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Production and Characterisation of Cellulase from Solid State Fermentation of Rice Straw by *Trichoderma harzianum* SNRS3

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ABSTRACT

Research on production and the use of cellulase and xylanase by commercial microbial strains is widely reported. However, research on production of cellulase and xylanase by local isolates of *Trichoderma harzianum* known as potential cellulase producers is still very limited. *T. harzianum* SNRS3 was used for cellulase and xylanase production from rice straw under solid state fermentation. Our study revealed that unlike *Trichoderma* sp. that is normally associated with low amounts of β-glucosidase, insufficient to perform an efficient hydrolysis, *T. harzianum* SNRS3 could be considered as a potential β-glucosidase producer, but not an efficient xylanase producer. As a result of storage of the crude cellulase at room temperature, β-glucosidase activity only decreased to above 80% of its original activity at the end of the 3rd week of storage. The crude cellulase produced by *T. harzianum* SNRS3 could be industrially applied as the enzyme is still highly active at 60°C and over a wide range of acidic pH.

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INTRODUCTION

Plant biomass is composed primarily of cellulose, hemicelluloses, and lignin (Kuhad et al., 1997; Carpita et al., 2001). Lignocellulosic waste materials

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obtained from energy crops, wood and agricultural residues represent the largest global renewable reservoir of potentially fermentable carbohydrates (Mtui & Nakamura, 2005; Talebnia et al., 2010). Therefore, Lignocellulosic wastes are regarded as attractive substrates for the production and recovery of a large number of value-added products such as enzymes (Mtui & Nakamura., 2005).

Having occupied 2% of the world's cultivated area; rice ranks the second most major crop worldwide. Rice is an extensive crop of Asia and Southeast Asia, which dominates tropical and sub-tropical belts (Devendra & Thomas, 2002; Leff et al., 2004). Rice straw is an abundant lignocellulosic waste in the world with several characteristics that make it an appropriate feed stock for biofuel production (Binod et al., 2010). Among the lignocellulosic crop residues, rice straw is the largest biomass feedstock in the world (Talebnia et al., 2010).

A variety of microorganisms such as bacteria and fungi are plant biomass decomposers in nature. This makes them interesting sources for enzyme discovery (Allgaier et al., 2010). Lignocellulosic enzymes are important commercial products of lignocellulosic wastes bioprocessing used in many industrial applications including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Howard et al., 2003; Rahnama et al., 2014). Currently, cellulases rank as the third largest volume of industrial enzyme with a wide range of applications in industry. Cellulases are employed in cotton processing, paper recycling, as detergent enzymes, in juice extraction, and as animal feed additives apart from their immense use in the hydrolysis of lignocellulosic biomass as a prerequisite for biofuel production (Wilson, 2009; Singhania et al., 2010). The use of lignocellulosic biomass-derived sugar for biofuel production has been shown to be promising (Park et al., 2012). In fact, cellulase will become the largest volume industrial enzyme, in case fermentation-derived fuel such as bioethanol, and biobutanol could replace the current fossil-based transportation fuel (Wilson, 2009).

Solid state fermentation (SSF) is known as the fermentation whereby an insoluble substrate is fermented in the absence or almost absence of free moisture (Chahal, 1985). Submerged fermentation (SmF) and SSF are both the common techniques applied for production of cellulases. SSF is advantageous over SmF due to the production of the enzymes at a higher yield and higher pH or temperature stability (Holker & Lenz, 2005; Singhania et al., 2009; Saqib et al., 2010).

Cellulases are the most extensively studied multiple enzyme complexes. As hydrolytic enzymes, cellulases cleave the β-1,4- glucosidic bonds in the cellulose structure (Singhania et al., 2010). The major cellulase components include cellobiohydrolases (exo-1,4-β-D-glucanase, CBH), endoglucanases (endo-1,4-β-D-glucanase, EG) and β-glucosidase (Hong et al., 2001; Li et al., 2006; Sun et al., 2008; Singhania et al., 2010). Nonetheless,

β-glucosidase does not act upon cellulose chain directly. Instead, β-glucosidase is responsible for the cleavage of cellobiose into two glucose monomers and this characteristic of β-glucosidase is of great significance since accumulation of cellobiose causes a product inhibition on both cellobiohydrolase and endoglucanase. Therefore, in addition to glucose production, β- glucosidase reduces product inhibition by cellobiose, which in turn causes cellulolytic enzymes to function more efficiently (Workman & Day, 1982; Lymar et al., 1995; Joo et al., 2009). Hence, β-glucosidase is the rate-limiting factor in the enzymatic hydrolysis of cellulose (Lymar et al., 1995; Fadel, 2000; Elyas et al., 2010). Cellulases with low levels of β-glucosidase hydrolyse cellulose slowly, and in such cases, the addition of β-glucosidase enhances cellulose hydrolysis (Johnson et al., 1982; Lymar et al., 1995; Fadel, 2000; Elyas et al., 2010). Unfortunately, the majority of cellulolytic fungi, including hypercellulase-producing mutants of *Trichoderma reesei* show low production of β- glucosidase (Saha et al., 1994; Skory & Freer, 1995; Riou et al., 1998; Elyas et al., 2010).

Xylanases, the xylan degrading enzymes, are present everywhere and are diverse by nature (Collins et al., 2005). Xylan hydrolytic enzymes have been reported mainly from bacteria (Gilbert & Hazlewood, 1993), fungi (Jin et al., 2012) and yeast (Hrmova et al., 1984; Liu et al., 1998). Microbial xylanases are preferable since they are highly specific, reaction conditions are mild, and substrate loss and generation of side products are almost negligible (Kulkarni et al., 1999). As the xylan molecule contains several substituted groups and side chains synergistic action of multiple hydrolytic enzymes might be essential for the molecule to be completely hydrolysed (Dhiman et al., 2008). Xylanases have many biotechnological applications and are therefore categorised among the most important enzymes in industry. Xylanases have numerous applications in biotechnology, and are widely used in food, animal feed, paper, and pulp industries, as well as in bioconversion of hemicelluloses to value-added products (Dhiman et al., 2008; Chapla et al., 2010).

Since understanding the action of cellulolytic enzymes under different conditions is of great importance, many researchers have focused on the characterisation of cellulase in terms of various physio-chemical parameters including temperature and pH (Farinas et al., 2010).

This study provides a comparison between cellulase production from rice straw under SSF by the local *T. harzianum* SNRS3 and *A. niger* ATCC 6275 as a model fungus. Crude cellulase enzymes produced by *T. harzianum* SNRS3 were characterised in terms of physio-chemical parameters such as optimum temperature, and pH. Thermal stability and the effects of storage at room temperature (28ºC), 4ºC, −20ºC, and −40ºC on the enzyme activity have also been studied.

