ENZYMATIC HYDROLYSIS OF CELLULOSE FROM STEAM-PRETREATED *LESPEDEZA* STALK (*LESPEDEZA CRYTOBOTRYA*) WITH FOUR *TRICHODERMA* CELLULASES

Yue Feng, Hui-Qin Liu, Run-Cang Sun, and Jian-Xin Jiang *

The hydrolytic potential of cellulases produced by *Trichoderma viride, Trichoderma pseudokoningii, Trichoderma koningii*, and *Trichoderma reesei* with addition of exogenous β-glucosidase was evaluated on cellulose of steam-pretreated *Lespedeza*. The *T. viride* enzyme achieved the highest glucose conversion (90.09%), while *T. pseudokoningii* cellulase achieved the highest ratio of cellobiose to glucose (4.94%) at the end of hydrolysis. Enzymatic adsorption on the substrate was evaluated on filter paper activity and β-glucosidase activity in the corresponding digest with the obtained *T.* cellulases. *T. viride* cellulase possessed an efficient adsorption-desorption on the substrate and reached the highest FPA difference (0.72 U/mL) among enzyme activities, indicating to its excellent hydrolysis capability. However, β-glucosidase in *T. viride* cellulase system showed close bonding on the substrate, suggesting that efficiencies of adsorption-desorption on the cellulose are different between the entire cellulase system and β-glucosidase. *T. viride* cellulase, with active endogenous β-glucosidase (1.60 U/mL), has compatible synergism with the additional exogenous β-glucosidase.

Keywords: Trichoderma cellulase; Steam-pretreated Lespedeza cellulose; Adsorption and desorption; Hydrolysate evaluation; Enzymatic activity

*Contact information: Department of Chemistry and Chemical Engineering, Beijing Forestry University Beijing 100083, China; * Corresponding author: jiangjx2004@hotmail.com*

INTRODUCTION

The inexpensive and renewable lignocelluloses from agriculture and forestry are potential raw materials for large-scale bio-ethanol production (Olofsson et al. 2008; Petersson and Liden 2007). *Lespedeza crytobotrya*, a perennial shrub species of the leguminous genus *Lespedeza*, is a drought-resistant and frost-hardy substantial biomass with a well developed root system. It acts as a ground cover to prevent loss of soil and water in varying regions. Its branches and leaves have been used as manure and forage, and its stalks could be an appropriate stuff for biomass conversion (Wang et al. 2009). Separation of the steam-pretreated *Lespedeza* stalks into its lignin, hemicelluloses, and cellulose components allows the development of new bio-refined products and cost effective bio-ethanol production. The cellulose of steam-pretreated *Lespedeza* can be readily converted to ethanol.

The relatively high cost of the cellulase preparation for cellulose hydrolysis remains one of the major obstacles to the large-scale commercial production of bio-

ethanol with the cellulosic biomass (Stenberg et al. 1998). The key issue in cellulase utilization is the difficulty in converting the specific cellulose to fermentable sugars at high yield and in an efficient way (Kovacs et al. 2008).

As an approach to addressing this problem, four *Trichoderma* strains have been selected to excrete the cellulase with high-performance extracellular multi-enzymatic system and substrate specificity (Zhang et al. 2006; Mandelset al. 1971; Latifian et al. 2007; Domingues et al. 2000). *Trichoderma reesei*, which has been extensively studied and reported to produce highly active endoglucanase and exoglucanase, is therefore of great interest. *Trichoderma viride*, a good producer of cellulase, hemicellulase, and β-glucosidase, allows for a comprehend-sive study in the rate and extent of lignocellulosic material hydrolysis (Gomes et al. 1992; Gong et al. 1977; Marques et al. 2003; Manonmani and Sreekantiah 1987). Also, *Trichoderma pseudokoningii* and *Trichoderma koningii* represent two capabilities in secretion of extracellular cellulase system with relatively high β-glucosidase and endoglucanase activities, respectively (Chandra et al. 2009; Sidhu et al. 1985; Kubicek 1982; Wood and McCrae 1978). However, not only the endoglucanase and exoglucanase but also the β-glucosidase activity plays an important role in the cellulose degradation. A high content of β-glucosidase from other sources is supplemented in the excreted cellulases to increase the final glucose yield (Chandra et al. 2009).

