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Note

Miniaturized enzyme production and development of micro-assays for cellulolytic and xylanolytic enzymes

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ABSTRACT

Miniaturized fungal cultivation and enzyme assays were developed. Cultivation for enzyme production was performed in 50 mL conical tubes. In addition, the miniaturized enzyme assays reduced the amount of enzymes and reagents necessary. These procedures can be adopted in screening fungi to determine if they produce cellulolytic and xylanolytic enzymes.

Cellulases and xylanases are used in the pulp, food, beer, and agricultural industries (Bhat, 2000), and are essential for the efficient conversion of lignocellulosic biomass to ethanol. Recently, worldwide interest in the use of these enzymes to produce bioethanol has been increasing, and studies on the production and utilization of cellulolytic and xylanolytic enzymes are in progress (Bhat, 2000; Li et al., 2009). A variety of fungi secrete large amounts of these enzymes into the culture medium. Fungi are normally cultured in a homogeneous culture medium by submerged fermentation with continuous stirring (Cianchetta et al., 2010). However, this requires large amounts of reagents, substrates, and water. In addition, the standard assays to measure the activities of enzymes produced are time-consuming and waste necessary reagents and enzymes. Hence, Xiao et al. (2004, 2005) developed a microplate-based filter paper assay (to detect total cellulase activity) and carboxymethylcellulose assay (to detect endoglucanase activity). The aims of this study were (1) to develop a small-scale method to cultivate fungi in 50 mL conical tubes, and (2) to develop miniaturized enzyme assays (micro-assay) for carboxymethylcellulase (CMCase), xylanase, β-glucosidase, β-xylosidase, and cellobiohydrolase. These methods were optimized and suitable for screening fungi that produce cellulolytic and xylanolytic enzymes.

All commercial enzymes and other chemicals were purchased from Sigma-Aldrich (USA). *Phanerochaete chrysosporium* KCTC6293 was supplied by the Korean Collection for Type Cultures (KCTC, Daejeon, Korea) and *Trichoderma harzianum* KUC5182 was selected from the Korea University Culture Collection (KUC, Seoul, Korea). Both strains of fungi were grown and maintained on malt extract media (MEA; 2%, w/v) at room temperature. For production of enzymes, one agar plug with mycelium (*P. chrysosporium*) or 200 μL of a spore suspension (10^6 spores/mL; *T. harzianum*) was inoculated in 10 mL of Mandels' medium (Juhász et al., 2005) containing 5% (w/v) rice straw as an energy source and substrate. In small-scale cultivation, the sterile rice straw (0.5 g) and culture medium (10 mL) were dispensed into each 50 mL sterilized conical tube (SPL, Korea). The culture medium was inoculated with the appropriate fungal culture and the tubes were sealed with silistoppers. The tubes were placed into a steel wire conical tube rack (50-holes) that was installed on a stand placed at a 50° angle on a rotary shaker. Cultivation was performed with agitation (100–250 rpm). In contrast, standard cultivation was performed in 250 mL Erlenmeyer flasks containing 50 mL medium. Cultures of fungi were incubated on a rotary shaker at 150 rpm (Domingues et al., 2000; Teoh and Mashitah, 2010). To allow comparison between small-scale and standard cultivation, the optimal rate of agitation (rpm) for growth in conical tubes was selected and the two cultivation methods were performed at 28 °C for 7 days simultaneously.

For miniaturized enzyme assay (micro-assay), the reagents and the enzyme volume used for CMCase and xylanase assays were reduced by 20-fold, and those for β-glucosidase, β-xylosidase, and cellobiohydrolase assays were reduced by 5-fold as described below relative to the standard assay (macro-assay) protocols (Bailey et al., 1992; Ghose, 1987; Rasmussen et al., 2006; Ximenes et al., 1996). CMCase assay for endo-β-1, 4-glucanase activity was performed using carboxymethyl cellulose. A volume of 25 μL of enzyme and 25 μL of 2% CMC (C5678) were incubated at 50 °C for 30 min. After incubation, 150 μL of DNS reagent (Bailey, 1988) was added and boiled for 5 min. Following the

