A novel Swollenin from *Talaromyces leycettanus* JCM12802 exhibits cellulase activity and enhanced cellulase hydrolysis towards various substrates

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Research

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Abstract

Background

Swollenins are present in some fungal species involved in the biodegradation of cellulosic substrates. They appear to promote a rearrangement in the network of non-covalent interactions between the cell wall polysaccharides, thus making it more accessible for degradation by hydrolytic enzymes. Here, we have reported a detailed characterization of a recombinant swollenin with respect to its disruptive activity on cellulosic substrates and synergistic effect with cellulases.

Results

In the present study, a novel swollenin gene *Tlswo* consisting of an open reading frame encoding 503 amino acids was identified from *Talaromyces leycettanus* JCM12802 and successfully expressed in *Trichoderma reesei* and *Pichia pastoris*. Similar to other fungal swollenins, *Tl*SWO contained a N-terminal family 1 carbohydrate binding module (CBM1) followed by a Ser/Thr rich linker connected to expansin-like domain which includes a family 45 endoglucanase-like domain and group-2 grass pollen allergen domain. *Tl*SWO demonstrated disruptive activity on Avicel and displayed a high synergistic effect with cellobiohydrolases, enhancing its hydrolytic performance up to 132%. The activity of *Tl*SWO on various substrates and biomass was also examined. It was shown that *Tl*SWO could release reducing sugars from lichenan, barley β-glucan, carboxymethyl cellulose sodium (CMC-Na) and laminarin. The specific activity of *Tl*SWO towards the substates above is 9.0 ± 0.100 U/mg, 8.9 ± 0.100 U/mg, 2.3 ± 0.002 U/mg and 0.79 ± 0.002 U/mg respectively. Moreover, *Tl*SWO exhibits maximum activity at pH 4.0 and 50 °C.

Conclusion

This study reported on a novel swollenin with highly efficient for biomass conversion. It also reveals the functional diversity of swollenin with activity on various substrates. Although the exact mechanism of swollenin catalytic action activity still remains unknown, the functional diversity of *Tl*SWO makes it a good candidate for industrial applications.

Background

Bioconversion of lignocellulosic materials still represents a challenge regarding the use of renewable feedstocks for fuel and chemical production. A number of non-glycoside hydrolase enzymes, such as expansins, loosenins, cerato-platanin proteins, certain carbohydrate binding modules, a swollenins are known to enhance plant biomass deconstruction [1]. Expansins have been widely identified in plant cell walls and are implicated in loosening the cell wall structure [2]. It has been proposed that expansins could disrupt hydrogen bonding between cell wall polysaccharides without hydrolyzing them [3]. Swollenins from fungi show sequence similarity to expansins and are collectively called expansin-like proteins. In

2002, the first expansin-like protein SWOI from fungi was discovered in *Trichoderma reesei* [4]. The functional assays indicated that SWOI could disrupt the structure of cotton fibers and filter paper without detectable reducing sugars [4]. In the past years, more than 10 swollenins from fungi have been identified and reported [5–8]. However, the exact mode of action of swollenin is not well understood.

In general, expansins have a length of no more than 250 amino acids which constitute a two domain structure. Domain 1 resembles glycoside hydrolase family 45 (GH45) which preserves some sequence features of the GH45 catalytic site. Domain 2 with a flat aromatic-rich surface has homology to group-2 grass pollen allergens and is proposed to function as a CBM [9, 10]. Domain 1 and domain 2 are connected by a short linker and both domains are essential for plant cell-wall loosening activity. Swollenins differ from expansins in that swollenins have an additional CBM domain which shows homology to fungal cellulases in the N-terminal [11]. In cellulases, CBM's that direct the enzymes on cellulosic surfaces and thus enhance lignocellulose degradation have been well studied [12–14]. The CBM and two domains are connected by a linker which is rich in serine and threonine residues which in fungi result in *O*-glycosylation. Although more and more swollenins are reported, little is known about the specific roles of each domain.

Previous studies suggested that several swollenins have been successfully expressed in several expression hosts, such as *T. reesei*, *Aspergillus niger*, *Pichia pastoris* [7, 8, 15]. Moreover these, swollenins were shown to disrupt the structure of cotton fibre, and reduce the strength of filter paper without detectable reducing sugars [4, 16–18]. In microorganisms, expression of swollenins and expansins appears to increase the bacterial colonization of plant tissues [19, 20]. Additionally, various authors have described the synergy of swollenin with glycoside hydrolases in releasing soluble sugars from substrate. For example, Zhou *et al*. expressed swollenin SWO2 in *Aspergillus niger*, and showed that simultaneous incubation of SWO2 with cellulases resulted in a significant synergistic activity in cellulose hydrolysis, and an even greater increase in the synergistic activity was obtained when cellulose was pretreated with swollenin followed by cellulase hydrolysis [7]. The synergy of swollenin with xylanase was also investigated. Santos *et al*. found that an swollenin *Th*SWO from *T. harzianum* created a rough and amorphous surface on Avicel and displayed a high synergistic effect with a commercial xylanase from *T. viride*, enhancing its hydrolytic performance up to 147% [6]. Moreover, *Anthony* et al. indicated that a chimeric enzyme with the swollenin from *T. reesei* fused with the feruloyl esterase A from *A. niger* was also found to result in a significant increase in ferulic acid release from lignocellulose [21]. In some studies, swollenins were also found that could release reducing sugars from cellulosic materials. For example, swollenin SWO2 from *T. pseudokoningii* and *Af*Swo1 from *Aspergillus fumigatus* were both found to exhibit very low level of endoglucanase activity [7, 22]. And in 2015, Andberg *et al*. demonstrated that the swollenin SWOI from *T. reesei* was shown to have activity on substrates containing β-1,4 glucosidic bonds, showing a unique mode of action with similarities with action of both endoglucanases and cellobiohydrolases [23]. It is also noteworthy that the solved of the crystal structure of maize EXP1 expansin showed that domain 1 and domain 2 were packed together, thus could form a long, shallow groove with potential to bind a glycan backbone [24]. However, to date, none of crystal structure of fungal

swollenin has been solved. And although these swollenins have been shown to be active on various substrates, the mechanistic details of swollenin function are still unknown.

In our previous study, thermophilic *T. leycettanus* strain JCM12802 has been identified as an excellent CAZyme source, from which a few genes with good characterization have been cloned and functionally characterized [25–28]. Here, we present a novel swollenin protein *Tl*SWO that was cloned from *T. leycettanus* JCM12802, and successfully expressed in *T. reesei* and *Pichia pastoris*. We have examined the activity of *Tl*SWO on various substrate and biomass. We demonstrate that *Tl*SWO can release reducing sugar from barley β-glucan, lichenan, laminarin and carboxymethyl cellulose sodium (CMC-Na). Also, *Tl*SWO can reduce the particle size and the surface structure of Avicel. Moreover, we have used pre-treated corn stover (PCS), cellulose nanocrystals (CNC), and phosphoric acid swollen cellulose (PASC) to test the synergy of swollenin with other cellulases. Notably, when using PCS as the substrate, the synergistic activity of *Tl*SWO with cellulase lead to cellulose conversion of as high as 72.2%, 32.0% higher than that of the control without *Tl*SWO.

Methods

Strains and Plasmids

T. leycettanus JCM12802 as the donor strain was purchased from Japan Collection of Microorganisms RIKEN BioResource Center (Tsukuba, Japan). *Escherichia coli* Trans I-T1 (TransGen, Beijing, China) was used for routine gene cloning. *T. reesei* AST1116 and *P. pastoris* GS115 (Invitrogen, Carlsbad, CA, USA) were both used as the host for gene expression. The plasmids of pPIC9 (Invitrogen) and pTrEno were used for driving *Tlswo* gene expression in *P. pastoris* and in *T. reesei*. The plasmid construction of pTrEno was according to the description of Linger et al. [29].

