Appl Biochem Biotechnol (2012) 168:638–650
DOI 10.1007/s12010-012-9806-5

Directed Evolution of *Penicillium janczewskii zalesk* α-Galactosidase Toward Enhanced Activity and Expression in *Pichia pastoris*

Yiqun Chen · Bo Zhang · Honglei Pei · Junnan Lv · Wenhan Yang · Yunhe Cao · Bing Dong

Received: 13 December 2011 / Accepted: 11 July 2012 /
Published online: 26 July 2012

Abstract In this study, the activity of an α-galactosidase obtained from *Penicillium janczewskii zalesk* was improved via modifying its gene by error-prone PCR and DNA shuffling. The mutated DNA was ligated to pBGP1, an autonomous-replicating vector, which was subsequently transformed into *Pichia pastoris* X-33. The expressed enzyme activities were measured after single colonies were cultured in yeast–peptone–dextrose medium in deep-well plates. After two rounds of screening, two mutants with higher activity were obtained. By PCR analysis, four mutation sites (S167G, P455L, N637S, and P490L/P490H) were found in these two variants (mutant-59 and mutant-8). Mutant-59 showed the highest activity at pH 5.0 and 40 °C with an increased V_{max} value of 769 μmol/min and the specific activity of 667 U/mg against p-nitrophenyl α-D-galactopyranoside. The two mutant enzymes also showed similar resistance to the metal ions of Cu^{2+}, Fe^{2+}, and Zn^{2+}. In a 10-L fermenter, the supernatant enzyme activity reached the maximum of 550.2 U/mL upon the methanol induction for 96 h. This fermentation activity of the mutant was improved approximately two more folds than the wild type α-galactosidase. This mutant of α-galactosidase is prospective in feed manufacturing as feed additives to improve nutrient digestibility in monogastric animals.

Keywords Directed evolution · α-Galactosidase · Improved activity · *Pichia pastoris*

Introduction

α-Galactosidases (EC 3.2.1.22) are a series of enzymes that play various physiological roles in wide applications. For example, α-galactosidase can selectively degrade α-D-galactose

Y. Chen · B. Zhang · H. Pei · J. Lv · W. Yang · Y. Cao (✉) · B. Dong (✉)
National Key Laboratory of Animal Nutrition, China Agricultural University,
No. 2 Yuanmingyuan West Road, Beijing 100193, People's Republic of China
e-mail: caoyunhe@126.com
e-mail: dongb@mafic.ac.cn

B. Zhang
Beijing Research Institute for Nutritional Resources,
No. 4 Dongbinhe Road Youanmenwai, Fengtai District, Beijing 100069, People's Republic of China

residues released from galactomannans, a component of hemicellulose [1]. α-Galactosidases have been used to eliminate oligosaccharides such as raffinose and stachyose in legume seeds which are considered as flatulence-causing factors [2]. They are also capable of converting type B red blood cells to type O [3] and provide an enzyme replacement therapy against Fabry disease [4].

The enzymatic activity of α-galactosidases varies much according to their origins. Most commercially available α-galactosidases show relatively low specific activities and limited yields [5–7], which must be improved before large-scaled applications. In vitro directed evolution is a regular and efficient method in protein engineering, which has been successfully applied in improving of catalytic activities of glycoside hydrolases such as xylanase and glucanase [8–10], as well as in some work conducted by Reetz group [11, 12]. Unfortunately, the directed evolution of α-galactosidases has not been systematically performed and only rare reports are available to date [13].

Our laboratory has cloned a novel α-galactosidase gene from *Penicillium janczewskii zalesk* and it was later expressed in *Pichia pastoris* [14]. The mature α-galactosidase, consisting of 722 amino acids, has a predicted molecular weight of 79 kDa. In the present study, we aimed to improve the α-galactosidase specific activity and enzyme activity by directed evolution through error-prone PCR and DNA shuffling. The mutant enzymes with better activity were subsequently characterized.

Materials and Methods

Strains, Vectors, and Reagents

P. pastoris X-33 and pPICzαA were purchased from Invitrogen (San Diego, CA, USA). pBGP1 was kindly provided by Dr. Charles C. Lee (Western Regional Research Centre, USDA, Albany, CA). *Escherichia coli* Top10, obtained from Tiangen Corporation (Beijing, China), was used as the host strain. Yeast extract and tryptone were purchased from Oxoid Limited (Hampshire, England). p-Nitrophenyl α-D-galactopyranoside was purchased from Sigma-Aldrich (St. Louise, USA). Taq polymerase and restriction enzymes were obtained from Takara Biotechnology (Dalian, China). All other reagents were obtained from Sigma-Aldrich (St. Louise, USA).

Construction of an Error-Prone PCR Library

The plasmid template of pPIC-agl1 encoding wild-type α-galactosidase (AGL1) [14] was amplified by PCR using the following primers: 5′-AGAGAGGCTGAAGCTGAATTC-3′ (S-F-1) and 5′-TGAGATGAGTTTTTGTTCTAGA-3′ (S-R-2). Error-prone PCR was carried out in a mixture containing 7 mM $MgCl_2$, 0.3 mM $MnCl_2$, 1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, and 0.2 mM dGTP. Regular Taq polymerase (Takara Biotechnology, Dalian, China) was used to achieve sufficient mutation rate. The PCR program was 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min for 45 cycles. The corresponding product (~2,300 bp), Agl1, was isolated from 0.6 % agarose gel and purified using a gel extraction kit (Tiangen Corp., Beijing, China), followed by restricted digestion with *Eco*RI and *Xba*I. The resulting fragment was ligated into pBGP1 to form Agl1-pBGP1, which was introduced into *E. coli* Top10 and directly incubated in 5 mL of liquid low-salt Luria-Bertani medium (0.5 % NaCl, 1 % tryptone, 0.5 % yeast extract, pH 7.0) containing 25 μg/mL of Zeocin (Invitrogen, San Diego, CA, USA) at 37 °C overnight.