MATERIALS AND METHODS

Substrate Preparation and Pretreatment

Rice straw was obtained from a paddy field in Sekinchan, Selangor, Malaysia. The dried rice straw was ground to 2 mm lengths using an electric grinder (Model CW-1, Hsiang Tai, Taiwan), and kept in a cold room at 4°C prior to use.

Microorganism and Inoculum Preparation

A local isolate of *T. harzianum* SNRS3 (isolated from rice straw collected from a rice field in Sekinchan, Selangor, Malaysia) and *A. niger* ATCC 6275 (used as model fungus) were used as inoculum, respectively. The fungal spores were kept in 30% (v/v) glycerol at minus 20°C. Reactivation of the spores was performed by growing on Potato Dextrose Agar (PDA) for 7 to 9 days. Spore suspension was freshly prepared prior to fermentation experiment by washing the agar surface with sterilised distilled water. The spores were then quantified and adjusted to 1×10^6 spores mL^{-1} by using a haemocytometer (Rahnama et al., 2013).

Fermentation

Cellulase enzyme was produced by solid state fermentation. A series of 250 mL Erlenmeyer flasks with cotton stoppers were autoclaved and used for the production and collection of the enzymes. Three grams of untreated rice straw was placed in different flasks. Mandels medium (Mandels et al., 1974) was added to each flask containing the rice straw, and the moisture content was kept at 65% (w/v). Mandels medium (1 L) contained 1.4 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.63 g urea, 0.3 g $CaCl_2$, 0.3 g $MgSO_4$. $7H_2O$, 1 mL Trace elements, 0.75 g peptone, and 2 mL Tween 80. The pH of the medium was adjusted to 5. The flasks were then incubated at 30°C prior to the extraction of crude enzyme. The extraction of crude enzyme mixture was carried out by adding 30 mL of 50 mM citrate buffer (pH 4.8) into each flask, followed by agitation for 30 min at 150 rpm and 30°C. The mixture was then centrifuged at 4°C and 1000 × g for 10 min. The supernatant was filtered and kept at 4°C prior to use (Rahnama et al., 2013).

Crude Cellulase Enzyme Characterisation

The crude cellulase enzyme produced from rice straw by *T. harzianum* SNRS3 was characterised in terms of temperature and pH optima, thermal stability and storage stability at various temperatures [room temperature (28°C, 4°C, −20°C, and −40°C)]. In order to study the optimum incubation temperature for the crude cellulase activity, the reaction mixtures were reacted in a temperature range of 40-90°C and incubated for 1 h for FPase and for 30 min for CMCase and β-glucosidase; the three major components of cellulases that act synergistically for the complete hydrolysis of cellulose. It was then followed by the assay under the standard assay conditions at pH4.8, as described in detail in the analytical procedure. CMCase activity was determined by estimating the reducing sugars produced from 1% (w/v) carboxymethylcellulose,

whereas FPase activity was determined by measuring the reducing sugars released from Whatman filter paper No.1. For β-glucosidase assay, the ρ-nitrophenol liberated from ρ-nitrophenyl-β-D- glucopyranoside (PNPG) was determined spectrophotometrically (Wood & Bhat, 1988).

The effect of pH on the activity of crude cellulase was determined in a pH range of 2.5-7.5 by using citric acid-disodium hydrogen phosphate (McIlvaine) buffer solutions, pH 2.6-7.6. The activity was measured under the standard assay conditions. In order to study the crude enzyme thermal stability, the crude enzyme was incubated at 50 and 60°C. The crude enzyme solution in the screw-capped glass tubes was withdrawn periodically at the time intervals of 30 min, 1 h, 2 h, 3 h, and 4 h. It was then cooled on ice and the residual enzyme activity was assayed under standard assay conditions. In order to investigate the crude enzyme stability over storage, the crude cellulase was aliquoted in four bottles. The bottles were each kept in different temperatures including room temperature (28°C), 4°C, −20°C, and –40°C. The enzyme activity was assayed under the standard assay conditions on a weekly basis up to two months.

Analytical Procedure

Crude cellulase activity was assayed according to the standard method (Wood & Bhat, 1988). Carboxymethylcellulase (CMCase) activity was determined by estimating the reducing sugars produced from 1% (w/v) carboxymethylcellulose, whereas Filter Paperase (FPase) activity was determined by measuring the reducing sugars released from Whatman filter paper No.1. The liberated reducing sugars were measured using the DNS method (Miller, 1959). One unit of CMCase or FPase activity was defined as the amount of enzyme that liberated 1 µmol reducing sugars/min under assay conditions and expressed as a unit of enzyme activity per gram fermented dry substrate (U/g).

For FPase, substrate blank contained 2.0 mL of 50 mM sodium citrate buffer (pH 4.8) in the presence of Whatman No.1 filter paper as the substrate. Conversely, the enzyme blank contained 1.8 mL of 50 mM sodium citrate buffer (pH 4.8), 0.2 mL of the crude enzyme mixture in the absence of Whatman filter paper, whilst the test was with the presence of filter paper. The reactions were carried out for 1 h at 40ºC.

As for CMCase assay, 0.2 mL of the crude enzyme was added to 1.8 mL of 1% (w/v) carboxymethylcellulose in 50 mM sodium citrate buffer (pH 4.8), and incubated at 40ºC for 30 min. The enzyme blank contained 0.2 mL of the enzyme and 1.8 ml 50 mM pH 4.8 sodium citrate buffer only [without 1% (w/v) carboxymethylcellulose], while for the substrate blank, 0.2 ml sodium citrate buffer (pH 4.8) was used instead of the crude enzyme.

For β-glucosidase assay, the reaction mixture, which consisted of 0.2 mL crude enzyme added to 2.0 mL of 0.5 mM ρ-nitrophenyl-β-D- glucopyranoside in 50 mM sodium citrate buffer (pH 4.8) was incubated at 40°C for 30 min. The reaction

was stopped by the addition of 2.0 mL of 1 M sodium carbonate (Na_2CO_3) immediately after the incubation time (Wood & Bhat, 1988). One unit of β-glucosidase was defined as the amount of enzyme that liberated 1 µmol ρ-nitrophenol/min under assay conditions and expressed as a unit of enzyme activity per gram fermented dry substrate (U/g). The substrate blank contained 0.2 mL of 50 mM sodium citrate buffer (pH 4.8) instead of the crude enzyme, while enzyme blank was prepared by adding 0.2 mL of crude enzyme into the sodium citrate buffer only (i.e., without ρ-nitrophenyl-β-D- glucopyranoside).

The xylanase activity was assayed by estimating the reducing sugars released from 1% (w/v) Birchwood xylan (Dong et al., 1992). The reaction was carried out by adding 0.2 mL of the crude enzyme to 1.8 mL of 1% (w/v) Birchwood xylan in 50 mM sodium citrate buffer pH 4.8 and incubated at 40ºC for 30 min. As for the substrate blank, 0.2 mL of 50 mM sodium citrate buffer (pH 4.8) was added to replace the crude enzyme, while for the enzyme blank, the sodium citrate buffer (pH 4.8) did not contain any xylan. One unit of xylanase activity was defined as the amount of enzyme that liberated 1 µmol reducing sugars/min under assay conditions and expressed as a unit of enzyme activity per gram fermented dry substrate (U/g). Reducing sugars released as a result of the reaction of the enzyme (FPase, CMCase, and xylanase) and the substrate was determined by using DNS method.

