Original Article

XYLANASE GENE MUTATION BY ERROR-PRONE PCR AND EXPRESSION IN *Pichia pastoris*

MUTAÇÃO DO GENE DA XILANASE POR PCR PROPENSA A ERROS E EXPRESSÃO EM Pichia pastoris

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ABSTRACT: Xylanase can hydrolyze xylan for reducing its anti-nutritional impact and improving nutrient availability, so obtaining suitable xylanase to degrade xylan is essential. Error-prone PCR and gene transformation were used in this study to obtain the ideal xylanase for degrading xylan effectively. The result showed that one mutant xylanase gene with high xylanase expression was obtained. After the mutant xylanase gene was connected with pGAPZαA and transformed into *Pichia pastoris* (*P. pastoris*), the recombinant *P. pastoris* with mutant gene was found to produce higher xylanase activity (0.1480 U/mL) than that with the native xylanase gene (0.1360 U/mL) after 12 h incubation ($p<0.05$). The optimal temperature and pH of xylanase expressed by native and mutant genes were the same, i.e. 40°C and 5.50 ($p<0.05$). In addition, adding 0.2% Tween 80 during recombinant *P. pastoris* incubation could significantly increase xylanase yield by about 30-35% ($p<0.05$). The mutant xylanase could significantly increase xylose yield from wheat meal more than the native xylanase ($p<0.05$).

KEYWORDS: Error-prone PCR. Xylanase. Gene mutation. Gene expression. *P. pastoris*

INTRODUCTION

Xylan is a major component of hemicellulose, which widely presents in the plant cell wall and is also a big renewable resource in nature (COLLINS et al., 2005). A variety of feed grains such as wheat, barley and oats contain rich xylans (SAINI and HENRY, 1989; CLEEMPUT et al., 1993), which affect the utilization and absorption of nutrients. Xylanase is able to hydrolyze xylan, thus obtaining the optimal xylanase is urgent for xylan degradation. Xylanase has been used in pulp and paper industry, animal feeding, food and other fields (BEG et al., 2001; LI et al., 2005; XIONG et al., 2005). Based on the primary sequence homology and hydrophobic cluster analysis, xylanases can be divided into two families, i.e. family F/10 and family G/11 (HENRISSAT, 1991).

The xylanase secreted by cereal belongs to family F/10, while xylanase produced by microorganisms belongs to both families (SIMPSON et al., 2003). In general, the native xylanase activity from microbes is low; therefore, using genetic engineering techniques to obtain ideal xylanase resources has become hot research point.

Error-prone PCR technique was reported in 1985, it has been accordingly improved (CADWELL and JOYCE, 1992). Error-prone PCR easily causes mutation of gene with less than 800 bp, which can change the natural parameters of the native enzymes. It was reported that two mutant protease genes of *Bacillus subtilis* were obtained by error-prone PCR, their catalytic efficiency was respectively 256 and 131 folds higher than the native enzyme (CHEN and ARNOLD, 1991 and 1993). SHIM et al. (2004) cloned a cyclodextrin glucose transferase gene from *Bacillus* I-5, and got a mutant gene containing three-point mutations (M234T, F259I and V591A) by error-prone PCR; as a result its cyclic activity was dramatically reduced by 10 folds, and the hydrolytic activity was enhanced by 15 folds (SHIM et al., 2004). Error-prone PCR and point-mutation method were used to obtain high acid resistance of mutant (T353I and H400R) of alpha amylase from *Bacillus licheniformis*, it was found that the mutants had stronger resistance than the native amylase under low pH value (LIU et al., 2012). Error-prone PCR has also been used to increase xylanase thermostability and alkaline stability (ZHANG et al., 2010; SHIBUYA et al., 2005); however, the study on xylanase activity and its hydrolysis ability caused by error-prone PCR has few reports.

The present study aimed to obtain the ideal xylanase for degrading xylan effectively. So, in order to obtain the ideal xylanase for high xylan degradation, error-prone PCR was used to reduce anti-nutrition impacts of xylan.

