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Characterization of Selected Cellulolytic Activities of Multi-enzymatic Complex System from *Penicillium funiculosum*

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The presence of endo-1,4-β-D-glucanase, cellobiohydrolase, and β-glucosidase activities in a multi-enzymatic complex system from *Penicillium funiculosum* was investigated. The interesting feature of these enzymes is their synergistic action for the hydrolysis of the native cellulose into glucose units. Both endo-1,4-β-D-glucanase and cellobiohydrolase showed broader pH activity profiles, with pH optima of 4.0 and 4.0−5.0, respectively. However, β-glucosidase activity showed a narrow pH-activity profile, with an optimum pH of 4.5. The different cellulolytic activities were stable in the acidic pH range of 2.5−6.0 and showed a similar optimal temperature of 60 °C. Although β-glucosidase has shown a close catalytic efficiency as that of endo-1,4-β-D-glucanase, its thermal stability was lower. However, the thermal stability profile of cellobiohydrolase was close to that of endo-1,4-β-D-glucanase. The results also revealed the presence of high levels of endo-1,3-1,4-β-D-glucanase, endo-1,3-β-D-glucanase, and pectinase activities in the multi-enzymatic cellulolytic complex system. Moreover, the investigated multi-enzymatic complex system was effective in degrading the nonstarch polysaccharides of soybean meal.

KEYWORDS: Cellulolytic complex; *Penicillium funiculosum*; endo-1,4-β-D-glucanase; cellobiohydrolase; β-glucosidase

INTRODUCTION

There has been an increasing interest in the complete degradation of plant cell-wall polysaccharides, because of their high-energy content in food and feedstuffs (*1, 2*). Because of the complexity and diversity of structural plant cell-wall polysaccharides, their complete degradation requires a set of synergistically acting enzymes (*1*). Bacteria and filamentous fungi were found to be capable of degrading the cell-wall polysaccharides by producing a mixture of functionally distinct glycosyl hydrolases (*3, 4*). However, only a few fungi, including *Penicillium*, *Aspergillus*, and *Trichoderma* species, are able to produce high levels of complex sets of glycosyl hydrolases (*2, 5*).

Much of the research interest has been directed toward the development of a biocatalytic process, whereby the cellulose could be hydrolyzed into glucose units by a microbial multi-cellulolytic complex system, involving many cellulases, such as endo-1,4-β-D-glucanases, cellobiohydrolases, and β-glucosidases (*6, 7*). The efficiency of the synergistic action of cellulases in a multi-enzymatic complex system was reported (*8, 9*) to be higher than that of their individual action. Indeed, endo-1,4-β-D-glucanases randomly hydrolyze the internal β-1,4-glycosidic linkage in the cellulose polymer, whereas cellobiohydrolases are involved in the hydrolysis of either nonreducing or reducing terminals of cellulose and cleave off the cellobiose (*10, 11*). Because cellobiohydrolases attack only the ends of the glycosidic chains, their activities are generally dependent upon those of endo-1,4-β-D-glucanases. On the other hand, β-glucosidases hydrolyze the cello-oligosaccharides and cellobiose into glucose units. Moreover, some of cellulolytic microorganisms have shown the ability to produce other glycosyl hydrolases, including pectinases, xylanases, and endo-1,3-1,4-β-D-glucanases, which could facilitate the access of cellulases to their substrates by hydrolyzing the associated plant polysaccharides (*12, 13*).

Most reported fungi secreting the multi-enzymatic cellulolytic complex system are *Trichoderma viride*, *Trichoderma reesei*, *Aspergillus niger*, and *Penicillium funiculosum* (*4, 14, 15*). However, the efficiency of the multi-enzymatic complex systems, produced by *T. viride* and *T. reesei*, was limited by their relatively low cellulolytic activity and thermostability (*15*). Moreover, the low β-glucosidase activity, obtained from *T. reesei*, has led to a strong enzyme inhibition, as a result of the accumulation of cellobiose (*16*). *P. funiculosum* is recognized as a potential and important source of highly active multicellulolytic complex system capable of degrading cellulose (*6, 14, 17*).

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The overall objective of the present study was the characterization, in terms of pH, temperature, thermal stability, and other kinetic properties, of selected endo-1,4-β-D-glucanase, cellobiohydrolase, and β-glucosidase activities, present in the multi-enzymatic cellulolytic complex system produced by *P. funiculosum*. The levels of other selected endoglucanase and pectinase activities in the multi-enzymatic complex system were also investigated. In addition, the capacity of this multi-enzymatic complex system of degrading the nonstarch polysaccharides of the soybean meal was evaluated.

MATERIALS AND METHODS

Materials. Substrates, including *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-β-D-lactoside, laminarin, citrus pectin, glucose, and 3,5-dinitrosalicylate, as well as amylase from *Bacillus licheniformis* and amyloglucosidase from *A. niger* were purchased from Sigma Chemical Co. (St. Louis, MO). Carboxymethyl cellulose and barley β-glucan were obtained from Megazyme (Wicklow, Ireland). Chemical reagents, including KCl, H_2SO_4, NaOH, potassium sodium tartrate, *p*-nitrophenol, sodium carbonate, sodium acetate, sodium citrate, monopotassium phosphate, dipotassium phosphate, and Tris, were purchased from Fisher Scientific (Fair Lawn, NJ). Nylon bags (F57, pore size 50 μm) were obtained from Ankom Technology Co. (Fairport, NY).

Production and Preparation of the Multi-enzymatic Complex Extract. The *P. funiculosum* strain (IMI 378536), used to produce the multi-enzymatic complex system, was obtained from the International Mycological Institute (Bakeham Lane, Englefield Green, Egham, Surrey, U.K.). The fungus was grown in batch culture at 30 °C on a modified medium of Steiner et al. (*18*), containing cellulose and corn steep liquor as the main carbon and nitrogen sources, respectively. The pH of the medium was adjusted, within the range of 3.0−6.0, by the addition of H_2SO_4 and NaOH. After 7 days of culture, the liquid medium was recovered by vacuum filtration of the mycelia and concentrated by tangential flow ultrafiltration through a membrane with a cutoff of 10 kDa. The multi-enzymatic complex extract was stabilized by the addition of sorbitol (5%, w/v) and potassium sorbate (3%, w/v) as an antimicrobial agent.

