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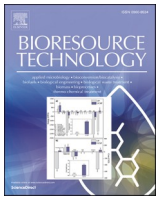

BsEXLX of engineered *Trichoderma reesei* strain as dual-active expansin to boost cellulases secretion for synergistic enhancement of biomass enzymatic saccharification in corn and *Miscanthus* straws

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HIGHLIGHTS

- A desirable engineered TrEXLX10 strain secretes active BsEXLX/expansin protein.
- The expansin is of highly binding activity with wall polysaccharides and lignin.
- TrEXLX10 secretes active cellulases and xylanases by expansin stabilization.
- High yield hexoses released by enzymatic hydrolysis and expansin coactivation.
- A model for expansin dual-active roles in enzymes secretion and biomass digestion.

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ABSTRACT

In this study, bacterial *BsEXLE1* gene was overexpressed into *T. reesei* (Rut-C30) to generate a desirable engineered TrEXLX10 strain. While incubated with alkali-pretreated *Miscanthus* straw as carbon source, the TrEXLX10 secreted the β-glucosidases, cellobiohydrolases and xylanses with activities raised by 34%, 82% and 159% compared to the Rut-C30. Supplying EXLX10-secreted crude enzymes and commercial mixed-cellulases for two-step lignocellulose hydrolyses of corn and *Miscanthus* straws after mild alkali pretreatments, this work measured consistently higher hexoses yields released by the EXLX10-secreted enzymes for synergistic enhancements of biomass saccharification in all parallel experiments examined. Meanwhile, this study detected that the expansin, purified from EXLX10-secreted solution, was of exceptionally high binding activities with wall polymers, and further determined its independent enhancement for cellulose hydrolysis. Therefore, this study raised a mechanism model to highlight EXLX/expansin dual-activation roles for both secretion of stable biomass-degradation enzymes at high activity and biomass enzymatic saccharification in bioenergy crops.

1. Introduction

Crop straws provide enormous lignocellulose residues that are sustainable and transformable for biofuels and bioproducts (Schädel et al., 2010). Since cellulosic ethanol is assessed as admirable additive into petrol for less release of net carbon, various advanced technologies

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have been attempted to improve biomass enzymatic saccharification and bioethanol conversion (Madadi et al., 2021). Nevertheless, the natural lignocellulose recalcitrance basically decides strong physical and chemical biomass pretreatments and low efficient enzymatic digestibility, leading to an overpriced lignocellulose processing that is unapplicable for bioethanol production along with possible secondary-waste liberation (Hu et al., 2023; Menon et al., 2012; Schutyser et al., 2018). Therefore, it remains to explore lignocellulose-degradation enzymes at high activity and biomass pretreatments under mild conditions (Liu et al., 2016).

Due to its superior secretion ability, *Trichoderma reesei* has been broadly applied as a major industrial fungus strain to produce commercial lignocellulose-degradation enzymes (Bischof et al., 2016; Couturier et al., 2012). As lignocellulose is of complicated structures and assorted interlinkages, the *T. reesei* strain needs to secret different types of enzymes enabled for complete lignocellulose digestion (Gupta et al., 2016; Liu et al., 2021; Peng et al., 2022). For instance, *endo*-β-1,4-glucanases (EGs; EC 3.2.1.4) act to release oligosaccharides by cleaving amorphous chains of cellulose microfibrils, whereas cellobiohydrolases (CBHs; EC 3.2.1.91) break cellulose chains to produce cellobioses (Druzhinina et al., 2017). Hereby, β-glucosidases (BGL, EC 3.2.1.21) are finally required to digest cellobioses for fermentable glucose production (Cragg et al., 2015). Meanwhile, *T. reesei* strain also secret various xylanases for hemicellulose hydrolyses (Hamid et al., 2023; Pauly et al., 2016). Nevertheless, as all types of enzymes consist of several isoforms, the optimal cocktails of biomass-degradation enzymes are largely varied, which is dependent on lignocellulose characteristics (Hamid et al., 2022; Liu et al., 2016; Zhang et al., 2023a).

Alternatively, the proteins with non-hydrolytic activity are explored to improve lignocellulose feature and digestibility (Florencio et al., 2016; Madadi et al., 2021; Zhang et al., 2020; Zheng et al., 2021). In general, those types of proteins have been found in different organisms such as bacteria, fungi and plants, and they mostly play an auxiliary role for lignocellulose enzymatic hydrolysis (Karimi et al., 2014). In comparison, some proteins can either break crystalline regions of cellulose microfibrils or disassociate hydrogen bonds between cellulose and hemicellulose, whereas other proteins preferentially interact with lignin to block its adsorption with cellulases (Zhang et al., 2020). Notably, a bacterial expansin (BsEXLE) protein is characterized with highly binding activity with pretreated lignocellulose residues, but little is known about its cascading-like applications for *T. reesei* secretion of lignocellulose-degradation enzymes *in vivo* and biomass enzymatic saccharification *in vitro* (Kim et al., 2013).

In this study, the *EXLX1* gene encoded as expansin was initially cloned from *Bacillus subtilis*, and then expressed in *T. reesei* strain (Rut-C30) driven under *pdc1* promoter (see supplementary materials). After the enzyme-secretion system was optimized to screen out engineered *T. reesei* lines, the desirable strain termed as TrEXLX10 was identified, due to its secreted enzymes with much raised activities including filter paper activity, *exo*-glucanase, xylanase and β-glucosidase, compared to its control (empty vector). Meanwhile, this work determined significantly increased proteins level in the TrEXLX10 strain, and further used the crude enzymes secreted by TrEXLX10 to detect much augmented biomass saccharification of corn and *Miscanthus* straws after mild alkali pretreatments were performed. More importantly, this study purified EXLX protein *in vitro* from the TrEXLX10 strain and characterized its distinct interactions with three major wall polymers, which led to raising a novel hypothesis model for understanding of expansin activation roles in both *T. reesei* secretion of lignocellulose-degradation enzymes and biomass enzymatic saccharification of bioenergy crops.