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MATERIAL AND METHODS

Materials, microbes and incubating media

The pMD19-T plasmid, *E. coli* DH5α, *Xba I*, *EcoR I*, *Bln I*, Premix Taq Ver.2.0 plus dye, DNA Marker, dNTP, T4 DNA ligase, DNA gel recovery kit were purchased from Takara (Dalian, China). *Pichia pastoris* (*P. pastoris*) X33, pGAPZαA and zeocin were purchased from Invitrogen, USA. Oat spelt xylan was purchased from Sigma. Instant error-prone PCR kit was purchased from Beijing Enzyme Gene Cloning Technology Co. Ltd., China. *Aspergillus niger* for native xylanase gene isolation was preserved in our laboratory.

E. coli was grown in LB medium containing 1% yeast extract, 2% tryptone and 1% NaCl or low-salt LB medium (0.5% NaC1) by incubating at 37°C and 120 rounds per min (RPM) for 24 h. *Aspergillus niger* was cultivated in a medium containing 0.6% soluble starch, 2.0% glucose, 0.2% yeast extract, 0.5% peptone, 0.2% KH_2PO_4 and 0.03% $MgSO_4$ by incubating at 30°C and 120 RPM for 72 h. *Pichia pastoris* was cultivated in YPD medium containing 1% yeast extract, 2% tryptone and 1% glucose by incubating at 30°C and 120 RPM for 24 h. *Pichia pastoris* with xylanase expression vector for xylanase yield were cultivated in the modified YPD medium with 1% yeast extract, 2% tryptone and 2% wheat bran. Xylanase from *Aspergillus niger* was obtained by solid-state fermentation at 30°C for 72 h, and then xylanase was isolated with 0.9% normal saline.

Xylanase gene isolation and vector construction

The cells of *Asperigillus niger* were collected by centrifuge, frozen in liquid nitrogen, and then ground in mortar. The total RNA was extracted by using RNA Extract Kit (Takara, Dalian, China). Double strand cDNA of xylanase gene were synthesized by RT-PCR. The First Strand cDNA Synthesis Kit (TransGen, Beijing, China) was used in reverse transcription. This transcription reaction mixture contained 2.0 μL total RNA, 10.0 μL 2×ES reaction mix, 1.0 μL Oligo$(dT)_{18}$, 1.0 μL RT enzyme mix and 6.0 μL RNase-free water, which was carried out at 42°C for 30 min and 99°C for 5 min.

According to the previous report (KORONA et al., 2006), the primers were designed by DNAMAN software and synthesized by Shanghai Sangon Biological Engineering Company, China. The primer sequences were as follows:

Up-stream primer：CG GAATTC ATGCTC ACCAAGAACCTTCT
EcoR I

Down-stream primer：GC TCTAGA TCAC TGAACAGTGATGGA
Xba I

PCR was carried out in a total volume of 50 μL mixture containing 2.0 μL reverse transcription product as template, 25 μL PCR SuperMix, 1.0 μL up-stream primer, 1.0 μL down-stream primer, 21 μL ddH_2O. The PCR process was initiated at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 100 s, and a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis with 1.0% agarose gel and purified by DNA gel recovery Kit (TransGen, Beijing, China). The purified PCR product was ligated with pMD19-T vector and transformed into *E. coli* competent cells by heat shock method. The transformants were selected on LB plates containing IPTG (24 mg/mL) and X-gal (20 mg/mL). The positive colonies were obtained after 48 h plate culture, and incubated in LB medium at 37°C and 120 RPM for 24 h. The pMD19-T with xylanase gene was isolated with kits.

Error-prone PCR of xylanase gene

The reaction mixture (30 μL) for error-prone PCR contained 1 μL each primer, 3 μL dedicated dNTP, 3 μL mix of error-prone PCR, 1 μL plasmid with xylanase gene (10 ng/μL), 3 μL $MnCl_2$ (5 mM), 1 μL Taq DNA Polymerase (5 U/μL) and 17 μL ddH_2O. The error-prone PCR conditions were 94°C for 3 min, and then 30 cycles of 94°C for 1 min, 60°C for 40 s, 72°C for 1 min, and finally 72°C for 10 min. The PCR product was purified as above.