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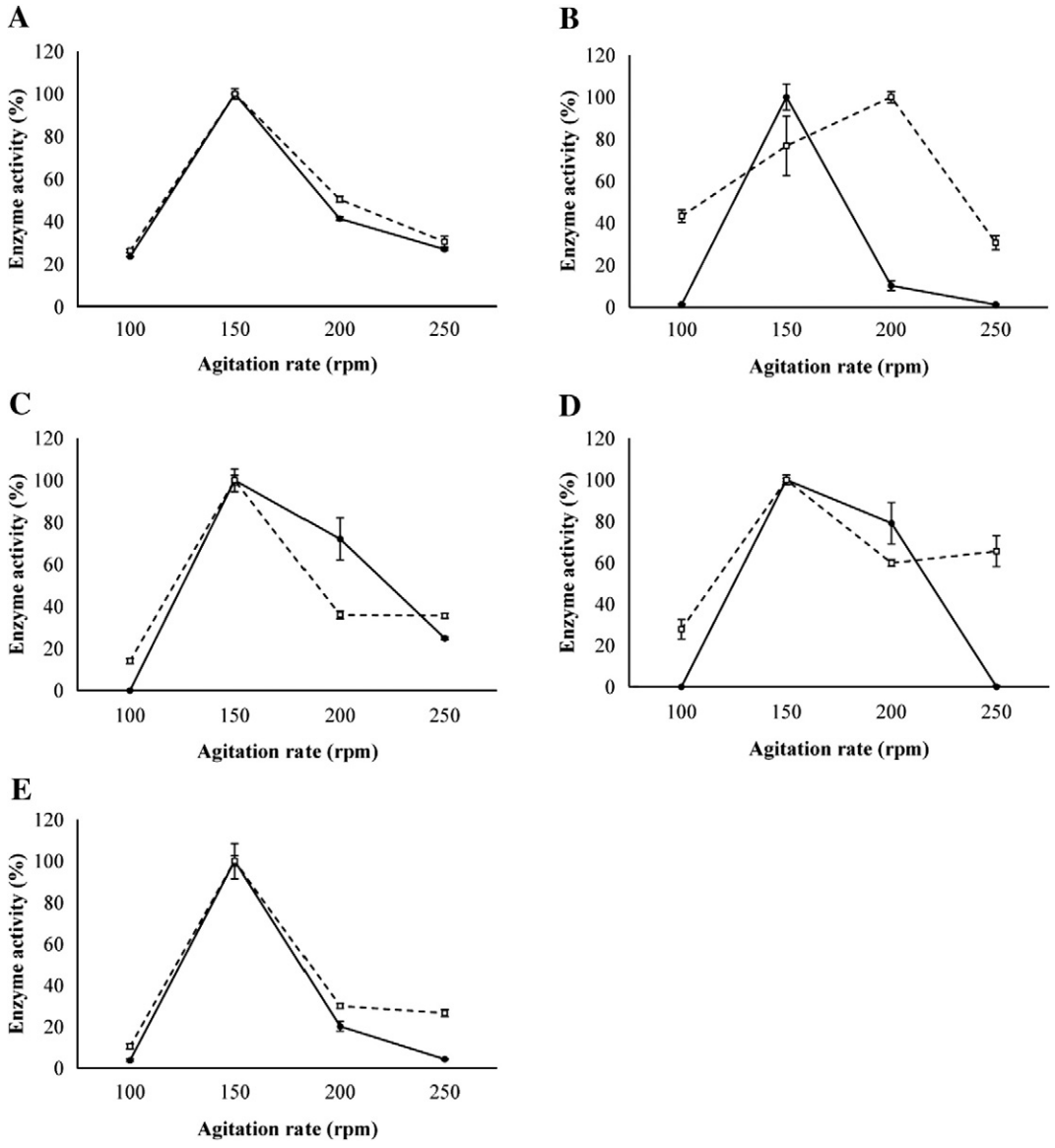

Fig. 1. Cell culture agitation rate and optimal production of various enzymes secreted from Phanerochaete chrysosporium KCTC6293 (–) and Trichoderma harzianum KUC5182 (--). A, CMCase; B, xylanase; C, β-glucosidase; D, β-xylosidase; and E, cellobiohydrolase.

