Full Paper

Effect of introducing disulfide bridges in C-terminal structure on the thermostability of xylanase XynZF-2 from *Aspergillus niger*

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In this study, a mutant xylanase of high thermostability was obtained by site-directed mutagenesis. The homologous 3D structure of xylanase was successfully modeled and the mutation sites were predicted using bioinformatics software. Two amino acids of XynZF-2 were respectively substituted by cysteines (T205C and A52C) and a disulfide bridge was introduced into the C-terminal of XynZF-2. The mutant gene xynZFTA was cloned into pPIC9K and expressed in *P. pastoris*. The optimum temperature of the variant XynZFTA was improved from 45°C to 60°C, and XynZFTA retained greater than 90.0% activity (XynZF-2 retained only 50.0% activity) after treatment at 50°C for 5 min. The optimum pH of mutant xylanase was similar to XynZF-2 (pH = 5.0). The pH stability span (5.0~7.0) of the mutant xylanase was increased to 3.0~9.0. Overall, the results implied that the introduction of a disulfide bridge in the C-terminal structure improved the thermostability and pH stability of XynZF-2.

Key Words: *Aspergillus niger*; disulfide bridges; site-directed mutagenesis; thermostability; xylanase

Introduction

Xylan is an important component of hemicellulose (Ramanjaneyulu et al., 2017), which is composed of galactomannan, xylose, konjac glucomannan, and arabia galactomannan and it is rich in natural cell wall polysaccharides (Zeng et al., 2016). Its skeleton is composed of a plurality of D-xylose residues which are con-

nected by β -1,4 glycosidic bonds (Walia et al., 2017). The efficient enzymatic degradation of xylan requires the synergistic action of several key enzymes, such as xylanase (EC 3.2.1.8) and xylosidase (EC 3.2.1.37).

Xylanase (EC 3.2.1.8) are glycoside hydrolases that catalyze the hydrolysis of β -1,4-xylosidic linkages of xylan backbone. Xylanases can be widely used for various applications, such as pulp bleaching (Kumar et al., 2017; Uday et al., 2017), the preparation processing of beverages (Kumar et al., 2014; Wang et al., 2014), feed additives, and can bring huge economic and social benefits (Bu et al., 2018; Kumar et al., 2018; Qian et al., 2015).

However, for practical industrial applications, xylanases need to have enhanced thermostability (Shah et al., 2017), therefore molecular modification techniques have been used to improve the thermal stability of xylanase (Kumar et al., 2016), and three examples are shown as follows. Aromatic residues were introduced into the N-terminus of the enzyme by Bai et al. (2014) and the mutant optimum temperature was increased by 5°C. A double mutant, S80T-S149T, was constructed and expressed in *Escherichia coli* by Zouari et al. (2015), and the thermostability of *Trichoderma reesei* xylanase was improved (Zouari). Six mutants of *G. thermodenitrificans* C5 were constructed through site-directed mutagenesis by Irfan et al. (2018), and the half-life of xylanases was increased 13 times at 60°C.

It has been confirmed that the thermostability of xylanase is associated with hydrophobic (Liang et al., 2018; Ni et al., 2018; Shukla et al., 2017; Wang et al., 2018), hydrophilic interaction, surface charge (Deller et al., 2018; Scsuková et al., 2000; Vidya et al., 2014), hydrogen bonds, ionic bridges, and disulfide bridges (Chang et al., 2017; Kumar et al., 2000). The introduction of disulfide bridges in the N-terminal have shown to be an appropriate method to improve the thermostability of

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Table 1. The names and sequences of the mutant primers.

Primer name	Prime sequence (5'-3')
205-52F	CCG <u>GAATTC</u> GTTCCCCACGACTCTGTGCG <i>EcoR'</i> site
A52C-F	GCTGGTTGTTACACTATTGAGTG
A52C-R	AGTGTAACAACCAAGCATCTCCG
205-52R	ATAAGAAT <u>GCGGCCGC</u> TTACTGAACACAGATGGA <i>NotI</i> site

The mutant codons are underlined, and the restriction enzyme sites are boxed.

xylanase (Nakamura et al., 2018; Zhou et al., 2016), while few experiments involved in the addition of disulfide bridges in the C-terminal have been previously reported. In order to investigate the effect of the introduction of disulfide bridge in the C-terminal on xylanase thermostability, a disulfide bridge was introduced in the C-terminal domains (T205C and A52C) by site-directed mutagenesis, and, subsequently the mutant gene named *xynZFTA* was transformed into *Pichia pastoris* GS115. An enzymatic characterization of this enzyme was performed, and the effects of temperature and pH on enzyme activity were investigated.