Enzymatic Assays. Endo-1,4-β-D-glucanase, endo-1,3-β-D-glucanase, endo-1,3-1,4-β-D-glucanase, and pectinase activities were assayed, according to a modification of the method of Wood and Bhat (*19*), using carboxymethyl cellulose (0.5%, w/v), laminarin (1%, w/v), barley β-glucan (1%, w/v), and citrus pectin (0.5%, w/v) as substrates, respectively. The enzymatic reaction was initiated by the addition of 0.25 mL (0.2−20 mg of protein) of the multi-enzymatic complex system extract suspension to 0.75 mL of the polysaccharide substrate solution, prepared in the sodium acetate buffer (0.2 M, pH 4.0). The reaction mixture was incubated in a water bath shaker at 50 °C with continuous agitation of 150 rpm. After 20 min, the enzymatic reaction was halted (*20*) by the addition of 1.5 mL of 1% (w/v) dinitrosalicylate reagent, prepared in 1.6% (w/v) NaOH. The reaction mixture was then placed in a boiling water bath for 5 min, for the development of reducing sugar color. Prior to cooling at room temperature, 0.5 mL of potassium sodium tartrate solution (50%, w/v) was added to the mixture. The absorbance of the resulting mixture was measured spectrophotometrically at 540 nm, against reagent blank, using DU-65 spectrophotometer (Beckman Instruments, Inc., San Ramon, CA). Two blank assays, without substrate or without enzyme, were conducted in tandem of the trials. The amount of the released reducing sugar was determined from the standard curve, constructed with the standard solutions of glucose and galacturonic acid. One unit of enzyme-specific activity was defined as the amount of enzymatic protein required to release 1 μmol of reducing sugar per min of reaction under the assay conditions.

β-Glucosidase and cellobiohydrolase activities were assayed, according to a modification of the method described previously by Deshpande et al. (*21*), using *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-lactoside as substrates, respectively. For β-D-glucosidase activity, the assay was performed by mixing 0.2 mL of 4 mM *p*-nitrophenyl-β-D-glucoside and 0.2 mL (0.1−5 mg of protein) of the multi-enzymatic complex system extract suspension, prepared in the sodium acetate buffer (0.2 M, pH 4.5). For cellobiohydrolase activity, the reaction mixture consisted of 0.2 mL of 4 mM *p*-nitrophenyl-β-D-lactoside, 0.02 mL of 0.2 M gluconolactone, and 0.18 mL (1−10 mg of protein) of the multi-enzymatic complex system extract suspension. After 20 min of incubation at 40 °C, the enzymatic reaction was halted by the addition of 1.2 mL of 1 M sodium carbonate. The absorbance of the reaction mixture was measured at 400 nm, using the Beckman spectrophotometer. Two blank assays, without enzyme or without substrate, were prepared in tandem of the trials. Both enzymatic reactions and blanks were run in triplicate. One unit of enzyme specific activity was defined as the amount of enzymatic protein that liberates 1 μmol of *p*-nitrophenol per min of reaction, under the assay conditions. The molar extinction coefficient of *p*-nitrophenol was estimated at 21.6 mM^{-1} cm^{-1}, using the standard solution of *p*-nitrophenol.

Determination of Optimum pH and pH Stability. To determine the optimum pH of the selected cellulolytic activities, including endo-1,4-β-D-glucanase, cellobiohydrolase, and β-glucosidase, the enzymatic assays were carried out at pH values ranging from 2.0 to 9.0, using different buffer systems (0.2 M), including KCl−HCl buffer (pH 2.0–2.5), sodium citrate buffer (pH 3.0–3.5), sodium acetate buffer (pH 4.0–5.5), potassium phosphate buffer (pH 6.0–7.0), and Tris-HCl buffer (pH 7.5–9.0). The pH stability of the selected cellulases was determined by a preincubation of the multi-enzymatic complex system extract in various pH buffer solutions for 2 h at 25 °C. After readjustment to the optimal pH, the residual cellulolytic activities were assayed as described above.

Determination of Optimum Temperature and Thermal Stability. To determine the optimum temperature, the selected cellulolytic activities were assayed at a wide range of temperatures (25−85 °C) in 0.2 M sodium acetate buffer at the optimum pH. The thermal stability was investigated by incubating the multi-enzymatic complex system extract in sodium acetate buffer (0.2 M, at its optimum pH stability) at selected temperatures (25−70 °C) for 2 h. After cooling to room temperature, the residual cellulolytic activities were measured, using the standard assay conditions as described above.

Determination of Kinetic Parameters. The kinetic parameters (K_{mapp} and V_{maxapp}) of the selected cellulases were investigated at the optimized conditions. The specific activities were measured, using various substrate concentrations. K_{mapp} and V_{maxapp} values were estimated, mathematically from the Lineweaver–Burk plot, using the Sigma Plot program (Jandel Scientific, Germany).

Determination of Nonstarch Polysaccharide Degradability. Prior to the enzymatic degradation, the soybean meal was defatted with the mixture of chloroform/methanol (2:1, v/v) at a ratio of 1:4 (w/v, meal per solvent) using the Soxhlet extractor system. The defatted soybean meal (∼1 g/bag) was introduced into the nylon bags (ANKOM Technology, pore size of 25 μm) to eliminate the filtration step and to allow the analysis of the residue rather than the filtrate. Six sealed bags were placed in 1 L glass bottle containing 400 mL of sodium acetate buffer (0.2 M, pH 4.5). The bottle was placed horizontally in a shaker water bath (40 °C, 1 h). A total of 80 mL of the multi-enzymatic complex system extract (50−433 mg of protein) was introduced into the bottle. A blank assay, without the enzymatic treatment, was run in tandem of the trials. After 4 or 24 h incubation, the buffer solution was decanted off and the bags were rinsed 6 times with deionized distilled water and dried in the oven at 50–55 °C for 48 h. The dried residual solid materials were subjected to nonstarch polysaccharide (NSP) analysis, according to a modification of the Uppsala total dietary fiber method (*22*). To remove the starch component, along with monosaccharides and oligosaccharides, the sample was sequentially digested with commercial amylase from *Bacillus licheniformis* and amyloglucosidase from *A. niger*. A defined amount of sample (0.4 g) was suspended in 5.0 mL of sodium phosphate buffer (0.2 M, pH 6.0) and incubated with 50 μL of amylase suspension at 95 °C for 1 h. After cooling to 60 °C, the pH of the digested mixture was adjusted to 4.1–4.8 with 0.3 N HCl, before the addition of 100 μL of amyloglucosidase suspension. After incubation (1 h, 60 °C), 20 mL of ethanol (95%) were added to yield a final concentration of 800 mL of ethanol per 1 L of digested solution and the resulted mixture was stored overnight at 4 °C. The precipitated pellets were recovered by centrifugation (3000*g*, 20 min) and hydrolyzed by a two-stage H_2SO_4