Sequence Analysis

The DNA and amino acid sequences of *Tl*SWO were analyzed using the BLASTx and BLASTp programs (http://www.ncbi.nlm.nih.gov/BLAST), respectively. The introns and exons were predicted using the GENSCAN Web Server (http://genes.mit.edu/GENSCAN.html). SignalP 3.0 was used to predict the signal peptide sequence (http://www.cbs.dtu.dk/services/SignalP). The potential N-glycosylation sites were predicted online (http://www.cbs.dtu.dk/services/NetNGlyc). Sequence assembly and estimation of the molecular mass and *p*I of the mature peptide were performed using the Vector NTI Suite 10.0 software (Invitrogen). Protein molecular weight and molar extinction coefficients were estimated at the ExPASy tools page (http://www.expasy.org/tools). Multiple sequence alignments were performed with Clustal W program from MEGA software 4.0.

Gene Cloning And Recombinant Protein Expression

T. leycettanus JCM12802 was cultured at 42 °C for 3–5 days in medium containing 5.0 g/L $(NH_4)_2SO_4$, 1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4·7H_2O$, 0.2 g/L $CaCl_2$, 10.0 mg/L $FeSO_4·7H_2O$, 30.0 g/L wheat bran, 30.0 g/L soybean meal, and 30.0 g/L corncob. Genomic DNA of *T. leycettanus* JCM12802 was extracted with the DNA isolation kit (Tiangen) following the manufacturer instructions and was used as a template for PCR amplification. Total RNA isolation and first strand cDNA synthesis were carried out as previously described [30]. PCR amplification was carried out using Fastpfu DNA polymerase and which purchased from TransGen. The fragment of *Tlswo* corresponding to the 21–503 amino acid sequence was amplified with primers P1 (5'- GCTCGTGCTCAGAGCAGCTGTGCAGG-3') and P2 (5'- CTAGATTACCTAGGTAAACTGCACC-3'). The amplified PCR product was cloned into pPIC9 and pTrEno vector using Gibson Assembly (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol for cloning and expression. *Escherichia coli* Trans I-T1 (TransGen, Beijing, China) which used for routine gene cloning was grown at 37 °C overnight in Luria-Bertani (LB) medium supplemented with 50 μg/mL of ampicillin (Sigma, St. Louis, MO).

When using *P. pastoris* GS115 as the expression host, the recombinant plasmids of pPIC9-*Tlswo* was linearized with *Bgl*II (New England Biolabs, UK) and transformed into the expression host via electroporation. The positive transformants were screened on minimal dextrose medium (MD) at 30 °C for 3 or 4 days until the single colonies appeared and placed into shaking tubes for enzyme production according to the protocol described in the *Pichia* Expression Kit (Invitrogen). The large-scale fermentation was performed as the previously described [31]. The recombinant *P. pastoris* GS115 containing pPIC9-*Tlswo* was grown at 30 °C in 400 mL BMGY medium in a 1 L shaking flask for 48 hours. Cells were collected and re-suspended again with 200 mL buffered methanol-complex medium (BMMY) medium with 0.5% (v/v) methanol and cultured at 30 °C for 72 h with shaking (200 rpm). Methanol was added into the medium every 24 hours.

When using *T. reesei* AST1116 as the expression host, the recombinant plasmids of pTrEno-*Tlswo* were linearized with *Sbf*I (New England Biolabs, UK) and then transformed into *T.reesei* AST1116 via electroporation. Potato dextrose (PD) plates were used for spore production and PDHX plates (PD plates with hygromycin and TritonX-100 at a final concentration of 100 μg/mL and 0.1% respectively) were used for the screening of potential *T. reesei* which allowed to grow for 2 to 3 days at 30 °C. Growth medium for *TlSWO* expression was Mandels and Andreotti medium with 5% glucose (MAG). Subsequently complete medium lactose (CML) was used for the overexpression of the transformants. The medium protocols of MAG and CML were performed following published protocol [29]. For large-scale fermentation, the positive transformants spore stocks were streaked on potato dextrose agar plates and allowed to grow 2 to 3 days. Then the spore was extracted from the plate and deposited into 1.0 L of MAG medium in a 2.8 L shake flask. The culture was grown at 28 °C with at 225 RPM for 24 h, after which the entire 1.0 L was transferred to 7.0 L of the same medium in a bioreactor.

Then the culture broth were extracted for further analysis by SDS-PAGE and activity assay. Culture broths were clarified via centrifugation and transferred to microcentrifuge tubes. Broths were diluted 3:1 in 4 × LDS sample buffer (Life Technologies Corp., Carlsbad, CA, USA) with 50 μL/mL β-mercaptoethanol as a

reducing agent. Samples were incubated at 95 °C for 5 min prior to loading onto NuPAGE SDS gels with MOPS buffer, electrophoresed at 200 V constant for approximately 40 min.

Protein Purification

Fermentation broths were harvested and sequentially vacuum-filtered. This filtered broth was then concentrated by tangential ultrafiltration with a 10 kDa MWCO. The broths were roughly concentrated to 100 mL. The final concentrated volume was exchanged with at least 2.0 L of 20 mM Bis-Tris pH 6.5 to remove residual peptides and other low molecular weight debris. The following purification steps were then performed according to previous publications [29]. The crude enzyme was purified through hydrophobic interaction chromatography (HIC) using a 26/10 Phenyl Sepharose Fast Flow column (GE Healthcare, Chicago, USA). Then the protein went through anion exchange chromatography using a 10/100 anion exchange column packed with Source 15Q (GE Healthcare, Chicago, USA) followed by HIC using a Source15 iso 10/100 column (GE Healthcare, Chicago, USA) and SEC using a 26/60 Superdex 75 column (GE Healthcare, Chicago, USA) and 20 mM acetate buffer pH 5.0, 100 mM NaCl as mobile phase.

SDS-PAGE were performed to assess purity of the protein *Tl*SWO. Electrophoretically separated on a 12% SDS-PAGE and visualized by Coomasie Blue staining. Protein concentration was measured with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Rockford, USA) and the Bradford protein assay kit (Bio-Rad).

Tl SWO activity assays

The activity of *Tl*SWO was measured using the 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1959). Enzyme activity was assayed in a final volume of 1.5 mL, with 1% (w/v) barley β-glucan (Megazyme Co., Bray, Ireland), lichenan (Megazyme Co., Bray, Ireland), laminarin (Megazyme, Wicklow, Ireland) and carboxymethyl cellulose sodium (CMC-Na) (Sigma-Aldrich, St. Louis, MO) as the substrates, and 10 µg/mL of enzyme at optimal conditions for 10 min. The effect of pH was studied using 100 mM citric acid-Na_2HPO_4 (pH 3.0−7.0) buffers at 50 °C and the effect of temperature was evaluated by incubation at pH 4.0, between 30 °C and 90 °C.

Effect of pH and temperature on TlSWO activity

Further, the effects of pH and temperature on the activities of *Tl*SWO were measured and compared. To determine the optimum pH of *Tl*SWO, the activities were assayed with 1% lichenan (w/v) in buffer of different pH, 100 mM glycine-HCl (pH 1.0−3.0), McIlvaine buffer (pH 3.0−8.0) and glycine−NaOH (pH 9.0−12.0). For pH stability, *Tl*SWO was preincubated at 37 °C for 1 h in buffers of different pH (1.0−12.0) and subjected to the residual activity assay. For the optimum temperature of *Tl*SWO was determined at optimal pH over the temperature range from 30 °C to 80 °C. The thermostability assay of *Tl*SWO (100 µg/mL) was carried out by preincubating at 37 °C, 50 °C, 60 °C or 70 °C for 0−60 min, and aliquots of 100 µL were withdrawn at different time points for residual activity assay.