Plasmids were extracted and electroporated by Gene Pulser Xcell total system (Bio-Rad, Hercules, CA, USA; 2,000 V, 25 μF, 2 mm cuvette) into *P. pastoris* X-33 following manual instruction. The transformed mixture was subsequently spread on the yeast–peptone–dextrose–sorbitol plates (2 % peptone, 2 % glucose, 1 % yeast extract, 1 M sorbitol, and 2 % agar, pH 6.5) containing 100 μg/mL of Zeocin for screening as the method described below.

Construction of a DNA Shuffling Library

Episomal plasmids from selected variants were extracted using a yeast plasmid extraction kit (Tiangen Corp., Beijing, China) and amplified in *E. coli* Top10 followed by plasmid purification as above described. A first round of PCR was performed by using two sets of primers designed near the middle of agl1 gene: 5′-GAATATCCGCGTGTCGACGAT-3′ (M1), 5′-ATCGTCGACACGCGGATATTC-3′ (M2), S-F-1, and S-R-2, which amplified two products generated from the primer sets of S-F-1 and M2 and S-R-2 and M1, respectively. The PCR program was set as 94 °C for 5 min, 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 45 s for 35 cycles, and the final extension at 72 °C for 8 min. These two fragments were purified and digested by DNase I followed by purification and reassembly according to Stemmer [15]. Briefly, after DNase I treatment, fragments in the range of 100–250 bp were purified by 2 % agarose electrophoresis gel and the full-length gene was reassembled using two terminal primers (S-F-1 and S-R-2). Subsequent procedures were the same as those used for the construction of the error-prone library.

Screening for Variants with High Activity

Single colonies from the mutant library were obtained with sterile toothpicks and cultured in 150 μL of yeast–peptone–dextrose medium (2 % peptone, 2 % glucose, 1 % yeast extract, pH 6.5) containing Zeocin (50 μg/mL) in sterile deep-well plates and incubated at 28 °C with shaking at 270 rpm for 24 h. Then, the plates were centrifuged at 3,000 rpm for 5 min to collect supernatant for the assay of enzyme activity. The assays were carried out in 96-well plates in which 10 μL of supernatant was mixed with 90 μL of p-nitrophenyl α-D-galactopyranoside prepared in 50 mM NaAc buffer (pH 5.0), and incubated at 40 °C for 10 min. The reactions were terminated by addition of 100 μL Na_2CO_3 (0.5 M). The absorbance was measured at 405 nm in a Synergy4 Hybrid Microplate Reader (BioTek, Winooski, VT, USA). The supernatant with the highest absorbance value was selected for next steps.

Shaking-Flask Expression of Mutant Enzymes

The plasmid with the highest activity was extracted from selected mutants, sequenced and digested with *Eco*RI and *Xba*I followed by ligation into pPICzαA. The recombinant plasmid was then linearized and transformed into *P. pastoris* X-33. Positive colonies were incubated in 30 mL of buffered glycerol-complex medium (BMGY, 100 mM potassium phosphate, pH 6.0, 0.34 % yeast nitrogen base, 4×10^{-5} % biotin, 1 % glycerol, 1 % yeast extract, 2 % peptone) at 28 °C and shaken at 250 rpm for 24 h. Thereafter, the cultures were centrifuged and re-suspended in 30 mL of buffered methanol complex medium, where 0.5 % methanol replaced 1 % glycerol in BMGY, to induce the expression of α-galactosidase. Methanol was added at 24 h intervals to reach a final concentration of 0.5 % for a total incubation time of 72 h. Samples (1 mL) were then taken before methanol addition for enzymatic assay, as the method described below.

Ten-Liter Bioreactor Fermentation

The mutant with the highest activity was cultured in a 10-L fermenter. The seed media contained 50 g glucose, 5 g KH_2PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4$, 1.5 g KOH, and 50 g $NH_4H_2PO_4$ per liter (pH 5.0). Glycerol (50 % *w*/*v*) was added into the medium as the supplemental carbon source when media glucose was exhausted. When the biomass reached 230 g/L, methanol was added to induce the expression of galactosidase. Fermentation biomass was sampled at 12 h intervals for activity measurement and supernatants were analyzed by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the measurement of protein expression.

Protein Purification

Two milliliters of supernatant sample obtained from fermentation was loaded on an anion-exchange column (UNOsphere Q Strong Anion Exchange Support, 1×20 cm, Bio-Rad, Hercules, CA, US) pre-equilibrated with five column volumes of buffer A (20 mM Tris–HCl buffer, pH 8.5). Subsequently, a wash with buffer A was carried out to remove unbound proteins. Proteins were eluted with a linear gradient from 0 % to 100 % of buffer B (20 mM Tris–HCl buffer, 1 M NaCl, pH 8.5) at a flow rate of 1.5 mL/min. The active fractions were pooled and examined by 12 % SDS-PAGE. Protein concentration was measured using the Coomassie Plus Protein Assay (Pierce, Rockford, IL, USA). Bovine serum albumin (BSA) was used as standards. All samples and standards were measured in triplicates.