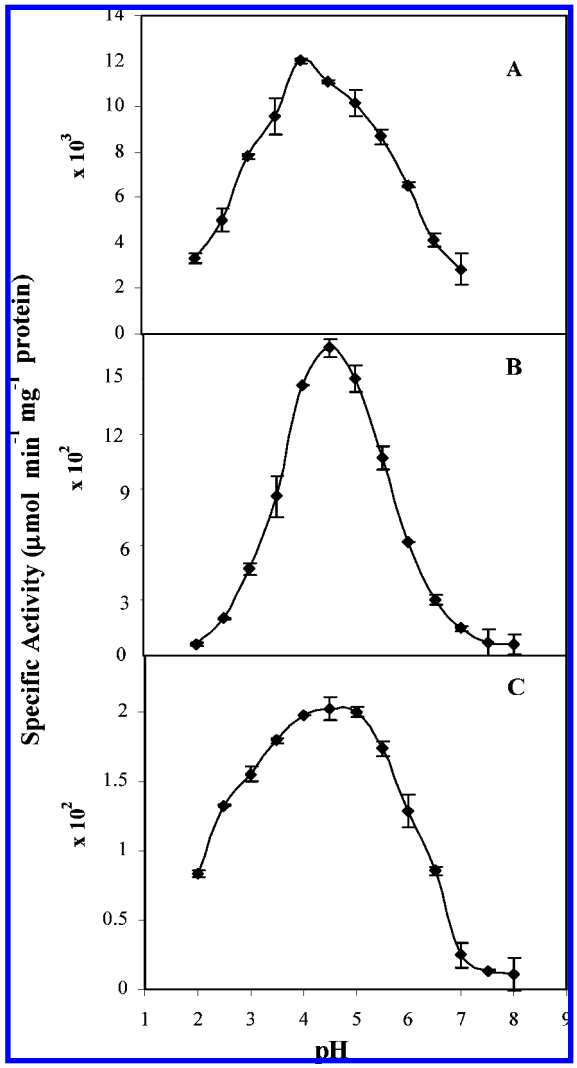

Figure 1. Effect of pH on selected cellulolytic specific activities of the multi-enzymatic complex system from *P. funiculosum*: (**A**) endo-1,4-β-D-glucanase, (**B**) β-glucosidase, and (**C**) cellobiohydrolase.

treatment; the precipitated pellets were first suspended in 4.0 mL of 12 N H_2SO_4 and settled at room temperature for 1 h, before its dilution to 2 N H_2SO_4. The suspension was incubated at 60 °C for an additional 1 h; the supernatant was then recovered by centrifugation (6000*g*, 10 min). The pH of the recovered supernatant was adjusted to 4.5–4.7 with 6 N NaOH and analyzed for its reducing sugar content, using dinitrosalicylate assay (*20*).

RESULTS AND DISCUSSION

Effect of pH on Selected Cellulolytic Activities. The effect of pH on the selected cellulolytic activities is shown in **Figure 1**. The results indicate that the multi-enzymatic complex system, produced by *P. funiculosum*, displayed endo-1,4-β-D-glucanase, cellobiohydrolase, and β-glucosidase activities over a broad pH range (2.0−8.0) but remained highly active in the acidic region. Dependent upon the type of cellulase, different pH-activity profiles were obtained. Endo-1,4-β-D-glucanase exhibited its maximal specific activity at pH 4.0 and retained more than 50% of its maximum activity in the pH range of 3.0−6.0 (**Figure 1A**), whereas β-glucosidase was most active at pH 4.5 and maintained more than 50% of its maximum specific activity in a narrow pH spectrum of 3.5−5.5 (**Figure 1B**). With regard to the cellobiohydrolase activity, its optimum pH was in the range of 4.0−5.0 and retained more than 60% of its maximum specific activity at a wide pH range of 2.5−6.0 (**Figure 1C**).

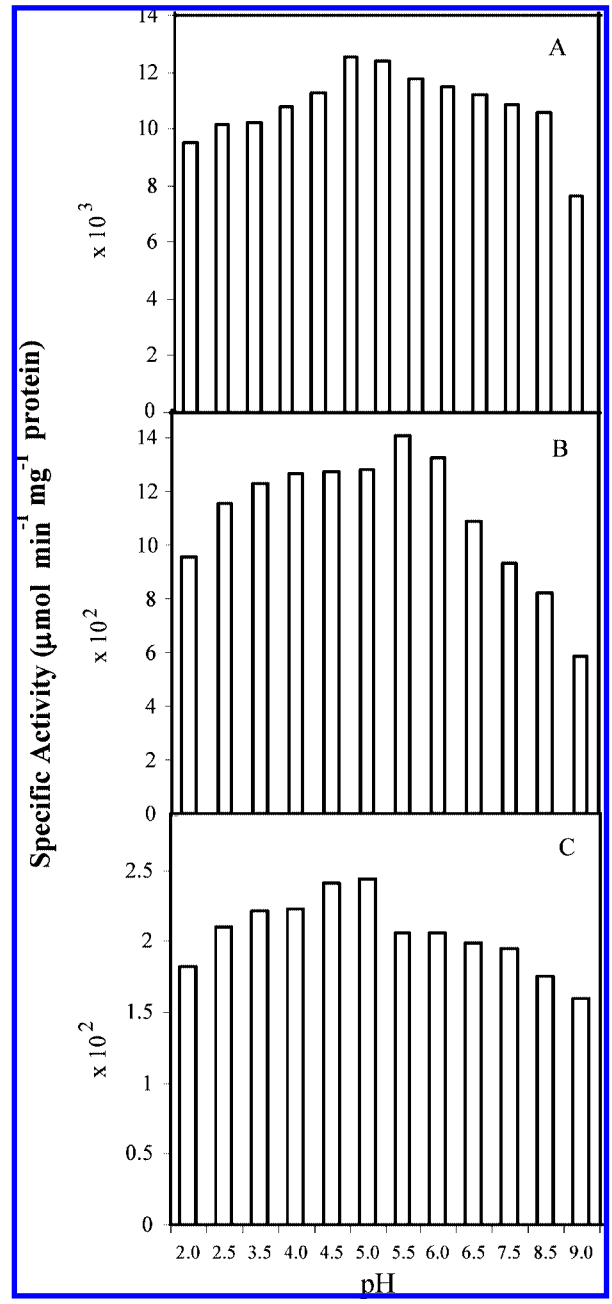

Figure 2. pH stability of selected cellulolytic specific activities of the multi-enzymatic complex system from *P. funiculosum*: (**A**) endo-1,4-β-D-glucanase, (**B**) β-glucosidase, and (**C**) cellobiohydrolase.

