°C) in potato dextrose agar slant and malt extract agar slant, respectively. The spores in the slant were suspended in 2 mL of medium (10^6–10^7 spores/mL) and transferred into a 250-mL Erlenmeyer flask containing 50 mL of medium. The subculture

medium was Mandel salts solution *(17)* supplemented with 2 mL/L of Tween-80, 1 g/L of peptone, and 10 g/L of glucose. Fungal cells were subcultured in an orbital shaker (175 rpm) at 30°C for one to two generations with the mycelium used for inoculum.

For cellulase production experiments, the composition of the medium was manure (10 g/L, unless specified) with 2 g/L of KH_2PO_4, 2 mL/L of Tween-80, and 2.0 mg/L of CoCl2. Homogenized manure was dispensed into 250-mL flasks containing 50 mL of medium. The pH of the medium was adjusted to 5.5 before autoclaving the medium at 121°C for 15 min. For pure culture, either *T. reesei* or *A. phoenicis* subculture (at 10% inoculum ratio [v/v]) was inoculated into each flask. For mixed culture, 10% (v/v) *T. reesei* and 10% (v/v) *A. phoenicis* inoculum was inoculated into each flask simultaneously. The flasks were incubated in an orbital shaker (175 rpm) at 27°C.

Enzymatic Hydrolysis of Manure Cellulose

The commercial enzyme Celluclast-1.5L (Sigma, St. Louis, MO), the crude enzyme broth from the pure culture of *T. reesei*, and the mixed culture of *T. reesei* and *A. phoenicis* were used for hydrolyzing the manure cellulose. Before enzymatic hydrolysis, manure samples were pretreated with dilute sulfuric acid to remove the protective hemicellulose-lignin layer. The pretreatment procedures were the same as those described previously *(5)*.

Hydrolysis was performed in 125-mL Erlenmeyer flasks containing 50 mL of enzyme solution and 5% (w/v) substrate (pretreated manure solid). The pH and temperature were adjusted to 4.8 and 50°C, respectively. For the three types of enzyme used, the enzyme loading was adjusted to the same filter paper activity (500 filter paper units [FPU]/L). At this FPU level, β-glucosidase activities of the commercial enzyme (Celluclast-1.5L) and crude enzyme broth of *T. reesei* were <25 IU/L, whereas the acivity of the mixed culture broth was about 250 IU/L. The flasks were incubated in an orbital shaker (175 rpm), and glucose concentration in the hydrolysate was determined to evaluate the efficiency of hydrolysis.

Analysis

Manure DM was determined by drying the samples at 105°C until the weight became constant. The content of cellulose, hemicellulose, and lignin can be determined by the analysis of NDF, ADF, and ADL, *(18)*. NDF is used to estimate the total lignocellulosic materials (including cellulose, hemicellulose, and lignin), ADF is used to estimate the content of lignin and cellulose, and lignin can be directly estimated from the ADL value *(18)*. Total carbon and total nitrogen were measured using automatic combustion (LECO CNS-2000). Ammonium was determined by the titrimetric method *(19)*. EPA method 3050/6010 was used to analyze other elements (potassium, phosphorus, calcium, magnesium, sodium, sulfur, iron, manganese, zinc, cobalt, copper). Here, the 3050 method is a nitric/hydrochlo-

ric acid digest, and 6010 indicates metal analysis by inductively coupled argon plasma spectroscopy.

Filter paper activity and β-glucosidase activity were determined according to standard IUPAC procedures *(20)*. One unit of filter paper activity was defined as the amount of enzyme that releases 1 μmol of glucose equivalents from Whatman no. 1 filter paper in 1 min. One unit of β-glucosidase activity was defined as the amount of enzyme converting 1 μmol of cellubiose to produce 2 μmol of glucose in 1 min. The glucose concentration in the cellubiose hydrolysate was measured using an enzyme assay kit (GAGO-20, Sigma). The saccharides produced in the enzymatic hydrolysate were analyzed by a Dionex ion chromatograph as described previously *(5)*.

