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The Effects of Wheat Bran Composition on the Production of Biomass-Hydrolyzing Enzymes by *Penicillium decumbens*

Xianyun Sun · Ziyong Liu · Yinbo Qu · Xuezhi Li

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Abstract The effects of the starch, protein, and soluble oligosaccharides contents in wheat bran on the extracellular biomass-hydrolyzing enzymes activities released by *Penicillium decumbens* mycelia grown in batch fermentations have been examined. The results showed increased starch content correlated directly with an increase in released amylase activity but inversely with the levels of secreted cellulase and xylanase. High amounts of protein in wheat bran also reduced the activities of cellulase, xylanase and protease in the culture medium. The effects of the soluble and insoluble components of wheat bran and cello-oligosaccharides supplements on production of extracellular cellulase and xylanase were compared. The soluble cello-oligosaccharides compositions in wheat bran were proved to be one of the most significant factors for cellulase production. According to the results of this research, determining and regulating the composition of wheat bran used as a fermentation supplement may allow for improved induction of cellulase and xylanase production.

Keywords *Penicillium decumbens* · Wheat bran · Biomass · Cellulase · Xylanase

Introduction

Penicillium species with the ability to produce high cellulase and hemicellulase titres have been described [1, 2]. Also, they have the advantage of containing higher glucosidase activity than *Trichoderma* [2]. So, *Penicillium* species have great potential for hydrolysis of lignocellulosic materials. *Penicillium decumbens* 114-2 was fast-growing cellulolytic fungus that isolated from soil [3] and its catabolic repression-resistant mutants have been used industrially for biomass hydrolysis [4, 5]. In previous study, wheat bran supplements have been shown to promote the growth of *P. decumbens*, and increase the cellulase and β-glucosidase activities released by *P. decumbens* mycelia [6], but the basis of this stimulation is still unknown.

X. Sun · Z. Liu · Y. Qu (✉) · X. Li
State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, China
e-mail: quyinbo@sdu.edu.cn

Wheat bran is the outer ~15% of the wheat seed and is composed predominantly of non-starch carbohydrates (~58%), starch (~19%) and crude protein (~18%), with the non-starch polysaccharides being primarily ~70% arabinoxylans, ~24% cellulose and ~6% β-(1,3)(1,4)-glucan [7–10]. Reports have showed wheat bran or acid-hydrolyzed wheat bran can increase cellulase production by filamentous fungi [6, 9, 11, 12]. Also, wheat-bran culture gave the richest gene expression profile of hydrolytic enzymes from *Aspergillus oryzae* among the three tested media [13]. However, which factor in wheat bran being important for cellulase synthesis is unknown yet.

It is generally believed that oligosaccharides play an important role in regulating the synthesis of wood-degrading enzymes [14, 15], and oligosaccharides has been proved to be converted to inducer (such as sophorose and gentiobiose) by transglucosylation [16–19]. So, we supposed that oligosaccharides may exist in autoclaved wheat bran, which may play an important role in increasing the production of the extracellular biomass-hydrolyzing enzymes by *P. decumbens*. The experiments reported here were undertaken to identify the components in wheat bran responsible for the stimulation of growth and the increased production of the industrially important biomass-degrading enzymes by *P. decumbens*. The knowledge obtained may provide a scientific platform for further improvement of the processes that employ this fungus for biomass conversion.

Materials and Methods

Microorganism and Culture Conditions

P. decumbens 114-2 stock cultures were grown on wheat bran extract agar slants for 6 days at 30 °C to allow sporulation. Spores were then washed from the agar surface using sterile water, and aliquots of the resulting spore suspensions were inoculated to liquid media of 50 ml in 300 ml flasks sterilized by autoclaving at 115 °C for 30 min. The media used contained one or more carbon sources, as noted in the text and figure legends, dissolved in Mandel's solution [3], namely (per liter): 3 g KH_2PO_4, 2.6 g $NaNO_3$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g $CaCl_2$, 0.5 g urea, 7.5 mg $FeSO_4{\cdot}7H_2O$, 2.5 mg $MnSO_4{\cdot}H_2O$, 3.6 mg $ZnSO_4{\cdot}7H_2O$, 3.7 mg $CoCl_2{\cdot}6H_2O$ and 1 g peptone (pH 5.5). The inoculated media were incubated for 4.5 days (the time point of maximum enzyme activities and steady growth phases of *P. decumbens*) at 30 °C, 180 rpm on rotary shakers.