For the soluble protein concentration analysis, a modified method (Lowry et al., 1951) was used with bovine serum albumin as a standard.

The impacts of temperature, pH, and storage on crude cellulase enzyme activity were expressed as relative activity that is a percentage of the maximum activity. Thermal stability of the crude cellulase was expressed as the residual activity that is a percentage of the original activity.

Statistical Analysis

The data were analysed by using one-way analysis of variance (ANOVA). Meanwhile, t Tests (LSD) was used to compare the difference of means among treatment groups. Differences of $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Cellulase from *T. harzianum* SNRS3 and *A. niger* ATCC 6275 as a Model Fungus

Cellulase production from local *T. harzianum* SNRS3 was compared to that by *A. niger* ATCC 6275 as a model fungus. Untreated rice straw was used as the fermentation substrate for both fungi. Figure 1 presents the production of FPase by both fungi studied over a period of 10 days. FPase production by *A. niger* and *T. harzianum* was not significantly different ($p>0.05$) during the first 5 days of fermentation. However, on day 6 of fermentation, FPase production by *A. niger* was significantly higher ($p<0.05$) than that by *T. harzianum* and the maximum activity of FPase was obtained at 7.06 U/g substrate and 6.25 U/g substrate using *A. niger* ATCC 6275 and *T. harzianum* SNRS3, respectively.

CMCase and β-glucosidase production profiles were also studied over a period of 10 days. A comparison between the CMCase and β-glucosidase production from rice straw by *T. harzianum* SNRS3 and *A. niger* ATCC 6275 is presented in Figure 2. The CMCase production by *A. niger* was significantly higher (p<0.05) than that by *T. harzianum* between days 1 and 3 of fermentation. However, on day 6, the CMCase obtained (111.31 U/g substrate) was significantly higher (p<0.05) than the maximum CMCase production (86.35 U/g substrate) when *A. niger* was used. β-glucosidase production in *T. harzianum* was the highest on days 7 and 8 of fermentation (p<0.05), with a yield of 173.18-173.71 U/g. However, the highest production of β-glucosidase by *A. niger* ATCC 6275 only gave an activity of 17.41 U/g (Day 7). *Trichoderma* sp. is normally associated with insufficient production of β-glucosidase to perform efficient hydrolysis, whereas *Aspergillus* sp. has been reported as the most efficient producer of β-glucosidase (Wen et al., 2005). According to the results of this study, however, *T. harzianum* SNRS3 proved to be a better β-glucosidase producer than *A. niger* ATCC 6275.

In comparison, a previous study on SSF of rice straw has reported the production of FPase (480.48 U/g substrate), CMCase (363.72 U/g substrate), and β-glucosidase (16.37 U/g substrate) by *A. terreus* ATTC 74135, and the production of 7.85 and 11.73 U/g substrate of FPase and CMCase respectively, by *A. niger* (Jahromi et al., 2011). On the other hand, using *A. niger* ATCC 6275 and palm cake under SSF, 23.8 U/g substrate CMCase was produced (Prasertsan et al., 1997).

The xylanase production profile was studied over a period of 10 days. A comparison between the xylanase production from rice straw using *T. harzianum* SNRS3 and *A. niger* ATCC 6275 is presented in Figure 3. As demonstrated in Figure 3, the xylanase production was increased significantly (p<0.05) when *A. niger* ATCC 6275 was used as the inoculum for enzyme

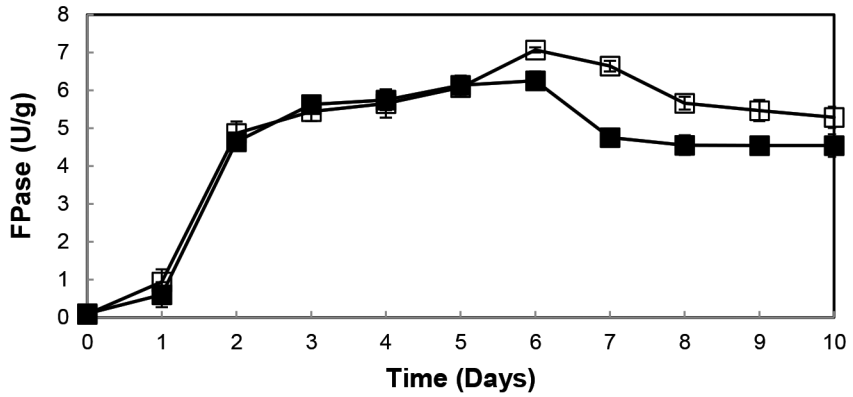

Figure 1. FPase from *T. harzianum* SNRS3 and *A. niger* ATCC 6275. Values are means of 3 replicates ± SD. Closed symbols represent: *T. harzianum* SNRS3; Open symbols represent: *A. niger* ATCC 6275

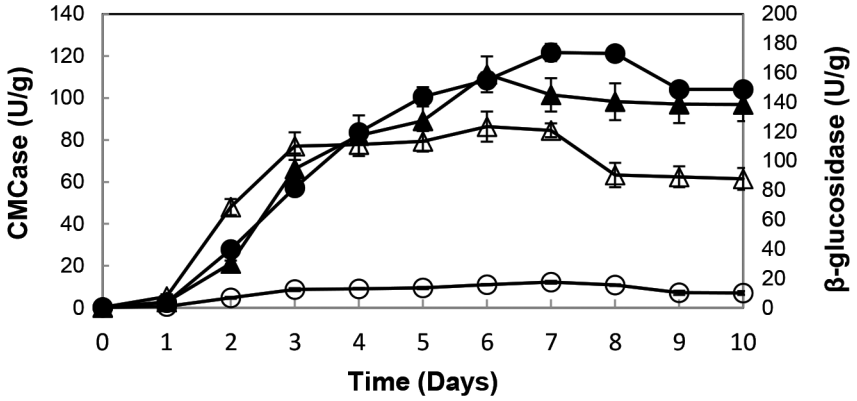

Figure 2. CMCase and β-glucosidase from *T. harzianum* SNRS3 and *A. niger* ATCC 6275. Values are means of 3 replicates ± SD. Symbols represent: (▲) CMCase activity; (●) β-glucosidase activity. Closed symbols represent: *T. harzianum* SNRS3; Open symbols represent: *A. niger* ATCC 6275