Results and Discussion

Manure Characterization

Table 1 gives the chemical characteristics of dairy manure. Raw manure contained 14.6% DM, half of which was lignocellulosics (hemicellulose, cellulose, and lignin). In terms of elemental composition, carbon was the most abundant, followed by nitrogen, calcium, and potassium. The manure also contained ideal nutrients for fungal culture; phosphorus, magnesium, sodium, sulfur, iron, manganese, zinc, cobalt and copper, each with less than 1% of dry weight. The diet of the cows consisted of (DM/[animal·day]): 17 lb of alfalfa hay, 16 lb of alfalfa haylage, 7 lb of cottonseed, 7 lb of wheatmill run, and 20 lb of grain, with the high lignocellulosic content derived from the high content of alfalfa in the diet.

Cellulase Production by the Fungus T. reesei

T. reesei was grown in medium containing different manure concentrations with full Mandel salts *(17)* and 2 mL/L of Tween-80. Filter paper activity increased with manure concentration from 3.35 to 10 g/L (dry basis) and was maintained at a high level for a range of 10–15 g/L of manure (Fig. 1). Specific studies have reported that high cellulose concentration could result in a higher cellulase activity. These studies include *Chaetomium globosum* on fruit fiber *(21)* and *Neurospora crassa* on wheat straw *(11)*. Additionally, Reczey et al. *(7)* reported that the cellulase yield increased with cellulose level in the medium when using wood as a substrate. The results obtained in our work are consistent with those reports. However, the effects of manure concentration on cellulase production did not solely depend on the amount of cellulose but also perhaps depended on other nutrients or ions. Ultimately, though, because 10 g/L (dry basis) was the optimal level for filter paper activity, this manure level was used as the optimal manure level for *T. reesei* culture.

Although the full Mandel salts solution was used in the aforementioned experiments, some of the salts may not be necessary as the corresponding nutrients contained in the manure may be sufficient for growth

Table 1
Major Composition of Freshly Collected Dairy Manure [a]

DM Component		14.60 ± 0.25 % (w/w) (% of DM)
Lignocellulosics	NDF	49.10 ± 1.300
	ADF [b]	37.83 ± 1.010
	ADL [c]	11.24 ± 1.020
	Hemicellulose (NDF-ADF)	11.27 ± 0.900
	Cellulose (ADF-ADL)	26.59 ± 0.280
	Lignin (ADL)	11.24 ± 1.020
Elements	Carbon	50.51 ± 1.220
	Total nitrogen	3.03 ± 0.580
	NH_4-N	0.44 ± 0.029
	Potassium	1.24 ± 0.017
	Phosphorus	0.81 ± 0.054
	Calcium	2.41 ± 0.184
	Magnesium	0.966 ± 0.061
	Sodium	0.243 ± 0.019
	Sulfur	0.496 ± 0.021
	Iron	0.134 ± 0.012
	Manganese	0.015 ± 0.001
	Zinc	0.013 ± 0.001
	Cobalt	0.0002 ± 0.000
	Copper	0.0046 ± 0.000

[a] Data are expressed as the mean ± SD of three replicates.
[b,c] Acid-detergent nitrogen (1.57% of DM) was formed via the nonenzymatic browning reaction when nitrogen-enriched manure was heated above 50°C *(18)*. The true values of ADF and ADL of acid-pretreated manure were obtained by subtracting acid-detergent nitrogen from the apparent values *(18)*.

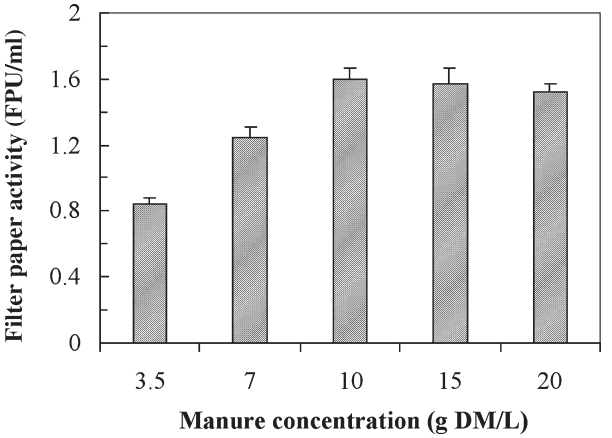

Fig. 1. Effects of manure concentration on cellulase production by the fungus *T. reesei*. Data are the means of three replicates and error bars show the standard deviation.