The digestion of cellulose to fermentable sugars is a process that involves the synergistic action of endoglucanase (EGI and EGII), exoglucanase or cellobio-hydrolyase (CBHI and CBHII), and β-glucosidase (cellobiase). Adsorption of cellulase on the lignocellulosic substrate during the hydrolyzing process has been proposed for the last 30 years (Várnai et al. 2010). Most studies concern the two-phase enzyme–substrate interaction that occurs within the first 30 min of incubation (Tu et al. 2009). However, the adsorption–desorption circular phenomenon of different enzyme components on the cellulosic substrate during the whole hydrolysis process is still not fully understood because of its complexity.

It is known that exoglucanase is competitively inhibited by the hydrolysis product cellobiose. Cellulase is stimulated more effectively by β-glucosidase components than by endoglucanase in hydrolyzing native cellulose, demonstrating that the β-glucosidase plays a significant role in the cellulose degradation (Berghem and Pettersson 1974). Therefore, bonding of enzymatic components on the substrate during the cellulose hydrolyzing process can be estimated based on the total activity (filter paper activity) and β-glucosidase activity in the hydrolysate.

Currently, great efforts are focused on the development of cellulases of high efficiency, considering both different sources and transgenic modification, and to explore new fields for their application (Hu et al. 2009). *T. viride*, *T. pseudokoningii*, *T. koningii*, and *T. reesei* were chosen to generate the enzyme complexes for hydrolysis of the specific substrate in present study. The aim of this study is to investigate the hydrolytic specificities of the secreted cellulase preparations from *Trichoderma* strains to the cellulose of steam-pretreated *Lespedeza*. The relation between the cellulolytic enzyme activities in the hydrolysate and the corresponding amount of released glucose was also evaluated.

EXPERIMENTAL

Substrate Preparation

Lespedeza stalk used in this experiment was kindly provided by the experimental farm of Beijing Forest University. It was composed of 37.6% cellulose, 20.3% hemicelluloses, 22.0% lignin, and 2.1% wax. The stalk was steam-pretreated at 212°C for 4 min, and contents of cellulose, hemicellulose, lignin, and wax in the pretreated sample were found to be 68.9%, 8.2%, 11.5%, and 1.7%, respectively (Jiang et al. 2006). The pretreated material was collected and oven-dried at 60 °C until a constant weight was achieved, usually after a minimum of 12 h. Then the steam pretreated samples were ground using a laboratory knife mill until the material passed through a 60-mesh screen with a size of 0.3 mm. Removal of lignin and hemicelluloses fractions from the milled material were carried out with sodium chlorite and sodium hydroxide. The cellulose fraction was composed of 95.8% cellulose and 2.5% hemicelluloses. Degree of polymerization and crystallinity index of the cellulose fraction were 486.7 and 50.7%, respectively. It was used as substrate for enzymatic hydrolysis experiments. Whatman No. 1 filter paper, microcrystalline cellulose Avicel PH101, and para-nitrophenyl-β-D-glucopyranoside (pNPG) were purchased from Sigma-Aldrich Company (Beijing).

Microorganism

T. viride (CGMCC no. 3.2941) and *T. pseudokoningii* (CGMCC no. 3.3002) were purchased from China General Microbiological Culture Collection Center. *T. koningii* (CICC no. 13007) and *T. reesei* (CICC no. 13052) were provided by China Center of Industrial Culture Collection. The fungi were inoculated in the PDA liquid medium for 3 days as original strains. Subsequently, the entire original microorganisms cultivated in the PDA medium were conserved for the following cellulase-produced fermentation.

Cellulolytic Enzymes Preparation

Shake flask fermentations were carried out in 250-mL cotton-plugged Erlenmeyer flasks containing 90 mL of Mandels medium (Mandels et al. 1971) supplemented with 2% (w/v) microcrystalline cellulose (MCC) and 0.5% (w/v) peptone for the cellulase production. After autoclaving at 121 °C for 20 min, the flasks were allowed to slowly cool to room temperature and then inoculated with 10 mL original microorganism in the PDA liquid medium of each fungal strain. Flask cultivation was performed at 30 °C on a rotary shaker at 150 rpm. After 5 days of cultivation, samples were centrifuged at 10000 × g for 30 min and the clear supernatants were ethanol-precipitated at minus 20 °C and resolved in the 0.1 mol/L sodium acetate buffer (pH 4.8). The four cellulase preparations were used for the subsequent enzymatic hydrolysis. Novozym 188, a β-glucosidase preparation from *Aspergillus niger*, was kindly donated by Novozymes A/S (Bagsvaerd, Denmark).

Cellulose Hydrolysis with the Cellulase Preparations

The enzymatic hydrolysis was accomplished with an enzyme loading of 10 filter paper activity unit/g substrate in duplicate to evaluate the hydrolytic potential of the cellulose from steam-pretreated *Lespedeza* by the cellulase preparations from *T.* strains.