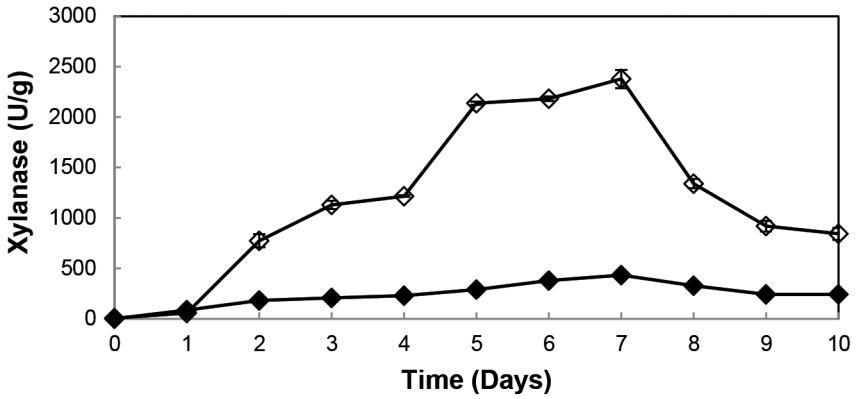

Figure 3. Xylanase from *T. harzianum* SNRS3 and *A. niger* ATCC 6275. Values are means of 3 replicates ± SD. Closed symbols represent: *T. harzianum* SNRS3; Open symbols represent: *A. niger* ATCC 6275

production. On day 7 of fermentation, xylanase was produced at the activity of 433.75 U/g substrate by *T. harzianum* SNRS3, as compared to 2378.64 U/g substrate obtained by *A. niger* ATCC 6275. Among the xylanolytic fungi, *Aspergillus* is known as an efficient and high xylanase producer (Fang et al., 2010). *A. terreus* ATCC 74135 was cultivated on untreated, ground rice straw under SSF and a very high xylanase production of 6,166 U/g substrate was obtained. Table 1 shows cellulase and xylanase production by different fungi grown on various agricultural wastes under solid state fermentation.

The profiles of extracellular protein production by *T. harzianum* SNRS3 and *A. niger* ATCC 6275 grown on untreated rice straw studied over a period of 10 days were also monitored. Figure 4 indicates that

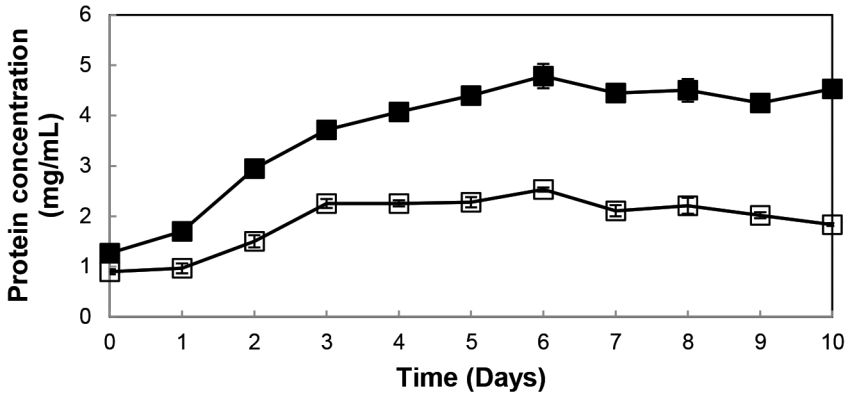

Figure 4. Extracellular protein profile of *T. harzianum* SNRS3 and *A. niger* ATCC 6275. Values are means of 3 replicates ± SD. Closed symbols represent: *T. harzianum* SNRS3; Open symbols represent: *A. niger* ATCC 6275

protein concentration increased significantly with time for *T. harzianum* ($p<0.05$), giving a higher protein concentration of 4.78 mg/mL on day 6 of fermentation compared to the maximum protein concentration of 2.43 mg/mL obtained on day 6 of fermentation for *A. niger*.

Characterisation of Crude Cellulase by *T. harzianum* SNRS3

Effect of Temperature on Crude Cellulase Activity

Figure 5 illustrates the effects of temperature on the activity of crude cellulase in a temperature range of 40-90°C**.**The temperature profile of the enzyme shows an optimal temperature of 50°C for FPase, CMCase, and β-glucosidase produced by *T. harzianum* SNRS3. The temperature profile of FPase illustrates an optimum temperature plateau ranging from 50-60°C**.** The same feature has been reported for FPase produced by *Penicillium notatum* NCIM NO-923 (Das & Ghosh, 2009) and *Penicillium funiculosum* (Karboune et al., 2008) with an optimum temperature of 50°C and 60°C, respectively. It is worth noting that FPase produced from *T. harzianum* SNRS3 can remain almost up to 100% active at 60°C, and this characteristic could be considered as a major advantage.

Unlike FPase that exhibited almost a similar optimum temperature plateau between 50-60°C, CMCase showed a different trend and the relative activity dropped sharply between 50-60°C. Similar to CMCase produced by *T. harzianum* SNRS3, CMCase produced by *Penicillium notatum* NCIM NO-923 (Das & Ghosh, 2009) and *Streptomyces* transformant T3-1 (Jang & Chen, 2003) has been reported to be optimally active at 50°C**.** However, a lower optimal temperature of 40°C has been reported for endoglucanase produced by *Aspergillus niger* Z10 (Coral et al., 2002). The highest activities of CMCase from *Penicillium* sp. CR-316 have been reported

at 65°C (Picart et al., 2007). Whereas, CMCase from *T. aurantiacus* (Kalogeris et al., 2003a) and *Bacillus* sp. (Rastogi et al., 2010) have been reported to be optimally active at 75°C.

Similar to β-glucosidase from *T. harzianum* SNRS3, *Stachybotrys* sp β-glucosidase have also been reported to be optimally active at 50°C (Amouri & Gargouri, 2006). Interestingly, a closer look at β-glucosidase temperature profile revealed that at 60°C, β-glucosidase activity was still above 70% of its maximum activity. This is considered an advantage of the crude cellulase enzyme produced by local *T. harzianum* SNRS3. Its ability to retain high activity at 60°C, when other cellulases are inactivated, is an important characteristic for cellulases as industrial enzymes. Table 2 summarises the optimum temperature of the crude cellulase produced by *T. harzianum* SNRS3 and various other microorganisms.

Effects of pH on Crude Cellulase Activity

As illustrated in Figure 6, cellulolytic enzyme complex system produced by *T. harzianum* SNRS3 displayed cellobiohydrolase, endoglucanase, and β-glucosidase activities over a broad range of pH. This characteristic of the crude cellulase by *T. harzianum* SNRS3 is considered as an advantage for cellulases that are important industrial enzymes. However, the cellulose degrading enzymes are highly active in the acidic region (Table 2). Depending on the type of cellulase, the pH-activity profiles obtained were different. FPase retained above 50% of its maximum activity in a broad pH range of 3.5-6.5, whereas CMCase retained almost above 70% of its maximum activity in the pH range of 3.5-7.0. However, β-glucosidase showed the highest activity at pH 5.0 and remained highly active in a narrow pH spectrum of 4.5-5.5.