2. Materials and methods

2.1. Collection of crop biomass, enzymes and microorganisms

Mature straws of two *Miscanthus* species (*M. sacchariflorus*/Msa01; *M. lutarioriparius*/ Mlu11) and two corn cultivars (B73, Y18) were harvested from the experimental station of Huazhong Agricultural University. The crop straws were dried at 60 °C, ground into powders through a 40 mesh sieve (425 μm) and stored in a dry container until in use.

Avicel paper was purchased from China National Pharmaceutical Group Co., Ltd. The mixed-cellulases enzymes (termed as HSB) was obtained from Imperial Jade Biotechnology Co., Ltd. Ningxia, China. β-Glucosidase, β-xylanse, endoglucanase and exoglucanase were bought from Megazyme. The *T. reesei* (Rut-C30) strain was obtained from the ARS (NRRL) Culture Collection.

2.2. Vector construction for BsEXLX1 expression

The *BsEXLX1* (AL009126.3) sequence of *Bacillus subtilis* was synthesized *in vitro* according to the codon preference of *T. reesei*. The vector was used for *BsEXLX1* expression in *T. reesei* (Rut-C30) as described (see supplementary materials), which contains Ppdc promoter, resistance gene of hygromycin B, signal peptide of CBH I and His-tag encoding sequence. The amplified pdc1 promoter, *EXLX1* gene, and pdc1 terminator were fused to pCZF according to the manufacturer's instructions (Sangon Biotech, Shanghai, China), and the pCZF-EXLX1 vector was generated and verified by sequencing. The vector pCZF-EXLX1 was transformed into *T. reesei* (Rut-C30) by *A. tumefaciens*-mediated transformation as previously described (Michielse et al., 2008). Twenty transformants were verified by PCR analysis (see supplementary materials).

2.3. Screen of engineered T. Reesei strains

All engineered *T. reesei* strains termed as TrEXLX1-10 and control strain (Rut-C30) were respectively grown on potato dextrose agar at 30 °C for 7 d. The spore suspensions were collected with flask culture under shaken for 7 d, and the flask cultivation conidia were then counted on haemocytometer. Before micro fluidic examination and sorting, the spore germination rates were evaluated for optimal incubation time. The spores were adjusted for the density at 6×10^6 spores/mL in enzyme-inducing medium, and about 500 μL spore suspension was shaken under 200 rpm/min at 30 °C for 7 d. The Mandels-Andreotti medium was incubated with 1% (g/L) NaOH-pretreted *Miscanthus* substrates or Avicel as carbon source.

2.4. Detection of filter paper activity and protein content

The filter paper activity (FPA) assay was previously described (Ghose, 1987). The well-mixed solution was incubated for 60 min at 50 °C, and the reaction was stopped by adding 2 mL DNS followed by boiling water for 10 min. Protein content of the crude enzymes secreted by engineered strains were detected as described (Bradford, 1976) using UV–vis spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd. Shanghai).

2.5. Exoglucanases, Endoglucanases, xylanases and β-glucosidase activity assay

Biomass-degradation enzyme assays were *in vitro* accomplished as previously described (Liu et al., 2021; Peng et al., 2022; Song et al., 2016; Zhang et al., 2023b). Activities of exoglucanase (CBH), endoglucanases (EG), xylanase (XYN) and β-glucosidase (BGL) were respectively detected by using *p*-nitrophenol-D-cellobioside (pNPC), carboxymethylcellulose (CMC-Na), beechwood xylan and D(-)-Salicin (Sigma-Aldrich Co. LLC., China National Pharmaceutical Group Co., Ltd., Shanghai Yuanye Bio-Technology Co., Ltd, China). The reducing sugars were accounted by the dinitrosalicylic acid method. The enzymes were treated at 100 °C for 10 min as the controls. One enzyme activity unit (U) was accounted by 1 μmol glucose or xylose per minute released from

enzymatic hydrolysis.

2.6. Biomass pretreatment and enzymatic hydrolysis

Biomass samples of corn and *Miscanthus* straws were pretreated with 5 mL 0.5%, 1.0% and 4% NaOH (w/v) under 150 rpm shaken at 50 °C for 2 h as previously described (Xu et al., 2012; Yu et al., 2022). The pretreated substrates were supplied with the 10 FPU/g of enzyme cocktails secreted by engineered strains and commercial mixed-cellulases (HSB), and the enzymatic hydrolyses were accomplished (Alam et al., 2019; Ding et al., 2022). For the two-step enzymatic hydrolyses, the firs-step hydrolysis was conducted for 48 h to collect the supernatant from 5 min centrifugation (3000 *g*), and the lignocellulose residues were further used for the second-step hydrolysis for another 48 h to collect the supernatant again. The supernatants were respectively applied to determine their hexoses, pentoses and protein contents. The hexose and pentose were respectively measured by anthrone/H_2SO_4 and orcinol/HCl methods (Fry, 1988) and all assays were accomplished under independent triplicates.

2.7. Yeast fermentation and ethanol measurement

The activated yeast (Angel yeast Co., Ltd., China) was incubated with the supernatants of enzymatic hydrolyses as described above, and the fermentation reaction was accomplished at 37 °C for 48 h as previously described (Fu et al., 2022). The fermentation solution was distilled, and the ethanol was accounted by the $K_2Cr_2O_7$ method at independent triplicate.