Table 2
The Distribution of Nutrients in Manure and Salts Solution[a]

Nutrient	From manure	From salts solution	Total concentration in medium	Nutrient ratio of manure to salts solution
Calcium	0.241 g/L	0.110 g/L	0.350 g/L	2.19:1
Magnesium	0.097 g/L	0.029 g/L	0.126 g/L	3.34:1
Iron	13.400 mg/L	1.000 mg/L	14.400 mg/L	13.4:1
Manganese	1.500 mg/L	0.521 mg/L	2.021 mg/L	2.88:1
Zinc	1.300 mg/L	0.317 mg/L	1.617 mg/L	4.10:1
Cobalt	0.020 mg/L	0.908 mg/L	0.928 mg/L	0.02:1
Nitrogen	0.303 g/L	0.436 g/L	0.739 g/L	0.69:1
Potassium	0.124 g/L	0.573 g/L	0.697 g/L	0.22:1
Phosphorus	0.081 g/L	0.456 g/L	0.537 g/L	0.18:1

[a]The calculation was based on 10 g/L (DM) of manure and the composition of Mandel salts solution *(17)*.

Table 3
Experimental Design for Eliminating Various Nutrients From Mandel Salts Solution *(17)* and Corresponding Cellulase Activity[a]

	Nutrient					Filter paper activity (IU/mL)
Run	KH_2PO_4	$CaCl_2$	$MgSO_4$	$(NH_4)_2SO_4$ and urea	Trace elements	
1	–	+	+	+	+	0.797 ± 0.085
2	+	–	+	+	+	1.74 ± 0.037
3	+	+	–	+	+	1.71 ± 0.057
4	+	+	+	–	+	1.61 ± 0.123
5	+	+	+	+	–	1.58 ± 0.109
6	+	+	+	+	+	1.59 ± 0.066

[a]–, The corresponding nutrient is eliminated from the salts solution; +, the corresponding nutrient is included in Mandel salts solution.

of the fungus. Thus, if identified, such nutrients could be eliminated from the medium recipe, without negative influence on the fungal cellulase production. To test this hypothesis, the nutrients distribution in manure (at 10 g/L) and in Mandel salts was analyzed (Table 2). It was found that the amounts of calcium, magnesium, iron, manganese, and zinc contained in the manure were much higher than those in the salt solution and that the nitrogen level from manure was lower but comparable (70% of) with that from the salts solution. However, the contributions of potassium and phosphorus from the manure were much lower (<23%) than those from the salt solution (Table 2).

The possibility of elimination of nutrients from the Mandel salts solution was further tested experimentally. As shown in Table 3, the elimi-

nation of nitrogen, calcium, magnesium, and trace elements from the salts solution had no negative influence on the cellulase production; cellulase production was almost the same (runs 4 and 5) or even better (runs 2 and 3) than that of the control. Additionally, even though nitrogen from manure was lower than that from the salts solution, it proved to be sufficient for the culture of fungus, and, therefore, elimination of a nitrogen source had no negative influence (Table 3). For KH_2PO_4-eliminated medium (run 1), however, it was found that *T. reesei* produced much lower cellulase than the control (Table 3). This is probably due to the insufficiency of potassium and phosphorus within the manure to support the growth of fungus (Table 2). Another reason may be that most of the manure phosphorus is in the form of organic phosphate and polyphosphates, which makes the utilization more difficult *(1,2,22)*.