α-Galactosidase Activity Assay

α-Galactosidase activities were assayed with p-nitrophenyl α-D-galactopyranoside as the substrate as described by Rezessy-Szabó et al. [16]. The 800 μL of reaction mixture contained 400 μL of diluted enzyme solution and 400 μL of p-nitrophenyl α-D-galactopyranoside (10 mM) in NaAc–HAc buffer (50 mM, pH 5.0). The enzyme was diluted to approximately 30 ng/mL in the reaction mixture to optimize the measuring precision. The reaction was incubated at 40 °C for 10 min before 3.2 mL of Na_2CO_3 (0.5 M) was added. The absorbance at 405 nm was monitored and converted to the quantity of p-nitrophenol release. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol p-nitrophenol from the substrate solution per minute under the assay condition.

Characterization of Mutant Enzymes

To determine the optimal pH for mutants, the enzyme and p-nitrophenyl α-D-galactopyranoside were prepared with Na_2HPO_4–citric acid buffers ranging from pH 4.0 to 8.0. The relative enzyme activities were measured as above described.

The determination of optimal temperatures was performed at 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, and 60 °C. The relative activities of the mutants were determined at the determined optimal pH for 10 min using NaAc–HAc buffer.

To investigate the stability of mutants under different pH conditions, the enzymes were incubated in Na_2HPO_4–citric acid buffers over the range from pH 4.0 to 8.0 for 30 min at room temperature. The standard activity assay was conducted at pH 5.0 and 40 °C for 10 min.

The enzyme thermal stability was measured by incubation of enzyme solution at 40 °C, 50 °C, 55 °C, or 60 °C. When the enzymes were incubated at 40 °C and 50 °C for 2 h and

samples were taken at 30 min intervals. When the enzymes were incubated at 55 °C, only 30 min of incubation was performed and samples were taken at 10 min intervals. For the 60 °C incubation, the time span was 20 min and sampling interval was 5 min. The residual activity was assayed as described above.

The effects of various metal ions and chemicals on the activity of mutant enzymes were tested at pH 5.0 and 40 °C for 10 min following the treatment in 10 mM ion solutions for 1 h. Reaction mixtures without the metal ions or chemicals were set as control.

To determine kinetic constants, reaction velocities were measured in the presence of series concentrations of p-nitrophenyl α-D-galactopyranoside solution (0.05, 0.10, 0.15, 0.20, 0.25, 0.40, and 0.50 mM). Lineweaver–Burk plot were graphed according to the determined velocities and the corresponding substrate concentrations, from which the K_m and V_{max} values were derived. All samples were assayed in triplicate.

Structure Modeling

The previously determined galactosidases structures were obtained from Protein Data Bank (PDB), with the entries of 2FYO, 2XN2, and 3MI6. The structure modeling of *P. janczewskii zalesk* α-galactosidase was accomplished in SwissModel [17], using 2FYO as the template. Structural comparison and graphics was performed with the PyMOL Molecular Graphics System (Schrödinger, LLC.).

Results

Screening for Mutants with High Activity

After the error-prone PCR, about 900 colonies were tested using 96-well plates assay which were triplicated. Among them, 18 showed higher absorbance values than that of the wild type. Plasmids from the top 8 mutants were extracted and used in DNA shuffling. Subsequently, two mutants (mutant-8 and mutant-59) were identified from about 500 variants, which showed 42 % and 45 % increase in activity compared with the wild type enzyme. By sequence analysis, the amino acid mutation sites were identified at S167G, P455L, N637S, and P490L in mutant-8, while mutant-59 contained S167G, P455L, N637S, and P490H.

Ten-Liter Fermentation of Mutant-59

Mutant-59 was selected for further study in a 10-L bioreactor. After being cultured for 136 h and induced for 96 h, the wet biomass and enzymatic activity reached the maximum of 360 g/L and 550.2 U/mL (Fig. 1). SDS-PAGE showed that the molecular weight of mutant-59 was about 100 kDa (Fig. 2). Three tubes of eluent with α-galactosidase activity were collected and the peak concentration of the purified enzyme was 36.5 μg/mL (Fig. 3). The purified enzyme showed a single band in SDS-PAGE (Fig. 4) with a specific activity of 667 U/mg with p-nitrophenyl α-D-galactopyranoside as substrate.

Properties of Mutant-59 and Mutant-8

As shown in Fig. 5, both of the two mutants showed the optimal pH at 5.0. The activities of these two mutants were maintained above 60 % within the pH range of 4.5 to 6.5, but the catalytic activities decreased rapidly when pH was above 7.5 or below 4.0. Interestingly, the

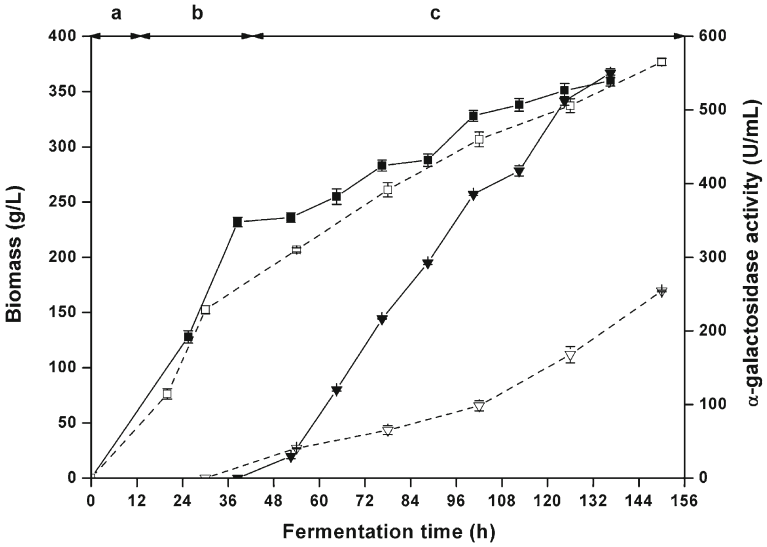

Fig. 1 Growth curves of *Pichia pastoris* harboring α-galactosidase genes and the corresponding α-galactosidase activities in 10-L fermenters. *Closed boxes* indicate wet biomass of mutant-59; *closed triangles* indicate α-galactosidase activity of mutant-59; *opened boxes* indicate wet biomass of wild type; *opened triangles* indicate α-galactosidase activity of wild type. **a** Glucose-fed batch phase. **b** Glycerol fed-batch phase. **c** Methanol induction phase