2.8. SDS-PAGE running and Western blot analysis

SDS-PAGE was run using Stain-Free precast gels (Beijing Zoman Biotechnology Co., Ltd.) based on the manufacturer's instructions. Western blotting was further accomplished using PVDF membrane for protein transforming as previously described (Liu et al., 2021). The membrane was blocked for 1.5 h in TBS buffer (20 mM Tris-HCl and 500 mM NaCl, pH 7.5) supplied with 5% nonfat dry milk, rinsed with TTBS buffer (0.05% Tween-20 in TB) and incubated with primary antibody serum at room temperature for 1 h by using HRP Rabbit Anti-His Tag at 1:1000 dilution. After TTBS wash for three times, the membrane was treated for 1 h at room temperature with secondary antibody (goat anti-rabbit secondary antibody IgG-HRP at a 1:5000). Both antibodies were purchased from Beijing Zoman Biotechnology Co., Ltd. The reactions were measured by the ECL Plus Western Blotting Detection, and protein immunoblot bands were scanned with a GeneGnome XRQ (Syngene Inc., Maryland, US).

2.9. Proteomic analysis

The TrEXLX10-induced enzymes were characterized by LC-MS/MS (Jingjie PTM BioLab Co.Ltd, Hangzhou, China; Orbitrap Elite LC-MS/MS, Thermo, USA) as described (Peng et al., 2022). The peptides were sorted out by the NSI source followed by tandem mass spectrometry (MS/MS). Liquid chromatography-MS/MS data were recognized by searching the *T. reesei* Rut-C30 protein sequence databases downloaded from Uniprot (http://www.uniprot. org).

2.10. Characterization of EXLX protein

For His-intein-based EXLX purification, the supernatant solution secreted by engineered strain was filtered by 0.22 μm membrane, and loaded onto a pre-equilibrated Ni-NTA Beads 6FF column at 25 °C for 30 min. The purified EXLX protein was eluted with PBS buffer (40 mM Bis-Tris, pH 7.0) at 37 °C for 12 h. Protein binding assay was completed according to the method (Madadi et al., 2021). Meanwhile, the zeta potential of supernatants was examined according to the method (Ding et al., 2012; Madadi et al., 2021).

2.11. Statistical analysis

All experimental assays were accomplished at independent triplicate unless indicated, and Student's *t*-test were subjective to the SPSS 23 software (Inc., Chicago, IL).

3. Results and discussion

3.1. Selection of desirable engineered T. Reesei strain (TrEXLX10) for high-activity enzymes production

In this study, the *BsEXLX1* gene was initially cloned to overexpress into *T. reesei* strain (Rut-C30) driven with Ppdc promoter (see supplementary materials). By screening out total ten transgenic *T. reesei* lines termed as TrEXLX1-10, this work collected their secreted crude enzymes, and determined largely varied filter paper activities (FPAs) *in vitro*. As a comparison, the TrEXLX10 line was selected as a desirable engineered strain for the following experiments, due to its highest PFA examined (Fig. 1). By means of our previously-established methods (Liu et al., 2021; Peng et al., 2022), this study determined five types of enzymes activities *in vitro* secreted by the engineered TrEXLX10 and Rut-C30 strains, respectively (Fig. 1A-E). Except for the similar CMCase activity, the activities of other four enzymes were significantly increased in the TrEXLX10 strain than ones of the Rut-C30 at $p < 0.01$ levels ($n = 3$). In particular, the TrEXLX10 strain was of the xylanase and β-glucosidase activities raised by 159% and 82%, whereas its filter paper and pNPC activities were augmented by 32% and 34%, respectively. Meanwhile, the TrEXLX10 strain also secreted the higher total protein level than that of the Rut-C30 by 40% (Fig. 1F). Taken together, the results appeared to be similar to the previous findings that either the activities of major lignocellulose-degradation enzymes or total protein levels are raised by the *T. reesei* (Rut-C30) incubation with the desirable de-lignin substrate of *Miscanthus* or the distinct cellulose nanofibrils substrate of rice nature mutant (Liu et al., 2021; Peng et al., 2022). As BsEXLX has been characterized to be the active expansin enabled to bind with lignocellulose substrates (Payne et al., 2015), the findings thus suggest that the TrEXLX10 strain may initially secrete sufficient expansin to activate *Miscanthus* lignocellulose substrate for facilitating *T. reesei* digestion of cellulose and hemicellulose as carbon source, consequently leading to an efficient secretion of cellulases and xylanases by TrEXLX10 strain as examined.

3.2. Consistently independent enhancements of biomass saccharification by TrEXLX10-secreted enzymes

As the TrEXLX10 strain could secrete multiple biomass-degradation enzymes at high activities as described above, this work implemented two-step enzymatic hydrolyses of corn and *Miscanthus* straws by supplying commercial mixed-cellulases followed with total crude enzymes secreted by TrEXLX10 or Rut-C30 strain (Fig. 2). After mild alkali pretreatments were performed with corn (0.05% NaOH) and *Miscanthus* (1% NaOH) straws, this work initially applied the commercial mixed-cellulases to digest pretreated lignocellulose residues for 48 h, and the remaining pellets were further incubated with the crude enzymes secreted by TrEXLX10 or Rut-C30 strain. Meanwhile, parallel experiments were conducted by using the secreted crude enzymes followed with the commercial mixed-cellulases. As a result, both corn and *Miscanthus* samples showed significantly higher hexoses yields released by TrEXLX10-secreted enzymes than those of the Rut-C30 at $p < 0.01$ level ($n = 3$) in the two parallel experiments performed (Fig. 2A, B). However, despite the TrEXLX10 strain could secrete higher-activity xylanase than that of the Rut-C30 as described above, both strains released similar pentoses yields in the corn and *Miscanthus* samples examined (Fig. 2C, D), suggesting that either commercial mixed-cellulases or secreted crude