As shown in Figure 6, the pH profile of the three components of the crude cellulase showed an increasing trend for the activity with the rise in pH value. In particular, the activity of FPase and CMCase increased sharply in the pH range between 2.5-4.0. For β-glucosidase, however, the activity increased drastically in the pH range between 3.5-4.50. Based on a relative activity of 100% for β-glucosidase at pH 5.0, the activity decreased to 12.2 %, and 8.1 % at pH3.5 and pH7, respectively.

Thermal Stability of Crude Cellulase

The results of thermostability of the crude cellulase produced by *T. harzianum* SNRS3 at 50 and 60°C are presented in Figure 7. As expected and can be observed, the higher the temperature, the higher the activity of the enzyme is likely to be lost. As shown in Figure 7, 30 min incubation of the crude enzyme at 50°C resulted in a reduction in the activity of FPase to around 70% of its original activity. However, incubation at 60°C for 30 min reduced the FPase activity to almost 40% of its initial activity. It is worth noting that increasing the incubation period up to 4 h did not further decrease the enzyme activity.

Unlike CMCase produced by *Penicillium notatum* NCIM NO-923 (Das & Ghosh, 2009) and *Penicillium citrinum* (Dutta et al., 2008) that proved to be more themostable at 50 and 60°C than FPase, CMCase produced by *T. harzianum* SNRS3 showed less thermal stability compared to FPase. The incubation of the crude cellulase at 50 and 60°C for 30 min caused the activity of CMCase to drop to almost 40%, and 30% of its original activity, respectively. At 50°C, however, and with the increase in the incubation time up to 4 h, no more reduction in the enzyme activity was detected. Consequently, in the incubation period range of 30 min to 4 h, a thermal stability plateau was observed. Meanwhile at 60°C, increasing the incubation time of the crude enzyme for up to 4 h steadily decreased the CMCase activity to less than 15% of its initial activity.

As depicted in Figure 7, β-glucosidase exhibited above 50% of its original activity after incubation at 50°C for 30 min and after that, any longer incubation of the enzyme up to 4 h did not have a significant effect on the residual activity of the enzyme. However, the enzyme was found to be less stable at 60°C and after 2-h incubation, and that almost all activities were lost probably due to the enzyme denaturation. Table 2 provides a comparison between thermostability of the crude cellulase produced by *T. harzianum* SNRS3 and that of cellulase by various microorganisms.

Effects of Storage on Crude Cellulase Activity

Results of the effects of storage on the activity of crude cellulase enzyme are depicted in Figures 8.A, 8.B, and 8.C.

Regardless of the storage temperature of the crude enzyme, the FPase activity was almost stable within the first week (Figure 8.A), and only at room temperature, a slight decrease occurred in the FPase activity from 100% up to 94%. Meanwhile, the activity of FPase for the crude samples kept at 4°C**,** − 20°C, and −40°C almost remained stable within the 2nd week, but the activity dropped to 77% at room temperature. After weeks 3 and 4 at 4°C**,** −20°C, and −40°C, the activity of FPase slightly decreased. However, at room temperature, the FPase activity of the crude enzyme decreased drastically at the end of week 4 and reached 27% of its original activity. Interestingly, the storage of crude enzyme at room temperature showed that FPase activity was still almost 60% of its original activity at the end of week 3. The FPase activity was still above 85% of its original activity after keeping the crude enzyme for 3 weeks at 4°C, −20°C, and −40°C.

As shown in Figure 8.B, CMCase proved to be more sensitive to storage temperature compared to FPase. At room temperature, the CMCase activity dropped sharply from 71% after week 1 to merely 25% and 7% at the end of weeks 3 and 4, respectively. However, CMCase retained 60% of its original activity at the end of the 2nd week of storage at room temperature. The effect of storage of the crude enzyme at

Table 1
Cellulase and Xylanase Production by Different Fungi Grown on Various Agricultural Wastes under Solid State Fermentation

Enzyme source	Carbon source	Enzyme activities (U/g dry substrate)				References
		FPase	CMCase	β-glucosidase	Xylanase	
Aspergillus niger ATCC 6275	Palm cake	_	23.8	_	282.9	(Prasertsan et al., 1997)
Aspergillus niger KK2	Rice straw	19.5	129	100	5070	(Kang et al., 2004)
Aspergillus terreus M11	Corn stover	243	581	128	_	(Gao et al., 2008)
Aspergillus terreus MTCC 8661	Palm oil fiber	_	_	_	115,269	(Suvarna Lakshmi et al., 2009)
Aspergillus terreus ATCC74135	Rice straw	480.48	363.72	16.37	6,166.01	(Jahromi et al., 2011)
Aspergillus ustus	Wheat bran	3.78	11.84	60.00	615.26	(Shamala &Sreekantiah, 1986)
Aspergillus ustus	Rice straw	5.82	12.58	15.82	740	(Shamala & Sreekantiah, 1986)
Myceliophthora sp. IMI 387099	Corn cob	0.31	11.38	5. 49	411.6	(Badhan et al., 2007)
Myceliophthora sp. IMI 387099	Bagasse	0.70	6.62	2.01	620.1	(Badhan et al., 2007)
Myceliophthora sp. IMI 387099	Wheat bran	0.74	26.6	3.83	128.9	(Badhan et al., 2007)
Myceliophthora sp. IMI 387099	Wheat straw	1.37	30.8	6.78	656.6	(Badhan et al., 2007)
Myceliophthora sp. IMI 387099	Rice straw	2.44	32.9	7.48	900.2	(Badhan et al., 2007)
Thermoascus aurantiacus	Wheat straw	4.3	956	46.1	2973	(Kalogeris et al., 1999)
Trichoderma aurantiacus	Wheat straw	_	1572	101.6	_	(Kalogeris et al., 2003a)
Trichoderma aurantiacus	Wheat straw	5.5	1709	79	4490	(Kalogeris et al., 2003b)
Trichoderma koningi F244	Wheat bran	94	287.3	184	_	(Li et al., 2004)
Trichoderma longibrachiatum	Wheat bran and wheat straw	_	_	_	592.7	(Azin et al., 2007)
Trichoderma reesei MCG77	Rice bran	2.314	_	_	_	(Latifian et al., 2007)
Trichoderma reesei ZU-02	Corncob	158	_	_	_	(Xia & Cen, 1999)
Aspergillus niger ATCC 6275	Rice straw	7.06	86.35	17.41	2378.64	Present study
Trichoderma harzianum SNRS3	Rice straw	6.25	111.31	173.71	433.75	Present study

