RESEARCH LETTER

Error-prone PCR of a fungal xylanase for improvement of its alkaline and thermal stability

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Abstract

Random mutagenesis was used to improve the alkaline and thermal stability of the xylanase (XynA) from *Thermomyces lanuginosus*. Error-prone PCR reactions were carried out; the PCR products were cloned into *Escherichia coli* and a library of 960 clones was selected on xylan-containing agar plates. The crude filtrates of positive xylanase producers were screened at 80 °C and tested separately at pH 10 for alkaline tolerance. The native XynA lost 80% activity after 90 min at 80 °C and lost 70% activity at pH 10. Conversely, the most thermostable variant, G41, retained 75% activity after 90 min at 80 °C and the best alkali-stable variant, G53, retained 93% activity at pH 10. Sequence analysis revealed four amino acid substitutions in G41 and a single substitution in G53. These variants, therefore, have improved thermal and alkaline stability and are ideal candidates for DNA shuffling experiments to produce a robust xylanase for industrial application.

Introduction

Directed evolution has proved to be useful in enhancing enzyme performance in non-natural environments. The method mimics the process of Darwinian evolution in a test tube, combining random mutagenesis and, sometimes, recombination with screening or selection for enzyme variants that have the desired properties (Arnold & Volkov, 1998; Johannes & Zhao, 2006). A significant advantage of this approach over rational design methods is that structural information is not required to guide the directed evolution experiment. It also allows the rapid and simultaneous exploration of sequence, structure and function space and thus provides a powerful tool to address biochemical questions involving protein stability and function (Kuchner & Arnold, 1997). The success of this strategy depends on the size, quality and diversity of the libraries and, crucially, on the sensitivity, efficiency and discriminatory power of the screening technique available (Fernandez-Gacio *et al.*, 2003; Turner, 2003).

An important step in a directed evolution experiment is to efficiently explore sequence space through random mutagenesis. Among random mutagenesis methods, error-prone PCR methods, based on the inaccurate amplification of genes, have been very successful and are generally used in directed evolution experiments due to their simplicity and versatility (Wong *et al.*, 2004).

Xylanase pretreatment of paper pulps is an attractive alternative to traditional methods of pulping that generate large amounts of chlorinated organic byproducts, which are harmful to the environment (Pokhrel & Viraraghavan, 2004). Xylanase treatment is carried out after the initial alkali extraction of the pulp at high temperatures; therefore, thermostable and alkalophilic xylanases are highly desirable (Raghukumar *et al.*, 2004), and have been the subject of intensive research. *Thermomyces lanuginosus* DSM 5826 produces high levels of cellulase-free, thermostable xylanase, which is catalytically active over a broad pH range (Singh *et al.*, 2003), which made it an attractive candidate for random mutagenesis. The objective of this study was to create xylanase variants tolerant to alkaline and thermophilic conditions for eventual application in the pulp and paper industry.

Materials and methods

Plasmids, strains and growth conditions

xynA from *T. lanuginosus* DSM 5826 was cloned from a genomic library into pBluescript (Schlacher *et al.*, 1996),

and the resulting plasmid was named pX3. Extraneous fungal DNA upstream and downstream of *xynA* and the *lacZ* fusion partner was removed by PCR using newly designed primers containing appropriate restriction sites. After PCR, cleavage and recloning, the new plasmid was called pX4. *Escherichia coli* XL1 Blue MRF' (Stratagene) served as the host organism and was grown overnight at 37 °C on Luria–Bertani (LB) medium containing ampicillin (100 μg mL^{-1}).

Random mutagenesis

Various conditions, including those from published research (Xu *et al*., 1999; Chen *et al*., 2001; Matsumura & Ellington, 2001) as well as a commercial mutagenesis kit (Diversify Random Mutagenesis Kit, Clontech), were used for mutagenesis of *xynA* and are presented in Table 1. The plasmid pX4 served as the template DNA for all mutagenic conditions tested. Standard T3 and T7 primers (Integrated DNA Technologies) were used for all reactions. The amount of template DNA (10 ng) and the primers (200 ng) were kept constant in all PCR reactions in a total volume of 50 μL. PCR was carried out in a PCR Genius thermocycler (Techne) and using the programs listed in Table 1. The PCR product (786 bp) was isolated from 0.8% agarose gels and purified using a GFX PCR DNA and Gel Band Purification Kit (Amersham). The purified PCR products were digested with PstI and XhoI and ligated into the similarly digested pBluescript vector (Stratagene) and subsequently transformed into competent *E. coli* XL1 Blue MRF' (Stratagene) cells using standard molecular biology procedures (Sambrook *et al*., 1989). Transformation mixtures were plated onto 0.4% RBB-xylan LB plates containing 100 μg mL^{-1} ampicillin and incubated overnight at 37 °C.