Figure 2 shows the patterns of the pH stability of selected cellulolytic activities, present in a multi-enzymatic complex system from *P. funiculosum*. Endo-1,4-β-D-glucanase (**Figure 2A**) and cellobiohydrolase (**Figure 2C**) specific activities were found to be stable in the pH range of 2.5−7.5, where they maintained more than 80% of their maximum specific activity after 2 h of incubation at 25 °C; at pH 2.0 and 9.0, these enzymes still showed 75 and 62% of their maximum specific activity, respectively. Although β-glucosidase (**Figure 2B**) was stable in the pH range of 2.5−6.0, retaining more than 82% of its maximum specific activity after 2 h of incubation at 25 °C, only 68 and 41% of its maximum specific activity were maintained at pH 2.0 and 9.0, respectively. The overall experimental findings indicate that, in the alkaline pH range (7.5−9.0), the endo-1,4-β-D-glucanase and cellobiohydrolase activities showed a higher pH stability than that of β-glucosidase.

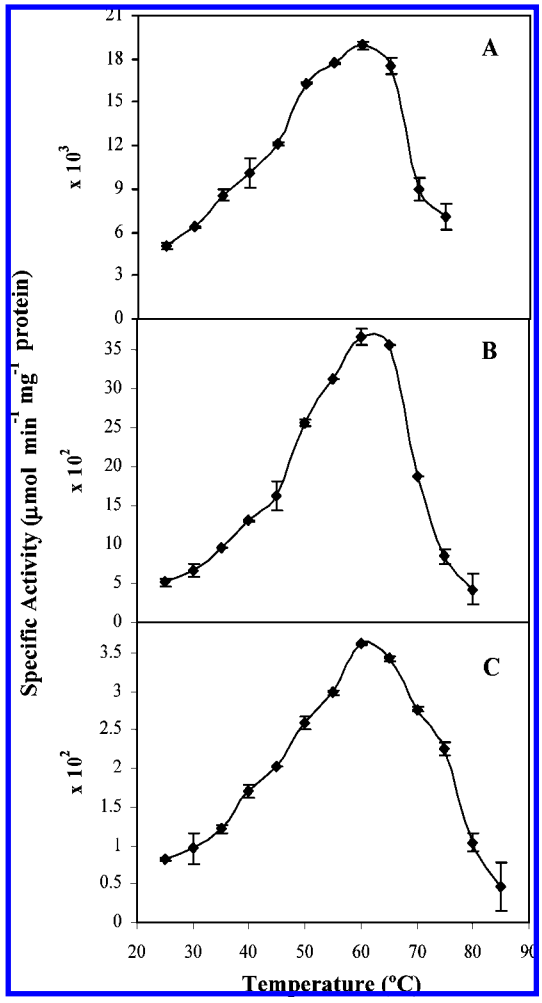

Figure 3. Effect of temperature on selected cellulolytic activities of the multi-enzymatic complex system from *P. funiculosum*: (**A**) endo-1,4-β-D-glucanase, (**B**) β-glucosidase, and (**C**) cellobiohydrolase.

Similarly, Deshpande et al. (*21*) reported that endo-1,4-β-D-glucanase, from *P. funiculosum*, displayed a higher pH stability than that of β-glucosidase, which lost 40% of its initial activity at pH above 6.0.

Overall, the pH activity and stability profiles of the selected cellulases, present in the multi-enzymatic complex system from *P. funiculosum*, showed common features as those reported for other cellulolytic fungal species (*6, 15, 21, 23*). Indeed, *Penicillium* sp. cellulase (*15*) has found to exhibit an optimal enzyme activity at pH 4.5. On the other hand, β-glucosidase from *Thermoascus aurantiacus* (*23*) has showed an optimal enzyme activity at pH 4.5, whereas β-glucosidase from *Penicillium purpurogenum* (*24*) was optimally active at pH 3.5. Cellobiohydrolase from *P. funiculosum* has been reported (*6*) to display relatively a high activity in the pH range of 4.0−5.5, whereas that of *Clostridium thermocellum* (*25*) was optimally active in the pH range of 6.0−8.4. With regard to the endo-1,4-β-D-glucanase, its optimal pH of 4.0 (**Figure 1A**) is similar to that reported (*26*) for the purified enzyme from *T. aurantiacus*. Moreover, close pH optima have been reported for the endo-1,4-β-D-glucanases from *Penicillium pinophilum* (*27*), *Fusarium oxysporum* (*8*), and *T. viride* (*28*), which were optimally active at pH 4.5−5.0 and stable at those below 6.0.

Effect of Temperature on Selected Cellulolytic Activities. **Figure 3** illustrates the effect of temperature on the selected cellulolytic activities, present in the multi-enzymatic complex system produced by *P. funiculosum*. The results (**Figure 3A**) show that the specific activity of endo-1,4-β-D-glucanase increased by a factor of 3.7 at temperatures ranging from 25 to 60 °C and then decreased dramatically at higher temperatures; at 75 °C, the residual specific activity was only 38% of the maximum value. The specific activity of β-glucosidase increased by a factor of 7.2 by increasing the temperature from 25 to 60 °C and then remained unchanged at 65 °C; however, above this temperature, the specific activity decreased dramatically, where the enzyme lost up to 78% of its maximum activity at 75 °C (**Figure 3B**). The results (**Figure 3C**) also indicate an increase in the specific activity of cellobiohydrolase by a factor of 4.4 by increasing the temperature from 25 to 60 °C, which was followed by a slight decrease at higher temperatures; at 75 °C, cellobiohydrolase still exhibited 62% of its maximum specific activity. The overall results indicate that the specific activity of β-glucosidase was more sensitive to the changes in the reaction temperatures than those of endo-1,4-β-D-glucanase and cellobiohydrolase. However, the three cellulolytic activities showed a similar optimal temperature of 60 °C. This optimal temperature falls within the range of 50−60 °C, as reported for cellulases from other fungi (*8, 24, 28–31*). Nevertheless, higher optimum temperatures of 70−80 and 50−70 °C have been reported for the endo-1,4-β-D-glucanases from *T. aurantiacus* (*26*) and *P. pinophilum* (*27*), respectively. Similarly, β-glucosidase from *T. aurantiacus* (*23*) was optimally active at a higher temperature of 80 °C.