Based on the experimental data in Table 3, a further experiment was conducted by growing the fungi in medium containing manure (10 g/L) with KH_2PO_4 (2 g/L), $CoCl_2$ (2 mg/L), and Tween-80 (2 mL/L) added. Although elimination of cobalt-containing trace elements solution appeared to have no negative influence on cellulase production (Table 3), cobalt was still included in the medium to avoid the possible negative effect of cobalt deficiency on fungal growth, because the manure contained very little of this element. The inclusion of cobalt at this level was considered an insignificant cost factor for the medium. Nitrogen, calcium, magnesium, and trace elements (except cobalt) were simultaneously eliminated from the salt solution. The medium containing manure with full Mandel nutrients added was used as a control. To give a detailed cellulase profile of the fungi under this condition, the time course of filter paper activity and β-glucosidase activity was monitored. As shown in Fig. 2, the three enzymes were in a similar pattern and increased in parallel with incubation time. Filter paper activity and β-glucosidase activity reached their highest levels at d 6. It was also found that the enzyme activities were at the same level as the control, suggesting that medium with reduced nutrients could sufficiently support a high cellulase production by *T. reesei* (Fig. 2).

The highest filter paper activity and β-glucosidase activity produced by *T. reesei* was 1.7 FPU/mL and 0.08 IU/mL, respectively, corresponding to a ratio of β-glucosidase to total cellulase of 0.047 (Fig. 2). It has been reported that an ideal ratio of β-glucosidase activity to filter paper activity is 0.12–1.5, depending on the source of enzyme and types of substrate *(6)*. For hydrolysis of manure cellulose, though, the optimal ratio of β-glucosidase activity to filter paper activity was about 0.38 *(5)*. This suggested that the β-glucosidase contained in *T. reesei*-derived cellulase was very low and, thus, insufficient to hydrolyze cellubiose to glucose.

The deficiency of β-glucosidase is common to most strains of *Trichoderma*. Several approaches have been attempted to overcome this deficiency. For example, a temperature and pH cycling strategy was applied to the culture of *T. reesei* RUT-C30 to increase β-glucosidase production *(23)*. In another study, the mutant *Trichoderma* E12 was grown on microcrystalline

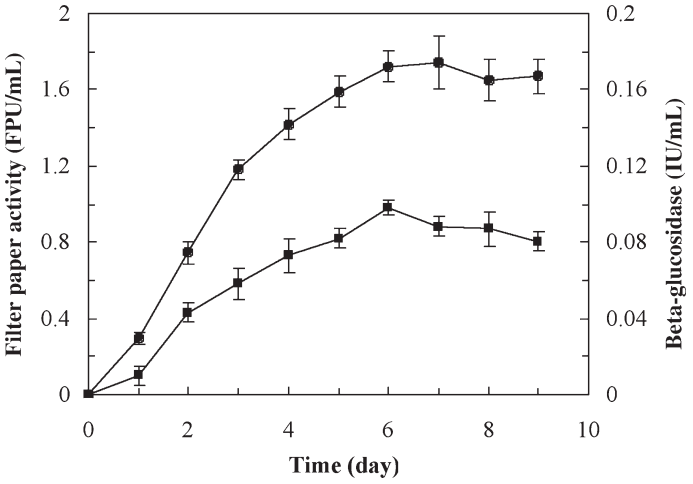

Fig. 2. Time course of filter paper activity (●) and β-glucosidase activity (■) in medium containing manure with KH_2PO_4, $CoCl_2$, and Tween-80 added. Data are the means of three replicates and error bars show the standard deviation.

cellulose with peanut cake used as a nitrogen source for a high C/N ratio. Results for that study showed a well-balanced ratio of β-glucosidase activity to filter paper activity *(24)*. *Trichoderma* could also be cocultured with the fungi *Aspergillus*, which is a good producer of β-glucosidase *(25–29)*. Such a mixed culture technique was therefore employed in the present work by growing *T. reesei* and *A. phoenicis* on dairy manure to achieve a high level of β-glucosidase.