Enzyme Assays

Accumulated *P. decumbens* biomass was removed by centrifugation, and aliquots of the resulting supernatants were diluted and assayed for enzyme activities. One unit of all enzyme activities is defined as the amounts of the enzyme that liberates 1 μmol of product per minute under the assay conditions used.

(1) Cellulase activity was measured by reducing sugar released from filter paper (Whatman No.1) and from carboxymethylcellulose with glucose as the standard [20–22].
(2) β-glucosidase activity was assayed using salicin as the substrate [23]. Dilutions of supernatant (0.5 ml) were incubated with 1 ml of 1% (*w*/*v*) solution of salicin in 0.2 M acetate buffer (pH 4.8) at 50 °C for 30 min and the DNS method was used to quantify the reducing sugar released.

(3) Xylanase activity was assayed by measuring the reducing sugars liberated from 1% (*w*/*v*) xylan (from oat spelts, Sigma) suspended in acetate buffer (0.2 M, pH 4.8) when incubated with aliquots of diluted supernatant at 50 °C for 30 min [24]. The amounts of reducing sugars released were determined using the DNS assay with xylose as the standard sugar.
(4) Amylase activity was measured using 1% (*w*/*v*) soluble starch dissolved in acetate buffer (0.2 M, pH 4.8) as the substrate. Reaction mixtures were incubated for 10 min at 40 °C and the reducing sugars released were measured using the DNS assay with maltose as the standard sugar.
(5) Protease activity was measured using 1% (*w*/*v*) casein as substrate dissolved in 0.1 M phosphate buffer (pH 3.0). Aliquots (2 ml) of diluted supernatant were mixed with 2 ml of 1% (*w*/*v*) casein and the reaction mixtures incubated at 40 °C for 10 min. An equal volume of trichloroacetic acid (0.4 l) was added to the reaction mixture, after filtration, the tyrosine released was quantified by the absorbance of the filtrate at 275 nm (A_{275}).

Biomass Determination

The biomass was measured using the perchloric acid method [25]. Mycelium suspension was mixed with an equal volume of 1-M perchloric acid, and the mixture was incubated at 100 °C for 20 min. After centrifugation at 10,000 g for 10 min, the absorbance at 260 nm (A_{260}) of dilutions of the resulting supernatant was measured. For control experiments, the minimal amounts of biomass in cell-free supernatants were measured. Standard curve was plotted by known amounts of biomass.

Determination of Starch, Soluble Sugars and Protein Contents of Wheat Brans

The starch content in wheat bran was determined enzymatically as described by Holm et al. [26]. To determine the contents of soluble sugars and protein in 10% (*w*/*v*) wheat bran, microcrystalline cellulose (MCC, from cotton, Shanghai Hengxin Co.) and CF11 cellulose powder (from cotton, Whatman), these polymers were suspended in water and autoclaved at 115 °C for 30 min. The resulting solutions were filtered through 0.45-μm filters and the mono- and oligosaccharide contents of the filtrates were identified and quantified by high performance liquid chromatography (HPLC) at 75 °C using a Bio-Rad HPX-42C column with water as the eluent. The amounts of protein present in the filtrates were determined by using Bradford assay [27] with bovine serum albumin used as the standard.

Data Analysis

All results are given as the mean ± standard deviation, with the numbers of independently repeated experiments listed. Statistical analyses were fulfilled by the *t* test.