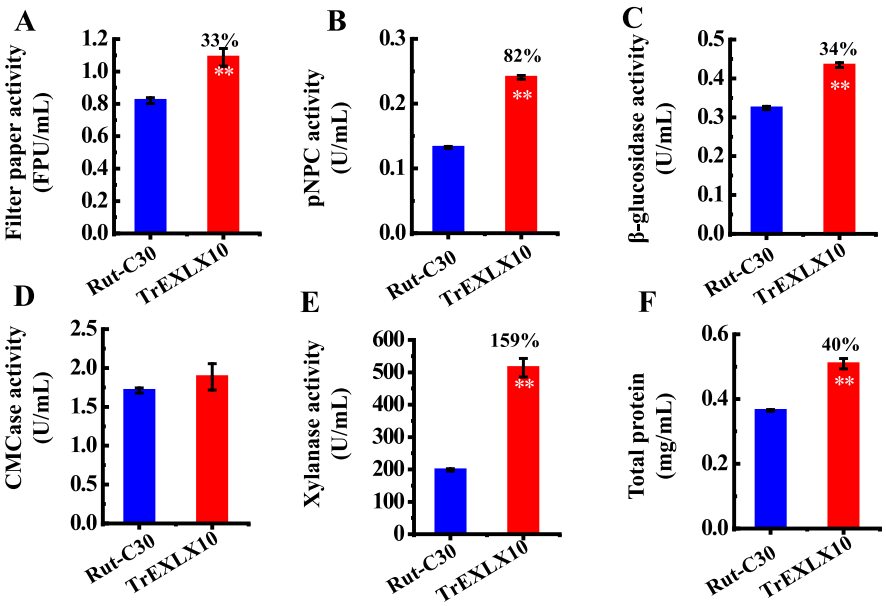

Fig. 1. Enzymatic activity assay *in vitro* using standard substrates and enzymes secreted by engineered *T. reesei* strain (TrEXLX10) and wild type (Rut-C30). (A-E) Activity assay of five biomass-degradation enzymes; (F) Total protein level; Data as mean ± SD (n = 3); ** As significant difference between TrEXLX10 and Rut-C30 samples by *t*-test at $p < 0.01$.

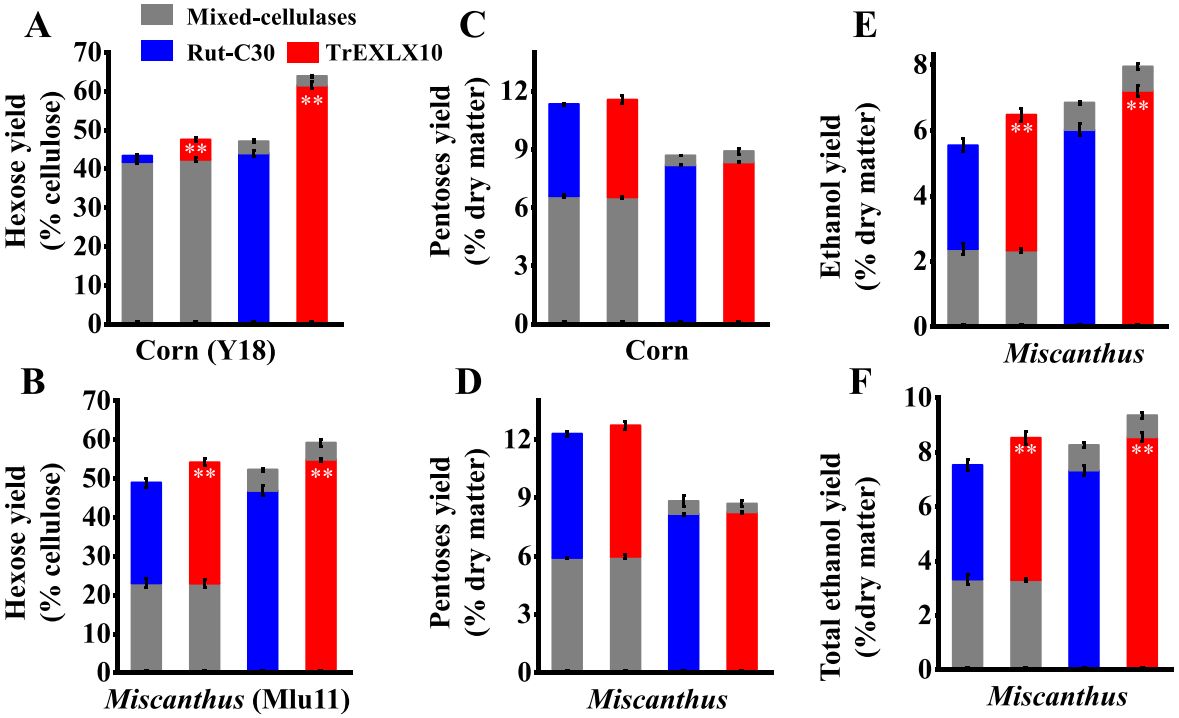

Fig. 2. Biomass saccharification for bioethanol production from two-step enzymatic hydrolyses of alkali-pretreated corn and *Miscanthus* straws by using commercial mixed-cellulases and enzymes secreted from engineered strain (TrEXLX10) and wild type (Rut-C30). (A, B) Hexoses yield against cellulose level; (C,D) Pentoses yield against total dry matter; (E) Bioethanol yield by yeast fermentation using hexoses released; (F) Total bioethanol yield using hexoses and pentoses (by in-theory pentoses-ethanol conversion rate as previously reported); Down and up sections of each column represented the first and second step enzymatic hydrolyses; Corn and *Miscanthus* straws were respectively pretreated with 0.5% NaOH and 1% NaOH; Data as mean ± SD (n = 3); ** As significant difference between TrEXLX10 and Rut-C30 samples by *t*-test at $p < 0.01$.

enzymes could be sufficient for hemicellulose hydrolyses in this reaction system. Furthermore, yeast fermentation was accomplished by using sugars released from enzymatic hydrolyses, and the corn and *Miscanthus* samples produced significantly higher bioethanol yields (% dry matter) by TrEXLX10-secreted enzymes hydrolyses than those of the Rut-C30 at $p < 0.01$ levels (Fig. 2E), consistent with their distinct enzymatic saccharification. Based on the pentose-ethanol conversion rate in theory, significantly raised total bioethanol yield was also calculated in the TrEXLX10-secreted enzymes hydrolyses (Fig. 2F).