Materials and Methods

Strains and plasmids. Expression vector pPIC9K and *Pichia pastoris* strain GS115 were purchased from the Invitrogen company (USA). The recombinant plasmid pET-28a-*xynZF-2* was constructed and preserved in our laboratory.

Reagents. Ampicillin (Amp), a DNA Plasmid Extraction Kit, and a DNA fragment Recovery Kit were purchased from the Sangon company (Shanghai, China); and a restriction endonuclease, a DL1000 DNA marker, a 1 kb DNA marker, and a T4 DNA ligase were purchased from TaKaRa (Dalian, China).

Medium. A YPD medium was used for the culture *P. pastoris* GS115, and a MD medium was used for screening the positive recombinant GS115. In order to obtain high density mycelium, a BMGY medium was used for the culture the recombinant GS115 before expression, and BMMY medium was used during target protein expression. A detailed formulation was prepared according to the *Pichia* expression manuals (Invitrogen).

Homologous sequence alignment and homology modeling. Phyre2 was used to simulate the homology of the three-dimensional structure of XynZF and XynZFTA (<http://www.sbg.bio.ic.ac.uk/phyre2/html/help.cgi?id=help/fag>). DiANNA was used to predict disulfide bridges and the mutant sites (Wang, 2015). The mutation energy was analyzed by Sketch Marvin (<http://www.chemaxon.com/marvin/sketch/index.jsp>).

Primers for PCR. The primers were designed based on the characteristics of the mutant amino acids and the characteristics of the polyclonal region of pPIC9K (Table 1).

Construction of recombinant expression vector. Using pET-28a-*xynZF-2* as the template, overlap extension PCR amplification was performed with primers 205-52F, A52C-

R, A52C-F and 205-52R under the following conditions: a denaturation at 94°C for 2 min; 35 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 1 min; and an elongation at 72°C for 10 min. The mutant gene *xynZFTA* was confirmed by DNA sequencing before being inserted into the *EcoRI/NotI* site pPIC9K vector. Finally the clones containing the recombinant plasmid were screened out, and the recombinant plasmid pPIC9K-ZFTA was constructed. Detailed manipulation was essentially as described by Sambrook (2012).

Transformation of the expression vector into *P. pastoris* GS115. The inducible expression plasmid (pPIC9K-ZFTA) was linearized with *SalI* and transformed into *P. pastoris* GS115 by electroporation according to the *Pichia* expression manuals. The parent pPIC9K without insert, linearized with *SalI*, was also transformed as a control. The transformants harboring the AOX1 factor promoter-controlled xylanase expression cassette were selected on the MD plates.

Expression of recombinant xylanase. Single colonies of the transformants were initially inoculated into a flask containing 25 ml of BMGY medium. After 24 h at 30°C and 220 r/min, the cultures were centrifuged at 3000 r/min for 5 min and resuspended by 30 ml of BMMY medium to induce expression. The cells were allowed to grow for 72 h at 30°C, and methanol was added every 24 h to a final concentration of 1.0% (v/v) for inducing the expression of the target protein. Culture aliquots were collected and cells were removed by centrifugation at 5000 r/min for 3 min. The supernatant fluids were assayed for the xylanase activity.

SDS-PAGE protein electrophoresis detection. The XynZFTA secreted from GS115-pPIC9K-ZFTA was detected by SDS-PAGE with a 5% concentration glue and a 10% separating gel, and the method was taken from the literature (Wang, 2015).