Results

The Effects of Starch and Wheat Bran on Synthesis of Cellulase and Xylanase

The amounts of biomass and cellulase and xylanase activities released into the supernatant of *P. decumbens* grown in media that contained a total of 3% (*w*/*v*) polymeric carbon

substrate were shown in Fig. 1. All growth media contained 1% microcrystalline cellulose (MCC) plus 2% wheat bran, or 2% wheat starch or a mixture of wheat bran and wheat starch (in the ratios 2:1, 1:1 or 1:2) that in total constituted 2% carbon source substrate. With the increase of wheat bran content, mycelial biomass, cellulase, β-glucosidase and xylanase activities in the supernatant increased but amylase activity reduced. The cultures grown on 1% MCC and 2% wheat starch with no wheat bran produced the lowest levels of cellulase, β-glucosidase, xylanase and amylase activity. As expected, the presence of starch, including the starch in wheat bran, apparently promoted amylase synthesis but starch alone did not support robust growth or substantial release of xylanase and cellulase by *P. decumbens*. There were no detectable differences in the amounts of cellulase or xylanase activity present in the supernatants of the different cultures, when these activities were calculated in terms of the biomass. The lower levels of cellulase and xylanase activities in the supernatants of cultures grown with starch therefore resulted from the reduced mycelia growth and as opposed to a regulatory reduction in enzyme synthesis or secretion.

Effects of Wheat Bran Granule Size on Synthesis of Cellulase and Xylanase

There were no detectable differences in the cellulase and xylanase activities in the supernatants of *P. decumbens* cultures grown in media supplemented with wheat bran where particles were size-separated by passage through a 1.5-mm filter compared to samples where media were not size-separated (*A* and *B* of Fig. 2). However, at smaller granule sizes, the net starch content increased and this resulted in increased amylase production but reduced total mycelial growth and lowered levels of cellulase and xylanase released (Fig. 2).

Effects of Wheat Protein Plus Wheat Bran on Cellulase and Xylanase Synthesis

Cultures were grown in media containing 1% MCC plus 2% wheat bran, or 2% wheat protein, or a mixture of wheat bran and wheat protein (ratios of 2:1, 1:1 and 1:2) that together constituted 2% carbon polymer solutions. Cultures grown with only MCC and wheat protein grew less well than cultures supplemented with wheat bran. Increasing the wheat protein to wheat bran ratio did not reduce the amounts of mycelial growth (Fig. 3)

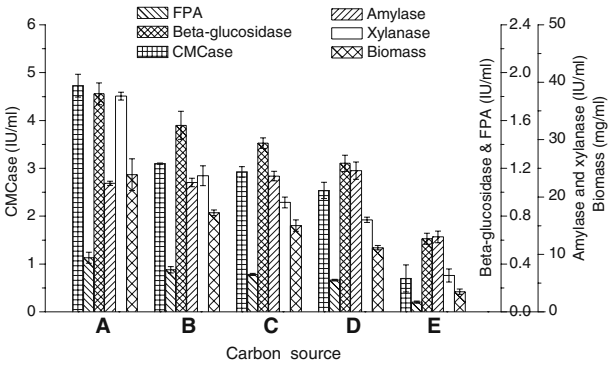

Fig. 1 Effects of starch and wheat bran on growth and production of extracellular cellulase, xylanase and amylase by *P. decumbens*. Cultures were grown and the levels of biomass and extracellular enzymes were measured as described in the "Materials and Methods" section. All cultures contained 1% MCC plus as additional carbon sources: *A* 2% wheat bran; *B* 0.7% wheat starch plus 1.4% wheat bran; *C* 1% wheat starch plus 1% wheat bran; *D* 1.4% wheat starch plus 0.7% wheat bran; and *E* 2% wheat starch