residual activities remained about 100 % after the mutants were pretreated in buffers over a wide range of pH (from 4.5 to 8.0) for 30 min and there was still more than 50 % activity in a buffer of pH 4.0 (Fig. 6).

The two mutants showed similar optimal temperature at 40 °C (Fig. 7). The activities remained at greater than 60 % in the narrow range from 30 °C to 45 °C. However, the activities declined rapidly when the temperature was higher than 50 °C. The activity could hardly be detected at 60 °C. The temperature stabilities of the mutants are presented in

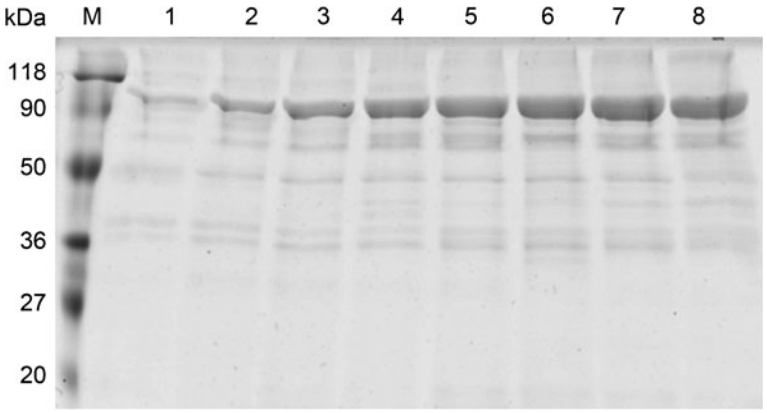

Fig. 2 SDS-PAGE of supernatants of mutant-59 in 10-L fermentation demonstrating the protein expression of α-galactosidase. *M* ladder; *lane 1–8* samples at 12, 24, 36, 48, 60, 72, 84, and 96 h. The loading volume of supernatant samples was 20 μL per lane

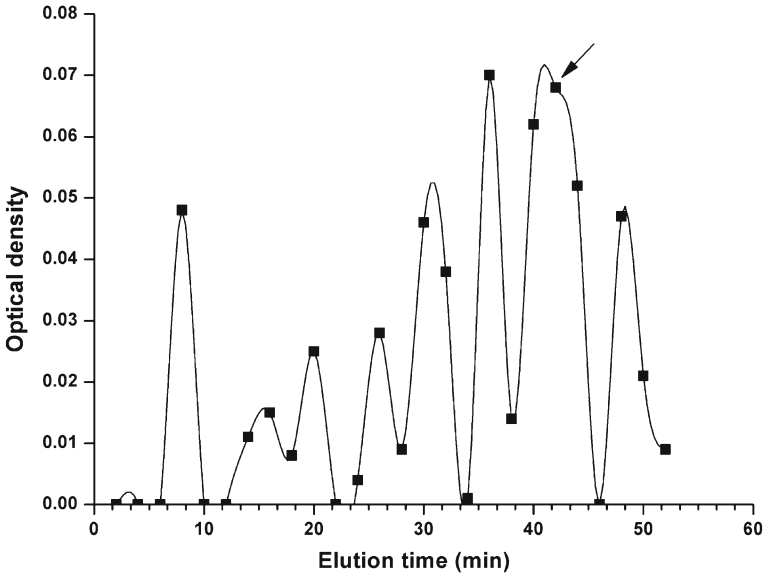

Fig. 3 Elution curves of α-galactosidase purification from fermentation supernatants drawn according to the OD values measured at 280 nm. The *arrow* indicates the peak of α-galactosidase protein

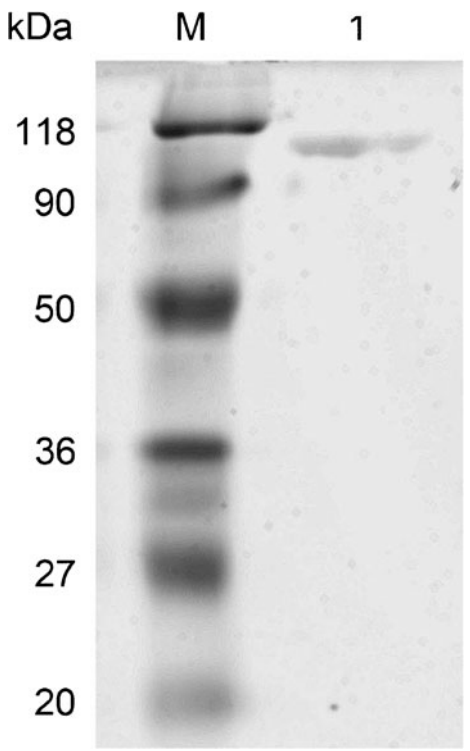

Fig. 4 SDS-PAGE of purified mutant-59. *M* ladder; *lane 1* purified α-galactosidase enzyme. The loading volume of supernatant samples was 20 μL per lane (approximately 0.73 μg)