Purification of expressed xylanase. The liquid cultures were precipitated with (NH₄)₂SO₄ (60% saturation), and left overnight. The resulting precipitate was harvested by centrifugation at 10,000 r/min for 10 min. The precipitated proteins were then resuspended in Na₂HPO₄-citric acid buffer (pH 6.0). The fractions containing xylanase were applied onto a Sephadex G-75 column (Amersham Pharmacia Biotech, Uppsala, Sweden; $\phi 1.6 \times 80$ cm) and eluted with the same buffer. The fractions containing the enzyme were concentrated in a dialysis bag surrounded by a thick layer of dry polyethylene glycol (average molecular weight 20,000). The concentrate was dialyzed for

Fig. 1. Homology modeling of XynZF-2 and XynZFTA.

A. Three-dimensional structure of XynZF-2 (Glu103 and Glu194 were the catalytic active centers). B. Three-dimensional structure of XynZFTA. C. Three-dimensional structure of original amino acids. D. Three-dimensional structure of disulfide-bridge.

Fig. 2. Construction of pPIC9K-ZFTA.

A. PCR amplification of the mutant gene *xynZF-T205C-A52C* with resistant sites. Lane 1 1000 bp DNA marker; Lane 2 *xynZFTA* with *EcoRI* and *NotI* site. B. Lane 1 The digestion products of *xynZFTA* with *EcoRI* and *NotI* site; Lane 2 1000 bp DNA marker; Lane 3 1 kb DNA Marker; Lane 4 The digestion products of pPIC9K with *EcoRI* and *NotI* site.

2 d against several changes of distilled water and lyophilized. All purification procedures were carried out at 4°C unless stated otherwise.

Properties of recombinant xylanase. Enzymatic properties of recombinant (XynZF-2) and mutant enzyme (XynZF-ZFTA) were analyzed by a standard procedure using 3,5-dinitrosalicylic acid (DNS), as described by Zhou et al. (2016). One unit of enzyme activity was defined as the quantity of enzyme required to liberate 1 mol of xylose equivalent per minute at 40°C, and specific activity was defined as units per mg protein. The results were the means of duplicate determinations of threefold independent measurements.

Fig. 3. SDS-PAGE analysis of XynZFTA.

Lane 1 Protein bands expressed by *P. pastoris* GSP. Lane 2 Protein molecular Marker. Lane 3 Protein bands expressed by *P. pastoris* XynZFTA.

Results

Homologous alignment and structural assay

Homology modeling of the three-dimensional structures was obtained through Phyre2 (Fig. 1). There are α -helix and β -folding structures of the recombinant protein molecule in Fig. 1 and they show the classic shape of a right hand half-grip which are possessed by a typical structure of the GH11 family. The active centers (Glu103 and Glu194) are indicated in Fig. 1A, and a disulfide bridge is shown in Fig. 1B. Two images of the partial three-dimensional structure of original amino acids and the disulfide bridge which was formed via mutation are presented in Figs. 1C and 1D, respectively. The mutation energy of XynZFTA was analyzed through Marvin Sketch, and found to be -0.9814 . A protein was judged to be more stable when the mutation energy was below -0.5 ; therefore, the enzyme introducing two mutant sites with A52C and T205C was predicted to possess more stable properties according to the molecular energy analysis presented above.

Construction of the recombinant expression vector

The gene XynZFTA was amplified and purified by agarose gel electrophoresis. The mutant gene and pPIC9K were digested by *EcoRI* and *NotI* and purified by agarose gel electrophoresis (Fig. 2). The mutant gene was connected with pPIC9K and the recombinant expression vector was successfully constructed.

Screening of *P. pastoris* transformants

The expression vector pPIC9K-xynZFTA was transformed into *P. pastoris* GS115. The colonies that showed resistance to over 4.0 mg mL^{-1} of G418 were selected for induction. The recombinant *P. pastoris* GS115 integrated with pPIC9K, named as *P. pastoris* GSP, was used as a negative control. By determining the enzyme activity, a xylanase yielding strain, named *P. pastoris* XynZFTA, was finally selected and used for further studies. No xylanase

Fig. 4. Effect of temperature on the activity of XynZFTA.

Fig. 6. Effect of pH on the activity of XynZFTA.

Fig. 5. Effect of temperature on the stability of XynZFTA.

Fig. 7. Effect of pH on the stability of XynZFTA.

activity was detected in the cultured supernatant of *P. pastoris* GSP under the same expression conditions.