Table 1. Mutagenic PCR conditions used for amplification of *xynA*

	Concentration (mM)			PCR		
Condition	Mg^{2+}	Mn^{2+}	dNTPs	Programme	No. of cycles	References
A (control)	1.5	–	0.1	94 °C – 1 min 42 °C – 1 min 72 °C – 2 min	35	–
B	4.8	0.5	0.2 AG 0.8 CT			Matsumura & Ellington (2001)
C	1.5	–	0.04 AG 0.2 CT			Chen *et al*. (2001)
DI*	2	0.04	0.02 AG 0.2 CT	94 °C – 1 min 46 °C – 1 min 72 °C – 2 min	20	Xu *et al*. (1999)
DII		–	0.02 AG 0.2 CT 0.04 dITP		30	
E†	3.5	–	0.04 G	94 °C – 1 min 46 °C – 30 s 68 °C – 1 min	25	Diversify kit
F†		0.64	0.04 G			
G†		0.64	0.2 G			
H† (Control)		–	0.2 AGCT			

*Two microlitres of unpurified PCR product of DI was used as template for DII.

†Diversify Random Mutagenesis Kit (Clontech) conditions. Special Diversify dNTP mix with unspecified concentrations was used for conditions E, F and G.

Growth of mutants and enzyme extraction

All clones exhibiting xylanase activity were inoculated into 5 mL LB broth containing 100 μg mL^{-1} ampicillin and incubated for 12–16 h at 37 °C in a shaking incubator at 170 r.p.m. One hundred and fifty microlitres of these overnight cultures were placed in sterile 1.5-mL eppendorf tubes containing an equal amount of sterile 30% glycerol and were stored at −20 °C to create the variant library. The rest of the culture was centrifuged at 5000 ***g*** and the media was discarded. The pellets were resuspended in 500 μL Bugbuster Protein Extraction Reagent (Novagen) and treated as described by the manufacturer. The lysates were then centrifuged at 15 000 ***g*** at 4 °C and the resulting pellets were discarded while the supernatants were stored at 4 °C for further analysis.

Screening for thermostable and alkali-stable xylanase variants

The clear lysate obtained after enzyme extraction contained the crude enzyme and was used to test the thermostability of the xylanase variants, which were analysed in duplicate. The protocol followed was a combination of the methods used by Giver *et al*. (1998) and Matsuura *et al*. (1999). A temperature of 80 °C was chosen for screening possible thermostable xylanase variants as XynA is documented to be stable up to 70 °C (Schlacher *et al*., 1996). Before incubation at 80 °C in a water bath, 0-min (untreated) samples were removed from the clear cell lysates and placed on ice. The crude enzymes were subsequently heated for 40 min at 80 °C, chilled on ice for 15 min and incubated for 30 min at room temperature to prevent low-temperature denaturation of the enzymes. The samples were centrifuged and the supernatants were assayed for residual xylanase activity (Bailey *et al*., 1992). Activities of the 0-min samples were considered as 100%, and enzyme activities for the 40-min incubation time were expressed as percentages of the untreated sample to determine the percentage residual activity after heat treatment. The wild-type XynA served as the control.

Screening for alkali-stable variants was conducted using a petri plate assay by creating wells in petri plates containing 0.1% birchwood xylan (Roth) and 1% agarose in 0.05 M glycine–NaOH buffer (pH 10). Ten microlitres of each crude enzyme was dispensed into the wells. Each screening plate also contained the XynA control. The plates were incubated at 60 °C for 2 h and treated as described previously (Teather & Wood, 1982; Béguin, 1990). Variants displaying zones

larger or more distinct than XynA were selected and the corresponding clones were grown and lysed as described earlier. Supernatants were diluted in 0.05 M glycine–NaOH buffer (pH 10) and incubated in a 60 °C water bath and residual activities of the enzymes were determined.