To confirm such enhanced hexoses yields by the TrEXLX10-secreted enzymes hydrolyses, this study further tested biomass saccharification of four corn and *Miscanthus* straws by performing one-step hydrolysis

with either commercial mixed-cellulases or the crude enzymes secreted by TrEXLX10 or Rut-C30 strain (Table 1). Consistently, the TrEXLX10-secreted enzymatic hydrolyses led to significantly higher hexoses yields than those of either the Rut-C30 or the mixed-cellulases with the raised rate of hexoses yield up to 55%. Nonetheless, similar pentoses yields were also determined in those three hydrolyses reactions. Furthermore, in this study performed alkali pretreatments at high concentrations with the corn (1% NaOH) and *Miscanthus* (4% NaOH) straws, and also determined significantly augmented hexoses yields from two-step enzymatic hydrolyses of TrEXLX10-secreted enzymes followed by commercial mixed-cellulases relative to the Rut-C30-secreted ones (Table 2). Notably, even though the secreted enzymes were mixed with the commercial mixed-cellulases at 1:1 proportion for one-step hydrolysis, the hexoses yield (89% cellulose) obtained in the TrEXLX10 sample was still higher than that of the Rut-C30 sample (69% cellulose) at $p < 0.01$ level (Table 2), providing consistent evidences to support for independent enhancements of biomass enzymatic saccharification by TrEXLX10-secreted enzymes. In addition, as the commercial mixed-cellulases have previously been characterized with high activities for all major lignocellulose-degradation enzymes (Cheng et al., 2019; Huang et al., 2019; Sun et al., 2020; Wang et al., 2016; Wu et al., 2019; Xu et al., 2012; Zahoor et al., 2017), all data obtained thus suggest that consistently-enhanced saccharification should not only be due to high-activity enzymes secreted by TrEXLX10 strain, but it may also be subjective to the TrEXLX10-secreted expansin that plays a non-catalytic role for specific improvement of lignocellulose recalcitrance during enzymatic hydrolysis (Xu et al., 2021). In addition, as corn and *Miscanthus* straws are of varied lignocellulose compositions (Alam et al., 2019; Wu et al., 2019), the different synergistic enhancements of biomass saccharification may be due to distinct expansin interactions with their lignocellulose substrates.

3.3. Stable cellulases complexes secreted by TrEXLX10 for high biomass enzymatic saccharification

To understand consistent enhancement of biomass saccharification by TrEXLX10-secreted enzymes, this study observed a time-course enzymatic hydrolysis of corn straw using crude enzymes secreted by TrEXLX10 or Rut-C30 strain (Fig. 3). As a comparison, the TrEXLX10-secreted enzyme hydrolysis constantly released higher hexoses yields than those of the Rut-C30 sample, and the hexoses yield was mostly elevated up to 26% after 48 enzymatic hydrolysis (Fig. 3A), which was supported by more soluble proteins determined in the supernatants of TrEXLX10-secreted enzyme hydrolyses (Fig. 3B). Meanwhile, this study detected relatively reduced Zeta potential values for the supernatants of TrEXLX10-secreted enzyme hydrolyses compared to the Rut-C30 sample (Fig. 3C), being accountable for relatively higher proteins levels detected in the TrEXLX10 sample (Madadi et al., 2021; Xu et al., 2021). Furthermore, the soluble proteins were separated by SDS-PAGE, and the TrEXLX10 samples obviously exhibited stronger bands corresponding for three enzymes (CBH, BGL and XYN) than those of the Rut-C30 sample (Fig. 3D), indicating that the higher proteins levels of TrEXLX10 sample should be mainly derived from more biomass-degradation enzymes secreted. It has thus provided direct evidence to explain why different types of enzymes activities were significantly raised in the TrEXLX10-secreted solution for higher biomass enzymatic saccharification as examined above. Notably, an additional band corresponding for 25 kD was observed only in the TrEXLX10 sample (Fig. 3D), which should be corresponding for the *BsEXLX*-encoded expansin. More importantly, this study performed LC-MS/MS assay for all potential enzymes and protein secreted by the TrEXLX10 strain, and more than 25 enzymes were identified from five major types of lignocellulose-degradation enzymes (Table 3). Based on mass spectra profiling (see supplementary materials), the expansin was verified as the EXLX protein secreted from the TrEXLX10 strain.

In addition, this study conducted control experiments of time-course enzymatic hydrolyses under the absent of corn straw (Fig. 3E). By contrast, the TrEXLX10 sample showed slightly reduced proteins levels in the supernatants during 48 h enzymatic hydrolyses, whereas total proteins were drastically decreased after 24 enzymatic hydrolysis in the Rut-C30 sample, suggesting that the enzymes secreted by the TrEXLX10 strain should be much more stable than those of the Rut-C30. It also suggests that the expansin secreted by the TrEXLX10 strain may play other role to stabilize cellulases enzymes in the supernatant, which should be another reason for consistently augmented biomass digestibility by the TrEXLX10-secreted enzymatic hydrolyses as examined above.

3.4. Synergistic enhancement of cellulose hydrolysis by TrEXLX10-secreted expansin

Since the engineered TrEXLX10 strain could secrete active expansin for non-catalytic enhancement of biomass enzymatic saccharification, this study purified the expansin from the crude enzymes solution by using the His-tag specific for link with EXLX protein (Fig. 4A). The isolated EXLX protein was confirmed by Western blot analysis to be corresponding for 25 kD band (Fig. 4B). Combined with the mass spectra profiling (see supplementary materials), the purified EXLX protein was finally identified as the typical expansin secreted by the TrEXLX10 strain. Further co-supplying the purified expansin into commercial mixed-cellulases, this study detected significantly raised FPA at $p < 0.01$ level ($n = 3$), relative to the control assay in the absent of expansin *in vitro* (Fig. 4C), which provides evidence to support for the assumption that the TrEXLX10 strain could also secrete active expansin for synergistic enhancement of biomass enzymatic saccharification of crop straws as examined.