Expression of recombinant xylanase

The activity of XynZFTA was up to 1169 U/mL, which was essentially consistent with that of XynZF-2 expressed in *P. pastoris* (Wang et al., 2015). Analysis of XynZFTA by SDS-PAGE is shown in Fig. 3. There is an evident band on the gel. The relative molecular mass was approximately 24.10 kDa which is consistent with XynZF-2 (Wang et al., 2015).

Analysis and comparison of enzymatic properties

Comparison of optimum temperature and thermostability.

The optimum temperature of the mutant XynZFTA, measured at pH 4.6 for 15 min, was 65°C, which was 20°C higher than that of the xylanase XynZF-2 (Fig. 4). To estimate the thermostability, the residual activities of the recombinant xylanases were measured after incubation at different temperatures for 60 min. The residual activities of XynZFTA were more than 80% after 1 h incubation at

50°C, whereas the activities of XynZF-2 almost vanished after the same treatment (Fig. 5). XynZFTA showed more than 80% activity after 1 h incubation at 50°C and 55°C; however, XynZF-2 was almost inactivated after incubation at 45°C and 50°C for 1 h. These results verified that the introduction of a disulfide bridge at C-terminal domains had a positive effect on the thermostability of XynZF-2.

Comparison of the optimum pH and pH stability.

The effects of pH on the activity and stability of the recombinant xylanases are presented in Figs. 6 and 7. The pH for higher catalytic activity were both at pH 5.0. It indicated the mutation with A52C and T205C had no impact on the optimum pH. In order to estimate the acid-base tolerance, residual activities of XynZF-2 and XynZFTA were measured after incubation in a different pH buffer at 40°C for 1 h. The mutant xylanase XynZFTA was relatively stable in the pH range of 3.0 to 9.0 which was broader than that of recombinant XynZF-2.

Effects of metal ions and other compounds. The activity of the purified recombinant enzyme in the presence of dif-

Table 2. Effects of metal ions and other compounds on mutant xylanase activity.

Metal ions	Relative active (%) [*]
Zn ²⁺	110.35±3.19
Mn ²⁺	66.94±2.57
Pb ²⁺	63.33±2.34
Ca ²⁺	119.57±1.82
Fe ²⁺	105.23±3.99
Fe ³⁺	50.51±3.42
Mg ²⁺	76.93±1.68
Li ⁺	85.38±2.74
Cu ²⁺	51.21±3.56
Gu ⁺	82.76±2.17
EDTA	113.63±2.39
Control	100±1.55

^{*}Mean of three independent readings, SD within 10%.

ferent metal ions or chemical reagents are indicated in (Table 2). The presence of Zn²⁺, Ca²⁺ and EDTA accelerated the activity of the mutant enzyme XynZFTA. The enzyme was inhibited by Fe³⁺, Cu²⁺, Mn²⁺, Pb²⁺ and Gu⁺. The addition of other reagents, such as Fe²⁺ had little effect on the activity. In addition, the effects of metal ions and EDTA on XynZFTA were roughly consistent with the effects on XynZF-2 (Wang et al., 2015).

Discussion

As an important stable structure in proteins (Zheng et al., 2016), disulfide bonds serve the function of maintaining protein stability in the form which can keep the bridge formation in the peptide residues as a complex biological reactions (Chen et al., 2017; Kisiela et al., 2018; Li et al., 2017; Nakamura et al., 2018; Xie et al., 2018). There exists a very stable core region of xylanase, so we can start this work in the C-terminal or the N-terminal which is far away from the core region (Letian et al., 2014). Studies concerning the improvement of the thermostability of xylanase have been carried out for many years (Chen et al., 2010), and most modifications are related to the N-terminal region, including the insertion of a homologous sequence of heat resistant protein, or the construction of mutants. However, to the best of our knowledge, improving the thermostability of xylanase by introducing disulfide bridges of the C-terminal region have never been reported. Therefore, the modification in this work was started from the α helix area near the C-terminal, a disulfide bridge was successfully constructed in order to achieve the purpose of improving the xylanase thermal stability.