In order to achieve effective cellulose hydrolysis, β-glucosidase supplementation was achieved with Novozym 188 at an enzyme loading of 20 β-glucosidase activity unit/g substrate. The pretreated cellulosic substrates were diluted with 0.1 mol/L sodium acetate buffer (pH 4.8) to 50 g substrate/L in a total reaction volume of 50 mL. The saccharification was cultivated at 45 °C on a rotary shaker at 180 rpm for 72 h. Samples were withdrawn and centrifuged at 10000 × g for 10 min. The hydrolysates were filtered through 0.2 μm filters and diluted properly for the further neutral sugar analysis.

Analytical Methods

The filter paper activity (FPA) was evaluated following the standard method of IUPAC (Ghose 1986). The β-glucosidase activity (BGA) was determined using the modified Berghem's method (Kovacset al. 2009). The assay mixture contained 1 mL 5 mmol/L pNPG in 0.05 M sodium acetate buffer (pH 4.8) and 100 μL appropriately diluted enzyme solution. After incubation at 50 °C for 10 min, 2 mL 1 mol/L Na_2CO_3 was added to the mixture.

The sample was diluted with 10 mL distilled water, and the liberated p-nitrophenol (PNP) was measured at 400 nm. One international unit (IU) of β-glucosidase activity liberates 1 μmol PNP per minute under the assay conditions. The above experiments were repeated three times, and data are presented as the mean values. Enzymatic protein concentration was determined by the Bradford method (Bradford 1976) using bovine serum album as a standard.

The sugars released from digest of the *Lespedeza* cellulose were analyzed using an ion exchange chromatography (Dionex, ICS 3000, USA) equipped with a pulsed amperometric detector. Glucose and cellobiose in the hydrolysates were separated using an anion exchange column (PA-10) with solution of 20 mmol/L NaOH for 40 min and 40 mmol/L NaOH for another 25 min at a constant flow rate (0.5 mL/min). The glucose yield was the detected glucose concentration in the digest multiplied by the dilution factor. The conversion expressed in percent was calculated based on the ratio of glucose released in the hydrolysate to the glucose weight of the loading cellulose.

RESULTS AND DISCUSSION

Trichoderma Cellulase Production

In the base-line experiment, evaluations of FPAs and BGAs of *Trichoderma* cellulase preparations were accomplished based on the standard method of IUPAC (Ghose 1986) and the modified Berghem's method (Kovacs et al. 2009), respectively. As shown in Fig. 1, *T. viride* and *T. pseudokoningii* cellulases achieved relative high FPA (4.34 and 4.33 U/mL, respectively). *T. viride* cellulase exhibited much higher BGA (1.60 U/mL) than the other *T.* cellulases.

The corresponding percentages of BGA in the total cellulolytic enzyme activity (FPA) from the four *Trichoderma* cellulase preparations were 36.88%, 5.90%, 6.54%, and 4.35%, respectively.

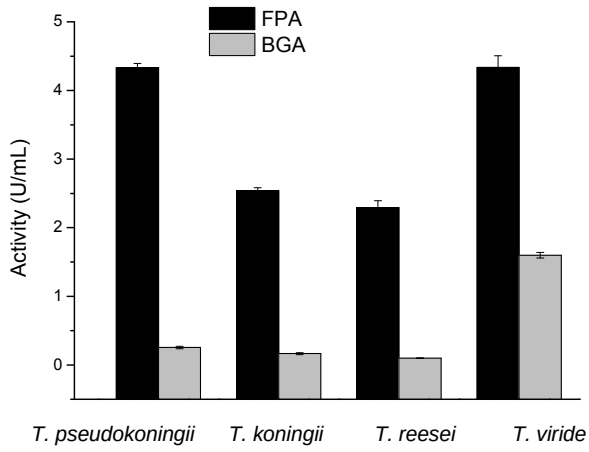

Fig. 1. The filter paper activities (FPA, U/mL) and β-glucosidase activities (BGA, U/mL) of the cellulases secreted by *Trichoderma pseudokoningii, Trichoderma koningii, Trichoderma reesei* and *Trichoderma viride*

Glucose Production

As a first step to evaluate the possible effect of cellulases secreted from *T.* strains, cellulose of steam-pretreated *Lespedeza* was subjected to enzymatic hydrolysis for 72 h. Experiments were carried out at a 5 wt% consistency with the enzyme loading of 10 FPA unit/g of cellulose. *T.* cellulase preparations were supplemented with exogenous β-glucosidase (Novozym 188) in order to equalize the BGA / FPA ratio to 2. The main role of β-glucosidase addition is to reduce the intermediate product inhibition during the processing.