With regard to the thermal stability, the results (parts A and C of **Figure 4**) show that there was no significant loss in the endo-1,4-β-D-glucanase and cellobiohydrolase specific activities at temperatures up to 55 °C; however, above this temperature, these two enzymes were less stable and retained only 20 and 34% of their initial specific activity, respectively, after their incubation at 70 °C. The experimental findings (**Figure 4B**) also show that the β-glucosidase specific activity remained stable after incubation at temperatures of 25−40 °C; however, an increase in the incubation temperature resulted by a drastic loss of its specific activity, where the enzyme retained only 51 and 6% of its initial specific activity at 60 and 65 °C, respectively. The overall results indicate that endo-1,4-β-D-glucanase and cellobiohydrolase specific activities exhibited a higher thermal stability than that of β-glucosidase. As a whole, the cellulolytic activities, present in the multi-enzymatic complex system from *P. funiculosum*, showed a thermal stability, quite similar to that of the majority of fungal cellulases described in literature (*8, 15, 25*), except for β-glucosidase (*23*) and endo-1,4-β-D-glucanase (*26*) from *T. aurantiacus*, which were mostly stable up to 70 °C. Furthermore, Van Wyk (*32*) showed that the endo-1,4-β-D-glucanase from *P. funiculosum* has higher thermal stability than that from *T. reesei*.

Kinetic Parameters. Although the multi-enzymatic complex system is present in a crude enzymatic extract, it was important to determine the kinetic parameters of the selected cellulolytic enzymes. The kinetic behaviors of endo-1,4-β-D-glucanase, β-glucosidase, and cellobiohydrolase, present in the cellulolytic complex system from *P. funiculosum*, were found to follow the Michaelis–Menten model (data not shown). The apparent Michaelis–Menten constants, K_{mapp} and V_{maxapp}, estimated by nonlinear regression are given in **Table 1**. The K_{mapp} of cellobiohydrolase was about 7.4 times higher that that of β-glucosidase, whereas the V_{maxapp} of β-glucosidase was 5.0 times higher. As a result, the catalytic efficiency, V_{maxapp}/K_{mapp} of β-glucosidase was substantially greater than that of cellobiohydrolase. The results also indicate that the catalytic ef-

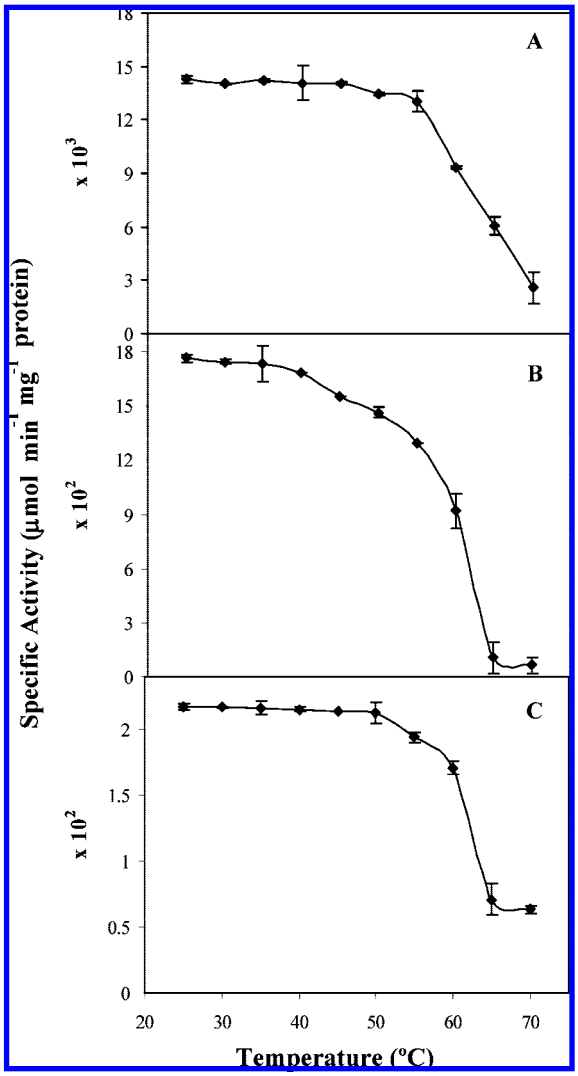

Figure 4. Thermal stability of selected cellulolytic activities of the multi-enzymatic complex system from *P. funiculosum*: (**A**) endo-1,4-β-D-glucanase, (**B**) β-glucosidase, and (**C**) cellobiohydrolase.

Table 1. Kinetic Parameters of Cellulases Present in the Multi-enzymatic Complex System Produced by *P. funiculosum*

enzymes	kinetic parameters		
	K_{mapp}[a]	V_{maxapp}[b]	catalytic efficiency[c]
endo-1,4-β-D-glucanase	3.55	29 239.8	2 059 140.8
β-glucosidase	0.35	2692.5	7692.9
cellobiohydrolase	2.59	533.2	205.9

[a] Michaelis constant was defined as mg mL^{-1} of carboxymethyl cellulose for endo-1,4-β-D-glucanase as well as mM of *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-lactoside for β-glucosidase and cellobiohydrolase, respectively. [b] Maximum velocity was defined as μmol of released reduced sugar per mg protein per min of reaction for endo-1,4-β-D-glucanase and as μmol of released *p*-nitrophenol per mg of protein per min of reaction for β-glucosidase and cellobiohydrolase. [c] Catalytic efficiency was defined as the ratio of V_{maxapp}/K_{mapp}, which was expressed in mL mg^{-1} min^{-1}.

ficiency of endo-1,4-β-D-glucanase was higher than those of β-glucosidase and cellobiohydrolase.