Xylanase gene expression in *E. coli* and *P. pastoris*

The purified mutant xylanase gene was ligated with pMD19-T vector and transformed into *E. coli*. The positive colonies were selected and the vectors were purified as above. DNA sequence of the mutant xylanase was determined in Sangon (Shanghai, China) and analyzed with DNAMAN (Lynnon Biosoft, USA). The vectors with mutant xylanase genes were digested by double endonucleases (*EcoR I* and *Xba I*), and the genes were isolated with 1% agarose gel, purified with kit. After the expression vector of pGAPZαA was digested with *EcoR I* and *Xba I*, it was then connected with the mutant xylanase gene by ligase. The vector was transformed into *E. coli* 5Hα, the transformants were selected on low salt LB plates containing zeocin (100 μg/mL). The vector was isolated and purified with kit, linearized with *Bln I*, and then transformed into competent *P. pastoris* with electroporation.

The competent cells of *P. pastoris* were prepared as follows: One fresh colony was selected and incubated overnight in 5 mL of YPD medium in a 50 mL flask with 120 RPM at 30°C, and then 0.1 mL was taken and put into 100 mL fresh YPD medium in a 500 mL flask for incubating until an optical density of 1.0 at 600 nm. The cells were centrifuged at 1500 RPM and 4°C for 5 min, washed twice with ice-cold sterile water and twice with ice-cold 1 M sorbitol. Finally, the cells were resuspended in 1 mL of ice-cold 1 M sorbitol. Eighty μL cell suspensions were mixed with 5-10 μg linearized pGAPZaA-xylanase gene and transferred into an ice-cold 0.2 cm electroporation cuvette. Parameters used for electroporation were 1.5 kV/cm, 25 μF and 400 Ω for inserting pGAPZaA-xylanase gene into *P. pastoris*. The transformed *P. pastoris* was sprayed on YPD plates with 0.5% xylan (w/v) and zeocin (100 μg/mL), incubated at 30°C for 48 h. The positive colonies were selected and incubated in another YPD plate with marking, and the original positive colonies were dyed with Congo red for 1 h, washed with normal saline for 30 min. The colonies with the big transparent zones were selected for the further analyses. In addition, the pGAPZαA with or without the native xylanase gene was also transformed into *P. pastoris* as control.

Xylanase activity determination of the transformed *P. pastoris*

The incubation of the transformed *P. pastoris* lasted for 36 h, during which 10 ml incubation was sampled at different culture time. The supernatant was prepared by centrifugation at 12000 RPM for 5 min. Xylanase activity was measured by DNS method. DNS reagent was prepared as follows: 3.15 g 3,5-dinitrosalicylic acid was put into 500 mL distilled water, mixed and heated to 45°C, added 100 ml sodium hydroxide (200 g/L), mixed again; added 91.00 g potassium sodium tartrate, 2.50 g phenol and 2.5 g sodium sulfite, mixed and heated to 45°C until dissolving, and then cooled to room temperature, the volume was adjusted to 1000 mL with distilled water. The xylanase reaction process was as following: 2 mL sample was put into a tube, added 5 mL DNS, mixed and then added 2 mL xylan (5 mg/mL). The reaction was conducted at 37°C and pH 5.5 for 30 min, terminated by putting the tube into boiling water for 15 min, and then the volume was adjusted to 25 mL with distilled water. Absorbance value was determined at 540 nm. One unit (U) is defined as the amount of enzyme that releases 1 μmol of reducing sugar from xylan solution (5 mg/mL) per minute. In order to increase xylanase yield, 0.2% Tween 80 and 2% wheat bran were added in the YPD medium.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of xylanase

Analytical SDS-PAGE was performed with 12% polyacrylamide gel. The gels were stained with coomassie brilliant blue R-250. The supernatant of the recombinant *P. pastoris* with xylanase gene was performed by boiling at 100°C for 5 min with equal volume of 5×SDS-PAGE loading buffer, and centrifuged at 10000 RPM for 3 min. The buffer consisted of 250 mM Tris-HCl, 10% SDS (w/v), 0.5% BPB (w/v), 50% glycerine (v/v), 5% β-mercaptoethanol (w/v). The electrophoresis was set with 50 V for the first 30 min and then kept at 100 V. Incubation supernatant of the recombinant *P. pastoris* without xylanase gene was used as control.