Cellulase Production by Mixed Culture T. reesei *and* A. phoenicis

The trends of filter paper activity and β-glucosidase activity were in a similar pattern and increased in parallel with incubation time although *T. reesei* and *A. phoenicis* demonstrated different abilities for producing total cellulase and β-glucosidase (Fig. 3). *T. reesei* produced a high level of total cellulase (Fig. 3A) with very low β-glucosidase (Fig. 3B). On the other hand, the total cellulase produced by *A. phoenicis* was very low (Fig. 3A) whereas β-glucosidase activity was much higher (Fig. 3B). The mixed culture resulted in a relatively high filter paper activity and β-glucosidase activity simultaneously, although its filter paper activity was 15% lower than the pure culture of *T. reesei* (Fig. 3A) and β-glucosidase activity was 18% lower than the pure culture of *A. phoenicis* (Fig. 3A).

The mixed culture of the fungi *Trichoderma* and *Aspergillus* has been studied on various substrates. Compared with the corresponding pure cultures, the enzyme levels produced by the mixed culture are dependent on the fungi species and substrates used. For example, when *T. reesei* LM-UC4 and *A. phoenicis* QM 329 were grown on bagasse, the filter paper

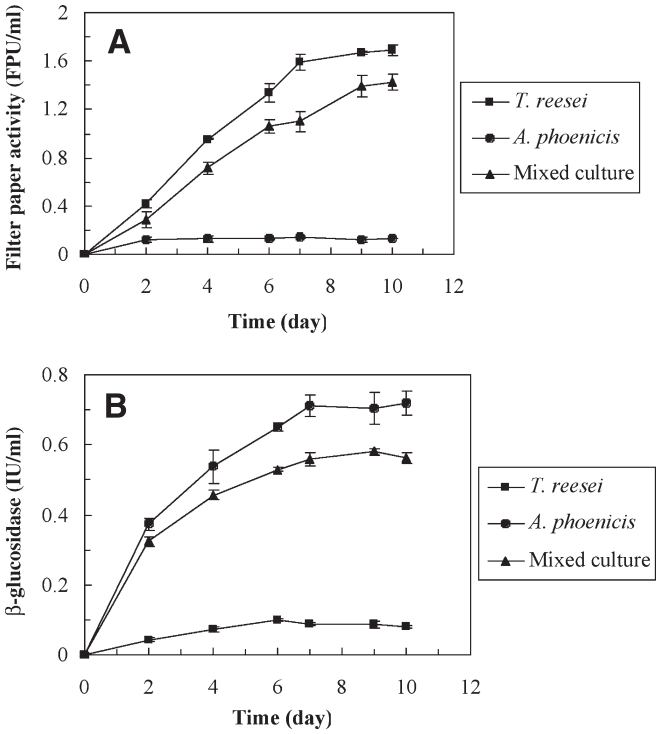

Fig. 3. Time course of **(A)** total cellulase (filter paper activity, FPU) and **(B)** total β-glucosidase production by pure culture of *T. reesei* and *A. phoenicis*, and mixed culture of the two fungi. Data are the means of three replicates and error bars show the standard deviation.

activity and β-glucosidase activity from the mixed culture were much higher than those of the corresponding pure cultures *(26)*. However, β-glucosidase produced by *Aspergillus niger* was higher than the mixed cultures of *T. reesei* LM-UC4 and *A. niger* when soymeal was added to the sugarcane bagasse *(29)*. Similar results were also observed in the mixed culture of *T. reesei* and *Aspergillus terreus* on bagasse *(30)*. When *T. reesei* RUT C30 and *A. phoenicis* were grown on starch substrate, both filter paper activity and β-glucosidase activity were lower than those of each pure culture *(25)*.

All of these findings suggest that the cellulase and β-glucosidase production from mixed fungi culture are species specific and dependent on the different substrates used. In the present work, the reduced filter paper activity and β-glucosidase activity of the mixed culture may be owing to the lack of synergism of the enzymes produced from the two species of fungi. Although the cellulase level was a little lower than that of the pure culture of *T. reesei*, the cellulolytic potential of the mixed culture could be markedly enhanced owing to the increased β-glucosidase.