Mutants displaying >60% residual activity after heat and alkaline treatments were inoculated into 5 mL LB broth containing 100 μg mL^{-1} ampicillin and incubated overnight. One millilitre of this culture was used to inoculate 300 mL LB medium containing 100 μg mL^{-1} ampicillin and shaken at 37 °C until the $OD_{600\,nm}$ values reached 0.5 absorbance units. The cells were induced for xylanase production by adding 1 mM isopropylthiogalactoside to the flasks and incubated at 37 °C for a further 24 h. Thereafter, the samples were centrifuged at 5000 ***g*** and the media were discarded. Each 50-mL pellet was resuspended in 2 mL of cold lysis solution, which comprised 150 U DNAse I (Roche Molecular Biochemicals), 2 g lysozyme (Roche Molecular Biochemicals) in 120 mL breaking buffer solution (6.80 g L^{-1} KH_2PO_4; 0.61 g L^{-1} $MgCl_2.6H_2O$; · 0.77 g L^{-1} dithiothreitol; 0.37 g L^{-1} EDTA, pH 6.8; 0.10 g L^{-1} phenylmethylsulphonylfluoride was added after autoclaving). The suspensions were left at 4 °C overnight and then centrifuged at 15 000 ***g***, and the supernatants containing the enzymes were stored at 4 °C until further use.

For determination of long-term thermal and alkaline stability, samples were incubated for 90 min at 80 °C and pH 10, respectively. Samples were removed every 15 min, incubated on ice, and then assayed for residual xylanase activity.

Sequence analysis of selected *xynA* variants

DNA sequencing was carried out to identify the mutations that were responsible for the observed changes in the stabilities of some xylanase variants. Automated sequencing of *xynA* variants was performed by Inqaba Biotechnical Industries (Pretoria, South Africa) using a Genetic Analysis System SCE2410 with 24 capillaries (SpectruMedix LLC). Chain terminating sequence reactions were performed using the BigDye® Terminator version 3.1 sequencing kit (Applied Biosystems). All sequencing reactions were performed according to the manufacturer's instructions, and preliminary analyses were performed using the BASESPECTRUM version 2.1.1 software (SpectruMedix).

The raw DNA-sequencing data were initially processed using the CHROMAS LITE software package (Technelysium, version 2.0) and both DNA strands were edited to yield complete gene sequences. The DNA sequences were then translated into their protein counterparts using the Translate tool from the ExPASy website (http://www.expasy.org/tools/dna.html) and aligned to the wild-type parent using the CLUSTALW (version 1.81) alignment program on the GenomeNet server (http://www.clustalw.genome.ad.jp).

Results and discussion

Screening for thermostable xylanase variants

Nine hundred and sixty positive xylanase producers were obtained after random mutagenesis, cloning and transformation. In this study, the library size was kept small as the effective size of a library is not the number of mutants reported or screened, but rather the number of unique mutants screened (Drummond *et al.*, 2005).

The variants that were amplified under all control or standard conditions displayed thermal and alkaline stability profiles similar to XynA. Of the 960 clones that were screened for improved thermal stability, only a small percentage displayed significant changes in this regard. Condition E (Table 1) generated the highest number of variants with improvements in their catalytic activities (90%), although a large majority (84%) remained unchanged in terms of their stability. Conditions C and F were found to be the least effective mutagenic conditions because the variants exhibited a 75% and 64% loss in activity with a concomitant 63% and 50% reduction in thermal stability, respectively. Condition D, however, generated the most thermostable mutants as 11% of the progeny retained >60% of their activity after heat treatment for 40 min. These variants, however, lost up to 90% of their catalytic activities.

Ten variants that had >60% remaining activity after heat treatment for 40 min were regrown and exposed to 80 °C for 90 min. Six of the 10 mutants exposed to 80 °C for 90 min displayed >60% remaining activity after 90 min, and three of their profiles are shown in Fig. 1. XynA lost almost 80% of its

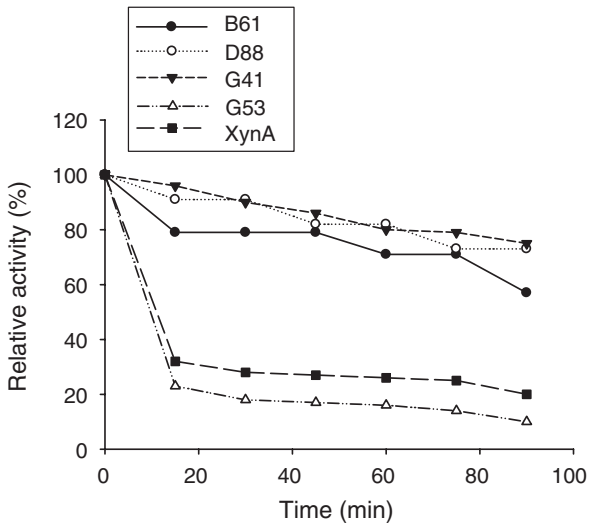

Fig. 1. Effect of temperature on the stability of wild-type XynA and its mutant progeny at 80 °C and pH 6.5. The remaining activities were expressed as percentages of the original activities, with each point representative of duplicate determinations.

activity under these conditions. Mutant G53 displayed an even lower stability and retained only 10% of its activity. The most stable mutant generated by initial random mutagenesis of *xynA* was G41, which displayed 75% retention of its total activity after 90 min of heat treatment. Mutant D88 had thermal stability similar to G41, but its activity was lower, while mutant B61 retained almost 65% of its activity. In general, all thermostable mutants displayed poor activity.