color development, 33 μL of each sample was then diluted with 165 μL of distilled water in a 96-well microtiter plate. The absorbance was measured at 540 nm. To measure the activity of xylanase, 7 μL of enzyme and 81 μL of 1% birchwood xylan (X0502) were incubated at 50 °C for 5 min, 135 μL DNS reagent was then added. After boiling for 5 min, the reaction products (200 μL) were measured at 540 nm. One unit per mL of CMCase or xylanase activity was described as the amount of enzyme required to release 1 μmol of glucose or xylose equivalents per milliliter of culture medium per minute. In addition, enzyme activities of β-glucosidase, β-xylosidase, and cellobiohydrolase were measured using *p*-nitrophenyl-β-D-glucopyranoside (N7006), *p*-nitrophenyl-β-D-xylopyranoside (N2132), and *p*-nitrophenyl-β-D-cellobioside (N5759), as substrates, respectively. The assay mixture of 200 μL containing the appropriate substrate (20 μL of a 10 mM solution in

Table 1
Enzyme activities (U/mL) and protein concentration (mg/mL) obtained from flask or conical tube cultivation of fungi at 150 rpm[a].

Enzymes	*Phanerochaete chrysosporium*		*Trichoderma harzianum*	
	Flask	Conical tube	Flask	Conical tube
CMCase	0.63 ± 0.13	1.47 ± 0.03	1.08 ± 0.25	1.39 ± 0.04
Xylanase	14.58 ± 1.68	159.96 ± 11.95	239.85 ± 43.88	1287.79 ± 102.12
β-Glucosidase	0.02 ± 0.00	0.30 ± 0.04	0.57 ± 0.12	1.68 ± 0.12
β-Xylosidase	0.01 ± 0.00	0.03 ± 0.00	0.06 ± 0.03	0.09 ± 0.01
Cellobiohydrolase	ND[b]	0.05 ± 0.00	0.04 ± 0.01	0.15 ± 0.02
Protein (mg/ml)	0.39 ± 0.09	0.46 ± 0.01	0.45 ± 0.02	0.32 ± 0.02

[a] All experiments were carried out in triplicate for each cultivation method.
[b] Not detected.