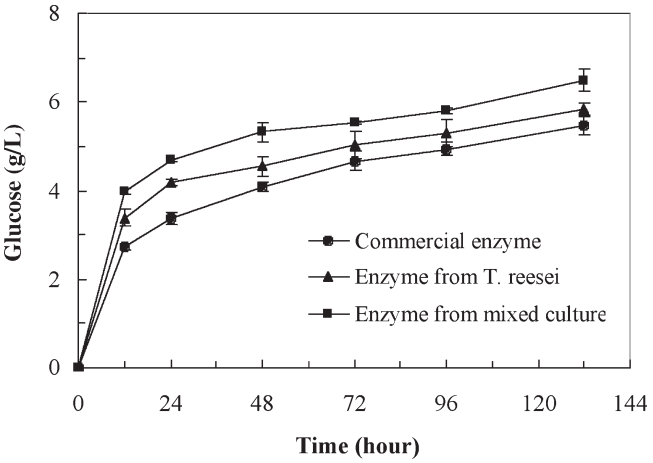

Fig. 4. Glucose concentration in hydrolysate during enzymatic hydrolysis of manure cellulose by using different enzyme sources. Data are the means of three replicates and error bars show the standard deviation.

Hydrolysis of Manure Cellulose by Different Enzyme Sources

To test the effectiveness of the enzymes produced from the mixed fungi culture, hydrolysis of manure cellulose was performed by using enzyme broth from the mixed culture, the pure culture of *T. reesei*, and commercial cellulase (Celluclast-1.5L), respectively. The concentration of glucose produced during hydrolysis was compared for different hydrolysis systems (Fig. 4). For each enzyme source used, the produced glucose followed a similar pattern; that is, glucose increased sharply for the first 12 h, and reached the highest levels at 96–132 h (Fig. 4). The produced glucose from mixed culture enzymes was significantly ($p < 0.01$; tested using the software SAS 8.0) higher than the levels obtained from the other two enzyme sources. The high glucose level was probably due to the high β-glucosidase contained in the mixed culture broth, with the ratio of β-glucosidase activity to filter paper activity being 0.41. In addition, it might be caused by other glycanase activities produced by *A. phoenicis*. The result suggests that the mixed fungi culture developed in the present work was an efficient method to produce cellulolytic enzymes in an effort toward utilization of manure cellulose.

Conclusion

The present work showed that dairy manure was a suitable substrate for cellulase production by both the fungi *T. reesei* and *A. phoenicis*. Manure at a concentration of 10 g/L (dry basis) could sufficiently support the growth of fungi by supplying not only cellulose (as carbon source), but also other nutrients including calcium, magnesium, iron, manganese, zinc and nitro-

gen. The mixed culture of *T. reesei* and *A. phoenicis* could produce cellulase containing a high level of β-glucosidase from dairy manure. β-Glucosidase activity and filter paper activity from the mixed culture system could reach 0.64 IU/mL and 1.54 FPU/mL, respectively, with a corresponding ratio of 0.41. The hydrolysis efficiency (in terms of glucose produced) by the mixed enzymes was higher than efficiencies by commercial enzyme and enzyme from the single culture of *T. reesei*. Our work demonstrated a possible way to develop an efficient cellulolytic system for the treatment of animal manure.

In the future, a technoeconomic analysis of the present cellulase production process using manure as a source of carbon and other nutrients should be performed, and its economic viability should be compared to the enzyme production on standard production medium in terms of both enzyme level and cost of the medium. Indeed, recent efforts by Genencor and Novozymes have reduced the enzyme cost significantly. Much of this reduction was achieved by improving production economics, in contrast to improving enzyme performance.