The biochemical parameter determination of the mutant xylanase

To measure the optimal temperature of the mutant xylanase, 5 mL enzyme solution was put into a 50 mL preheated tube and kept in a electric-heated thermostatic water bath at 30, 40, 50, 60, 70, 80 and 90°C for 15 min respectively, and then the tube was cooled to room temperature immediately with ice water. To measure optimal pH value of the mutant xylanase, the pH values of enzyme solution were adjusted to 3.50, 4.50, 5.50, 6.50, 7.50, 8.50 and 9.50. The residual xylanase activity was estimated with the above protocol.

Effect of xylanase on polysaccharide degradation in wheat meal

Wheat was ground to 40-60 meshes, and then mixed with the liquid enzyme at a proportion of 1:10 (g/mL), which was kept at 40°C for 2 h. The enzymatic hydrolysis solution was centrifuged at 1000 RPM for 5 min, and the supernatant was used for detecting reducing sugar concentrations. The content of reducing sugar was measured by ion chromatography of Dionex ICS-3000 with CarboPac PA10 separation column (4×250 mm) and guard column (4×50 mm). The eluent concentration of NaOH was 50 mmol/L, the setting flow rate was 0.8 mL/min.

Statistical analysis

The data were analyzed using the ANOVA procedures of Statistical Analysis Systems Institute, 2004. Duncan's multiple range test was used to evaluate treatment means. The results were considered statistically significance at $p<0.05$.

RESULTS AND DISCUSSION

Error-prone PCR and base modification of xylanase gene

The mutant xylanase genes were obtained by error-prone PCR amplification. After the mutant genes were connected with PMD19-T and transferred into *E. coli*, 32 positive colonies were obtained by the white-blue colony selection. The base sequence analyses of mutant xylanase genes indicated that some bases in 6 mutants (No. 1, 4, 5, 6, 7 and 8) were changed (Table 1). These base changes were possible to affect xylanase activity and properties.

Table 1. The modified base points of mutant xylanase genes and the corresponding changes of amino acids

Mutant xylanase genes	The modified base points	Amino acid changes
1	117th (GAC-AAC)	Aspartic acid-Asparagine
4	34th (CTC-GTC)	Leucine-Valine
5	43th (ACC-GCC)	Threonine-Alanine
6	116th (ACT-ATT)	Threonine-Isoleucine
7	179th (AAG-ACG)	Lysine-Threonine
8	53th (TTC-CTC)	Phenylalanine-Leucine
	107th (GGC-AGC)	Glycine-Serine

In order to increase gene mutant rate, the reaction system and loop condition of PCR were modified in this study including increasing magnesium ion concentration, adding manganese ions, and so on. For increasing the selection accuracy of positive colonies, Congo red is used because it can form a red compound with xylan. A transparent zone will be formed around the positive colony if the colony is able to secrete xylanase for degrading xylan to cause the red color disappeared. The previous researches have obtained some good mutant genes by error-prone PCR technique for alkali resistance and high stability of xylanase (CHEN et al., 2001; STEPHENS et al., 2009), in agreement with this study.