In addition to the complexity of the multi-enzymatic system, the variability of the enzymatic assays reported in literature made it difficult to compare the K_{mapp} values of cellulases from different sources. Nevertheless, the K_{mapp} (**Table 1**) of the endo-1,4-β-D-glucanase (3.55 mg mL^{-1}) was lower than that reported (*33*) for the same enzyme from another *P. funiculosum* strain

Table 2. Specific Activities and Kinetic Parameters of Selected Endoglucanases and Pectinase Present in the Multi-enzymatic Cellulolytic Complex System from *P. funiculosum*

enzymes	specific activity[a]	K_{mapp}[b]	V_{maxapp}[b]
endo-1,3-β-D-glucanase[c]	1450.2 ($\pm$98.2)[d]	0.18	1927.3
endo-1,3-1,4-β-D-glucanase[e]	40 247.7 ($\pm$1227.7)	2.31	49 751.2
pectinase[f]	1013.9 ($\pm$108.7)	16.7	24 639.6

[a] Specific activity is expressed in micromoles of released reduced sugar per milligram of protein per minute of hydrolysis. [b] Michaelis constant, K_{mapp}, was defined as mg mL^{-1} of polysaccharide, and maximum velocity, V_{maxapp}, was defined as micromoles of released reduced sugar per milligram of protein per minute of hydrolysis. [c] Endo-1,3-β-D-glucanase activity was assayed at pH 4.5 and 50 °C, using 1% laminarin as the substrate. [d] Data are average of more than three determinations, and standard deviations are given. [e] Endo-1,3-1,4-β-D-glucanase activity was assayed at pH 4.5 and 50 °C, using 1% barley β-glucan as the substrate. [f] Pectinase activity was assayed at pH 4.5 and 50 °C, using 0.5% citrus pectin as the substrate.

(20 mg mL^{-1}); however, close K_{mapp} values (2.0−4.8 mg mL^{-1}) have been reported (*27*) for two isoenzymes of endo-1,4-β-D-glucanase from *P. pinophilum*. On the other hand, the K_{mapp} of cellobiohydrolase (2.59 mM) was higher than that reported for the same enzyme from another *P. funiculosum* strain (0.85 mM), using cello-oligosaccharide as substrate (*6*). However, the K_{mapp} values (1.23−4.30 mM) of cellobiohydrolases from *Sporotrichum pulverulentum* (*10*) and *T. reesei* (*11*), using *p*-nitrophenyl-β-D-lactoside as substrate, were close to that obtained (2.59 mM) in the present study. The results (**Table 1**) also indicate that the K_{mapp} of β-glucosidase for *p*-nitrophenyl-β-D-glucoside was 0.35 mM; however, Kantham and Jagannathan (*17*) reported K_{mapp} values of 0.23 and 1.08 mM for two isoenzymes of β-glucosidase from another *P. funiculosum* strain. Lower K_{mapp}values of 0.085 and 0.11 mM have been reported for β-glucosidase from *P. purpurogenum* (*24*) and *T. aurantiacus* (*23*), respectively.

Investigation of Selected Endoglucanase and Pectinase Activities. To facilitate the access of cellulases to their substrates, cellulolytic microorganisms may produce other glycosyl hydrolases, which hydrolyze the associated polymeric cellulosic substrates (*12, 13*). **Table 2** shows that the multi-enzymatic cellulolytic complex system secreted by *P. funiculosum*, exhibited different levels of endo-1,3-1,4-β-D-glucanase, endo-1,3-β-D-glucanase, and pectinase activities. The level of endo-1,3-β-D-glucanase was lower than those of endo-1,3-1,4-β-D-glucanase and endo-1,4-β-D-glucanase. The experimental findings (**Table 2**) suggest that the multi-enzymatic cellulolytic complex system has a higher specificity for the hydrolysis of β-1,4 linkages in polysaccharides than that for β-1,3 linkages. The results also indicate that the multi-enzymatic complex system exhibited a high level of pectinase activity. The K_{mapp} of the endo-1,3-β-D-glucanase (0.18 mg mL^{-1}) was lower that those of endo-1,4-β-D-glucanase (3.55 mg mL^{-1}) and endo-1,3-1,4-β-D-glucanase (2.31 mg mL^{-1}), but it was in the same range as those reported (*34*) for other yeast and fungal endo-1,3-β-D-glucanases (0.11−0.33 mg mL^{-1}). As far as the authors are aware, there is limited information on pectinase from *P. funiculosum*. Nevertheless, the apparent value of K_{mapp} (16.7 mg mL^{-1}) was higher than those reported for pectinases from *Penicillium expansum* (*35*) and *A. niger* (*36*), with 9.0 and 12.8 mg mL^{-1}, respectively; however, lower K_{mapp} values of 2.28 and 3.30 mg mL^{-1} were obtained for other pectinases from *A. niger* (*36*) and *Pichia pinus* (*37*), respectively.

The literature (*35, 38, 39*) indicated that all *Penicillium* spp. have shown either cellulolytic or pectinolytic activities, with the exception of one *Penicillium* spp. from Japanese mandarin

Table 3. Degradability of Nonstarch Polysaccharides (NSPs) of Soybean Meal Supplemented with the Multi-enzymatic Cellulolytic Complex System from *P. funiculosum*

proportion of multi-enzymatic product (%)[b]	NSP degradability (%)[a]	
	4 h of incubation	24 h of incubation
7.22	16.1 (±0.3)[c]	21.0 (±0.1)
3.33	9.7 (±0.5)	15.6 (±1.2)
1.67	4.0 (±0.5)	10.0 (±0.5)
0.83	1.3 (±0.2)	6.8 (±0.5)

[a] NSP degradability is defined as NSPs in the control minus NSPs remaining in the residue after the enzymatic incubation divided by NSPs in the control, multiplied by 100. [b] Proportion of the multi-enzymatic complex supplement in the soybean meal is expressed in the percentage of (w/w) grams of protein of the enzymatic product per gram of soybean meal. [c] Data are average of more than three determinations, and standard deviations are given.

orange that showed both enzyme activities (*40*). On the other hand, endo-1,4-β-D-glucanase, endo-1,3-β-D-glucanase, and pectinase are widely distributed in bacteria, fungi, and plants, whereas endo-1,3-1,4-β-D-glucanases are known to be produced only by plants and bacteria (*41*), with the exception of anaerobic fungus *Orpinomyces* strain (*42*); considering these two facts together, the multi-enzymatic cellulolytic complex system, produced by *P. funiculosum*, could be considered as an appropriate biocatalyst for the degradation of various associated plant cellulosic polysaccharides.