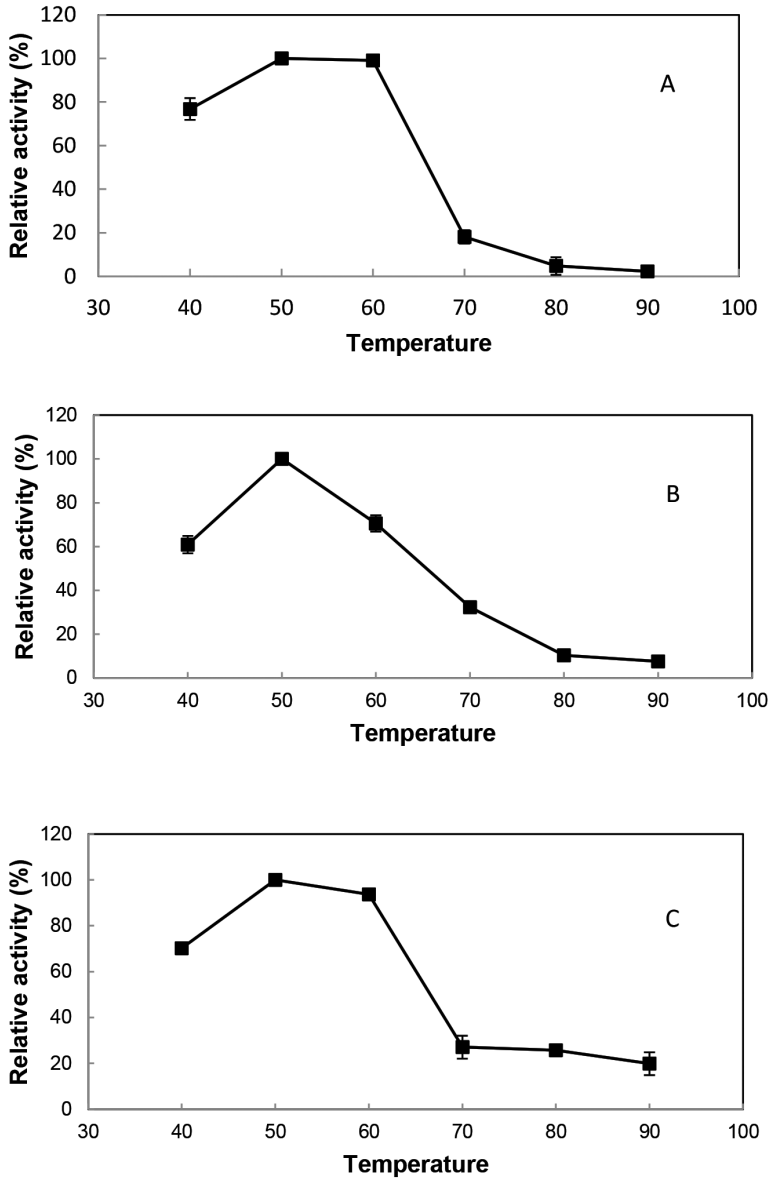

Figure 5. The effects of temperature on the activity of crude cellulase by *T. harzianum* SNRS3 from rice straw in SSF. Values are means of 3 replicates ±SD. A: FPase, B: CMCase, C: β-glucosidase

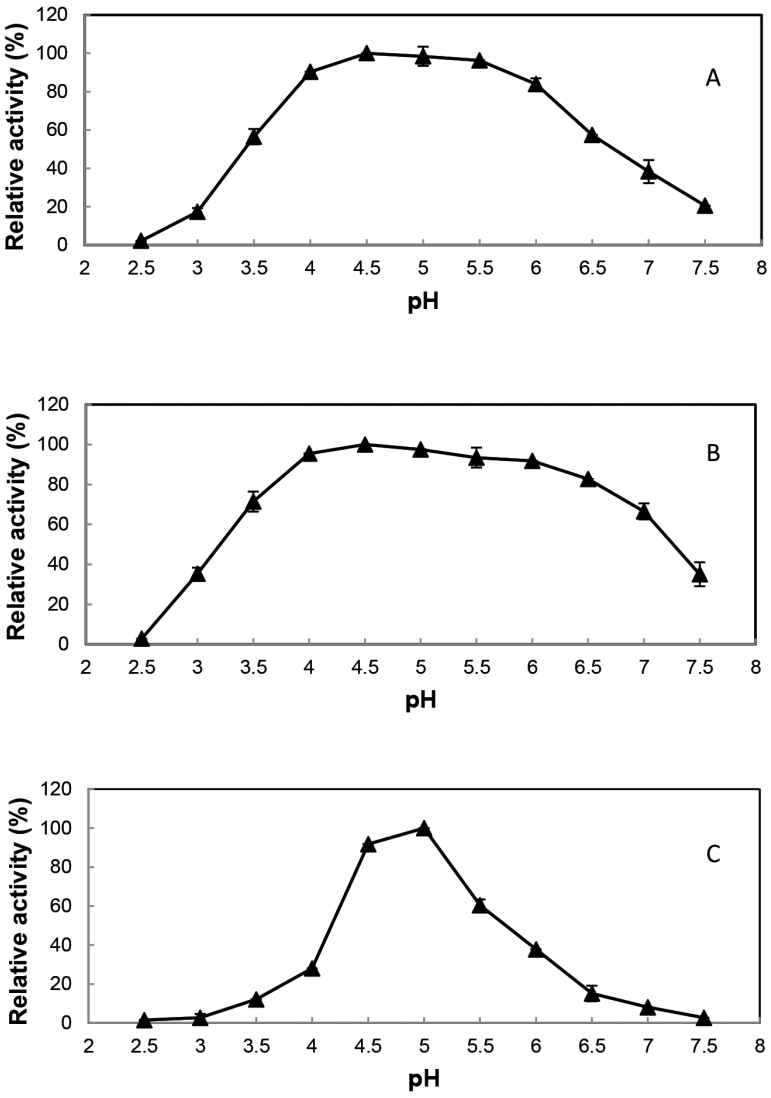

Figure 6. The effects of pH on the activity of crude cellulase by *T. harzianum* SNRS3 from rice straw in SSF. Values are means of 3 replicates ± SD. A: FPase B: CMCase C: β-glucosidase