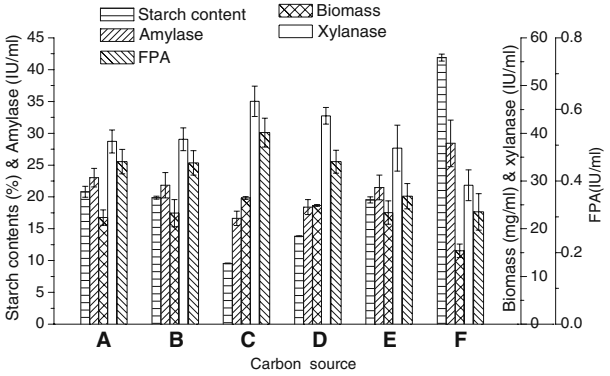

Fig. 2 Effects of granule size of wheat bran on growth and production of extracellular cellulase, xylanase and amylase by *P. decumbens*. All cultures contained 1% MCC plus 2% wheat bran: *A* all sizes of wheat bran granules; *B* wheat bran granules that passed through a 1.5-mm filter; *C* to *F* wheat bran granules with diameters of 0.45 to 0.8 mm, 0.3 to 0.45 mm, 0.2 mm to 0.3 mm, and <0.2 mm, respectively

but the amounts of cellulase, xylanase and protease released were reduced. Together, the fact that 0.1% peptone has presented in Mandel's solution, the obtained data indicated high amounts of protein in wheat bran repressed the synthesis or secretion of cellulase, xylanase and protease by *P. decumbens*, which was consistent with the observation that acid-hydrolyzed protein inhibited cellulase production by *Trichoderma reesei* [9].

Comparison of the Effects of the Soluble and Insoluble Components of Wheat Bran on Cellulase and Xylanase Synthesis

A 10% wheat bran suspension in water was autoclaved for 30 min at 115 °C and the resulting soluble (liquor) and insoluble components (residue) were separated by filtration. The liquor and residue fractions were then used alone or mixed with 1% MCC as carbon sources for *P. decumbens* growth. The biomass in cultures grown on wheat-bran liquor plus MCC was less than that on wheat-bran residues plus MCC, but the cellulase activity released was higher (Fig. 4). The liquor apparently contained a factor that stimulated cellulase synthesis and/or secretion. In contrast, the xylanase activity in the supernatants of cultures supplemented with wheat-bran liquor was lower than that in cultures supplemented with the wheat-bran residues, which were in consistent with the residues having higher hemicellulose content.

In the absence of MCC, total biomass accumulation, and also the cellulase and xylanase activities present in the supernatant were ~50% lower in cultures grown on only wheat bran

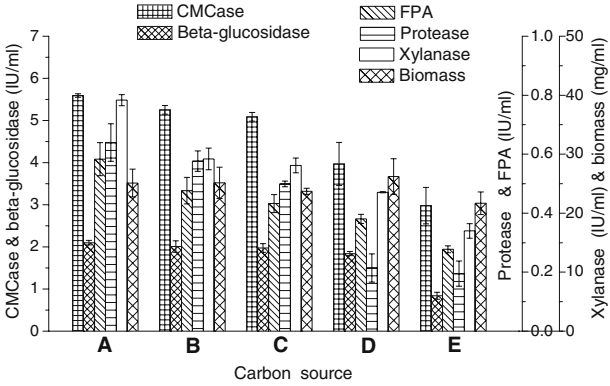

Fig. 3 Effects of protein and wheat bran on growth and production of extracellular cellulase, xylanase and protease by *P. decumbens*. All cultures contained 1% MCC plus as additional carbon sources: *A* 2% wheat bran; *B* 1.4% wheat bran plus 0.7% wheat protein; *C* 1% wheat bran plus 1% wheat protein; *D* 0.7% wheat bran plus 1.4% wheat protein; and *E* 2% wheat protein

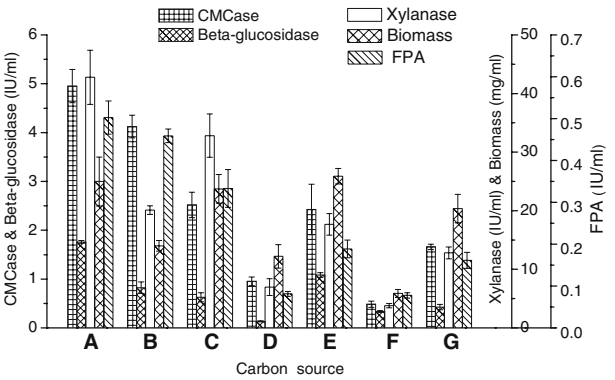

Fig. 4 Effects of the water soluble and insoluble components of wheat bran on growth and production of extracellular cellulase and xylanase by *P. decumbens*. The carbon sources were: *A* 1% MCC plus 2% wheat bran; *B* 1% MCC plus 2% wheat bran liquor; *C* 1% MCC plus 2% wheat bran residues; *D* 3% MCC; *E* 3% wheat bran; *F* 3% wheat bran liquor; *G* 3% wheat bran residues