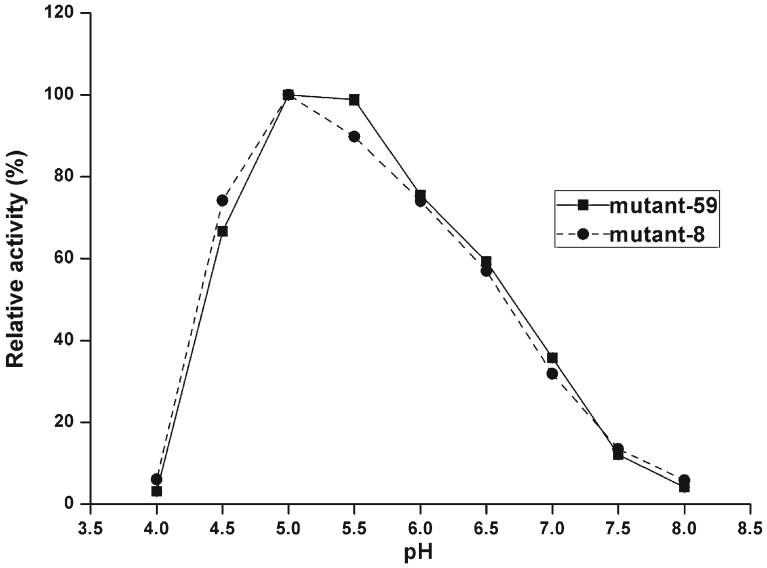

Fig. 5 Effects of pH on α-galactosidase activity of the mutants. *Boxes*=mutant-59, *circles*=mutant-8

Figs. 8 and 9. Both of them showed increasing activities at 40 °C. More than 50 % of the activity remained after being incubated for 2 h at 50 °C. However, the enzymatic activity decreased dramatically after 20 min at 55 °C and was completely lost at 60 °C for only 5 min.

The effects of various metal ions on mutant activity were measured. As shown in Table 1, these two mutants showed similar activity in most ion solutions except for Cu^{2+}, Fe^{2+}, and

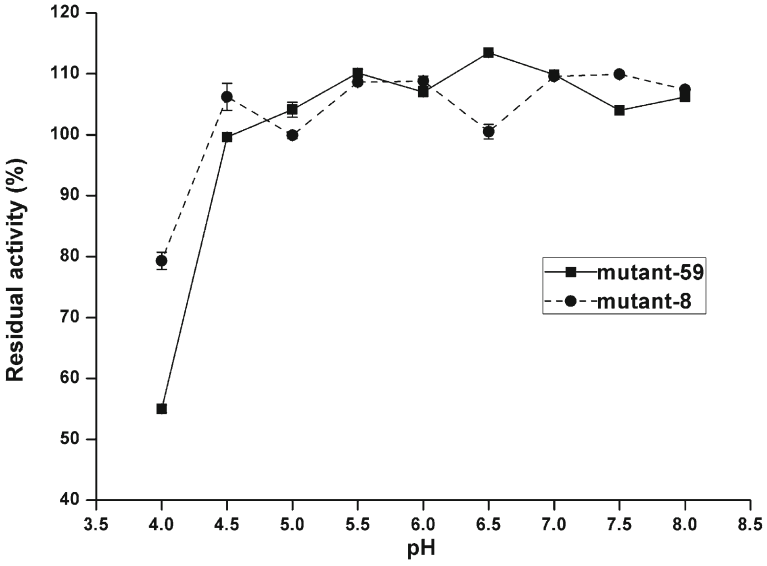

Fig. 6 pH stability of α-galactosidase of the mutants after being treated for 30 min. *Boxes*=mutant-59; *circles*=mutant-8

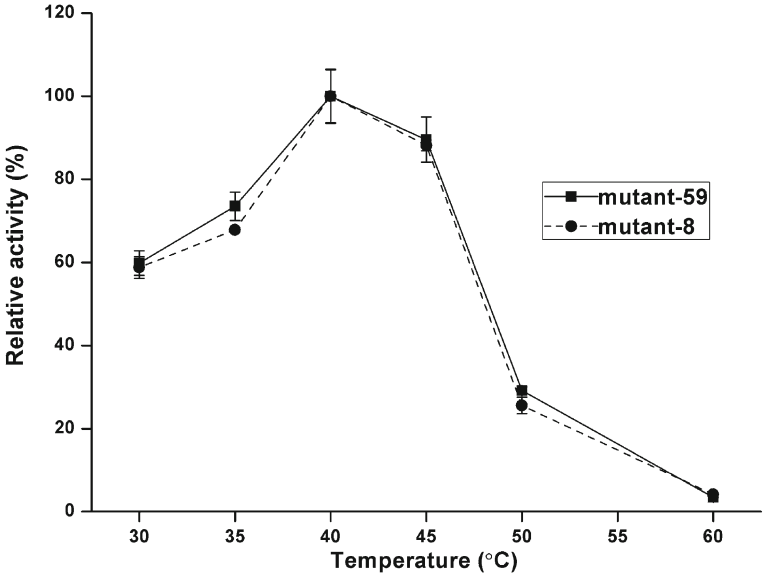

Fig. 7 Effects of temperature on α-galactosidase activity of the mutants. *Boxes*=mutant-59, *circles*=mutant-8

Zn^{2+}. Mutant-59 exhibited 40.6 % activity in the presence of Cu^{2+} and only 8.4 % after being treated with Fe^{2+}. Mutant-8 lost 76.5 % of its activity in response to Cu^{2+} and showed a slight decrease of 15.9 % when Zn^{2+} was present.