The introduction of a disulfide bridge may decrease the flexibility of the molecule, and this can explain the huge increase in K_m of XynZFTA compared with XynZF-2. According to the data we obtained from the experiment, it is clear that the introduction of disulfide bridges in the C-terminal brings a 15°C enhancement of the optimal temperature compared with original enzyme, and it was speculated that the conformation of the enzyme was changed, and furthermore that the intramolecular force was enhanced.

Molecular dynamics simulations also proved lower amounts of root and potential energy for the mutant

XynZFTA. Based on these results, it implied that the mutation energy might affect the thermostability of the enzyme and Pons et al. (1995), Jean-Luc, and Ehsan have demonstrated this view by Molecular dynamics simulation. Capability of high temperature tolerance of XynZF-2 has been advanced to a certain extent with the decrease of mutation energy value. Disulfide bridges were formatted inside the molecule, and the “hinge” region was made more compact in thermophiles by the addition of a disulfide bridge which was described by Kumar et al. (2000). This might be the reason for leading to a larger range for xylanase to showing pH stability.

Redox-inert metal ions have been reported to stabilize negative charges and to activate substrates by virtue of their Lewis acid properties used in enzymes (Claudia et al., 2008), whereas redox-active metal ions can be used both as Lewis acids and as redox centres. According to that, the acceleration of enzyme activity by the addition of Zn²⁺, Ca²⁺ and Fe²⁺ could be explained. The activity of the mutant enzyme inhibited by heavy metal ions, such as Pb²⁺ and Cu²⁺, might be due to the displacement of metal ions in the active centre of the enzyme.

The thermostability was enhanced via the introduction of disulfide bridges in the C-terminal of XynZF-2. The mutant protein can also maintain a high enzyme activity under acidic conditions. This provides an important theoretical basis for the further study of xylanase.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Research involving Human Participants and/or Animals

This research does not involve any studies with human participants or animals.

Informed Consent

Informed consent was obtained from all individual participants contributions to the study.

References

- Bai, W., Yang, L., and Ma, Y. J. (2014) Improving thermal stability of xylanase by introducing aromatic residues at the N-terminus. *Chin. J. Biotechnol.*, **30**, 1217–1224.
- Bu, Y., Cui, Y., Ying, P., Hu, M., Tian, Y. et al. (2018) Engineering improved thermostability of the GH11 xylanase from *neocallimastix*