liquor than on only wheat bran residues. Apparently in the liquor overall concentration of substrate, carbon was lower and levels of cellulose and hemicellulose were too low to stimulate cellulase and xylanase synthesis. Consistent with this interpretation, when *P. decumbens* cultures were grown on wheat bran with or without MCC, similar amounts of biomass accumulated but the cellulase and xylanase activities in the supernatant were much lower in the absence of MCC. Wheat bran alone did not therefore provide sufficient cellulose for maximum cellulase and xylanase production and release by *P. decumbens* mycelia.

Soluble Protein, Mono- and Oligosaccharide Composition of Wheat Bran and Cellulose Powder

HPLC and Bradford method analysis of the water soluble components from a 10% suspension of wheat bran revealed the presence of proteins (0.83 mg/ml), monosaccharides (glucose, xylose and arabinose), small oligosaccharides (xylobiose and xylotriose) and some larger oligosaccharides, apparently tetra-, penta-, and hexa-saccharides. In contrast, boiling suspensions of MCC and cellulose powder CF11 resulted in solutions that contained very little soluble protein, mono- or oligosaccharides (Fig. 5). Based on these results and the discovery that *P. decumbens* cultures grew better and released more cellulase and xylanase when grown with MCC plus wheat bran than on MCC plus soluble protein, it seems that the soluble oligosaccharides in wheat bran are the primary stimulators of cellulase and xylanase synthesis in *P. decumbens* cultures.

Stimulation of Growth and Production of Cellulase and Xylanase by the Soluble Components of Wheat Bran

Cultures of *P. decumbens* were grown with 2% MCC plus 0.5, 1, 2, 5 or 10% (*v*/*v*) of wheat-bran liquor. The total amounts of biomass accumulated, and the levels of cellulase and xylanase released to the supernatant did increase with increasing amounts of added wheat-bran liquor, but the increases were not directly proportional (Fig. 6).

Studies of 12 different *Penicillium* species have demonstrated that there was a coinduction between cellulolytic and xylanolytic activities whether the substrate was cellulose or xylan, whereas activities of cellulolytic enzymes were generally higher during growth on cellulose than on xylan [28]. The conclusion was further confirmed by our studies of *P. decumbens* (data not shown). Together with the above results of starch and protein experiments, it must be the high levels of water soluble cello-oligosaccharides

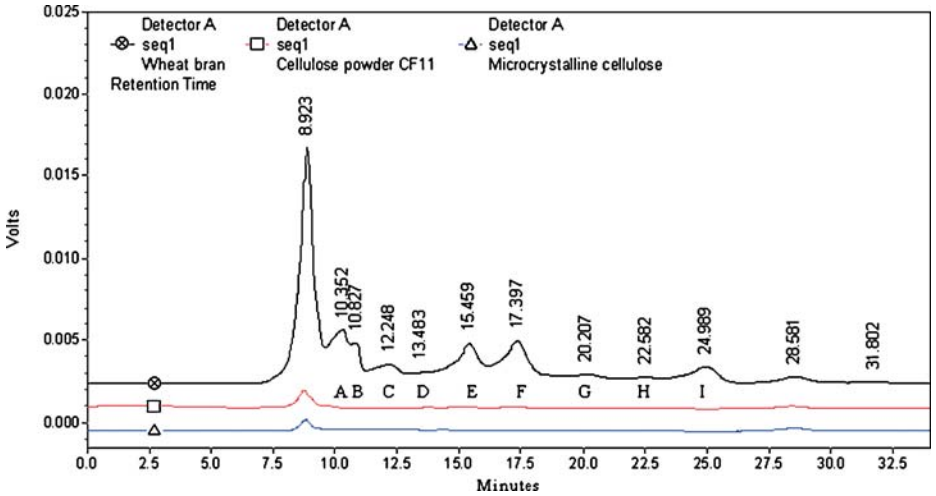

Fig. 5 HPLC separation of the water-soluble sugars extracted from wheat bran, MCC and cellulose powder CF11. Based on known standards, the elution peaks labeled A through I contained hextose, pentose, tetraose, xylotriose, xylobiose, glucose, xylose and arabinose, respectively