Light And Scanning Electron Microscopic Analyses

Avicel PH-101was used as solid cellulosic substrates. Ten milligrams of Avicel were incubated with different amount of purified TlSWO in 100 mM citric acid-Na_2HPO_4 buffer (pH 4.0). The experiment was carried out on a rotary shaker at 40 °C for different time interval. Control experiments without TlSWO were also performed under the same conditions. The physical structure of Avicel fibers was initially observed using light microscopy (Olympus TH4-200, Japan). Photomicrographs of the samples were captured using a scanning electron microscope (Hitachi SU8010, Tokyo, Japan) at a voltage of 15 kV.

Polysaccharide Depolymerization Analysis

Hydrolysis reactions on 1% barley β-glucan, 1% CMC-Na, 1% laminarin and 0.5% lichenan, were carried out overnight at pH 4.0, 37 °C using the enzyme in a final concentration of 100 μg/mL. High-performance anion-exchange chromatography (HPAEC) (Thermo Fisher Scientific, Sunnyvale, CA, USA) equipped with a Carbo-Pac PA200 column (3 × 250 mm) was used to determine the reaction products released from the polysaccharide.

Synergism between TlSWO and cellulases

The substrate used in this work were NREL dilute acid pre-treated corn stover (PCS) P120927, cellulose nanocrystals (CNCs), phosphoric acid swollen cellulose (PASC) and each substrate equiva to 8.5 mg of glucan. For CNC preparations from Avicel, about 2 g of Avicel were added to pre-heated HCl at 80 °C. Then run the acid hydrolysis for 4 hours, stirring every 15 minutes with a glass or Teflon rod followed by centrifugation sevral times, 1600 × g for 10 minutes. Decant supernatant and resuspend pellet in DI water until the pH reached 5.0. The CNCs were then suspended in the supernatant after centrifugation. Collect the translucent supernatant and resuspend pellet in DI water and shake vigorously to break up clumps until the supernatant is no longer translucent. The reaction mixtures were carried out in triplicate vials at 40 °C and each substrate was suspended in 20 mM sodium acetate buffer, pH 5.0. The enzyme cocktail comprised endoglucanase I from *Trichoderma longibrachiatum* (Megazyme Co., Bray, Ireland), cellobiohydrolases Cel7A from *Penicillium funiculosum* and β-glucosidase from *Aspergillus niger* (Megazyme Co., Bray, Ireland) at a concentration (mg protein/g of glucan) of 2, 13 and 1, respectively. The reaction was followed for 120 h, with sampling every 24 h. 100 μL samples containing both solids and liquid are removed from the mixtures and diluted for sugar analysis by high performance liquid chromatography (HPLC) with a BioRad HPX-87H column. Control experiments using BSA were also performed under the same conditions as mentioned above.

Results

Identification and characterization of Tswo gene

The ORF of putative *Tswo* consisted of six exons, encoding a deduced protein (*Tl*SWO) consisting of 503 amino acids and a signal peptide at the cleavage site between amino acids 20 and 21 (SignalP 4.1 server). The cloning sequence of putative *Tl*SWO was submitted to NCBI GenBank as MT180127. The predicted product shows the highest sequence similarity to the amino acid sequence of the known swollenin which are from *Aspergillus fumigatus* or *Penicillium oxalicum.* Further analysis by PROSITE (http://br.expasy.org/prosite) demonstrated that *Tl*SWO consists of three domains, fungal-type carbohydrate-binding module family 1 (CBM1) (amino acids 23–59), family 45 endoglucanase-like domain of expansin (Expansin_EG45) (amino acids 206–388) and a cellulose-binding-like domain of expansin (Expansin_CBD) (amino acids 400–492), which are typical of the swollenins from fungi (Fig. 1). In the CBM1 of *Tl*SWO, six cysteines were highly reserved same as GH6 cellobiohydrolase. Disulfide bond prediction by DiANNA (DiANNA 1.1 web server) showed that there are three disulfide bonds in the CBM1 of *Tl*SWO (Cys4-Cys21, Cys11-Cys28, Cys22-Cys28). CBM1 and Expansin_EG45 are connected by a Serine -Threonine rich linker domain. Although the function of linker has been well studied in cellulases, it was not clear if linker play the same role in swollenins as that of cellulases. Sequence alignment of swollenins showed that *Tl*SWO maintained the conserved HMD (histidine, methionine, aspartic acid) catalytic motif of GH45 cellulase (HFD, histidine, phenylalanine, aspartic acid), which is part of the active site (Fig. 1). In HFD, aspartic acid is the proton donor during the catalytic process in GH45 cellulase. However, the other catalytic active site aspartic acid is absent in swollenin and part of GH45 cellulases. In the C-terminal region of *Tl*SWO, Expansin_CBD is homology to pollen allergen. There are a few conserved aromatic amino acids in the sequence, i.e. Y400, Y401, F402, W429, Y447, W450, Y496 and F503, which may play a key role in binding substrates (Fig. 1).

Expression of Tl**SWO in** P. pastoris **GS115 and** T. reesei **AST1116**

The expression was done using the *aox1* promoter and *eno* promoter in *P. pastoris* and *T. reesei,*respectively. Following high-throughput fungal expression and screening, a large set of colonies resulting in SDS bands after transformation were identified. The results revealed that recombinant *Tl*SWO (483 amino acid residues, 51.1 kDa and a theoretical isoelectric point of 4.45) was successfully expressed using *P. pastoris* and *T. reesei* (Figure S1). The purified swollenin protein migrated as an about ~80 kDa protein in SDS-PAGE. Since the difference between the SDS-band and the theoretical molecular weight, the single band was analyzed by MALDI-TOF MS and the trypsin-digested peptide sequences were matched to the deduced amino acid sequences of *Tl*SWO (Figure S2). Sequence prediction suggested that there are five N-glycan sites on *Tl*SWO (Asn35, Asn154, Asn249, Asn366 and Asn436). After Endo H digestion, *Tl*SWO decreased in molecular weights to ~72 kDa□still higher than calculated value (Figure S1A). We speculate that the rest of the molecular weights increase is caused by heavily *O*-glycan glycosylation in the linker region which is rich in serines and threonines.

Activity of Tl**SWO on different substrates**

The cellulolytic activity of *Tl*SWO was measured with lichenan, barly β-glucan, CMC-Na, laminarin, avicel, glucomannan, and the xylanase activity was measured with birchwood xylan, and the mannase activity

was measured with locust bean gum. All the reaction were carried out overnight. As a result, TlSWO only showed significant activity on lichenan, barly β-glucan, glucomannan, CMC-Na and a very low activty on laminarin. However, we didn't observe any reducing sugars released from the other substrates. We further examined the specific activites of TlSWO with lichenan, barly β-glucan, CMC-Na as the substrate. TlSWO showed the highest activity on lichenan (9.0 ± 0.100 U/mg) and barley β-glucan (8.9 ± 0.100 U/mg) which were followed by CMC-Na (2.3 ± 0.002 U/mg). On the other hand, only slight activity against laminarin (0.79 ± 0.002 U/mg) was found (Fig. 2). These findings suggest that TlSWO mainly acts on cellulose rich substrates and showed a preference on the substrates that contain β-1,3 – 1,4 linkages.

Mode of action of TlSWO

The mode of action of TlSWO was also assessed using lichenan, barly β-glucan，CMC-Na and laminarin as substrates. As a result, CMC-Na was hydrolyzed to cellobiose and small amount of cellotriose (Fig. 3). Analysis of the hydrolysis products of lichenan, barly β-glucan showed that TlSWO preferentially hydrolyzed these two substrates to products with different degrees of polymerization, including cellobiose and cellopentose, followed by cellohexose and cellotetrose (Fig. 3). We detected no sugar release after incubating TlSWO with laminarin (Fig. 3). These results suggested that TlSWO may have a mixed function of endo-cellulase and exo-cellulase.