The glucose yields increased with increment of hydrolysis time, and they were 69.88%, 69.16%, 69.50%, and 90.09% in the hydrolysates at 72 h with *T. pseudokoningii*, *T. koningii*, *T. reesei*, and *T. viride*, respectively. The *T. viride* cellulase showed a similar trend of glucose concentration in curves of 12h and 72 h with the other *T.* cellulases. However, the hydrolysis with *T. viride* cellulase released more glucose than that with other *T.* cellulases (Fig. 2). This indicates that *T. viride* cellulolytic enzyme system has a significant effect on enzymatic hydrolysis of cellulose from steam-pretreated *Lespedeza*, which could be initially attributed to high endoglucanase and exoglucanase activities or hydrolytic specificity towards the substrate.

The cellulosic components were rapidly digested by the synergic action of the cellulolytic enzymes from *T. viride* to achieve the efficient polysaccharide degradation and glucose production in the digest. The hydrolysis was carried out under the typical endo–exo synergism of fungal enzymes, promoting the progressive peeling of cellulose molecules from the surface of the fiber without accumulating any detectable short cellooligomers in the hydrolysis mixture (Martins et al. 2008). Thus, the high level of glucose yield with *T. viride* cellulase also indicates that the enzymatic system plays an effective synergistic action among the endoglucanase, exoglucanase, and β-glucosidase in contrast with the other *T.* cellulases.

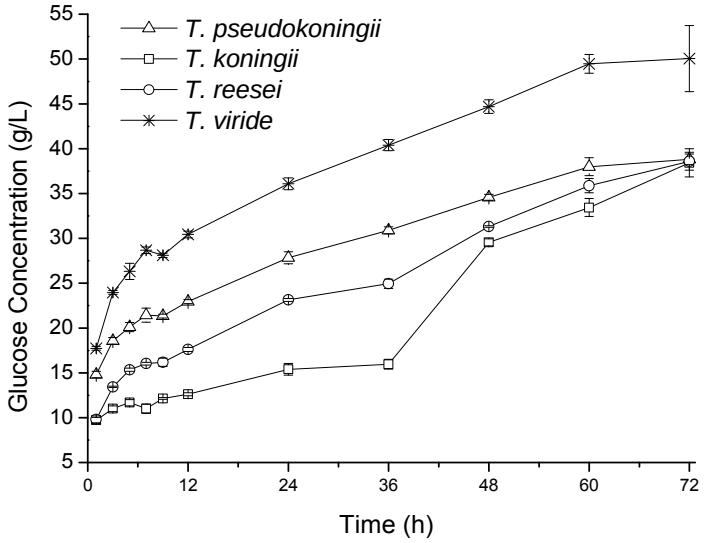

Fig. 2. The glucose concentration in the digests of cellulose from steam-pretreated *Lespedeza* with the obtained cellulase preparations

Cellobiose Production

Most cellobiose was hydrolyzed into glucose during saccharification with β-glucosidase supplementation at an enzyme loading of 20 BGA unit/g of substrate. The residual cellobiose in the hydrolysate of cellulose from steam-pretreated *Lespedeza* (Fig. 3) was largely uneven for different *T.* cellulase loading. The cellobiose concentration in the digest with *T. viride* cellulase increased to 0.76 g/L at 7 h and finally reached 1.47 g/L at the end of saccharification.

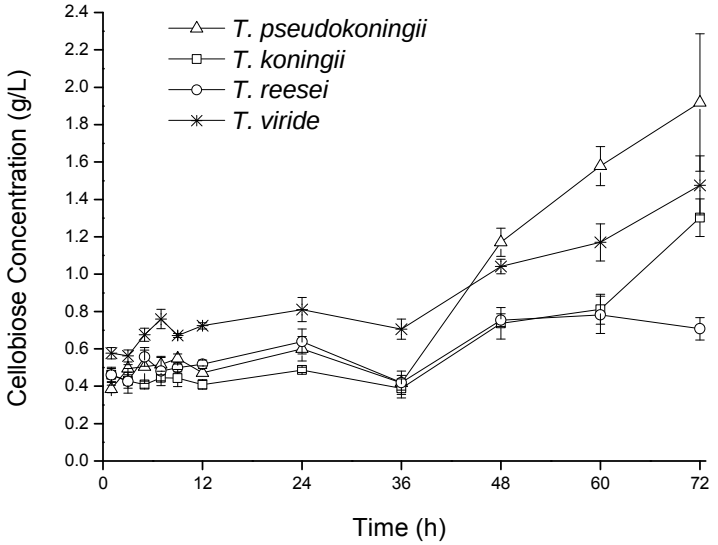

Fig. 3. The cellobiose concentration in the digests of cellulose from steam-pretreated *Lespedeza* with the obtained cellulase preparations