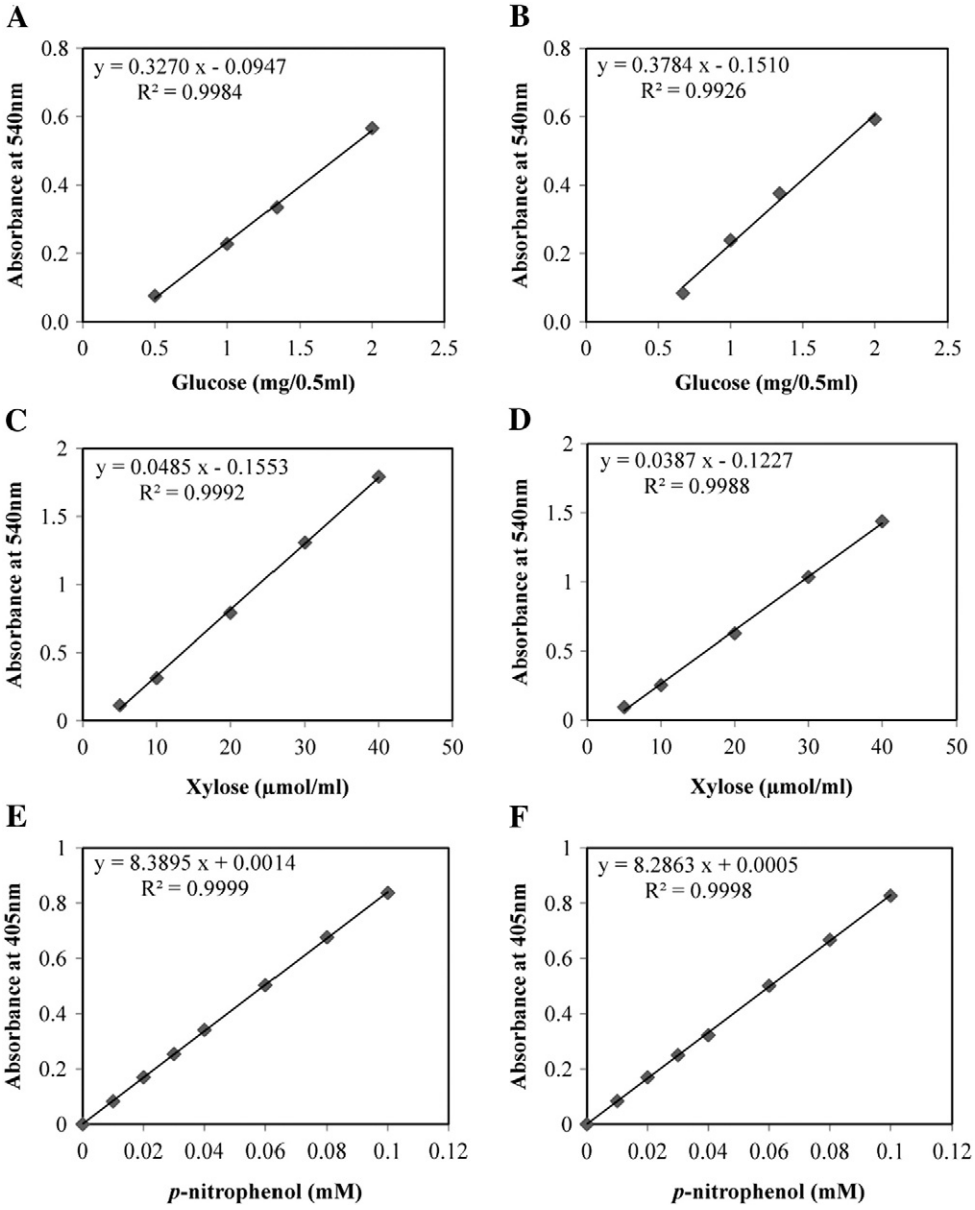

Fig. 2. Standard curves for measuring the release of glucose, xylose, and *p*-nitrophenol. A, C, and E curves for macro-assay; and B, D, and F curves for micro-assay.

100 mM sodium acetate buffer, pH 5.0) and 20 µL of the appropriate enzyme was incubated at 50 °C for 5, 10, and 30 min for β-glucosidase, β-xylosidase, and cellobiohydrolase, respectively. To stop the reaction, 20 µL 2 M Na_2CO_3 was added. The *p*-nitrophenol liberated was measured at 405 nm. One unit per mL of enzyme activity was expressed as the amount of enzyme required to liberate 1 µmol of *p*-nitrophenol from each substrate per milliliter of culture medium per minute. The protein content was determined using the method of Bradford (1976), with bovine serum albumin.

To evaluate the micro-assay procedure (Ramada et al., 2010; Xiao et al., 2004), commercial enzyme activities measured by macro- and micro-assays were compared. CMCase, cellobiohydrolase, and β-xylosidase activities were determined using Celluclast® 1.5 L (C-2730). Xylanase and β-glucosidase were measured using Xylanase (X-3876) and Novozyme® 188 (C-6105), respectively. Ten samples of each enzyme were assayed with all reactions performed in triplicate. Data were analyzed using the SAS System for Windows V8.2 (SAS Institute Inc., Cary, NC). The *t*-test was performed to analyze statistical differences between macro- and micro-assays with commercial enzymes.

Since no earlier studies concerning agitation rates during cultivation of fungi in conical tubes were found, an experiment was conducted to determine the optimal agitation rates (rpm) in conical tubes. The rates were chosen with reference to the ranges previously reported for culturing fungi using general conditions (Haltrich et al., 1994; Oguntimein et al., 1992): 100, 150, 200, and 250 rpm. In most instances, the enzymes examined showed the highest activity when the fungi were cultured at 150 rpm and most enzymes exhibited the lowest activity when the fungi were cultured at 100 rpm (Fig. 1). Therefore, the conical tube cultivation was performed at 150 rpm. As shown in Table 1, most of enzymes secreted from both *P. chrysosporium* and *T. harzianum* showed higher values of enzyme activities in the conical tube cultivation compared with the flask cultivation. In addition, by using a 50-place conical tube rack, enzymes could be produced from fifty different fungal samples at a time and there was no need to transfer the media into other vessels for centrifugation. Thus, our results indicate that this simple procedure minimizes reagent consumption, labor, and time required.