The mutant xylanase gene expression in *P. pastoris* and xylanase activity determination

The transformed *E. coli* and *P. pastoris* with pGAPZαA-mutant xylanase gene were selected with zeocin and color change indication of Congo red, respectively. The result showed that mutant 1, 4, and 6 had higher xylanase activity than other mutants ($p<0.05$). Table 2 indicated that the maximal xylanase activity was obtained after the recombinant *P. pastoris* was incubated for 12 h ($p<0.05$), and then it decreased ($p<0.05$). Among the three mutant xylanases, mutant 1 had the highest xylanase activity during incubating period ($p<0.05$), which was higher than the native xylanase ($p<0.05$). The further research indicated that adding 0.2% Tween 80 could significantly increase xylanase yield by 30% for the recombinant *P. pastoris* with mutant xylanase gene and 35% for the recombinant *P. pastoris* with native xylanase gene, compared with that without Tween 80 addition ($p<0.05$). In addition, the recombinant *P. pastoris* with or without native xylanase gene was used as the positive or negative controls.

Table 2. The effect of different incubating time on xylanase activity of recombinant *Pichia pastoris* (U ml^{-1})

Items	6 h	12 h	24 h	36 h
Mutant 1	0.0712 ± 0.0015^{a}	0.1480 ± 0.0014^{a}	0.0819 ± 0.0018^{a}	0.0443 ± 0.0009^{a}
Mutant 4	0.0502 ± 0.0012^{c}	0.1251 ± 0.0035^{c}	0.0603 ± 0.0014^{c}	0.0374 ± 0.0012^{b}
Mutant 6	0.0474 ± 0.0014^{d}	0.1043 ± 0.0021^{d}	0.0584 ± 0.0016^{d}	0.0374 ± 0.0008^{b}
Native xylanase gene	0.0565 ± 0.0011^{b}	0.1360 ± 0.0025^{b}	0.0674 ± 0.0016^{b}	0.0382 ± 0.0016^{b}
Control (without xylanase gene)	0.0112 ± 0.0006^{e}	0.0226 ± 0.0009^{e}	0.0198 ± 0.0011^{e}	0.0185 ± 0.0006^{c}

Note: Data with the different letters in the same columns mean significant difference ($P<0.05$), while data with the same letters in the same columns mean insignificant difference ($P>0.05$).

The result showed that xylanase genes were amplified from the genomic DNA of recombinant *P. pastoris*, indicating that the mutant xylanase genes were transformed into *P. pastoris* successfully.

Figure 1 indicated that all the supernatants of the recombinant *P. pastoris* with mutant xylanase genes had the bright bands at the points with the molecular weight of 24 kDa, which corresponded to the molecular weight predicted by the bases of xylanase gene. However, there was no band in the supernatant of the recombinant *P. pastoris* without xylanase gene in lane 7. The SDS-PAGE analysis proved that the mutant xylanase had been expressed successfully in *P. pastoris*.

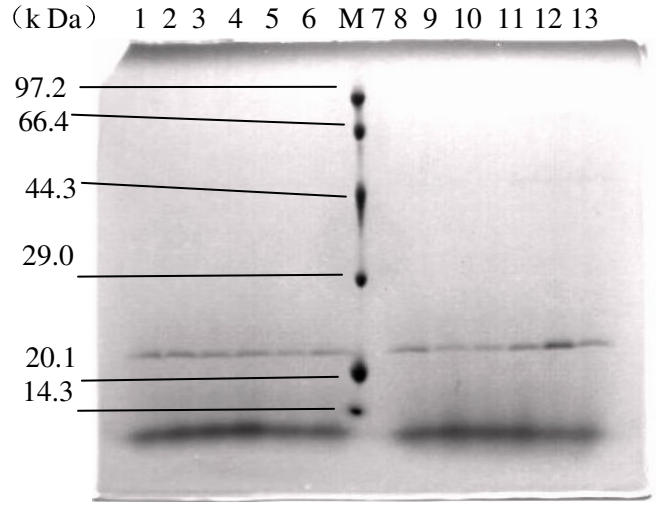

Figure 1. The SDS-PAGE of incubation supernatant of the recombinant *Pichia pastoris*
Note: Lane M: Protein molecular weight markers; Lane1 and Lane 2: incubation supernatant of mutant 1; Lane 3 and Lane 4: incubation supernatant of mutant 4; Lane 5 and Lane 6: incubation supernatant of mutant 5; Lane 7: incubation supernatant of recombinant *Pichia pastoris* without xylanase gene; Lane 8 and Lane 9: incubation supernatant of mutant 6; Lane 10 and Lane 11: incubation supernatant of mutant 7; Lane 12 and Lane 13: incubation supernatant of mutant 8. The above supernatants were concentrated for 12 folds.