Enzymatic hydrolysis of polysaccharide using *T. koningii* cellulase and Novozym 188 yielded 4.74% ratio of cellobiose to glucose within 12 h, while they were 2.66%, 4.70%, and 3.25% for *T. pseudokoningii, T. reesei,* and *T. viride* cellulases, respectively (Fig. 4). However, the ratio achieved a rapid increment from 36 to 72 h, and it reached 4.94% at 72 h for *T. pseudokoningii*. As for *T. viride* cellulase and Novozym 188, the ratio reached 2.73% at the end of hydrolysis.

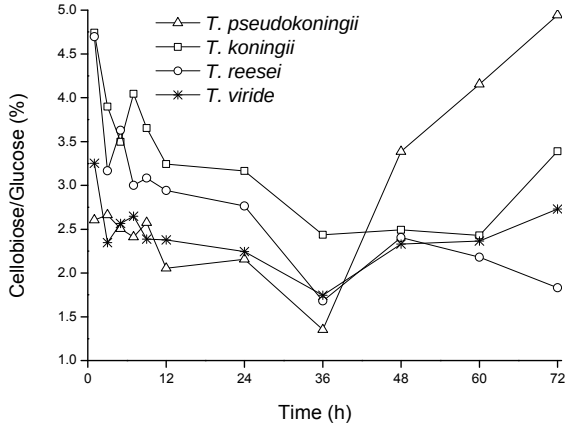

Fig. 4. The ratios of cellobiose to glucose in the digests of cellulose from steam-pretreated *Lespedeza* with the obtained cellulase preparations

Cellobiose created by the cellulase could not be digested in-process rapidly by the β-glucosidase within a few hours, leading to a cellobiose yield increase as a consequence of accumulation. The wavelike curves in Fig. 4 are reflective of the accumulation and consumption of cellobiose during saccharification. Differences among the ratios of cellobiose to glucose may be due to differences in their cellulolytic enzyme systems. An interesting observation during these experiments was that all the ratios reached their lowest point at 36 h, suggesting that the cellobiose released by the exoglucanase and endoglucanase is appropriately degraded into glucose by the β-glucosidase with minimum accumulation. *T. koningii* cellulase exhibited high cellobiose ratio in the 12 h digestion due to the low glucose concentration in the corresponding digest (Fig. 2), indicating that the additional β-glucosidase achieves the lowest hydrolytic efficiency in the digest with *T. koningii* cellulase within 12 h compared to other *T.* cellulases. *T. pseudokoningii* cellulase showed high cellobiose conversion compared to *T. viride* cellulase at the end of hydrolysis. The result indicates that cellobiose accumulation could result in lower glucose conversion. Cellulase from *T. viride* was superior to other *Trichoderma* cellulase preparations for glucose conversion, probably due to its high β-glucosidase activity in the cellulolytic enzyme system. Accumulated glucose in the hydrolysate may be another factor that inhibits the original β-glucosidase activity in the cellulolytic enzyme systems during the hydrolysis (Breuil et al. 1992). However, no attempt was made in this study to investigate the above hypothesis.

Protein Concentration

Protein concentrations, FPAs, and β-glucosidase activities of *T.* cellulases remaining in the digests of cellulose from steam-pretreated *Lespedeza* were measured to

evaluate the bonding performance of the enzymes. The loaded enzymes were diluted in the reacting solution. Concentrations of them were calculated to be 0.5 U/mL FPA and 1 U/mL BGA in the corresponding digests based on the initial *T.* cellulases and Novozym 188 loading. The detected values were higher than the theoretical values. Although the detected activity values were low, differences in hydrolysis broths allow direct comparisons of adsorption-desorption performance of the loaded enzymes on the selected substrate. Profiles of protein contents in the corresponding digests showed frequent fluctuation at 12 h (Fig. 5), which suggested that most adsorption-desorption of the loaded enzymes on the substrate existed at the foremost 12 h. Slight waves could also be observed after 12 h, implying that continuous bonding and release of the enzymatic proteins happened during the whole process of hydrolysis. The same result has been reported on delignified substrates previously (Boussaid and Saddler 1999). The protein of *T. viride* cellulase achieved the least concentration in the digest within 12 h, and the glucose and cellobiose yields after 12 h of hydrolysis by *T. viride* cellulase was higher than that obtained from other *T.* cellulases. The results indicated that the *T. viride* cellulase system performed the most effective degradation to the cellulosic substrate at the initial stage of hydrolysis. *T. viride* cellulase gained the least protein content and highest glucose yield in the corresponding digest at the end of hydrolysis, suggesting the more bonding of the enzyme on the substrate, the more glucose production would be accomplished.