These findings agree with those of a previous work where improvements in thermal stability have deleteriously affected catalytic activity (Schoichet *et al.*, 1995; Palackal *et al.*, 2004; Fenel *et al.*, 2006; Stephens *et al*, 2007). It seems that reduced flexibility is a necessary consequence of thermostabilization. Often either thermal stability or catalytic activity is impaired and it is indeed rare, but not impossible, to improve both properties simultaneously in a single enzyme. In a landmark study conducted on the laboratory evolution of the *p*-nitrobenzyl esterase from *Bacillus subtilis*, there was coevolution of both activity as well as thermostability in this enzyme (Giver *et al.*, 1998).

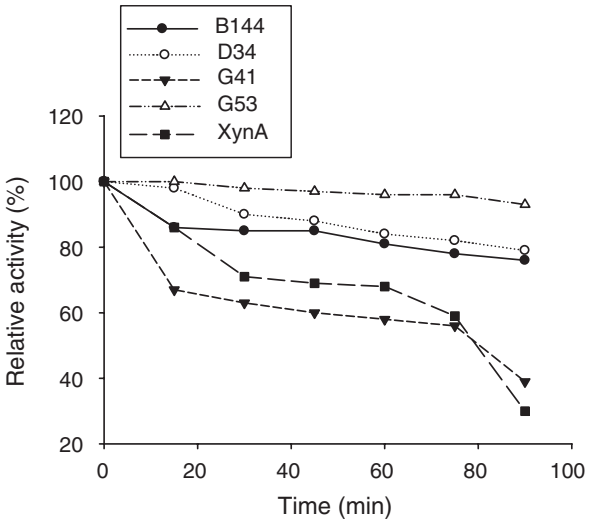

Fig. 2. Effect of alkalinity on the stability of wild-type XynA and its mutant progeny at pH 10 and 60 °C. The remaining activities were expressed as percentages of the original activities, with each point representative of duplicate determinations.

Screening for alkali-stable xylanase variants

The alkaline plate screen effectively eliminated xylanases with inferior or equivalent stability to XynA. However, a highly active xylanase could produce a large zone of hydrolysis, that could be easily misconstrued for superior stability. Thus, a liquid assay at pH 10 for 40 min resolved this problem. Only 62 mutant clones with significantly larger zones of hydrolysis than XynA were selected for further enzyme assay testing. It was observed that most of the 62 clones had activity similar to XynA, with only 24 showing improvements in catalytic activity and 23 clones showing >60% activity at pH 10 after 40 min at 60 °C.

Seven of the best clones were selected for long-term analysis. Six of the seven variants tested retained >60% of their activity after 90 min at pH 10. The alkaline stability profiles of four of these variants are depicted in Fig. 2. XynA lost 70% of its activity while B144 and D34 retained 76% and 79% of their activity, respectively. Mutant G41 lost nearly 60% of its activity under these extremely alkaline conditions. The most remarkable alkaline-stable mutant in this study however, was G53, which retained 93% of its activity after a similar treatment. Most alkaline-stable mutants did not exhibit a significant decline in catalytic activity.

Thirty-nine percent of the mutants displayed better activity than XynA while 34% had >60% remaining activity after 40 min of alkaline treatment. XynA retained 30% of its activity after 90 min at pH 10 while mutant G53 lost only 7% activity under the same conditions (Fig. 2). G41, on the other hand, had only 40% activity after treatment under identical conditions. A noteworthy observation was that the catalytic activity of the more alkaline-stable variants was comparable with XynA, which is in complete contrast to the observations regarding thermal stability in this study. Previous studies on the alkaline stability of other xylanases showed similar findings (Chen *et al.*, 2001; Turunen *et al.*, 2002; Palackal *et al.*, 2004). It appears that structural adaptation for temperature stability exerts a greater effect of lowering the catalytic activity compared with alkaline stability adaptation.