- patriciarum*, via computational library design. *Appl. Microbiol. Biot.*, **102**, 1–11.
- Chang, X., Xu, B., Bai, Y., Luo, H., Ma, R. et al. (2017) Role of N-linked glycosylation in the enzymatic properties of a thermophilic GH 10 xylanase from *Aspergillus Fumigatus* expressed in *pichia pastoris*. *PLoS One*, **12**, e0171111.
- Chen, X. Z., Xu, S. Q., Zhu, M. S., Cui, L. S., Zhu, H. et al. (2010) Site-directed mutagenesis of an *Aspergillus niger* xylanase B and its expression, purification and enzymatic characterization in *Pichia pastoris*. *Process Biochem.*, **45**, 75–80.
- Chen, Y., Li, T., Li, J., Cheng, S., Wang, J. et al. (2017) Stabilization of peptides against proteolysis through disulfide-bridge conjugation with synthetic aromatics. *Org. Biomol. Chem.*, **15**, 1921–1929.
- Claudia, A., Ivano, B., Gabriele, C., Gemma, L. H., and Janet, M. T. (2008) Metal ions in biological catalysis: from enzyme databases to general principles. *J. Biol. Inorg. Chem.*, **13**, 1205–1218.
- Deller, R. C., Carter, B. M., Zampetakis, I., Scarpa, F., and Perriman, A. W. J. (2018) The effect of surface charge on the thermal stability and ice recrystallization inhibition activity of antifreeze protein iii (afp iii). *Biochem. Biophys. Res. Commun.*, **495**, 1055–1060.
- Irfan, M., Gonzalez, C. F., Raza, S., Rafiq, M., Hasan, F. et al. (2018) Improvement in thermostability of xylanase from *Geobacillus thermodenitrificans* C5 by site directed mutagenesis. *Enzyme Microb. Technol.*, **111**, 38–47.
- Kisiela, M., Faust, A., Ebert, B., Maser, E., and Scheidig, A. J. J. (2018) Crystal structure and catalytic characterization of the dehydrogenase/reductase SDR family member 4 (DHRS4) from *Caenorhabditis elegans*. *FEBS J.*, **285**, 275–293.
- Kumar, L., Nagar, S., Mittal, A., Garg, N., and Gupta, V. K. (2014) Immobilization of xylanase purified from *Bacillus pumilus* vlk-1 and its application in enrichment of orange and grape juices. *J. Food. Sci. Technol.*, **51**, 1737–1749.
- Kumar, P. R., Eswaramoorthy, S., Vithayathil, P. J., and Viswamitra, M. A. (2000) The tertiary structure at 1.59 Å resolution and the proposed amino acid sequence of a family-11 xylanase from the thermophilic fungus *paecilomyces varioti*, bainer 1. *J. Mol. Biol.*, **3**, 581–593.
- Kumar, P. S., Yaashikaa, P. R., and Saravanan, A. J. (2018) Isolation, characterization and purification of xylanase producing bacteria from sea sediment. *Biotacal Biotransfor*, **13**, 299–303.
- Kumar, S., Tsai, C. J., and Nussinov, R. J. (2000) Factors enhancing protein thermostability. *Protein Eng.*, **13**, 179–191.
- Kumar, S., Haq, I., Prakash, J., Singh, S. K., Mishra, S. et al. (2017) Purification, characterization and thermostability improvement of xylanase from *Bacillus amyloliquefaciens* and its application in pre-bleaching of kraft pulp. *Biotechnology*, **7**, 19–30.
- Kumar, V., Marín-Navarro, J., and Shukla, P. (2016) Thermostable microbial xylanases for pulp and paper industries: trends, applications and further perspectives. *World J. Microbiol. Biotechnol.*, **32**, 1–10.
- Letian, S., Claire, D., Béatrice, S., Isabelle, A., Elena, E. et al. (2014) Impact of an N-terminal extension on the stability and activity of the GH11 xylanase from *Thermobacillus xylanilyticus*. *J. Biotechnol.*, **174**, 64–72.
- Li, G., Fang, X., Su, F., Chen, Y., Xu, L. et al. (2017) Enhancing the thermostability of *Rhizomucor miehei* lipase with a limited screening library by rational design point mutations and disulfide bonds. *Appl. Environ. Microbiol.*, **84**, 1–16.
- Liang, Y., Yu, K., Zheng, Q., Xie, J., and Wang, T. J. (2018) Thermal treatment to improve the hydrophobicity of ground cacao 3, particles modified with sodium stearate. *Appl. Surf. Sci.*, **436**, 832–838.
- Nakamura, H., Oda-Ueda, N., Ueda, T., and Ohkuri, T. (2018) A novel engineered interchain disulfide bond in the constant region enhances the thermostability of adalimumab Fab. *Biochem. Biophys. Res. Commun.*, **495**, 7–11.
- Ni, S., Wang, C., Bian, H., Yu, Z., Jiao, L. et al. (2018) Enhancing physical performance and hydrophobicity of paper-based cellulosic material via impregnation with starch and pei-kh560. *Cellulose*, **25**, 1365–1375.