To compare the accuracy of the macro- and micro-assays (Xiao et al., 2004, 2005), standard curves were produced. Fig. 2 indicates that both

Table 2
Statistical comparison of commercial enzyme activities (U/mL) between macro- and micro-assays[a].

Repetitions	CMCase		Cellobiohydrolase		β-Glucosidase		β-Xylosidase		Xylanase	
	Macro	Micro	Macro	Micro	Macro	Micro	Macro	Micro	Macro	Micro
1	476.4	452.5	14.0	14.2	352.5	337.1	64.8	63.2	5623.0	5262.0
2	473.8	463.2	14.0	14.5	348.7	344.8	66.0	63.3	5842.7	5184.0
3	490.7	466.8	14.3	14.5	364.3	327.2	65.7	62.3	6051.4	5292.3
4	489.6	472.6	14.3	14.4	352.2	339.5	65.6	63.0	6004.7	5305.3
5	483.6	471.0	14.4	14.4	348.4	362.7	65.3	62.7	5977.2	5162.4
6	476.0	483.3	14.5	14.8	349.3	370.4	66.0	63.1	5922.3	5192.7
7	481.3	483.3	14.4	14.4	357.9	364.1	66.2	63.7	5848.1	5456.8
8	489.2	480.4	14.7	14.7	356.6	346.3	65.8	64.6	5905.8	5326.9
9	490.0	488.2	14.2	14.7	366.8	364.6	65.0	64.1	5853.6	5487.2
10	478.1	490.1	14.7	14.6	350.9	366.5	65.6	64.4	5705.3	5322.6
Average	482.9 ± 6.6	475.1 ± 12.0	14.4 ± 0.2	14.5 ± 0.2	354.8 ± 6.5	352.3 ± 15.1	65.6 ± 0.4	63.4 ± 0.8	5873.4 ± 131.7	5299.2 ± 108.7
$t_{0.05}$	2.10[b]		2.10[c]		2.18[d]		2.10[e]		2.10[f]	

[a] All data obtained in triplicate. *T*-test: The data obtained from the micro-assay were compared to those obtained from the macro-assay.
[b] The calculated *t*- value is 1.78, which is below the critical *t*-value of 2.10 at $P = 0.05$ and DF = 18.
[c] The calculated t-value is 1.76, which is below the critical *t*-value of 2.10 at $P = 0.05$ and DF = 18.
[d] The calculated t-value is 0.47, which is below the critical *t*-value of 2.18 at $P = 0.05$ and DF = 12.3.
[e] The calculated t-value is 7.84, which is above the critical *t*-value of 2.10 at $P = 0.05$ and DF = 18.
[f] The calculated t-value is 10.63, which is above the critical *t*-value of 2.10 at $P = 0.05$ and DF = 18.

methods accurately measure glucose, xylose, and *p*-nitrophenol amounts in the range of concentrations tested.

Various commercial enzymes were used to verify that the enzyme activities obtained by micro-assays are similar to those obtained by the macro-assays. Table 2 shows that there is no significant difference between the mean values measured with the two methods (macro- vs. micro-assay) for CMCase, β-glucosidase, and cellobiohydrolase. On the other hand, the difference between the two methods for β-xylosidase and xylanase is statistically significant, but the cause can be due to the very low variance in enzyme activities among the 10 samples tested. It seems therefore, that the difference will not greatly affect screening performance. The advantages of the micro-assays developed in this study are that a number of different fungal samples can be handled and measured at once. The enzyme prepared can be easily measured using less reagents, substrates, and time resulting in cost savings (Berlin et al., 2006).

In conclusion, methods to simplify cultivation of fungi and enzyme assay were developed by miniaturizing standard methods. Small cultures grown in 50 mL conical tubes instead of 250 mL flasks conserve resources and are more convenient. Also, the micro-assays require less labor and time than the standard macro-assays. Hence, the miniaturized cultivation and enzyme assay methods are useful procedures for screening fungi to determine if they produce cellulolytic and xylanolytic enzymes.

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