As a summary, both of the mutants had subtle decreased optimal pH from 5.2 to 5.0, but the stability from pH 6.0~8.0 dramatically increased. The optimal working temperature of

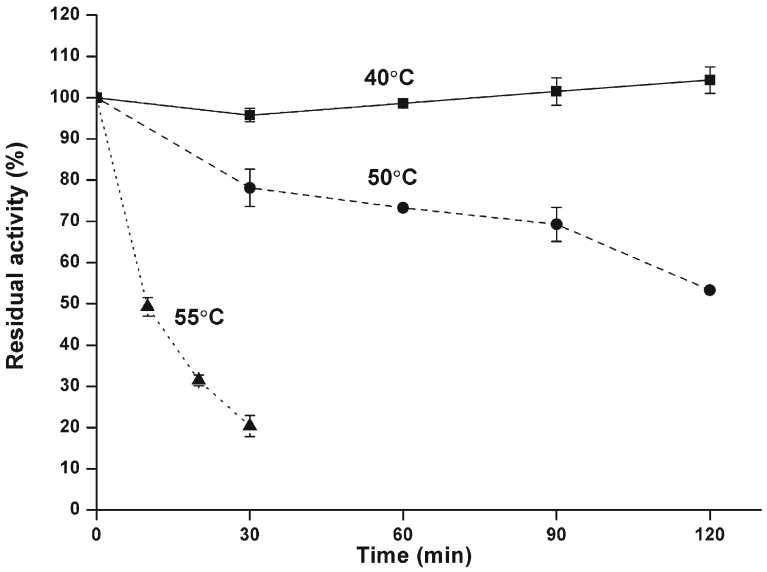

Fig. 8 Temperature stability of α-galactosidase of mutant-59 at 40 °C, 50 °C, and 55 °C. *Boxes* 40 °C, *circles* 50 °C, *triangles* 55 °C

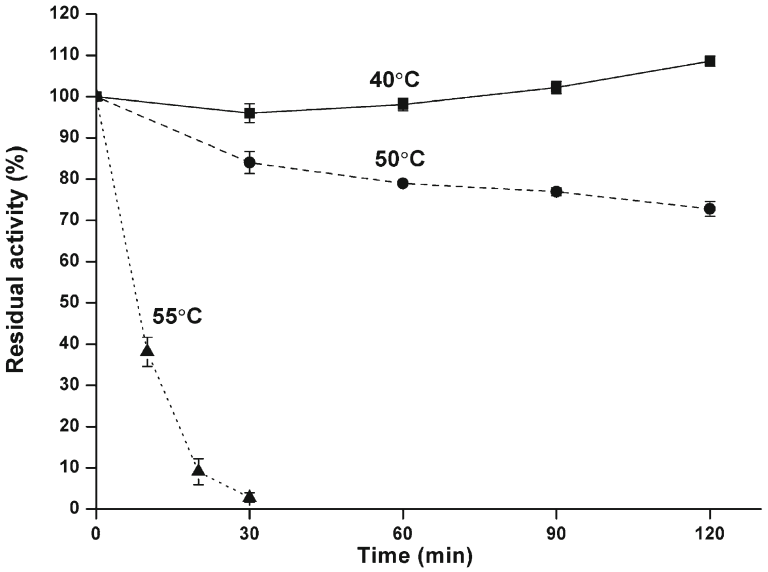

Fig. 9 Temperature stability of α-galactosidase of mutant-8 at 40 °C, 50 °C, and 55 °C. *Boxes* 40 °C, *circles* 50 °C, *triangles* 55 °C

these two mutants was exactly the same as the wild type but it was worth noticing that they showed outstanding stability at 40 °C although activity could not be detected above 60 °C. The two mutants were more sensitive to some ions than the wild type enzyme, especially Cu^{2+} and Fe^{2+}. How the substitutions of amino acid caused these changes requires further investigation.

The kinetic properties of these two mutants are listed in Table 2. Mutant-59 had the highest V_{max} value of 769.2 μmol/min, which was 338 % compared to the wild type (227.3 μmol/min), although K_m value increased to 4.1 mM. The V_{max} value of mutant-8 was only 173 % of the wild type.

Table 1 Effects of metal ions and chemical agents on relative activity of mutants

Metal ion or chemical agent	Relative activity (%)		
	Mutant-59	Mutant-8	Wild type
No addition	100.0	100.0	100.0
Cu^{2+} ($CuSO_4$)	40.6±1.0	23.5±0.6	92.2±0.6
Mn^{2+} ($MnSO_4$)	99.3±0.6	110.8±1.0	106.6±2.7
Ca^{2+} ($CaCl_2$)	102.9±0.6	100.5±0.4	102.6±3.4
Zn^{2+} ($ZnSO_4$)	100.4±1.5	84.1±0.1	96.1±2.5
Mg^{2+} ($MgCl_2$)	102.3±1.3	102.8±0.3	101.6±1.4
Fe^{2+} ($FeSO_4$)	8.4±0.4	97.5±3.6	94.0±2.0
Na^{+} (NaCl)	101.5±0.5	101.1±0.8	100.8±0.5
K^{+} (KCl)	101.2±1.8	104.2±1.1	101.6±2.7
EDTA	104.3±2.7	104.2±0.3	104.4±2.8

Table 2 Kinetic parameters of wild-type and mutants for pNPG

	K_m (mM)	V_{max} (μmol/min)
Wild type	1.0	227.3
Mutant-59	4.1±0.4	769.2±69.2
Mutant-8	1.0±0.3	392.2±52.0

Discussion

E. coli is widely applied in expression and screening systems of directed evolution. The successful examples in the studies of glycoside hydrolases are xylanase and glucanase [10, 18]. However, *E. coli* is a prokaryotic host, which cannot process post-translational modification such as glycosylation [19]. In this study, the cloned α-galactosidase in our laboratory is a highly glycosylated protein [14]. Therefore, the episomal replicating plasmid pBGP1 was used in this research since it contains an autonomous replication sequence that allows it to replicate as an episomal element in yeast cells [20]. This facilitates the transformation in *P. pastoris* and subsequent screening and sequencing [21].