3.5. Distinct interactions between TrEXLX10-secreted expansin and lignocellulose substrates

Because the TrEXLX10-secreted expansin could play a synergistic role for enhancing biomass enzymatic digestibility, this work further purified the expansin to *in vitro* incubate with three standard substrates (Avicel, xylan and lignin), which should be accountable for three major wall polymers of lignocellulose residues in major crop straws (Zafar et al., 2022). Using our recently-established approach for binding-activity assay (Madadi et al., 2021), this study measured that the TrEXLX10-secreted expansin had the highest binding activity with lignin

Table 1
Hexoses and pentoses yields released from T. reesei-secreted enzymes hydrolyses of pretreated crop straws.

Crop straw	Hexoses yield (% cellulose)			Pentoses yield (% dry matter)		
	Mixed-cellulases	Rut-C30	TrEXLX10	Mixed-Cellulases	Rut-C30	TrEXLX10
Corn (B73)*	28.23 ± 2.31^{a}	30.05 ± 2.12^{a}	35.2 ± 1.84^{b}	5.47 ± 0.17^{a}	7.09 ± 0.15^{b}	7.28 ± 0.13^{b}
Corn (Y18)	37.4 ± 0.88^{a}	43.8 ± 0.16^{b}	67.96 ± 0.67^{c}	5.56 ± 0.35^{a}	7.06 ± 0.12^{b}	7.02 ± 0.23^{b}
***Miscanthus sacchariflorus* (Msa01)**	10.2 ± 0.25^{a}	12.04 ± 0.96^{b}	17.56 ± 1.01^{c}	3.9 ± 0.09^{a}	6.34 ± 0.07^{b}	6.43 ± 0.06^{b}
***Miscanthus lutarioriparius* (Mlu11)**	12.86 ± 0.55^{a}	31.71 ± 0.97^{b}	35.04 ± 0.44^{c}	3.8 ± 0.1^{a}	6.23 ± 0.06^{b}	6.5 ± 0.33^{b}

* Corn and *Miscanthus* straws were pretreated with 0.5% NaOH. The data as mean ± SD (n = 3). ^{a,b,c} As significant difference among commercial mixed-cellulases and enzymes secreted by engineered strain (TrEXLX10) and RUT-30 by performing multiple *t*-test at $p < 0.05$ (n = 3).

Table 2
Hexoses yields (% cellulose) released by enzymatic hydrolyses using either T. reesei (RUT-30, TrEXLX10) secreted enzymes followed with commercial mixed-cellulases or mixture (1:1) of commercial cellulases and secreted enzymes in corn and *Miscanthus* straws.

Pretreated Sample[#]	Rut-30	Mixed-cellulases	Total	TrEXLX10	Mixed-cellulases	Total	Rut-30 + Mixed-cellulases (1:1)	TrEXLX10 + Mixed-cellulases (1:1)
	0–48 h	**48–96 h**		**0–48 h**	**48–96 h**		**0–48 h**	**0–48 h**
Corn (Y18)	82.69 ± 4.51	8.74 ± 0.93	91.43 ± 3.58	92.91 ± 2.46**	5.01 ± 0.22	97.92 ± 2.58**	80.11 ± 0.63	88.75 ± 1.97**
***Miscanthus* (Mlu11)**	64.64 ± 0.30	9.14 ± 0.46	73.78 ± 0.46	70.12 ± 0.54**	7.03 ± 0.37	77.16 ± 0.38 **	60.26 ± 0.91	69.26 ± 1.97**

[#]Corn and *Miscanthus* straws were respectively pretreated with 1% NaOH and 4% NaOH. Data as mean ± SD (n = 3); ** As significant difference between TrEXLX10 and RUT-30 samples by *t*-test at $p < 0.01$.

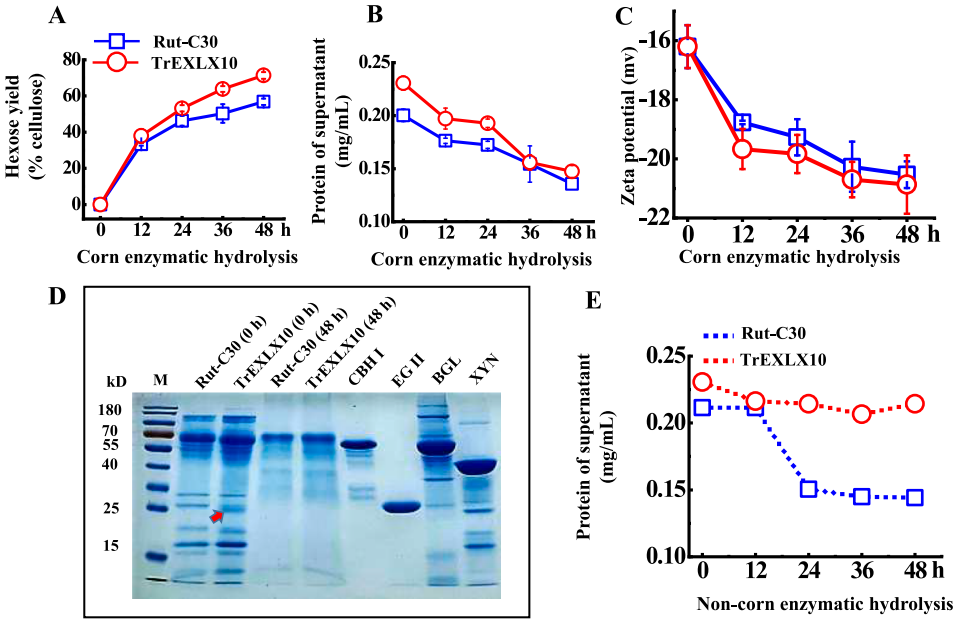

Fig. 3. Characterization of time-course enzymatic hydrolyses of 0.5% NaOH-pretreated corn straw using enzymes secreted from engineered strain (TrEXLX10) and wild type (Rut-C30). (A) Hexoses yield against cellulose level; (B) Protein level remained in the supernatant of enzymatic hydrolysis with corn straw; (C) Zeta potential assay of the supernatants; (D) SDS-gel profiling of supernatant proteins after 0 h and 48 h enzymatic hydrolyses; (E) Protein level remained in the supernatant of enzymatic hydrolysis without corn straw supply; Data as mean ± SD (n = 3); CBHI, EGII, BGL, XYN as standard individua enzyme markers.