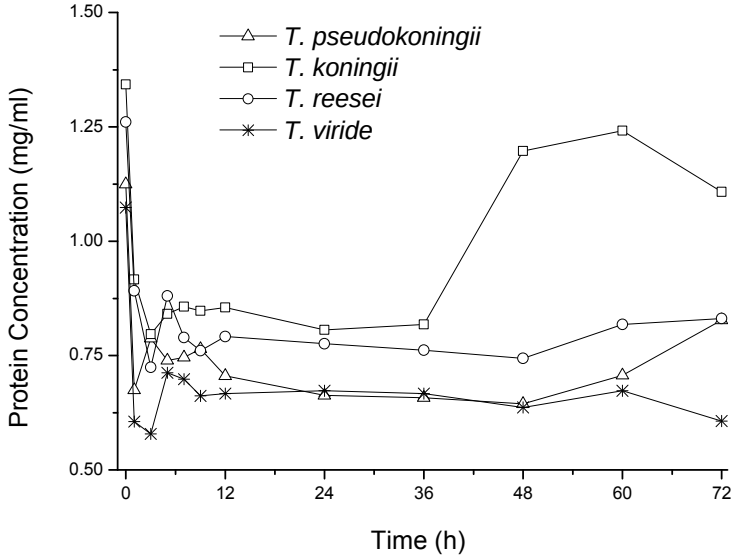

Fig. 5. The protein concentrations in the digests of cellulose from steam-pretreated *Lespedeza* with the obtained cellulase preparations

Filter Paper Activity

The FPAs of residual cellulases in the digests performed fluctuant profiles during the saccharification (Fig. 6). *T.* cellulases achieved significant adsorption-desorption on the cellulosic substrate during the course of hydrolysis. Cellulase from *T. viride* exhibited the most active adsorbing and desorbing performance within 12 h (Fig. 6A). The residual

cellulase reached a relatively low FPA value (0.73 U/mL) at 9 h, suggesting that a maximum amount of *T. viride* enzymes is adsorbed on the substrate in the hydrolytic process. Effective enzymatic hydrolysis of the substrate keeps going with the adsorption-desorption of cellulase according to the promoted glucose concentrations within 12 h. *T. viride* enzyme reached their minimum FPA point (0.80 U/mL) in the digest at 36 h (Fig. 6B). Also, the protein concentration and FPA value both showed a quick decrease after 60 h (Fig. 5 and 6B), indicating that the enzyme achieved adsorption on the substrate. Profiles of the *T. pseudokoningii* and *T. koningii* enzyme activities were almost same within 36 h (Figs. 5 and 6B). Both enzymes performed obvious adsorption-desorption on the substrate. Residual enzyme in the digest with *T. pseudokoningii* cellulase achieved its minimum FPA value (0.77 U/mL) at 48 h. The quick adsorption of *T. reesei* cellulase on the substrate after the start of enzymatic hydrolysis was revealed by the protein content and residual FPA analysis in the corresponding digest. The adsorption results are well in accordance with results of Zhou et al. using *T. viride* cellulase and microcrystalline cellulose substrate (Zhou et al. 2004). *T. reesei* cellulase in the digest obtained its minimum FPA value (0.60 U/mL) at 12 h, followed by slightly increase until the end of saccharification. *T. reesei* enzyme released less cellobiose after 36 h than that from the other *T.* strains. The result indicates that some enzymes remain inefficient bonding on the substrate.