***In Vitro* Nonstarch Polysaccharide Degradation.** The efficiency of the multi-enzymatic cellulolytic complex system from *P. funiculosum* in the degradation of NSPs of soybean meal was evaluated. In comparison to the majority of protein meal sources, soybean meal contains a higher NSP proportion (20−29%), which refers to the total polysaccharide fraction associated to the cell wall that are resistant to amylases and proteases (*43*). The degree of soybean meal NSP degradability was indirectly assessed by measuring the residual NSPs content after the enzymatic treatment.

Table 3 shows that the incubation of soybean meal with the multi-enzymatic cellulolytic complex system resulted in an increase of its NSP degradability by 1.3−21%, depending upon the incubation time and the proportion of enzyme supplement. Indeed, increasing the proportion of the multi-enzymatic complex supplement in the soybean meal from 0.8 to 7.2% (w/w) resulted in an increase of its NSP degradability from 1.3 to 16.1% after 4 h of incubation and from 6.8 to 21% after 24 h of incubation. The experimental findings show that the multi-enzymatic cellulolytic complex, from *P. funiculosum*, was effective as enzyme supplement in the degradation of NSPs of soybean meal; this degradation could make the endogenous digestive enzymes to interact with their substrates more easily and act on more efficiently (*44*).

The overall characteristics of the cellulolytic complex system from *P. funiculosum* could make it an appropriate biocatalyst for its application in the degradation of the cell-wall polysaccharides in food and feedstuffs. Parameters of optimum pH, reaction temperature, and kinetic parameters indicated that the endo-1,4-β-D-glucanase, cellobiohydrolase, and β-glucosidase activities share many similarities of those obtained from other sources.

LITERATURE CITED

(1) Enari, T. M. Microbial cellulases. In *Microbial Enzymes and Biotechnology*, 1st ed.; Fogarty, W. M., Ed.; Applied Science Publishers: London, U.K., 1983; pp 183–224.

(2) Lynd, L. R.; Weimar, P. J.; van Zyl, W. H.; Pretorius, I. S. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 506–577.

(3) Rao, M.; Mishra, C. Glucohydrolase from *Penicillium funiculosum*. *Appl. Microbiol. Biotechnol.* **1989**, *30*, 130–134.

(4) Poutanen, K.; Tenkanen, M.; Korthe, H.; Puls, J. Accessory enzymes involved in the hydrolysis of xylans. In *Enzymes in Biomass Conversion*, ACS Symposium Series 460; Leatham, G. F., Himmel, M. E., Eds.; American Chemical Society: Washington, D.C., 1991; pp 426–436.

(5) Hasper, A. A.; Dekkers, E.; van Mil, M.; van de Vondervoort, P. J. I.; de Graaff, L. H. EglC, a new endoglucanase from *Aspergillus niger* with major activity towards xyloglucan. *Appl. Environ. Microbiol.* **2002**, *68*, 1556–1560.

(6) Wood, T. M.; McCrae, S. I.; Macfarlane, C. C. The isolation, purification and properties of the cellobiohydrolase component of *Penicillium funiculosum* cellulase. *Biochem. J.* **1980**, *189*, 51–65.

(7) Henrissat, B.; Claeyssens, M.; Tomme, P.; Lemesle, L.; Mornon, J.-P. Cellulase families revealed by hydrophobic cluster analysis. *Gene* **1989**, *81*, 83–95.

(8) Christakopoulos, P.; Kekos, D.; Macris, B. J.; Claeyssens, M.; Bhat, M. K. Purification and characterization of a less randomly acting endo-1,4-β-D-glucanase from the culture filtrates of *Fusarium oxysporum*. *Arch. Biochem. Biophys.* **1995**, *316*, 428–433.

(9) Romfxo, M. D.; Aguado, J.; Rodriguez, L. Hydrolysis of cellobiose using β-glucosidase from *Penicilliurn funiculosum*: Kinetic analysis. *Acta Biotechnol.* **1999**, *19*, 3–16.

(10) Eriksson, K. E.; Pettersson, B. Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cellulose. Purification and physico-chemical characterization of an exo-1,4-β-glucanase. *Eur. J. Biochem.* **1975**, *51*, 213–218.

(11) Godbole, S.; Decker, S. R.; Nieves, R. A.; Adney, W. S.; Vinzant, T. B.; Baker, J. O.; Thomas, S. R.; Himmel, M. E. Cloning and expression of *Trichoderma reesei* cellobiohydrolase I in *Pichia pastoris*. *Biotechnol. Prog.* **1999**, *15*, 828–833.

(12) Ugwuanyi, J. O.; Obeta, J. A. N. Pectinolytic and cellulolytic activities of heat resistant fungi and their macerating effects on mango and African mango. *J. Agric. Food Chem.* **1999**, *79*, 1054–1059.

(13) Kohli, U.; Nigam, P.; Singh, D.; Chaudhary, K. Thermostable, alkalophilic and cellulase free xylanase production by *Thermoactinomyces thalophilus* subgroup C. *Enzyme Microb. Technol.* **2001**, *28*, 606–610.

(14) Persson, I.; Tjerneld, F.; Hahn-Hagerdal, B. Fungal cellulotic enzyme production: A review. *Process Biochem.* **1991**, *26*, 65–74.

(15) Castellanos, O. F.; Sinitsyn, A. P.; Vlasenko, E. Y. Comparative evaluation of hydrolytic efficiency toward microcrystalline cellulose of *Penicillium* and *Trichoderma* cellulases. *Bioresour. Technol.* **1995**, *52*, 119–124.

(16) Holtzapple, M.; Cognata, M.; Shu, Y.; Hendrickson, C. Inhibition of *Trichoderma reesei* cellulase by sugars and solvents. *Biotechnol. Bioeng.* **1990**, *36*, 275–287.

(17) Kantham, L.; Jagannathan, V. β-Glucosidase of *Penicillium funiculosum*. II. Properties and mycelial binding. *Biotechnol. Bioeng.* **1985**, *27*, 786–791.

(18) Steiner, J.; Socha, C.; Eyzaguirre, J. Culture conditions for enhanced cellulase production by a native strain of *Penicillium purpurogenum*. *World J. Microbiol. Biotechnol.* **1994**, *10*, 280–284.

(19) Wood, T. M.; Bhat, K. M. Methods for measuring cellulase activities. *Methods Enzymol.* **1988**, *160*, 87–112.