Effect of temperature and pH on TlSWO

The effect of pH and temperature on TlSWO activity were further investigated with lichenan as substrate. As a result, we determined that the optimum pH is 4.0 but the enzyme showed activity over a broad pH range of 2.0–12.0 (Fig. 4A). As for pH stability, TlSWO retained more than 80% of its activity in pH range 2.0–9.0 after incubation at 37 °C for 1 h, but lost 30% of its activity when incubated at pH 10.0–12.0 (Fig. 4B). In addition, TlSWO was optimally activity at 50 °C and retained more than 90% activity in the range of 40–60 °C, but rapidly lost its activity above 70 °C (Fig. 4C). As we can see from Fig. 4D, TlSWO was stable at a temperature of 37 °C and 50 °C after incubation for 1 h, however, when the temperature was raised to 70 °C, the activity was dramatically decreased to 40% after incubation for 10 min.

Disruptive action of TlSWO on Avicel

The disruptive effect of TlSWO on Avicel was evaluated using light microscopy and scanning electron microscopy. The result of light microscopy analysis showed that after incubation with different amounts of TlSWO for 24 h, the physical structure of Avicel tend to be significant different with the untreated Avicel (Fig. 5). As we increased the amount of TlSWO, Avicel was disrupted to small particles. The sample that was pretreated with 300 µg of TlSWO for 12 h was taken out for further analysis using scanning electron microscope. As we can see, TlSWO created a rough and amorphic surface on Avicel compared with the unpretreated sample (Figure S3).

Synergism between TlSWO and cellulases

To test the capacity of *Tl*SWO to enhance biomass hydrolysis by enzymatic cocktail, we hydrolyzed pretreated biomass using cellulases alone and also supplemented by *Tl*SWO. Biomass degradation experiments were performed using β-galactosidase (EC 3.2.1.21), cellobiohydrolase (EC 3.2.1.91) and endoglucanase (EC 3.2.1.4) in the presence of *Tl*SWO. The reactions with BSA or without *Tl*SWO were conducted as control. The total protein was 13 mg protein/g of glucan in all the reactions. Since endoglucanase randomly cleaves the internal β-1,4-glycosidic bonds, cellobiohydrolase processively acts on the chain termini to release cellobiose, and β-glucosidase that hydrolyzes cellobiose to glucose [31], thus the production of glucose was compared in different reactions.

The results showed that *Tl*SWO exhibited significant synergetic effects on cellobiohydrolase Cel7A when using PCS as the substrate. Initially, we measured the quantity of glucose of the reaction which contained only 13 (mg protein/g of glucan) of *Tl*SWO. As a result, no glucose was detected in the reaction, suggesting that PCS could not be hydrolyzed by *Tl*SWO (Fig. 6). The PCS conversion achieved in 24 h, 48 h, 72 h, 96 h and 120 h were 8.9%, 13.1%, 14.8%, 15.5% and 16.4% respectively when the reaction contained Cel7A and β-glucosidase alone, while they reached 11.2%, 18.2%, 21.6%, 23.8% and 26.4% when Cel7A and β-glucosidase was combined with 2 (mg protein/g of glucan) *Tl*SWO. Although *Tl*SWO action alone did not lead to any detectable levels of released glucose, the enzyme addition to Cel7A and β-glucosidase led to a significantly enhanced hydrolytic activity of the cocktail on PCS.

PASC and CNC exist in amorphous and crystalline forms, respectively, which may affect the binding of *Tl*SWO to them. Therefore, the action of *Tl*SWO for the two substrates were further examined. Similar to the results of PCS, *Tl*SWO could not release any sugars from PASC and CNC when used alone (Fig. 7). When utilizing Cel7A and β-glucosidase the conversion results showed that PASC conversion rate reached 9.9%, 19.6%, 29.2%, 37.5% and 40.2% in 24 h, 48 h, 72 h, 96 h and 120 h respectively (Fig. 7A). However, when Cel7A and β-glucosidase were supplemented by *Tl*SWO the conversion rates increased to 33.0%, 53.1%, 61.9%, 67.8% and 72.2% at the five time points respectively. Looking at the 120 h, time point the conversion rate of PASC was increased approximately 32% compared with Cel7A and β-glucosidase alone. This result suggests that *Tl*SWO exhibited significant synergetic effects with Cel7A. In addition, the synergetic effects of *Tl*SWO and endoglucanases were also explored during this research. When using endoglucanase and β-glucosidase alone the conversion rate was 51.4%, 65.7%, 76.5%, 82.0% and 85.6% at 24 h, 48 h, 72 h, 96 h and 120 h. When used in combination with *Tl*SWO, the conversion rate reached to 58.6%, 73.6%, 80.4% and 85.7% in the first four time points. Although these four values were a little higher than that of endoglucanase and β-glucosidase alone, the value at 120 h was 86.4%, which was close to 85.6%. Therefore, the addition of *Tl*SWO did not provide a significant increase of enzymatic hydrolysis of PASC when used in combination with endoglucanase.

The cellulose conversion rate of CNCs was significantly lower than that of PASC. Our results showed that on CNCs, the Cel7A and β-glucosidase achieved conversion in 24 h, 48 h, 72 h, 96 h and 120 h was 28.2%, 36.7%, 43.7%, 51.3% and 59.3% respectively (Fig. 7B). This conversion rate was also increased when *Tl*SWO was added. The glucose yields obtained by the cocktail enzyme systems containing *Tl*SWO and Cel7A and β-glucosidase were 31.9%, 45.1%, 54.3%, 62.4% and 68.9% respectively, higher in all cases

when compare with the control. This result suggests that *Tl*SWO showed a synergistic effect with processive cellobiohydrolases; however, when used in combination with endoglucanases, *Tl*SWO did not present any significant synergistic effect. After 120 h of enzymatic hydrolysis, the yield of glucose released from CNCs was 16.4% when combined use of *Tl*SWO, endoglucanase and β-glucosidase, which corresponds with the hydrolysis rate of using endoglucanase and β-glucosidase alone (14.9%). Comparing with the results of PASC, we suggest that *Tl*SWO will act on amorphous cellulose more efficiently than the crystalline form.

Discussion

Plant expansins are cell wall proteins, possessing the function of loosening plant cell walls, causing irreversible expansion, and promoting specific tissue or organ morphogenesis in plants [10, 32–34]. To date, several kinds of expansin-like proteins have been identified from bacteria and fungi [6, 20, 35]. Among which, the swollenin SWOI from *T.reesei* was reported as the typical one from fungi. Although the initial study showed that SWOI could disrupt the structure of the cell walls without producing detectable amounts of reducing sugar, subsequent studies have confirmed that SWOI exhibits hydrolytic activity against cellulosic substrates with features of both endoglucanases and cellobiohydrolases [4, 23]. Indeed, similarly to SWOI, the other two swollenins, *Af*SWO1 from *A. fumigatus*, SWO2 from *T. pseudokoningii* have also showed hydrolytic activity on various substrates [7, 22], thereby suggesting that these proteins interact with cellulose or hemicellulose. In this study, *Tl*SWO from *T. leycettanus* JCM12802 was found to have similar functions as the other fungal swollenins, and shares a relatively high sequence identity with SWOI, *Af*SWO1 and SWO2 (64.5%, 73.7% and 63.2%, respectively). Similar to SWOI, *Tl*SWO showed the highest activity against lichenan and barly β-glucan substrates, which have both β-1,4 and β-1,3 linkages, and also showed very slight activity towards laminarin which has only β-1,3 linkages. Based on previous reports, expansins from plant and bacteria are typically made of two domains, D1 and D2, which connected by a short linker. The D1 domain has homology to the family-45 glycoside hydrolases module, while the D2 domain shows sequence similarity to grass pollen allergens, which may play the role of carbohydrate binding module (CBM) [20, 24]. According to previously reports, expansins have higher similarity with GH45 subfamily C enzymes than with other members of the GH45 family [36, 37]. The conserved motif termed HFD is part of the conserved GH45 active site, and is also present in *Tl*SWO (HMD). The aspartic acid in this motif plays the role of proton donor in GH45. Neverthless, like GH45 subfamily C enzyme, the other key residues that are critical for catalytic activity are absent in expansins and swollenins. This suggests that expansins and swollenins maybe adopt an distinct mode of action with the normal catalytic process by inverting mechanisms. Recently, Nakamura et al. proposed that *Pc*Cel45A which belongs to GH45 subfamily C utilizes an imidic acid form of asparagine residue as general base in a "Newton's cradle" proton relay catalytic mechanism [38], with possible implications for elucidating the elusive catalytic mechanism in expansins. However, due to horizontal gene transfer (HGT) during the process of evolution, there is difference between fungal swollenins and bacterial or plant expansins in sequence [39]. Therefore, the catalytic mechanism of *Pc*Cel45A may be not suitable for swollenins. Besides, since crystal structures of swollenin are available