present in wheat bran that have positive effects on inducing higher levels of cellulase production.

Effects of Cello-oligosaccharides on Production of Extracellular Cellulase and Xylanase

To further confirm the conclusion, the effects of cello-oligosaccharides supplements on the production of cellulase and xylanase were examined. As shown in Table 1, the cultures containing 2% MCC plus 0.05% cellobiose or 0.05% cello-oligosaccharides (the mixture of cellotetraose, cellopentose and cellohextose) and the control with only 2% MCC were compared. There was no detectable difference in biomass accumulated among these three cultures, but the amounts of extracellular cellulase, β-glucosidase and xylanase increased with the supplement of cello-oligosaccharides or cellobiose in the medium. This further demonstrated that soluble cello-oligosaccharides composition of wheat bran was one of the most significant factors in enhancing cellulase production.

Discussion and Conclusions

The experiments reported were undertaken to understand the empirical observation wheat bran additions to industrial-scale batch fermentation improve biomass hydrolysis by *P. decumbens*. The results obtained have established that the starch content of wheat bran is an important, potentially negative factor, as high starch contents stimulate amylase production, but do not support high levels of mycelial growth, cellulase or xylanase production. Similarly, although the addition of high amount wheat protein did not inhibit growth, it did result in a reduction in the levels of cellulase, xylanase and protease released to the medium. In contrast, the high levels of water-soluble cello-oligosaccharides present in wheat bran, concentrations that are much higher than in cellulose-only preparations, have