Why were the maximal xylanase activity obtained after the recombinant *P. pastoris* was incubated for 12 h, and then decreased? The reason may be due to enzyme degradation and cell age declining with incubating period prolonging. It is predicted that amino acid change in xylanse protein from aspartic acid to asparagine may increase xylanase activity. Even though the xylanase activity of mutant 1 was higher than the native one, it was lower than the previous reports about xylanase gene expression in *P. Pastoris* (DENG et al., 2006; RUANGLEK et al., 2007; LIU; LIU, 2008). Why Tween 80 addition can increase xylanase yield, the reason may be that it can increase the permeability of cell membrane, accelerate the secretion of a variety of enzymes outside cells (ZHUANG et al., 2000).

The optimal pH and temperature determination of xylanase

Figure 2 showed that the optimal temperature of mutant xylanase was 40°C ($p<0.05$), which was the same as the native xylanase; however, at this temperature point, mutant xylanase activity was higher than the native one ($p<0.05$). Figure 3 indicated that the optimal pH values of both mutant and native xylanases were 5.5, in which the xylanase activity was significantly higher than that in other pH points ($p<0.05$).

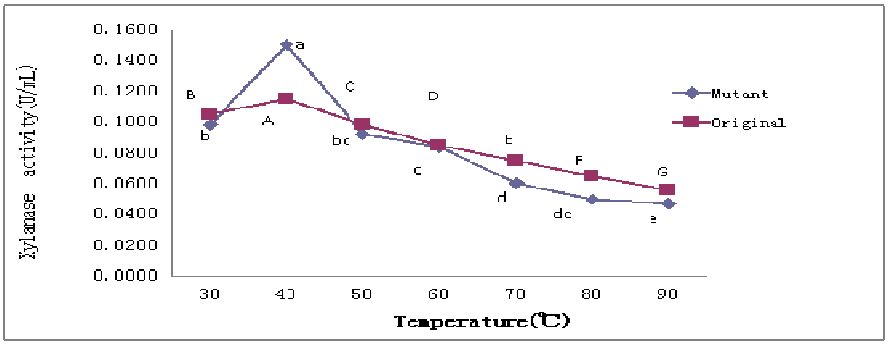

Figure 2. The effect of different temperatures on xylanase activity of recombinant *Pichia pastoris*
Note: Data with the different small or capital letters mean significant difference for the mutant or native xylanase ($P<0.05$), while data with the same small or capital letters mean insignificant difference for the mutant or native xylanase ($P>0.05$).

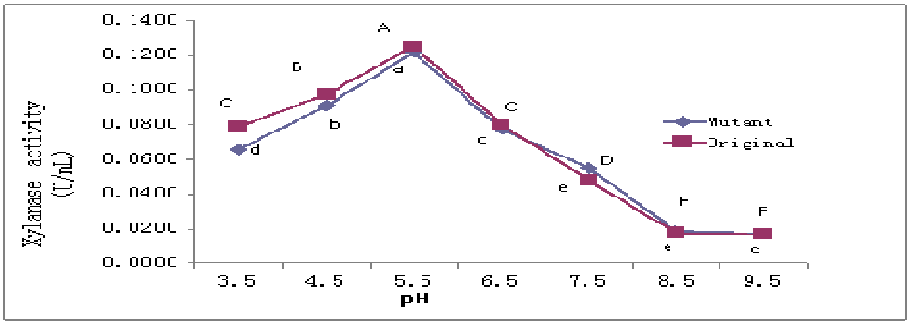

Figure 3. The effect of different pH values on xylanase activity of recombinant *P. pastoris*
Note: Data with the different small or capital letters mean significant difference for the mutant or native xylanase (P<0.05), while data with the same small or capital letters mean insignificant difference for the mutant or native xylanase (P>0.05).