in the Protein Data Bank (PDB; http://www.rcsb.org) to date, the precise mechanistic details of swollenin catalytic action activity still remain unknown.

Unlike expansins, fungal swollenins contain an additional N-terminal CBM followed by a typical linker which is rich in Ser/Thr, and several sequence insertions in the D1 and D2 domains can be found in fungal swollenins. These insertions result in an increase in size of fungal swollenins which are roughly twice the size of expansins from plants and bacteria. It is generally accepted that CBMs can increase the concentration of their parent enzyme on the surface of substrates, leading to more rapid degradation of the polysaccharide [40, 41]. Analysis of the primary amino acid sequence using BLAST indicates that *Tl*SWO contains an N-terminal CBM region (amino acid residues 21–59) which showed the highest similarity to the fungal GH6 family 1 CBMs. Six typical conserved cysteines which could form three pairs of disulfide bonds. Also present are three conserved aromatic residues which are typical in GH6 and GH7 cellulases CBMs in the *Tl*SWO CBM these are Trp28, Tyr54, and Tyr55. These two important sequence features play a key role in the stability and activity of cellulases. Moreover, it has been demonstrated that the length of linker is crucial for the activity of cellulases [42]. The linker region of *Tl*SWO is over 140 amino acids, longer than that of most of reported swollenins and fungal cellulases [43]. Although CBM and linker have been well studied in cellulases, little is currently known about the actual role of these two regions in swollenins.

The smooth surface structure of Avicel tended to show a rough texture after treatment with *Tl*SWO, suggesting a cell wall modification activity usually exhibited by swollenins. According to the previous reports, expansin or expansin-like proteins promote cellulase activity through disrupting of the non-covalent bonds or the surface structure of cellulosic substrates, thus enhancing cellulose accessibility by enzymes [44, 45]. So far, the synergistic effect between swollenins and hydrolytic enzymes have been explored in some research. For example, the swollenin *Th*Swo from *Trichoderma harzianum* displayed a high synergistic effect with a commercial xylanase, enhancing its hydrolytic performance up to $147 \pm 7\%$ [6]. Simultaneous reaction with both *Po*SWOI from *Penicillium oxalicum* and cellulases enhanced the hydrolysis of crystalline cellulose Avicel by approximately 50% [5]. Also, the swollenin *Af*Swo1 improved the glucose conversion rate of Avicel by 1.2 times when treating with both *Af*Swo1 and cellulase mixture [22]. The ability of *Tl*SWO to boost the hydrolysis of cellulosic substrates by different enzymes was also analyzed in this study. The results showed that, a greater increase in glucose yields was observed when the cellulose were incubated with *Tl*SWO and cellobiohydrolases, however, there were no significant synergistic effect between *Tl*SWO and endoglucanases. This result differs from the previous report, in which swollenin exhibited strong synergistic interaction with endoglucanases [1]. We propose that *Tl*SWO has better co-ordination with cellobiohydrolases. As the previously reported, C_1-C_x model hypothesized about the process of cellulose degradation (C_1: non-hydrolytic component, C_x: endo- or exo-acting cellulases). One proposal made by this model was that C_1 can decrystallize cellulose by displacing hydrogen bonds in the microfibril, leading to a more available structure for C_x [46, 47]. Based on this, Eibinger *et al*. and Kang *et al*. speculated that swollenins play the possible role of C_1 enzymes due to its disruptive activity in the enzymatic saccharification of lignocellulosic substrates, although the action

mode of C_1 enzymes need to be further explored [5, 11, 48]. On the other hand, amorphous cellulose (PASC) and cellulose nanocrystals (CNC) treated with or without *Tl*SWO were used to compare the effect of swollenin. The result suggested that the total glucose concentration was increased by 32% when PASC was incubated with *Tl*SWO and cellobiohydrolases, comparing to incubating with cellobiohydrolases only. However, when using CNC as the substrate, there is no significant difference between the group with *Tl*SWO and without *Tl*SWO. It appears like that swollenin prefers to bind and disrupt the amorphous cellulose than crystalline cellulose.

Conclusions

In the present study, we reported a new swollenin, *Tl*SWO, from *T. leycettanus* JCM12802. *Tl*SWO is an acidic and mesophilic swollenin, showing acitivity towards lichenan, barley β-glucan, carboxymethyl cellulose sodium and laminarin. A greater increase in glucose yields was observed when the cellulose were incubated with *Tl*SWO and cellobiohydrolases, however, there were no significant synergistic effect between *Tl*SWO and endoglucanases, suggesting that TlSWO has better co-ordination with cellobiohydrolases. Futher analysis indicated that *Tl*SWO exhibited synergetic effects on cellobiohydrolase when using PCS and PASC as the substrate. However, when using CNC as the substrate, there is no significant difference between the group with TlSWO and without *Tl*SWO, suggesting that TlSWO prefers to bind and disrupt the amorphous cellulose than crystalline cellulose.

Abbreviations

CMC-Na
carboxymethycellulose sodium
CBM
carbohydrate binding module
GH
glycoside hydrolase
PCS
pre-treated corn stover
CNC
cellulose nanocrystals
PASC
phosphoric acid swollen cellulose

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HZ and YW performed the experiments and performed the experiments. RB designed and performed the synergism experiments and analyzed the data. BY and XX designed the research and participated in the bioinformatics analysis. FZ and HL revised the manuscript. All authors read and approved the final manuscript.

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Additional Files

Fig. S1 Production and purification of recombinant *Tl*SWO in *Pichia pastoris* and *Trichoderma reesei*. A: SDS-PAGE analysis of the culture supernatant of *Tl*SWO produced by *Pichia pastoris*. Lane 1: the crude enzyme of *Tl*SWO; lane 2, the purified *Tl*SWO; lane 3, the deglycosylated *Tl*SWO; B: SDS-PAGE analysis of the culture supernatant of *Tl*SWO produced by *Trichoderma reesei*. Lane 1: the crude enzyme of positive control contained *Tr*Cel7; lane 2, the crude enzyme of *Tl*SWO; lane 3, the purified *Tl*SWO.

Fig. S2 MALDI-TOF-MS analysis of the recombinant protein *Tl*SWO.

Fig. S3 Scanning electron microscopic analyses of Avicel. Ten milligrams of Avicel was incubated with 300 μg of purified *Tl*SWO in 100 mM citric acid-Na_2HPO_4 buffer (pH 4.0) for 12 hours.