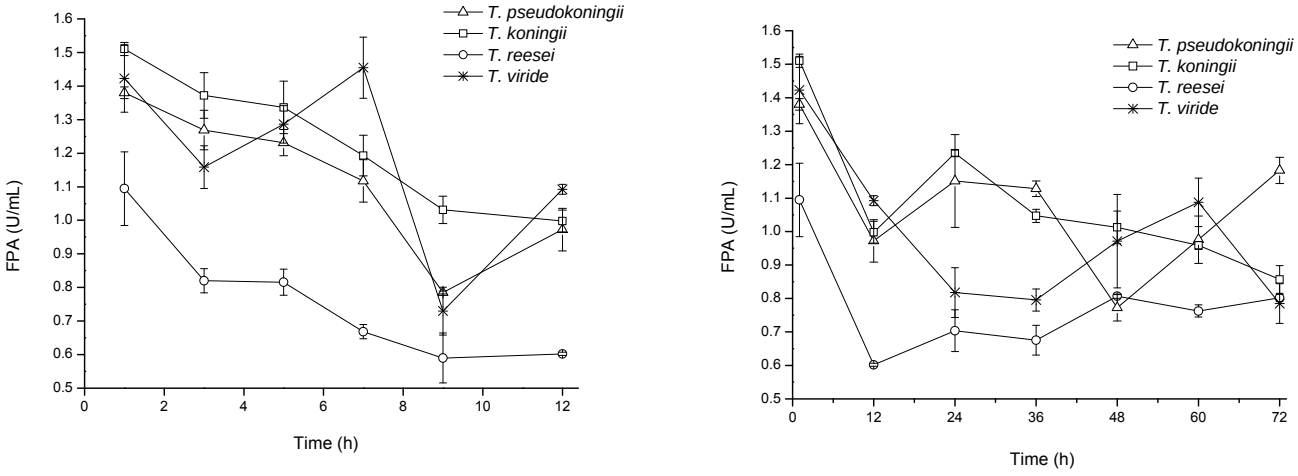

Fig. 6. The filter paper activities of the residual cellulases in the digests of cellulose from steam-pretreated *Lespedeza* with the obtained cellulase preparations within 12 h (A) and 72 h (B)

T. viride cellulase gave the highest FPA difference (0.72 U/mL) among the enzyme activities within hydrolytic process, compared to cellulases from *T. koningii, T. pseudokoningii* and *T. reesei* (0.65, 0.61 and 0.49 U/mL, respectively) in the digest (Fig. 7). The value indicates that *T. viride* enzyme has the most effective capabilities of adsorption and desorption to the cellulose from steam-pretreated *Lespedeza*. *T. viride* cellulase achieved more efficient glucose production than other *T.* cellulases, which also confirms its super hydrolysis efficiency to the substrate.

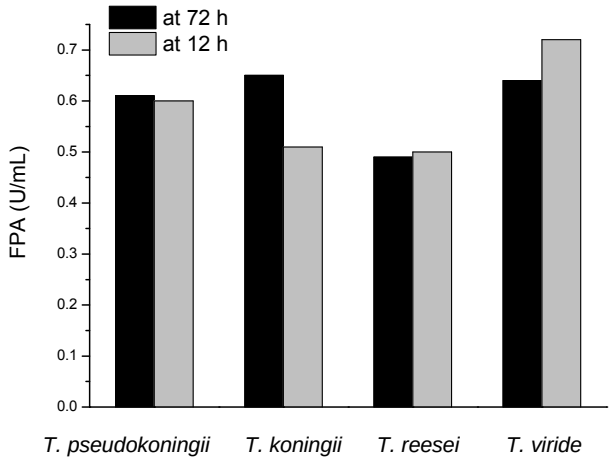

Fig. 7. The FPA difference between high and low activity values of the residual cellulases in the digests of cellulose from steam-pretreated *Lespedeza* with the obtained cellulase preparations

β-glucosidase Activity

Obvious increments of β-glucosidase activity were observed in the hydrolyzates with *T.* cellulases after 9 h (Fig. 8A). The profile of β-glucosidase activity exhibited by *T. viride* was almost constant in the corresponding digest during the saccharification (Fig. 8B). Similar phenomenon has been reported with AnCel3A β-glucosidase on catalytically delignified spruce hydrolyzation (Várnai et al. 2010). Other *T.* cellulases exhibited several obvious waves in profiles of β-glucosidase activity within 72 h, which suggests that these enzymes follow the trend of continual adsorption and desorption on the cellobiose.