- Pons, J., Planas, A., and Querol, E. (1995) Contribution of a disulfide bridge to the stability of 1,3-1,4-beta-D-glucan 4-glucanohydrolase from *Bacillus licheniformis*. *Protein Eng.*, **9**, 939–945.
- Qian, C., Liu, N., Yan, X., Wang, Q., Zhou, Z. et al. (2015) Engineering a high-performance, metagenomic-derived novel xylanase with improved soluble protein yield and thermostability. *Enzyme Microb. Technol.*, **70**, 35–41.
- Ramanjaneyulu, G., Ramya, A., Kumari, B. S. S., Kumar, K. D., and Reddy, B. R. J. (2017) Xylanase-producing microflora in eastern ghats of andhra pradesh, India. *J. Forestry Res.*, **28**, 291–298.
- Sambrook, J. (2012) Molecular cloning: a laboratory manual [Volume 3][M]. Cold Spring Harbor Laboratory Press, pp. 1109–1113.
- Scsuková, S., Ježová, M., Vranová, J., and Kolena, J. (2000) Involvement of membrane surface charge in thermal stability of the rat ovarian lh/hcg receptor. *BBA-Biomembranes*, **1468**, 15–19.
- Shah, V., Pierre, B., Kirtadze, T., Shin, S., and Kim, J. R. J. (2017) Stabilization of *Bacillus circulans* xylanase by combinatorial insertional fusion to a thermophilic host protein. *Protein Eng. Des. Sel.*, **30**, 281–290.
- Shukla, N., Pomarico, E., Hecht, C., Taylor, E. A., Chergui, M. et al. (2017) Hydrophobic interactions of sucralose with protein structures. *Arch. Biochem. Biophys.*, **639**, 38–43.
- Uday, U. S. P., Bandyopadhyay, T. K., Goswami, S., and Bhunia, B. J. (2017) Optimization of physical and morphological regime for improved cellulase free xylanase production by fed batch fermentation using *Aspergillus niger* (kp874102.1) and its application in bio-bleaching. *Bioeng. Bugs.*, **8**, 137–146.
- Vidya, J., Ushasree, M. V., and Pandey, A. J. (2014) Effect of surface charge alteration on stability of L-asparaginase II from *Escherichia* sp. *Enzyme Microb. Technol.*, **56**, 15–19.
- Walia, A., Guleria, S., Mehta, P., Chauhan, A., and Parkash, J. (2017) Microbial xylanases and their industrial application in pulp and paper biobleaching: a review. *Biotechnology*, **7**, 11–21.
- Wang, D. D., Zhou, C. Y., Li, T. B., Zhang, Z. Q., Wang, Y. J. et al. (2015) Efficient expression of the xylanase gene xynZF-2 from *Aspergillus niger* XZ-3S in *Pichia pastoris*. *Food Industry SCI*, **7**, 200–203.
- Wang, J., Tan, Z., Wu, M., Li, J., and Wu, J. (2014) Improving the thermostability of a mesophilic family 10 xylanase, auxyn10a, from *Aspergillus usamii* by in silico design. *J. Ind. Microbiol. Biot.*, **41**, 1217–1225.
- Wang, Q., Xiao, S., Shi, S. Q., and Cai, L. J. (2018) Effect of light-delignification on mechanical, hydrophobic, and thermal properties of high-strength molded fiber materials. *SCI Rep-UK*, **8**, 955–965.
- Wang, Z. H. (2015) Effects of Site-directed mutagenesis of Cry11Ba from *Bacillus thuringiensis* on the structure and function. Dissertation, Hunan Normal University.
- Xie, D. F., Fang, H., Mei, J. Q., Gong, J. Y., Wang, H. P. et al. (2018) Improving thermostability of (r)-selective amine transaminase from *Aspergillus terreus* through introduction of disulfide bonds. *Biotechnol. Appl. Biochem.*, **3**, 65, 255–262.
- Zeng, Y., Yarbrough, J. M., Mittal, A., Tucker, M. P., Vinzant, T. B. et al. (2016) In situ label-free imaging of hemicellulose in plant cell walls using stimulated raman scattering microscopy. *Biotechnol. Biofuels*, **9**, 256–273.
- Zheng, F., Huang, J., Liu, X., Hu, H., Long, L. et al. (2016) N- and C-terminal truncations of a GH10 xylanase significantly increase its activity and thermostability but decrease its SDS resistance. *Appl. Microbiol. Biotechnol.*, **100**, 3555–3565.
- Zhou, C. Y., Li, T. B., Wang, Y. T., Zhu, X. S., and Kang, J. (2016) Exploration of a N-terminal disulfide bridge to improve the thermostability of a GH11 xylanase from *Aspergillus niger*. *J. Gen. Appl. Microbiol.*, **62**, 83–89.
- Zouari, A. D., Hmida, S. A., Ben, H. H., Ben, M. S., Mezghani, M. et al. (2015) Improvement of *Trichoderma reesei* xylanase II thermal stability by serine to threonine surface mutations. *Int. J. Biol. Macromol.*, **72**, 163–170.