Figures

Figure 1

Sequence alignments of TlSWO with other 5 swollenins from fungi. AfSWO from Aspergillus fumigatus (GeneBank No. XP_747748), PdSWO from Penicillium decumbens (GeneBank No. ACH57439), PoSWO from Penicillium oxalicum (GeneBank No. ADZ74267), TrSWO from Trichoderma reesei (GeneBank No. CAB92328), TpSWO from Trichoderma pseudokoningii (GeneBank No. ABV57767.1).
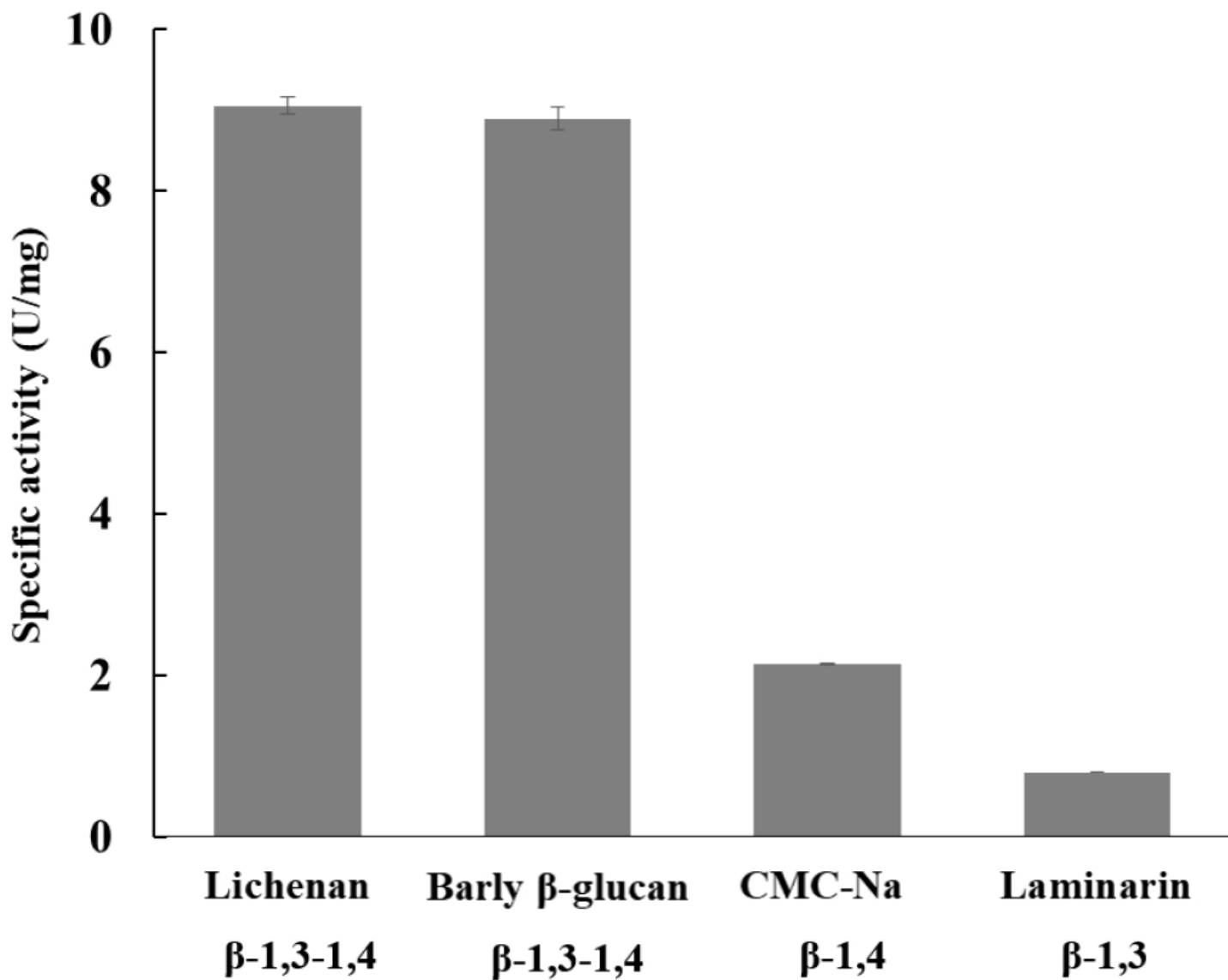
Figure 2

The substrate specificity of the TlSWO. Hydrolysis reactions on 1% barley β-glucan, 1% CMC-Na, 1% laminarin and 0.5% lichenan, were carried out overnight at pH 4.0, 50 °C. The linkage type of each substrate was labeled.
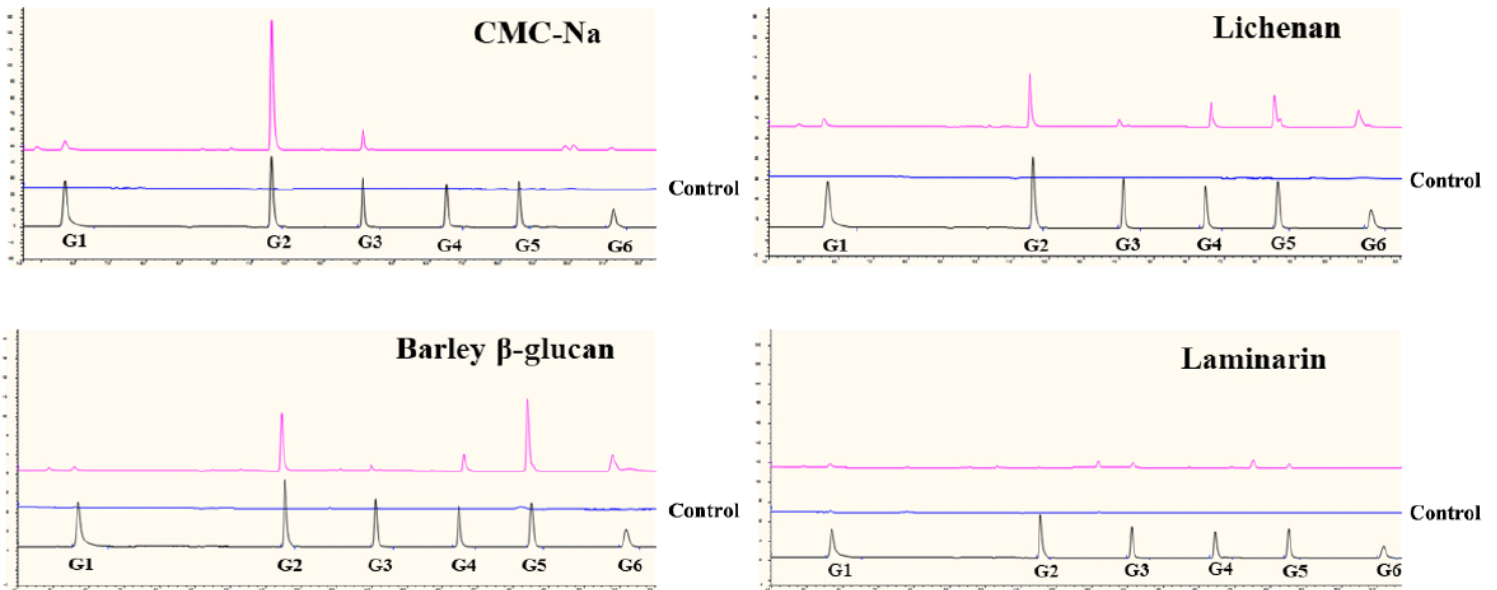
Figure 3