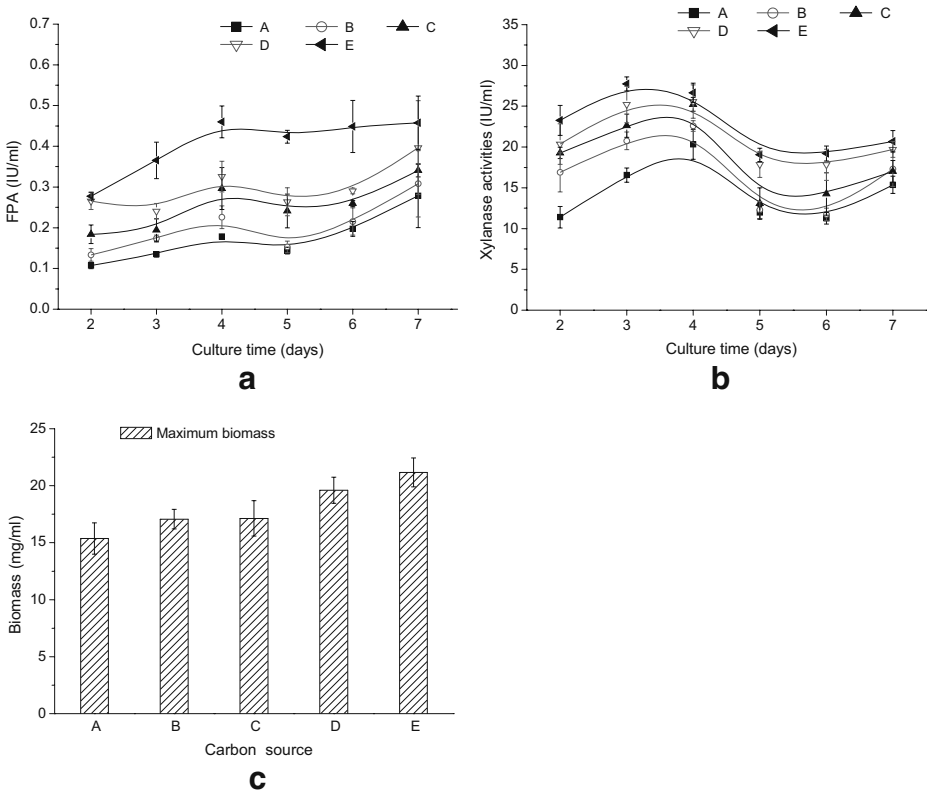

Fig. 6 Effects of the water soluble components of wheat bran supplements on growth and production of extracellular cellulase and xylanase by *P. decumbens*. Cultures were grown as described in the "Materials and Methods" and samples of the supernatant were collected assayed for (**a**) cellulase (FPA) and (**b**) xylanase over a 1 week period. (**c**) The maximum biomass accumulated was determined after 2 days. All cultures contained 2% MCC plus A through E additions of 0.5%, 1%, 2%, 5% or 10% soluble components of wheat bran, respectively

positive effects in terms of inducing higher levels of cellulase production during shorter fermentation time.

It had shown that the regulation of cellulase formation was significantly different between *Penicillium* and *Trichoderma* [16]. In *T. reesei* and *P. purpurogenum*, the inducers are sophorose and gentiobiose, respectively. In *P. purpurogenum*, constitutive cellulase

Table 1 Effects of cellobiose and cello-oligosaccharides supplements on growth and production of extracellular cellulase and xylanase (enzyme activities per gram mycelia mass).

	2% MCC	2% MCC+0.05% CB	2% MCC+0.05% COS
CMCase ($IU \cdot g^{-1}$)	115±27	147±27	133±25
FPA ($IU \cdot g^{-1}$)	8.06±0.99	9.25±1.61	9.04±0.74
β-glucosidase ($IU \cdot g^{-1}$)	8.82±2.59	11.13±1.68	12.89±2.35
Xylanase ($IU \cdot g^{-1}$)	800±66	1041±117	1121±148
Biomass ($g \cdot l^{-1}$)	9.52±0.94	9.67±1.53	9.10±0.19

MCC microcrystalline cellulose, *CB* cellobiose, *COS* cello-oligosaccharides (the mixture of cellotetraose, cellopentose and cellohextose)

degrades cellulose to cello-oligosaccharides and glucose as these saccharides enter the cell. The intracellular β-glucosidase converts cello-oligosaccharides to glucose, gentiobiose and other disaccharides. Gentiobiose induces mass synthesis of cellulase and then the synthesized cellulase is secreted to the outside of the cell [16, 17]. Experimental evidence was reported that the activity of extracellular basal cellulase is the rate-limiting event in induction of synthesis of the cellulase transcripts by cellulose [29]. This paper showed that wheat bran contained plenty of cello-oligosaccharides, and the amounts of extracellular cellulase, β-glucosidase and xylanase increased with the supplement of cello-oligosaccharides or cellobiose in culture medium. Therefore, higher content of soluble cello-oligosaccharides in culture medium of wheat bran can induce higher levels of cellulase production during shorter fermentation time. This is consistent with previous reports that acid-hydrolyzed wheat bran increased cellulase production by *T. reesei* and *P. decumbens*, and that the levels of released enzymes correlated with the mono- and oligosaccharide contents of the wheat bran [9, 11, 12]. It seems likely that inducers of cellulase synthesis, such as sophorose, are generated directly by acid-hydrolysis of wheat bran. Hrmova et al. [14] also reported that mixed disaccharides, could play an important role in regulating the synthesis of wood-degrading enzymes. Therefore, it can be seen from above discussions that cello-oligosaccharides can accelerate synthesis of filamentous fungi cellulase.

Taken together, the results of this research demonstrate the importance of knowing and regulating the starch and soluble protein contents of wheat bran supplements from different sources when adding wheat bran to stimulate cellulase and xylanase production by *P. decumbens*. Our results also predict that adding cello-oligosaccharides directly to *P. decumbens* fermentation could significantly improve industrial-scale biomass hydrolysis by *P. decumbens*.

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