It is reported that the combinatorial library method focusing on the amino acids that represent side-chain properties is effective for the directed evolution of smaller-sized enzymes [22]. Since our target protein is relatively large, we selected both error-prone PCR and DNA shuffling in this study. Error-prone PCR is relatively a low-efficient method to construct libraries, leading to many invalid proteins [23]. In fact, over 90 % of the mutants

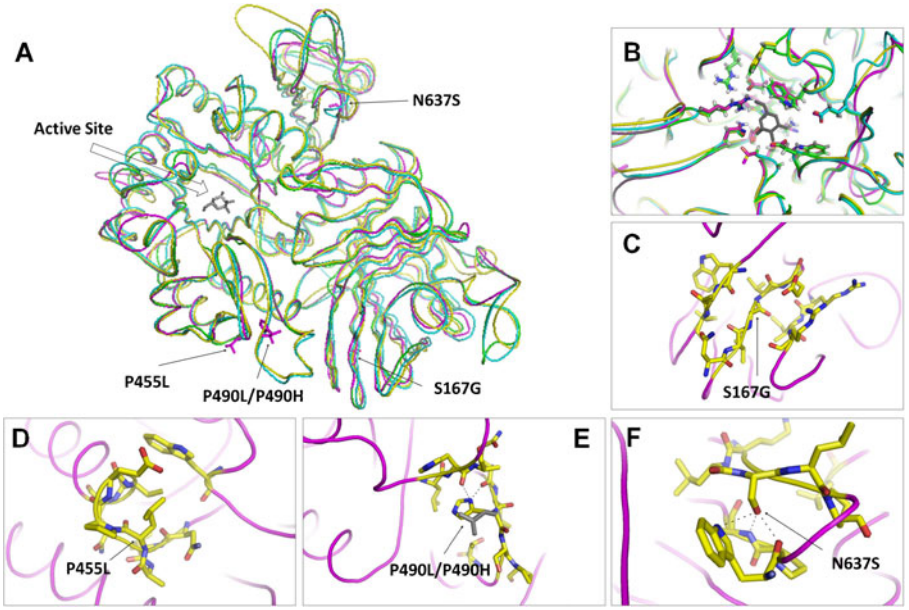

Fig. 10 Structures of galactosidases. **a** Superimposed structures of galactosidases (*green* 2FYO, *cyan* 2XN2, *yellow* 3MI6, *magenta* modeled *Penicillium janczewskii zalesk* α-galactosidase), with the active site containing a galactose molecule (*gray*) from 2FYO. The mutation sites were demonstrated as labeled. **b** Residues within the galactosidase active sites. **c–f** Micro-environment at the mutation sites of S167G, P455L, P490L/P490H, and N637S, respectively. The hydrogen bonds involving mutant residues are labeled as *dashed lines*, while the side chain of P490L is *labeled gray*

showed no activity at this step in our study. The second round of DNA shuffling can ensure the production of the sequences that over 1 k base pairs (kb) length without causing frame shifts [15].

As the mutants showed increased enzymatic activity, the underlying molecular mechanism was of great interests. Therefore, structural modeling was performed for *P. janczewskii zalesk* α-galactosidase. This enzyme belongs to the galacoside hydrolase family and is categorized into the GH36 subfamily, whose structure is represented by several galactosidases (PDB entries: 2FYO, 2XN2, and 3MI6). The structure of *P. janczewskii zalesk* α-galactosidase was successfully modeled in SwissModel, using 2FYO as the template. Compared with the real galactosidase structures, the modeled structure is highly similar in the overall folding and contains almost identical residues in the active site, both indicting the reliability of structure simulation (Fig. 10a, b). Interestingly, none of the mutation sites is identified near the active site. Instead, they are located in the peripheral regions. Close inspection revealed that these mutations altered the local micro-environment by establishing new interactions with nearby residues. For example, new hydrophobic cores are formed in the P455L and P490L mutants, which involve Val458/Trp433/Leu451/Leu455 and Leu489/Leu435/Leu490, respectively (Fig. 10d, e). Such hydrophobic interactions might increase local structural stability. In other mutants, such as S167G, N637S, and P490H, the hydrogen bonding network at the mutation sites would be affected (Fig. 10c, e, f). The above changes in local interactions are most likely to contribute to the rigidity of structural domains, resulting in subtle modulation on the overall flexibility to boost the enzymatic activity.

In protein engineering, the yield of target protein is often a major concern. In the case of α-galactosidase, previous studies did not provide satisfactory protocols for enzyme production. For example, Mi et al. [24] reported 111 U/mL of α-galactosidase from *Penicillium* sp. at 144 h of induction, while Chen et al. [7] acquired approximately 140 U/mL of the enzyme after 250 h induction. In this study, the yield of mutant-59 was 550.2 U/mL after induction for 96 h. This is about 4~5-fold higher yield, obtained with much shorter induction period compared with previous reports. The yield is 2.16-fold higher than the wild type enzyme as well [14]. The K_m value of mutant-8 was the same as the wild type (K_m=1.0 mM), while mutant-59 showed a subtle increased K_m of 4.1 mM. Therefore the yield increase was largely due to the elevated specific activity and V_{max} value.