Hydrolysis capacity of TlSWO to degrade CMC-Na, lichenan, barley β-glucan and laminarin. All the reactions were carried out overnight at pH 4.0, 37 °C using the enzyme in a final concentration of 100 µg/mL.
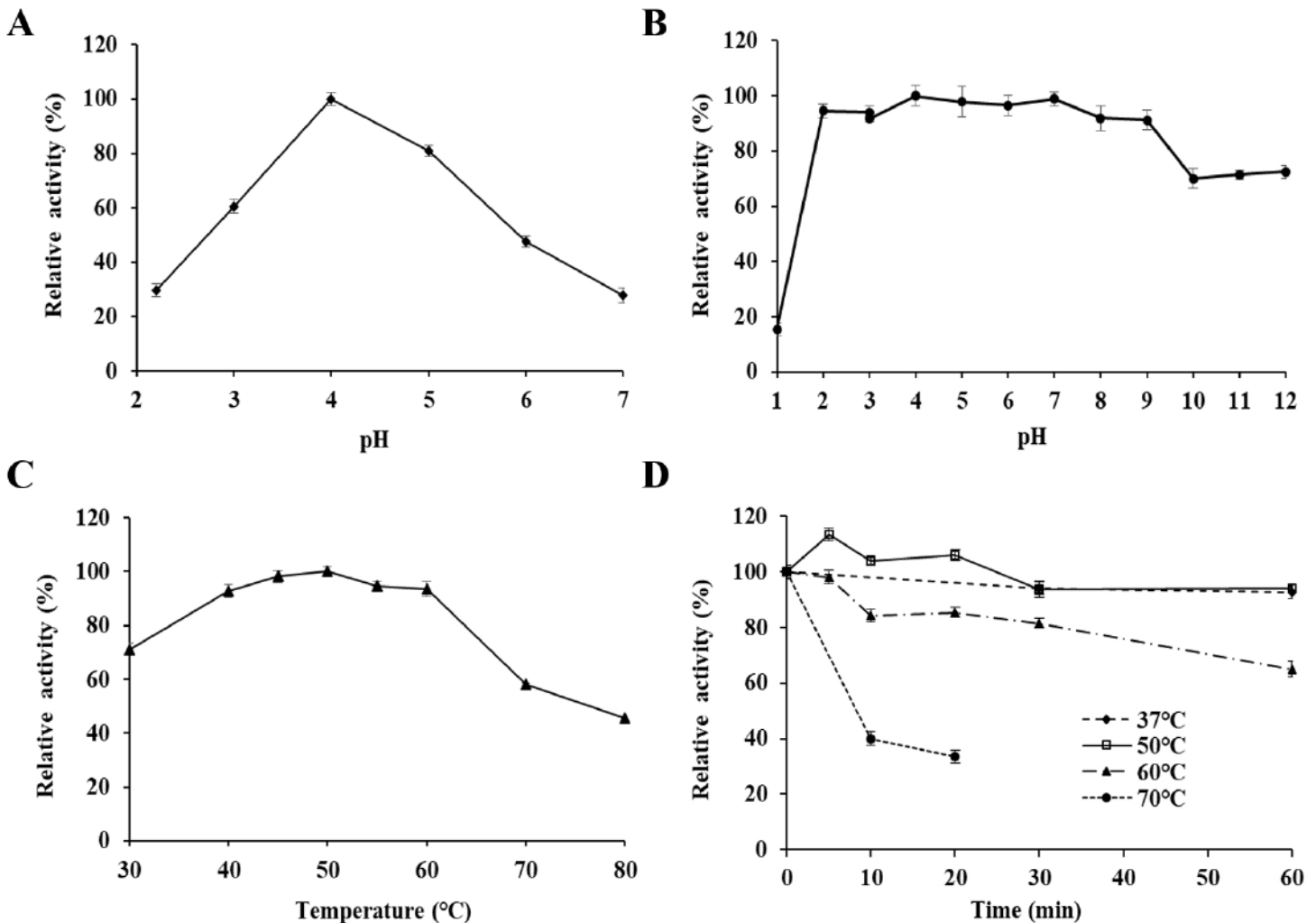
Figure 4

Enzyme properties of purified recombinant TlSWO. (A) pH-activity profiletested at 50 °C. (B) pH stability. After incubation of the enzymes at 37 °C for 1 h in buffers ranging from pH 1.0 to 12.0, the residual activities were determined at pH 4.0 and 50 °C. (C) Temperature-activity profiletested at the pH 4.0. (D) Thermostability. TlSWO was pre-incubated at 37 °C, 50 °C, 60 °C and 70 °C for different periods of time, and subjected to residual activity assay under optimal conditions.
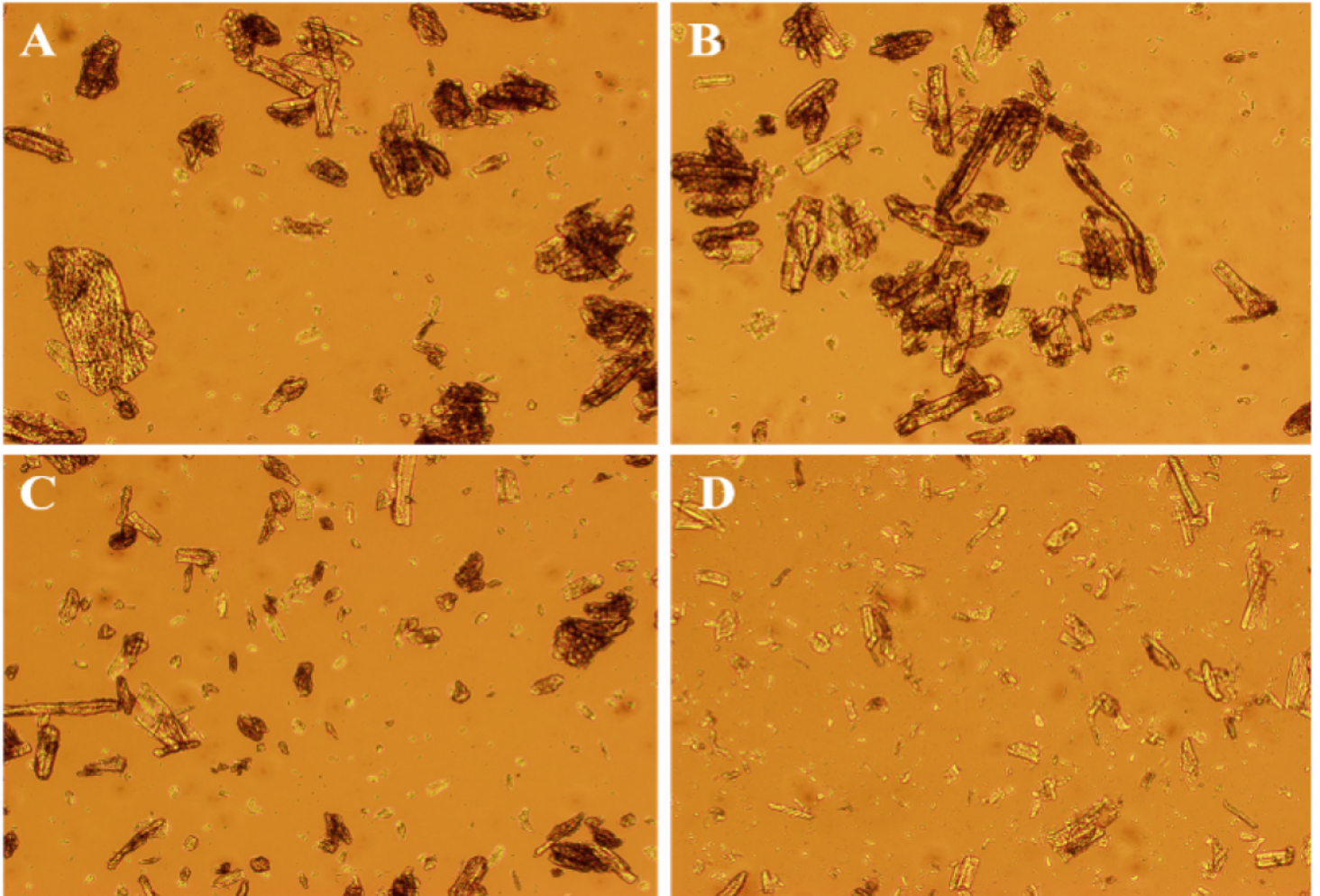
Figure 5