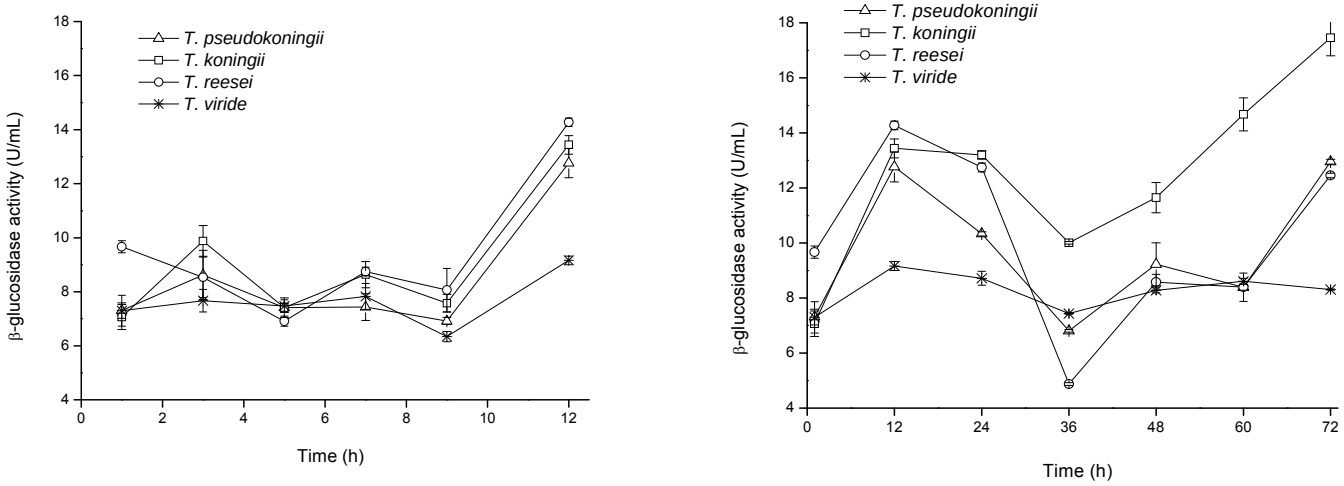

Fig. 8. The β-glucosidase activities of the residual cellulases in the digests of cellulose from steam-pretreated *Lespedeza* with the obtained cellulase preparations within 12 h (A) and 72 h (B)

Along with the highest glucose concentration and lowest protein concentraton in the digest (Fig. 2 and 5), *T. viride* cellulase had the lowest β-glucosidase activity at 72 h, indicating that β-glucosidase performed effective adsorption and degradation to cellobiose during the process of hydrolysis. The enzyme gave the most glucose yield compared with other three *T.* cellulases, implying that sustained adsorption of β-glucosidase on the substrate could result in more release of the end-product. *T. viride* cellulase showed the least fluctuation in the curves of the residual β-glucosidase activities, while the enzyme exhibited the most waves in FPA profiles among the residual cellulases in corresponding digests with the four *T.* cellulase. The results suggest that enzymatic adsorption and desorption on the cellulose from steam-pretreated *Lespedeza* have different efficiency for the entire cellulase system and the β-glucosidase. *T. viride* cellulase had the most active endogenous β-glucosidase (Fig. 1), which probably contribute to greater synergism with the additional exogenous β-glucosidase. Therefore, the enzyme achieved high hydrolysis capability as well as the efficient adsorption on the substrate.

Profiles of cellobiose concentration and β-glucosidase activity in the corresponding digests exhibited their low points at 36 h (Fig. 3 and 8B), which suggests that β-glucosidase achieved maximum amount of adsorption and effective degradation of cellobiose at that moment. The residual β-glucosidase activity with *T. koningii, T. reesei* and *T. pseudokoningii* cellulases increased from 36 to 72 h, meaning that β-glucosidase started to release from cellobiose. However, *T. viride* cellulase had most β-glucosidase adsorbed on the substrate, which confirmed the hypothesis of the better hydrolysis efficiency for the *T. viride* cellulase. The protein concentration and β-glucosidase activity in the digest with *T. koningii* cellulase increased rapidly after 36 h. Only FPA in the digest with *T. koningii* cellulase decreased continuously, which suggests that most additional β-glucosidase started to desorb from the substrate after 36 h, and cellulose from steam-pretreated *Lespedeza* is degraded by endoglucanase and exoglucanase in the *T. koningii* cellulase system.

CONCLUSIONS

This study demonstrates that the source of the strain used for cultivation of cellulase strongly influences the resulting cellulolytic enzyme capabilities. Evaluation of cellulase adsorption on the substrate was adequately achieved by determinations of protein concentration, filter paper activity, and β-glucosidase activity of residual cellulase in the corresponding digest. The addition of β-glucosidase will influence the extent of bonding and hydrolysis of cellobiose. The cellulase preparation from *T. viride* with additional exogenous β-glucosidase possessed high hydrolysis capability to the cellulose from steam-pretreated *Lespedeza* due to its efficient adsorption-desorption on the substrate and compatible synergism. Cellulase with excellent hydrolysis capability for a substrate can, thus, be produced by cultivation with *T. viride*. Further efforts should be aimed at investigating how the adsorption-desorption performance of β-glucosidase on cellobiose influences the release of cellobiose and glucose during the whole process of hydrolysis.

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