(20) Miller, G. L. Use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428.

(21) Deshpande, V.; Seeta, R.; Mishra, C.; Rao, M. Stability and recovery of *Penicillium funiculosum* cellulase under use conditions. *Appl. Biochem. Biotechnol.* **1988**, *19*, 11–19.

(22) Theander, O.; Aman, P.; Westerlund, E.; Andersson, R.; Pettersson, D. Total dietary fiber determined as neutral sugar residues, uronic acid residues, and klason lignin (the Uppsala method): Collaborative study. *J. AOAC Int.* **1995**, *78*, 1020–1044.

(23) Parry, N. J.; Beever, D. E.; Owen, E.; Vandenberghe, I.; Van Beeumen, J.; Bhat, M. K. Biochemical characterization and mechanism of action of a thermostable β-glucosidase purified from *Thermoascus aurantiacus*. *Biochem. J.* **2001**, *353*, 117–127.

(24) Hidalgo, M.; Steiner, J.; Eyzaguirre, J. β-Glucosidase from *Penicillium purpurogenum*: Purification and properties. *Biotechnol. Appl. Biochem.* **1992**, *15*, 185–191.

(25) Bhat, S.; Owen, E.; Bhat, M. K. Isolation and characterisation of a major cellobiohydrolase (S8) and a major endoglucanase (S11) subunit from the cellulosome of *Clostridium thermocellum*. *Anaerobe* **2001**, *7*, 171–179.

(26) Parry, N. J.; Beever, D. E.; Owen, E.; Nerinckx, W.; Claeyssens, M.; Beeumen, V. J.; Bhat, M. K. Biochemical characterization and mode of action of a thermostable endoglucanase purified from *Thermoascus aurantiacus*. *Arch. Biochem. Biophys.* **2002**, *404*, 243–253.

(27) Bhat, K. M.; McCrae, S. I.; Wood, T. M. The endo-(1→4)-β-D-glucanase system of *Penicillium pinophilum* cellulase: Isolation, purification, and characterization of five major endoglucanase components. *Carbohydr. Res.* **1989**, *190*, 279–297.

(28) Marques, S.; Pala, H.; Alves, L.; Amaral-Collaco, M. T.; Gama, F. M.; Girio, F. M. Characterisation and application of glycanases secreted by *Aspergillus terreus* CCMI 498 and *Trichoderma viride* CCMI 84 for enzymatic deinking of mixed office wastepaper. *J. Biotechnol.* **2003**, *100*, 209–219.

(29) Noomen, H. T.; Chaabouni-Ellouz, S.; Kammoun, A.; Ellouz, R. Hydrolytic efficiency of *Penicillium occitanis* cellulase. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 197–201.

(30) Sinitsyna, O. A.; Bukhtoyarov, F. E.; Gusakov, A. V.; Okunev, O. N.; Bekkarevitch, A. O.; Vinetsky, Y. P.; Sinitsyna, A. P. Isolation and properties of major components of *Penicillium canescens* extracellular enzyme complex. *Biochemistry (Moscow)* **2003**, *68*, 1200–1209.

(31) Montero, M.; Sanz, L.; Rey, M.; Monte, E.; Liobell, A. BGN 16.3, a novel acidic β-1,6-glucanase from mycoparasitic fungus *Trichoderma harzianum* CECT 2413. *FEBS Lett.* **2005**, *272*, 3441–3448.

(32) Van Wyk, J. P. H. Thermostability of cellulase from *Penicillium funiculosum*. *Biotechnol. Lett.* **1997**, *11*, 385–386.

(33) Sahasrabudhe, N. A.; Lachke, A. H.; Ranjekar, P. K. Characterization of the purified multifunctional cellulase components of *Penicillium funiculosum*. *Biotechnol. Lett.* **1987**, *9*, 881–886.

(34) Mishra, C.; Robbins, P. W. Specific β glucanases as tools for polysaccharide structure determination. *Glycobiology* **1995**, *5*, 645–654.

(35) Silva, D. O.; Attwood, M. M.; Tempest, D. W. Partial purification and properties of pectinlyase from *Penicillum expansum*. *World J. Microbiol. Biotechnol.* **1993**, *9*, 574–578.

(36) Acuña-Argüelles, M. E.; Gutiérrez-Rojas, M.; Viniegra-González, G.; Favela-Torres, E. Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid-state fermentation. *Appl. Microbiol. Biotechnol.* **1995**, *43*, 808–814.

(37) Moharib, S. A.; El-Sayed, S. T.; Jwanny, E. W. Evaluation of enzymes produced from yeast. *Nahrung* **2000**, *44*, 47–51.

(38) Rajal, V. B.; Carrillo, L.; Cuevas, C. M. Exocellular hydrolases from *Penicillium ulaiense*. *World J. Microbiol. Biotechnol.* **1996**, *12*, 413–414.

(39) Krogh, K. B. R.; Morkeberg, A.; Jørgensen, H.; Frisvad, J. C.; Olsson, L. Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes. *Appl. Biochem. Biotechnol.* **2004**, *114*, 389–401.

(40) Fujii, K.; Shintoh, Y. Degradation of mikan (Japanese mandarin orange) peel by a novel *Penicillium* species with cellulolytic and pectinolytic activity. *J. Appl. Microbiol.* **2006**, *101*, 1169–1176.

(41) Borriss, R.; Buettner, K.; Maentsaelae, P. Structure of the β-1,3-1,4-glucanase gene of *Bacillus macerans*: Homologies to other β-glucanases. *Mol. Gen. Genet.* **1990**, *222*, 278–283.

(42) Chen, H.; Li, X.-L.; Ljungdahl, L. G. Sequencing of endo-1,3-1,4-β-D-glucanase (lichenase) from the anaerobic fungus *Orpinomyces* strain PC-2: Properties of the enzyme expressed in *Escherichia coli* and evidence that the gene has a bacterial origin. *J. Bacteriol.* **1997**, *179*, 6028–6034.

(43) Van Soest, P. J.; Robertson, J. B.; Lewis, B. A. Methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597.

(44) Li, W.-F.; Feng, J.; Xu, Z.-R.; Yang, C. M. Effects of non-starch polysaccharides enzymes on pancreatic and small intestinal digestive enzyme activities in piglet fed diets containing high amounts of barley. *World J. Gastroenterol.* **1994**, *10*, 856–859.

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