Table 3
LC-MS/MS assay of all enzymes secreted by engineered strain (TrEXLX10).

	Protein	Gene	Accession No.	Mw (kDa)
Expansin	EXLX	*exlX/EXLX1*	A0A6M3ZBB1	25.91
	Swollenin	*swo1*	Q9P8D0	51.52
CBH	Exoglucanase 1	*cbh1*	P62694	54.07
	Exoglucanase	*TRIREDRAFT_72567*	G0RB58	49.65
	Exoglucanase	*cel7b*	G0RKH9	48.21
EG	Endoglucanase IV	*cel61a*	O14405	35.51
	Endo-beta-1,4-glucanase	*M419DRAFT_124438*	A0A024S2H5	22.33
	Endoglucanase II	*cel5a*	G0RB67	44.16
	Endoglucanase VIII	*cel5b*	Q7Z9M6	46.86
	Endoglucanase VII	*M419DRAFT_122518*	A0A024SFJ2	26.80
BGL	Glycoside hydrolase family 31	*TRIREDRAFT_82235*	G0RWC5	98.54
	β-D-Glucoside glucohydrolase	*bgl1*	Q12715	78.43
	β-Glucosidase	*cel3e*	Q7Z9M0	83.00
	β-Glucosidase	*cel3b*	Q7Z9M5	93.95
	β-Glucosidase	*TRIREDRAFT_47268*	G0RGP9	88.12
XYL XYN BXL	Xylan-1,4-β-xylosidase	*bxl1*	Q92458	87.19
	α-Glucuronidase	*aguA*	G0RV49	93.42
	Xyloglucanase	*M419DRAFT_111943*	A0A024S9Z6	87.13
	β-Xylanase	*xyn3*	Q9P973	38.08
	Endo-1,4-β-xylanase	*Xyn2*	A0A1L7H884	20.77
	Non-reducing end alpha-L-arabinofuranosidase	*TRIREDRAFT_55319*	G0RA39	53.13
	α-L-Arabinofuranosidase	*abf2*	Q7Z9N0	34.78
PG	β-Galactosidase	*bga1*	Q70SY0	111.37
	α-Galactosidase	*agl1*	Q92456	48.52
	α-Galactosidase	*TRIREDRAFT_72704*	G0RVN2	68.46
	α-Galactosidase	*TRIREDRAFT_124016*	G0RVT1	82.09
	Pectin lyase	*TRIREDRAFT_121746*	G0RJ25	83.09

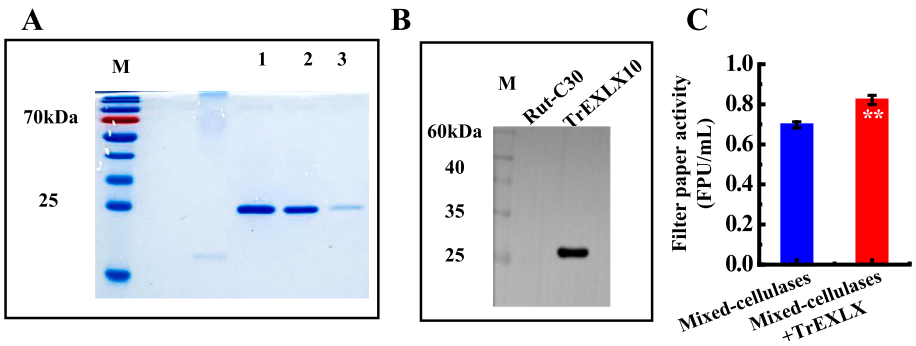

Fig. 4. Characterization of expansin activity purified from crude enzymes solution secreted by engineered strain (TrEXLX10). (A) SDS-gel profiling of EXLX protein eluates purified by His tag; (B) Western-blot analysis of EXLX protein; (C) Synergistic enhancement on filter paper activity *in vitro* by adding purified EXLX into commercial mixed-cellulases. Data as mean ± SD (n = 3); ** As significant difference between mixed-cellulases and mixed-cellulases co-supplied with purified EXLX samples by *t*-test at $p < 0.01$.

substrate, whereas the Avicel and xylan substrates were of a similar binding activity (Fig. 5A). However, the lignin-binding activity was only higher than those of both Avicel and xylan substrates by<40%, which was different from the previous reports that the BsEXLX1 is of more than 7-fold higher binding activity with lignin than that of the Avicel (Kim et al., 2013; Lin et al., 2013). Meanwhile, as a comparison with previously characterized proteins that show a dominated binding activity with lignin and slight interactions with wall polysaccharides (Madadi et al., 2021; Kim et al., 2009), the TrEXLX10-secreted expansin remained relatively higher binding activities with cellulose and hemicellulose substrates, confirming its distinct role for improving recalcitrant property of diverse lignocellulose substrates. It may also explain why the TrEXLX10 strain could secret diverse types of cellulases and xylanses (Table 3), mainly due to its highly binding activities with all major wall polymers.