Light microscopic analyses of Avicel. Ten milligram of Avicel was incubated with different amount of purified TlSWO in 100 mM citric acid-Na2HPO4 buffer (pH 4.0) for 24 hours. (A) 0 µg TlSWO (B) 10 µg TlSWO, (C) 100 µg TlSWO, (D) 300 µg TlSWO.
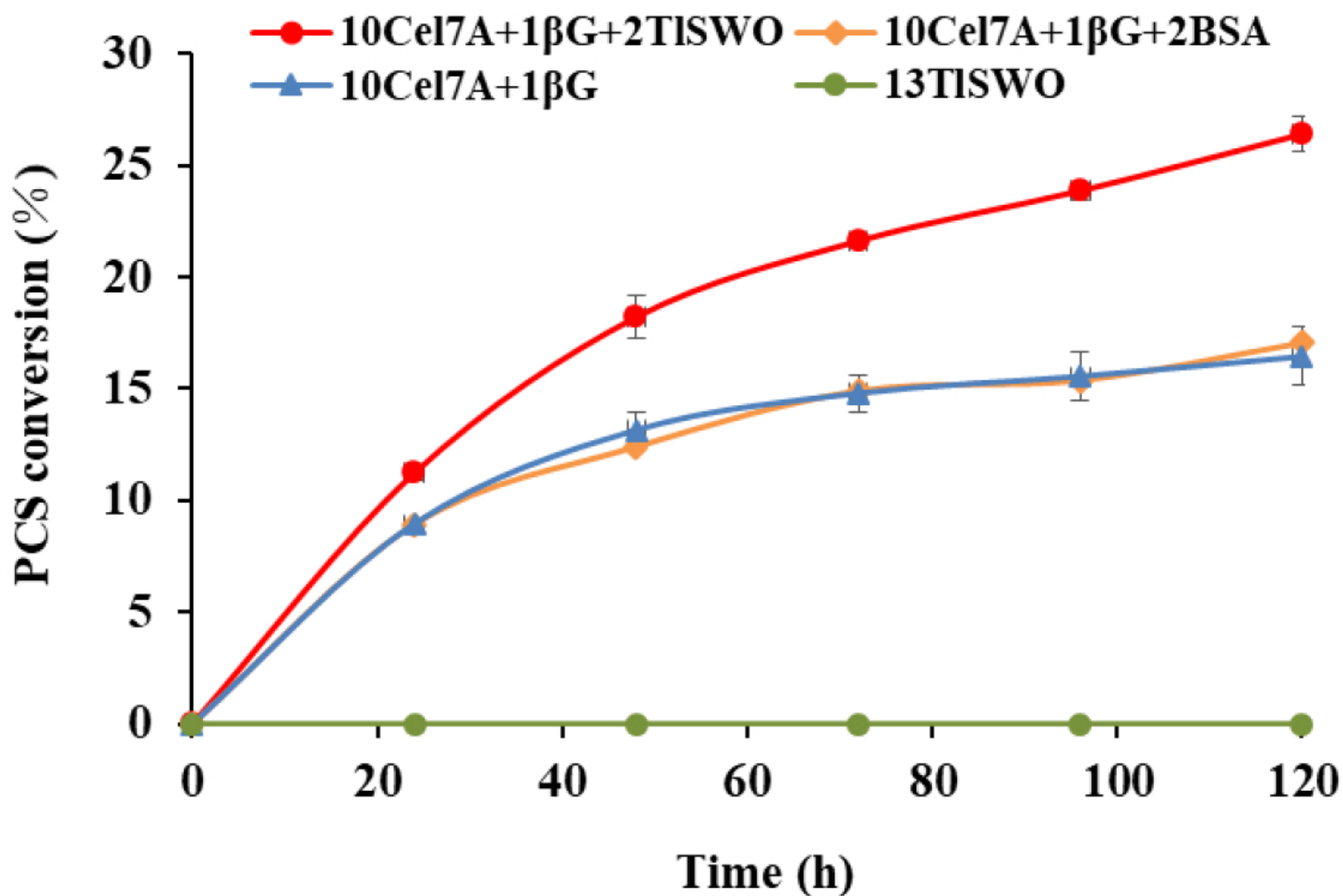
Figure 6

Conversion performance of TlSWO on PCS. PCS hydrolysis performed at 40 °C, β-D-glucosidase and cellobiohydrolases were added in the incubation mixture as described in Methods.
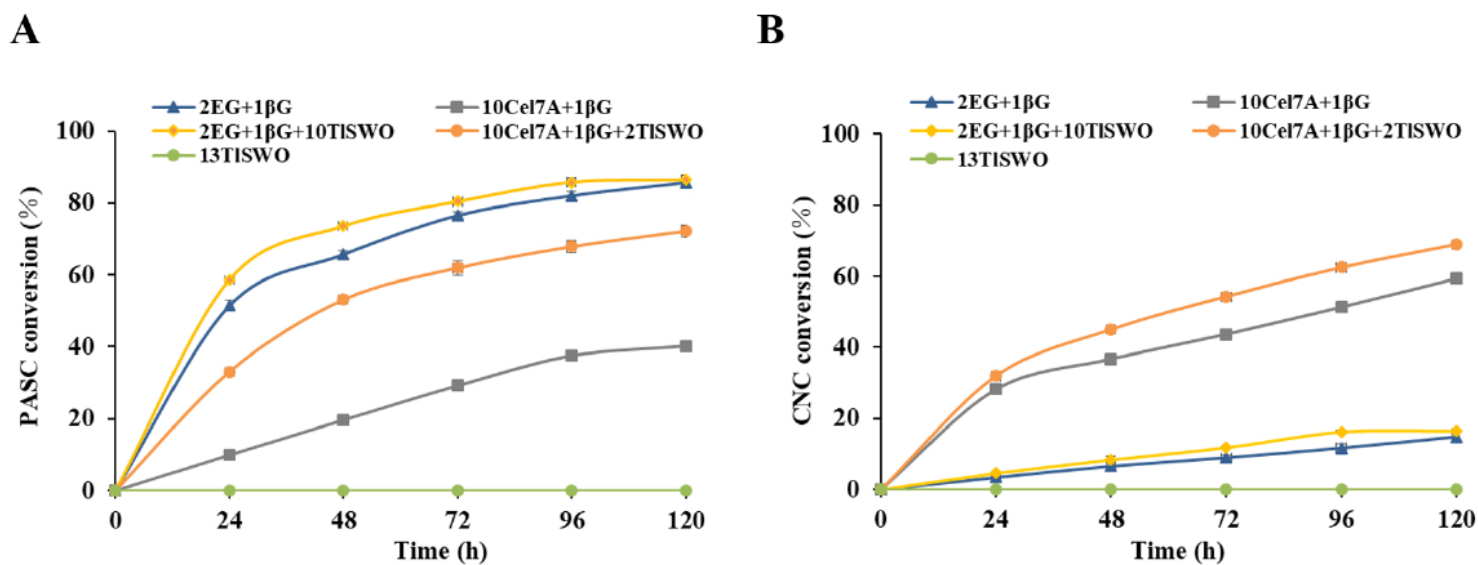
Figure 7

Conversion performance of TlSWO. (A) PASC hydrolysis, (B) CNC hydrolysis. All the reaction performed at 40 °C, β-D-glucosidase, cellobiohydrolases, and endoglucanase were added in the incubation mixture as described in Methods.