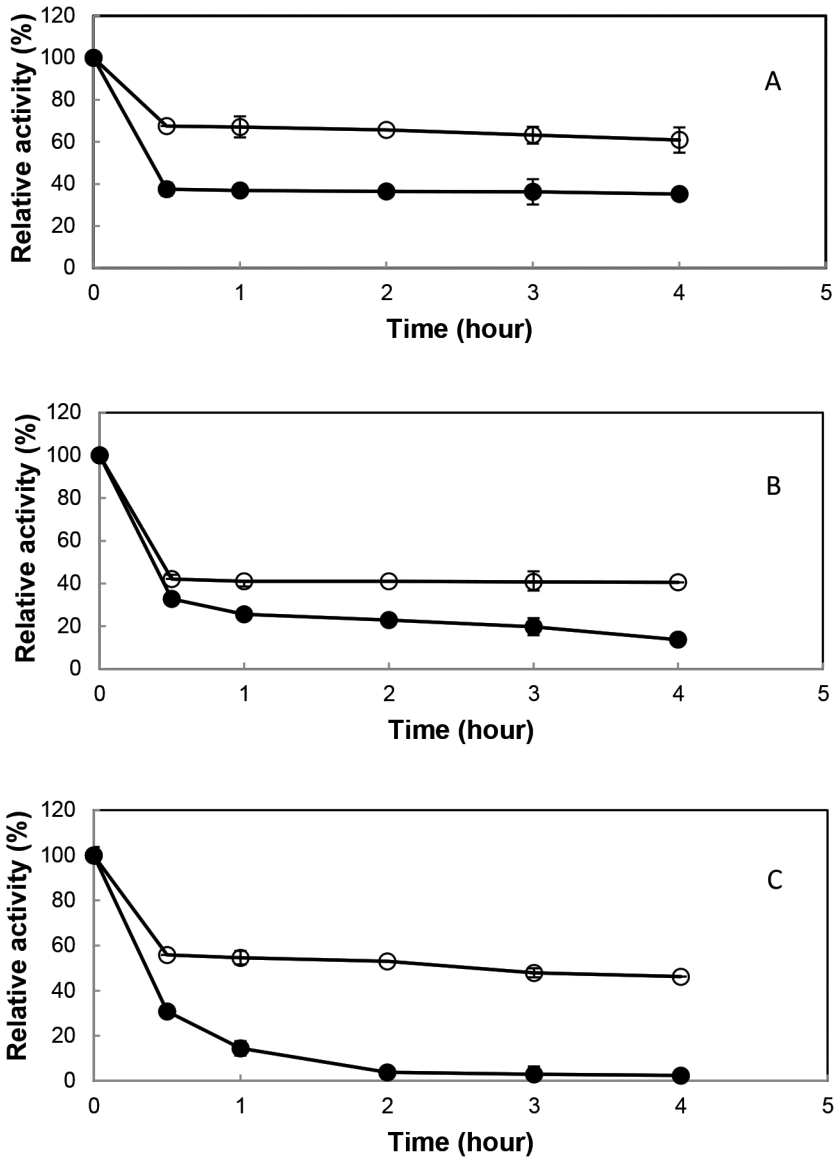

Figure 7. Thermostability of FPase (A), CMCase (B), and β-glucosidase (C) by *T. harzianum* SNRS3 from rice straw in SSF. Values are means of 3 replicates ± SD. Closed symbols represent: Residual activity at 60°C; Open symbols represent: Residual activity at 50°C

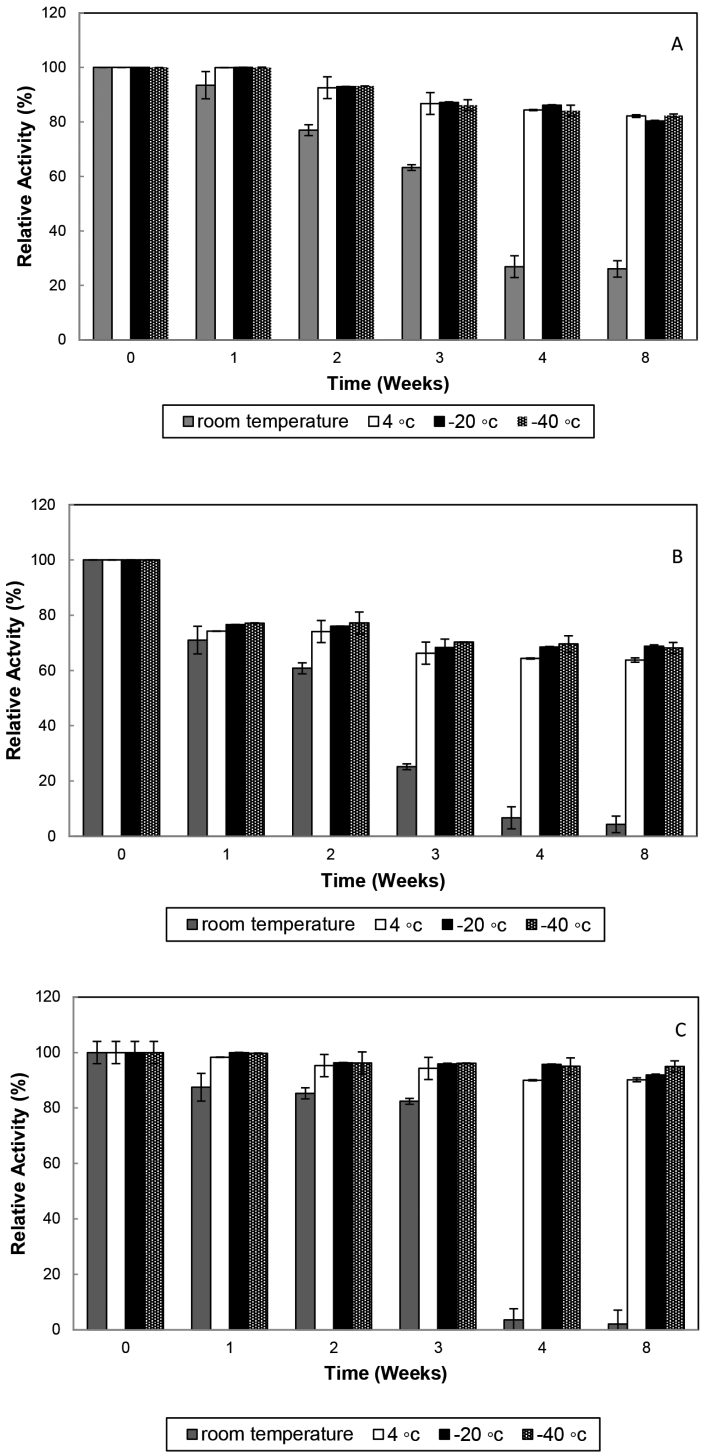

Figure 8. The effects of storage on the activity of crude cellulase by *T. harzianum* SNRS3 from rice straw in SSF. Values are means of 3 replicates ± SD. A: FPase B: CMCase C: β-glucosidase

Table 2
Optimum Temperature, pH, and Thermostability of Celluase Produced by Various Microorganisms