3.6. *Mechanisms about the expansin dual enhancements for biomass-degradation enzymes secretion and biomass enzymatic saccharification*

Based on all findings achieved above, this study raised a hypothetic model to elucidate how the TrEXLX10-secreted enzymes and expansin could cause synergistic enhancements of lignocellulose hydrolyses in corn and *Miscanthus* straws (Fig. 6). While the engineered TrEXLX10 strain was initially incubated with alkali-pretreated *Miscanthus* straw, the secreted expansin could not only block lignin, but also interact with cellulose and hemicellulose to maintain their effectively accessible and digestible by *T. reesei* to release carbon source, which should consequently lead to an integrated enhancement for lignocellulose-degradation enzymes secretion, compared to the control strain (Rut-C30) examine in this work (Liu et al., 2021; Peng et al., 2022). Even though the crude enzymes secreted by the EXLX10 strain were only incubated with alkali-pretreated crop straws, their biomass enzymatic

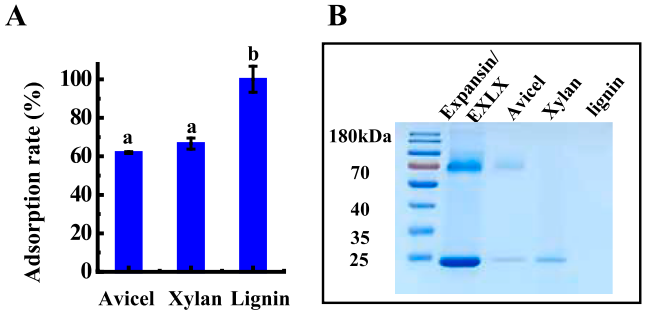

Fig. 5. Measurement of wall polymer adsorption with the EXLX protein purified from enzymes secreted from engineered strain (TrEXLX10). (A) Adsorption rate between expansin/EXLX protein and wall polymers; (B) SDS-gel profiling of EXLX protein after adsorption with standard lignocellulose substrates; Data as mean ± SD (n = 3); [a, b]As significant difference among three polymers samples by multiple *t*-test at $p < 0.05$.

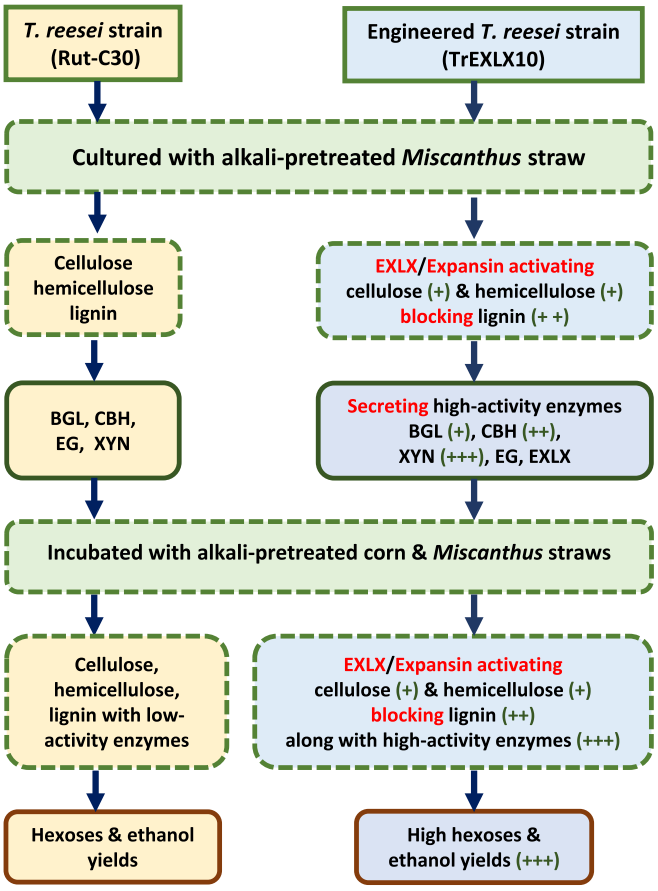

Fig. 6. A mechanism model about dual activation roles of the expansin/EXLX protein for the high-activity biomass-degradation enzymes secreted by engineered TrEXLX10 strain and high-yield sugars obtained from lignocellulose enzymatic hydrolyses by interaction with cellulose and hemicellulose and blocking lignin in crop straws.

saccharification could be much upgraded, mainly due to the synergistic roles of high-activity and well-stabilizing biomass-degradation enzymes and active expansin interactions with lignocellulose substrates. It also explains why either the commercial mixed-cellulases or the crude enzymes secreted Rut-C30 strain were of relatively low activities for less biomass enzymatic saccharification of crop straws examined, mainly subjective to the lack of either expansin interaction with lignocellulose substrates or expansin stabilization with cellulases and xylanases. Hence, this model has provided a powerful strategy for high-activity biomass-degradation enzymes and high-yield biomass saccharification by integrating engineered fungi strain with mild biomass process technology.

4. Conclusions

By selecting the desirable engineered TrEXLX10 strain that overexpressed bacterial *BsEXLE1* gene into *T. reesei* (Rut-C30), this study determined significantly raised activities of TrEXLX10-secreted enzymes such as β-glucosidases, cellobiohydrolases and xylanases. The EXLX10-secreted enzymes were supplied to release significantly higher hexoses yields than those of the Rut-C30 for synergistic enhancement of biomass saccharification in alkali-pretreated corn and *Miscanthus* straws. As EXLX10-secreted expansin was of exceptionally high binding and stabilizing activities with lignocelluloses or independent enhancement of cellulose digestion, this study has raised a mechanism model emphasizing expansin dual-activations for biomass-degradation enzymes secretion and biomass enzymatic digestibility in bioenergy crops.

CRediT authorship contribution statement

Chengbao Xu: Methodology, Formal analysis, Investigation, Writing – original draft. **Tao Xia:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Hao Peng:** Investigation, Validation, Writing – review & editing. **Peng Liu:** Investigation, Writing – review & editing. **Yihong Wang:** Investigation, Validation, Writing – review & editing. **Yanting Wang:** Supervision, Writing – review & editing. **Heng Kang:** Validation, Writing – review & editing. **Jingfeng Tang:** Supervision, Writing – review & editing. **Muhammad Nauman Aftab:** Supervision, Writing – review & editing. **Liangcai Peng:** Methodology, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.128844.

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