These results confirmed directed evolution is an efficient way to generate proteins with various properties. With high catalytic activity and fermentation yield, mutant-59 is potentially suitable in the industrial application as feed additives to improve diet digestibility such as soybean containing feedstuff.

Acknowledgments The authors sincerely thank Dr. P. A. Thacker for helpful suggestions and language revision. We thank Dr. Charles C. Lee for kindly providing pBGP1 plasmid. This work is supported by the Program for the New Century Excellent Talents in University (NCET-07-0807), the Scientific and Technical Supporting Programs (2011BAD26B02-2), and Bud Program of Beijing Academy of Science and Technology (2011-5).

References

1. van den Brink, J., & de Vries, R. P. (2011). *Applied Microbiology and Biotechnology, 91*, 1477–1492. doi:10.1007/s00253-011-3473-2.
2. LeBlanc, J. G., Ledue-Clier, F., Bensaada, M., de Giori, G. S., Guerekobaya, T., Sesma, F., et al. (2008). *BMC Microbiology, 8*, 22–30. doi:10.1186/1471-2180-8-22.
3. Zhu, A., Leng, L., Monahan, C., Zhang, Z., Hurst, R., Lenny, L., et al. (1996). *Archives of Biochemistry and Biophysics, 327*, 324–329. doi:10.1006/abbi.1996.0129.

4. Chiba, Y., Sakuraba, H., Kotani, M., Kase, R., Kobayashi, K., Takeuchi, M., et al. (2002). *Glycobiology, 12*, 821–828.
5. Cao, Y., Wang, Y., Luo, H., Shi, P., Meng, K., Zhou, Z., et al. (2009). *Journal of Microbiology and Biotechnology, 19*, 1295–1300.
6. Cao, Y., Wang, Y., Meng, K., Bai, Y., Shi, P., Luo, H., et al. (2009). *Applied Microbiology and Biotechnology, 83*, 875–884. doi:10.1007/s00253-009-1939-2.
7. Chen, Y., Jin, M., Egborge, T., Coppola, G., Andre, J., & Calhoun, D. H. (2000). *Protein Expression and Purification, 20*, 472–484. doi:10.1006/prep.2000.1325.
8. Kaur, J., & Sharma, R. (2006). *Critical Reviews in Biotechnology, 26*, 165–199. doi:10.1080/07388550600851423.
9. McHunu, N. P., Singh, S., & Permaul, K. (2009). *Journal of Biotechnology, 141*, 26–30. doi:10.1016/j.jbiotec.2009.02.021.
10. Zhang, Z. G., Yi, Z. L., Pei, X. Q., & Wu, Z. L. (2010). *Bioresource Technology, 101*, 9272–9278. doi:10.1016/j.biortech.2010.07.060.
11. Reetz, M. T., Kahakeaw, D., & Lohmer, R. (2008). *ChemBioChem, 9*, 1797–1804. doi:10.1002/cbic.200800298.
12. Reetz, M. T., Wang, L. W., & Bocola, M. (2006). *Angewandte Chemie International Edition, 45*, 1236–1241. doi:10.1002/anie.200502746.
13. Fridjonsson, O., Watzlawick, H., & Mattes, R. (2002). *Journal of Bacteriology, 184*, 3385–3391. doi:10.1128/JB.184.12.3385-3391.2002.
14. Zhang, B., Chen, Y. Q., Li, Z. M., Lu, W. Q., & Cao, Y. H. (2011). *Biologia, 66*, 205–212.
15. Stemmer, W. P. (1994). *Proceedings of the National Academy of Sciences of the United States of America, 91*, 10747–10751.
16. Rezessy-Szabó, J. M., Nguyen, Q. D., Hoschke, A., Braet, C., Hajós, G., & Claeyssens, M. (2007). *Biochimica et Biophysica Acta, 1770*, 55–62. doi:10.1016/j.bbagen.2006.06.022.
17. Arnold, K., Bordoli, L., Kopp, J., & Schwede, T. (2006). *Bioinformatics, 22*, 195–201. doi:10.1093/bioinformatics/bti770.
18. Liang, C., Fioroni, M., Rodríguez-Ropero, F., Xue, Y., Schwaneberg, U., & Ma, Y. (2011). *Journal of Biotechnology, 154*, 46–53. doi:10.1016/j.jbiotec.2011.03.025.
19. Cregg, J. M., Cereghino, J. L., Shi, J., & Higgins, D. R. (2000). *Molecular Biotechnology, 16*, 23–52. doi:10.1385/MB:16:1:23.
20. Lee, C. C., Williams, T. G., Wong, D. W., & Robertson, G. H. (2005). *Plasmid, 54*, 80–85. doi:10.1016/j.plasmid.2004.12.001.
21. Sandström, A. G., Engström, K., Nyhlén, J., Kasrayan, A., & Bäckvall, J. E. (2009). *Protein Engineering Design and Selection: PEDS, 22*, 413–420. doi:10.1093/protein/gzp019.
22. Hokanson, C. A., Cappuccilli, G., Odineca, T., Bozic, M., Behnke, C. A., Mendez, M., et al. (2011). *Protein Engineering Design and Selection: PEDS, 24*, 597–605. doi:10.1093/protein/gzr028.
23. Reetz, M. T., Kahakeaw, D., & Lohmer, R. (2008). *Chembiochem: A European Journal of Chemical Biology, 9*, 1797–1804. doi:10.1002/cbic.200800298.
24. Mi, S. J., Meng, K., Wang, Y. R., Bai, Y. G., Yuan, T. Z., Luo, H. Y., et al. (2007). *Enzyme and Microbial Technology, 40*, 1373–1380.