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References

1. Fontenot, J. P., Smith, L. W., and Sutton, A. L. (1983), *J. Anim. Sci.* **57 (Suppl. 2),** 221.
2. Moller, H. B., Sommer, S. G., and Ahring, B. K. (2002), *Bioresour. Technol.* **85,** 189–196.
3. Sun, Y. and Cheng, J. (2002), *Bioresour. Technol.* **83,** 1–11.
4. von Sivers, M. and Zacchi, G. (1995), *Bioresour. Technol.* **51,** 43–52.
5. Wen, Z., Liao, W., and Chen, S. (2004), *Bioresour. Technol.* **91,** 31–39.
6. Duff, S. J. B. and Murray, W. D. (1996), *Bioresour. Technol.* **55,** 1–33.
7. Reczey, K., Szengyel, Z., Eklund, R., and Zacchi, G. (1996), *Bioresour. Technol.* **57,** 25–30.
8. Ju, L. K. and Afolabi, O. A. (1999), *Biotechnol. Prog.* **15,** 91–97.
9. Rajoka, M. I. and Malik, K. A. (1997), *Bioresour. Technol.* **59,** 21–27.
10. Ogel, Z. B., Yarangumeli, K., Dundar, H., and Ifrij, I. (2001), *Enzyme Micro. Technol.* **28,** 689–695.
11. Romero, M. D., Aguado, J. Gonzalez, L., and Ladero, M. (1999), *Enzyme Micro. Technol.* **25,** 244–250.
12. Kalogeris, E., Christakopoulos, P., Katapodis, P., Alexiou, A., Vlachou, S., Kekos, D., and Macris, B.J. (2003), *Process Biochem.* **38,** 1099–1104.
13. Xia, L. and Cen, P. (1999), *Process Biochem.* **34,** 909–912.
14. Smits, J. P., Rinzema A., Tramper, J., van Sonsbeek, H. M., and Knol, W. (1996), *Appl. Microbol. Biotechnol.* **46,** 489–496.
15. Umikalsom, M. S., Ariff, A. B., Shamsuddin, Z. H., Tong, C. C., Hassan, M. A., and Karim, M. I. A. (1997), *Appl. Microbiol. Biotechnol.* **47,** 590–595.
16. Haddadin, M. S. Y., Abu-Reesh, I. M., Haddadin. F. A. S.. and Robinson, R. K. (2001), *Biores. Technol.* **78,** 225–230.
17. Mandel, M. and Weber, J. (1969), *Adv. Chem. Ser.* **95,** 391–414.

18. Goering, H.K. and Van Soest, P.J. (1970), in *Agricultural Handbook* No. 379, Agricultural Research Service, US Department of Agriculture, Washington, DC, pp. 1–20.
19. Eaton, A. D., Clesceri, L. S., and Greengerg, A. E. (1995), *Standard Methods for the Examination of Water and Wastewater*, 19th Ed., American Public Health Association, Washington, DC.
20. Ghose, T. K. (1987), *Pure Appl. Chem.* **59,** 257–268.
21. Umikalsom, M. S., Ariff, A. B., Zulkifli, H. S., Tong, C. C., Hassan M. A., and Karim, M. I. A. (1997) *Bioresour. Technol.* **62,** 1–9.
22. Henze, M., Harremoes, P., Jansen, J. L. C., and Arvin, E. (1996). *Wastewater Treatment: Biological and Chemical Process*, 2nd Ed., Springer, Berlin.
23. Tangnu, S. K., Blanch, H. W., and Wilke, C. R. (1981), *Biotechnol. Bioeng.* **23,** 1837–1849.
24. Cochet, N. (1991), *Enzyme Micro. Technol.* **13,** 104–109.
25. Duff, S. J. B., Cooper, D. G., and Fuller, O. M., (1987), *Enzyme Micro. Technol.* **9,** 47–52.
26. Duenas, R., Tengerdy, R. P., and Gutierrez-Correa, M. (1995), *World. J. Microbiol Biotechnol*. **11,** 333–337.
27. Gutierrez-Correa, M. and Tengerdy, R.P. (1997), *Biotechnol. Lett.* **19,** 665–667.
28. Flachner, B., Brumbauer, A., and Reczey, K. (1999), *Enzyme Micro. Technol.* **24,** 362–367.
29. Gutierrez-Correa, M., Portal, L., Moreno, P., and Tengerdy, R. P. (1999), *Bioresour. Technol.* **68,** 173–178.
30. Massadeh, M. I., Yusoff, W. M. W., Omar, O., and Kader, J. (2001), *Biotechnol. Lett.* **23,** 1771–1774.