This result showed that the subtle changes of amino acids did not make drastic changes for biochemical parameters of xylanase, even though xylanase activity has been improved by gene modification. This is consistent with the previous report, in which it was found that gene mutation did not change the optimal temperature and thermal stability of original genetics, compared with the native xylanase (ZHOU et al., 2008; CHEN et al., 2018); however, the other reports indicated that gene modification improved pH range and thermal stability of xylanase (CHEN et al., 2001; STEPHENS et al., 2009). Maybe the different modified points in xylanase gene will have different effects on biochemical parameters of xylanase.

Effect of xylanase on polysaccharide degradation in wheat meal

Table 3 showed that the mutant xylanase was more effective than the native one to degrade polysaccharide in wheat meal for the maximal xylose yield ($p<0.05$). However, the mutant xylanase was weaker for arabinose and glucose yields ($p<0.05$), compared with other resources of xylanases. The reason of the difference may be due to the different enzyme resources, xylanase purity or the modified xylanase gene (PUCHART et al., 2018). It was reported that xylanase gene from *Bacillus licheniformis* was expressed in *E.coli*, the enzyme was effective for sugarcane bagasse saccharification (AFTAB et al., 2018), in agreement with this study. In addition, the xylanase from *Aspergillus niger* could improve the yields of glucose and arabinose effectively ($p<0.05$), which may be due to the xylanase compound isolated from *Aspergillus niger* in this study contains xylanase as well as other enzymes.

Table 3. Effect of different resources of xylanases on polysaccharide degradation in wheat meal (μg ml^{-1})

Items	Arabinose	Glucose	Xylose
Mutant xylanase from recombinant *P. pastoris*	13.99±3.02^{c}	91.75±7.56^{c}	112.85±1.64^{a}
Incubation supernatant of recombinant *P. pastoris* without xylanase gene (Control)	105.53±0.06^{b}	1406.43±129.57^{b}	0.00^{d}
Native xylanase from recombinant *P. pastoris*	25.13±5.15^{c}	1374.89±135.73^{b}	28.29±8.06^{c}
Xylanase from *Aspergillus niger*	222.80±20.95^{a}	3860.92±337.90^{a}	45.36±2.44^{b}

Note: Data with the different letters in the same columns mean significant difference (P<0.05), while data with the same letters in the same columns mean insignificant difference (P>0.05).

CONCLUSIONS

One mutant xylanase gene has been obtained with error-prone PCR to yield higher xylanase activity for xylan degradation than the native gene.

The mutant xylanase has the same biochemical parameters as the native one.

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RESUMO: A xilanase pode hidrolisar o xilano para reduzir seu impacto antinutricional e melhorar a disponibilidade de nutrientes, portanto, obter xilanase adequada para degradar o xilano é essencial. A PCR propensa a erros e a transformação genética foram utilizadas neste estudo para obter a xilanase ideal para degradar eficazmente a xilana. O resultado mostrou que um gene mutante de xilanase com alta expressão de xilanase foi obtido. Depois que o gene mutante da xilanase foi conectado ao pGAPZαA e transformado em Pichia pastoris (P. pastoris), o recombinante P. pastoris com o gene mutante produziu maior atividade de xilanase (0,1480 U / mL) do que com o gene nativo da xilanase (0,1360 U / mL) após 12 h de incubação ($p < 0,05$). A temperatura e o pH ótimos da xilanase expressa pelos genes nativos e mutantes foram os mesmos, ou seja, 40 ºC e 5,50 ($p < 0,05$). Além disso, a adição de Tween 80 a 0,2% durante a incubação de P. pastoris recombinante poderia aumentar significativamente o rendimento de xilanase em cerca de 30-35% ($p < 0,05$). A xilanase mutante poderia aumentar significativamente o rendimento de xilose da farinha de trigo mais do que a xilanase nativa ($p < 0,05$).

PALAVRAS-CHAVE: PCR propensa a erros. Xilanase. Mutação de Gene. Expressão genetica. P. pastoris

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