Sequence analysis

Sequence alignments revealed that the mutations were scattered throughout the protein, but concentrated in the regions forming β-sheet B, with >50% of the total number of substitutions occurring in sheet A, which forms the hydrophilic, solvent-accessible part of *xynA* (Gruber *et al.*, 1998). A previous study of G/11 xylanases indicated that this long β-sheet is responsible for thermostability, because it stabilizes the overall xylanase structure (Hakulinen *et al.*, 2003). Alkali-stable mutant G53 has a single amino acid substitution (A54T) while thermostable mutant G41 has four such substitutions (K30E, W40R, T57A and K80R). These mutations are depicted in Fig. 3 and for these two variants, the mutations occurred within the first 80 amino acids of the protein.

The A54T mutation in G53 may have increased the polarity of XynA and allowed it to exhibit great stability at pH 10 (Fig. 2). Site-directed mutagenesis of the *Trichoderma reesei* endoglucanase, where threonine replaced asparagine, resulted in a shift of the pH optimum to the alkaline range (Wang *et al.*, 2005). A clear conclusion that can drawn from the other work is that very large stability differences, in some

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xynA    MVGFTPVALAALAATGALAFPAGNATELEKRQTTPNSEGWHDGYYYSWWSDGGAQATYTN  60
G53     MVGFTPVALAALAATGALAFPAGNATELEKRQTTPNSEGWHDGYYYSWWSDGGTQATYTN  60
G41     MVGFTPVALAALAATGALAFPAGNATELERRQTTPNSEGRHDGYYYSWWSDGGAQAAYTN  60
        ****************************:********* ************:**:***

xynA    LEGGTYEISWGDGGNLVGGKGWNPGLNARAIHFEGVYQPNGNSYLAVYGWTRNPLVEYYI  120
G53     LEGGTYEISWGDGGNLVGGKGWNPGLNARAIHFEGVYQPNGNSYLAVYGWTRNPLVEYYI  120
G41     LEGGTYEISWGDGGNLVGGRGWNPGLNARAIHFEGVYQPNGNSYLAVYGWTRNPLVEYYI  120
        ******************:*****************************************

xynA    VENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGT  180
G53     VENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGT  180
G41     VENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGT  180
        ************************************************************

xynA    VQTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG  225
G53     VQTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG  225
G41     VQTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG  225
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Fig. 3. Amino acid sequence alignment of xylanase variants G41 and G53 with each other and with wild-type *xynA*. The alignment was performed by using the CLUSTALW (version 1.81) alignment programme on the GenomeNet server (http://www.ebi.ac.uk/clustalw). Alignment sequence characters are indicated as follows: *positions that have a single, fully conserved residue; ':' shows conservation within a strong group of amino acids. The absence of an alignment character implies that an unrelated amino acid was substituted. Amino acid substitutions that differ from *xynA* are indicated in boldface. Catalytic residues in the active site of *xynA* are shown in italics.

cases, are due to one or very few point mutations (Hasegawa *et al*., 1999; Williams *et al*., 1999; Sandgren *et al*., 2003).

Conversely, thermostable G41 has a sum total of four mutations, which cumulatively conferred considerable thermal stability to the enzyme (Fig. 2), albeit with poor catalytic activity. It is interesting that two of these mutations are arginine substitutions. A sequence and crystal structure alignment study showed that highly thermostable xylanases have an increased number of charged residues, especially arginine, resulting in enhanced polar interactions (Hakulinen *et al*., 2003). Lysine → arginine mutations in a number of enzymes lead to enhanced thermal stability (Mrabet *et al*., 1992). Shallowly buried arginines are likely to influence the overall electrostatic potential of the protein surface. Several studies indicate that there is a correlation between protein stability and the number of arginines on the protein surface. The comparison of mesophilic proteins and their thermophilic counterparts has revealed that thermophilic proteins have, on average, a higher arginine content on the protein surface (Argos *et al*., 1979; Vogt *et al*., 1997). A change in polarity might also contribute to the thermostabilization of these variant xylanases by rearrangement of interactions such as those with hydrogen bonds and/or salt bridges (Murashima *et al*., 2002).

These findings highlight that enzymatic properties often diverge from each other during evolution, with one property taking precedence over the other. After screening the entire mutant library, only mutant G41 was sufficiently thermostable while mutant G53 was extremely alkali tolerant. In contrast, G41 was found to have low alkaline stability at pH 10 and G53 displayed dismal thermal tolerance at 80 °C. G53 had catalytic activity (197 nkat mL^{-1}) almost comparable to XynA (216 nkat mL^{-1}), while G41 had a much lower activity (22 nkat mL^{-1}). If random mutagenesis were to continue, the properties would diverge even further from each other on the 'directed' evolutionary tree. The perfect way to prevent this would be to assemble these properties into a single xylanase using DNA shuffling or sexual recombination, which is the basis for future studies.

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