Microorganism	Enzyme	Temperature optimum (ºC)	pH optimum	Thermostability	References
Penicillium citrinum	FPase	-	6.5	less thermostable than CMCase	(Dutta et al., 2008)
Penicillium funiculosum	FPase	60	4.0-5.0	thermostable at up to 55°C	(Karboune et al., 2008)
Penicillium notatum NCIM NO-923	FPase	50	4.0	stable at 40ºC; at 50 and 60ºC FPase less thermostable	(Das & Ghosh, 2009)
Alternaria alternate	CMCase	55-60	5.0-6.0	-	(Macris, 1984)
Aspergillus fumigatus	CMCase	64.3	-	-	(Saqib et al., 2010)
Aspergillus niger Z10	CMCase	40	4.5 and 7.5	above 40% of activity at 90°C after 15 min	(Coral et al., 2002)
Aspergillus terreus M11	CMCase	70	2.0	highly stable; retained 65% of activity after 6 h	(Gao et al., 2008)
Bacillus sp.	CMCase	75	5.0	Stable at 50°C after 1 day; 97% activity remained at 60°C after 1 day	(Rastogi et al., 2010)
Ceriporiopsis subvermispora	CMCase	60	3.5-5.0	rapid loss of activity at 40 and 50°C	(Heidorne et al., 2006)
Geobacillus sp.	CMCase	70	5.0	100% active at 50 and 60°C after 1 day; 7% loss of activity at 60°C after 2 days	(Rastogi et al., 2010)
Penicillium sp. CR-316	CMCase	65	4.5	stable at 60°C after 3 h; lost 75% of activity at 65°C after 1 h	(Picart et al., 2007)
Penicillium citrinum	CMCase	-	5.5 and 8	stable at 50-70°C after incubation for 2 h	)Dutta et al., 2008)
Penicillium funiculosum	CMCase	60	4.0	thermostable at up to 55°C	(Karboune et al., 2008)
Penicillium notatum NCIM NO-923	CMCase	50	4.0	stable at 40ºC	(Das & Ghosh, 2009)
Streptomyces drozdowiczii	CMCase	50-60	5.0	stable at 50°C after 1 h; 40% of activity remained after 2 h; 20% of activity remained after 8 h	(Grigorevski de Lima et al., 2005)
Streptomyces transformant T3-1	CMCase	50	7.0-8.0	stable at 50°C after 5 days; 98% of activity remained at 50°C after 7 days; half-life 15 h at 60°C ; half-life 5 h at 70°C	(Jang & Chen, 2003)

cont'd Table 2

T. aurantiacus	CMCase	75	3.5 and 4	half-life 42 min at 80°C; half-life 1 day at 70°C	(Kalogeris et al., 2003a)
Alternaria alternata	β-glucosidase	70-75	4.5-5.0	Half-life 3.5 days at 60°C , 1.8 h at 65°C, and 10 min at 70°C	(Macris, 1984)
Aspergillus niger	β-glucosidase	55	4.5	-	(Watanabe et al., 1992)
Aspergillus pullulans	β-glucosidase	75	-	-	(Saha et al., 1994)
Aspergillus terreus M11	β-glucosidase	70	3.0	highly thermo stable; retained 53% of original activity after 6 h	(Gao et al., 2008)
Aureobasidium sp.	β-glucosidase	80	4.0	stable at 80°C for 15 min	(Hayashi et al.,1993)
Aureobasidium pullulans	β-glucosidase	80	4-4.5	retained 98% of activity after 1 h incubation at 75°C	(Leite et al., 2007)
Ceriporiopsis subvermispora	β-glucosidase	60	3.5-5.0	rapid loss of activity at 40 and 50°C	(Heidorne et al., 2006)
Penicillium decumbens	β-glucosidase	65-70	4.5-5.0	96% of activity remained at 50°C after12 h; 50% of activity remained at 70°C after 4 h	(Chen et al., 2010)
Penicillium funiculosum	β-glucosidase	60	4.5	stable at 25-40	(Karboune et al., 2008)
Stachybotrys sp.	β-glucosidase	50	5.0	-	(Amouri & Gargouri, 2006)
Thermoascus aurantiacus	β-glucosidase	70	4.5	retained 98% of activity after 1 h at 70°C	(Leite et al., 2007)
T. aurantiacus	β-glucosidase	80	4.5	half-life 18 min at 80°C; half-life 2.5 days at 70°C	(Kalogeris et al., 2003a)
Aspergillus niger	Crude cellulase	35-60	4.0-5.5	-	(Farinas et al., 2010)

cont'd Table 2

Aspergillus niger ATCC-16404	Crude cellulase	54-57	5.0-5.5	-	(Machado de Castro et al., 2010)
Trichoderma harzianum IOC-4038	Crude cellulase	47- 54	4.9-5.2	rapid thermal denaturation at 60ºC corresponding to half-life < 1 h	(Machado de Castro et al., 2010)
Trichoderma reesei RutC30	Crude cellulase	52-57	5.1-5.3	-	(Machado de Castro et al., 2010)
T. harzianum SNRS3	FPase	50	4.5-5.5	At 50°C after 30 min and up to 4 h, 70% of activity remained, at 60°C after 30 min and up to 4 h, 40% of activity remained	Present study
	CMCase	50	4.0-5.0	At 50°C after 30 min up to 4 h, 40% of activity remained; at 60°C after 30 min 30% of activity remained and after 4 h, < than 15% of activity remained	
	β-glucosidase	50	5.0	At 50°C after 30 min up to 4 h, > 50% of activity remained; inactivated after 2h at 60°C	

4°C, −20°C, and −40°C was almost similar. After week 1, the activity first decreased to almost above 75% for the three samples kept at 4°C, −20°C, and −40°C. It is worth noting that storage at 4°C for 1 month caused the CMCase activity to drop to almost 65% of its initial activity.

Similarly, the β-glucosidase activity was also affected by storage temperature and duration (Figure 8C). Storing the crude enzyme at room temperature caused the activity to drop sharply from 82% at the end of week 3 to 4% at the end of week 4. However, the β-glucosidase activity was only slightly affected when the enzyme was kept at 4°C, −20°C, and −40°C for one month. Like FPase, β- glucosidase was also proven to be almost stable for the first three weeks of storage at room temperature. The β- glucosidase activity only decreased to above 80% of its original activity at the end of the 3rd week of storage at room temperature.

It is worth noting that after keeping the crude cellulase for 2 months at 4ºC, −20ºC and −40ºC, CMCase retained almost above 60% of its original activity. However, FPase and β- glucosidase remained active, i.e. above 80% and 90% of their original activity, respectively.

CONCLUSION

Unlike *Trichoderma* sp. that are normally associated with the production of low amount of β-glucosidase for an efficient hydrolysis, *T. harzianum* SNRS3 was shown to be a potential β-glucosidase producer. Meanwhile, *T. harzianum* SNRS3 produced β-glucosidase at a much higher activity (173.71 U/g substrate) compared to 17.41 U/g substrate β-glucosidase by *A. niger* ATCC 6275, belonging to a genus reported as the most efficient producer of β-glucosidase. However, *T. harzianum* SNRS3 was not an efficient xylanase producer (433.75 U/g substrate) compared to *A. niger* ATCC 6275 (2378.64 U/g substrate). FPase showed an optimum temperature plateau in the temperature range of 50 to 60ºC, indicating that this enzyme can remain active almost up to 100% at 60ºC. At 60ºC, the β-glucosidase activity was still above 70% of its maximum activity. This is a significant characteristic of cellulases with a wide range of industrial applications. Cellulose degrading enzymes were highly active in the acidic region and could be mostly applied over a wide range of acidic pH.

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