Journal of Industrial Microbiology & Biotechnology (2000) 25, 310–314

www.nature.com/jim

# Development of an *Escherichia coli* expression system and thermostability screening assay for libraries of mutant xylanase

R Ebanks[1], M Dupont, F Shareck, R Morosoli, D Kluepfel and C Dupont

*Centre de Microbiologie et Biotechnologie, INRS-Institut Armand-Frappier, Laval, Québec, Canada H7N 4Z3*

**A thermostability screening assay was developed using an *Escherichia coli* expression system to express *Streptomyces lividans* xylanase A (XlnA). The screening system was tested using mutants randomized at position 49 of the *S. lividans* XlnA gene, a position previously shown to confer thermostability with a I49P point mutation. The library was cloned into an *E. coli* expression vector and transformed into XL1-Blue bacteria. The resulting clones were screened for increased thermostability with respect to wild-type XlnA. Using this assay, we isolated the I49P mutant previously shown to be thermostable, as well as novel I49A and I49C mutants. The I49A and I49C mutants were shown to have 2.8- to 8-fold increase in thermostability over that of wild-type XlnA. The results show that the screening assay can selectively enrich for clones with increased thermostability and is suitable for screening small- to medium-sized libraries of 5000–20,000 clones.** *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 310–314.

**Keywords:** xylanase; thermostability; screening assay; mutagenesis; *Streptomyces lividans*

## Introduction

Xylanases (EC 3.2.1.8) catalyze the hydrolysis of the $\beta$-1,4-glycosidic bonds of xylan, the major cell wall polysaccharide component of hemicellulose. Xylanase A (XlnA) from mesophilic actinomycete *Streptomyces lividans* is a 47-kDa protein composed of two domains: a 33-kDa catalytic domain and a 14-kDa xylan-binding domain [6]. The catalytic domain of XlnA belongs to family 10 of the glycosyl hydrolases [3] and its three-dimensional structure shows that it folds into a $(\beta/\alpha)_8$ barrel protein [5].

Xylan-degrading enzymes have considerable commercial potential in several biotechnological applications, most notably their use in the bio- or pre-bleaching process in the pulp and paper industry [18]. A commercially viable xylanase must be stable at the high temperatures used in the Kraft pulping process (75–80°C). Xylanases from thermophilic organisms can function at these temperatures. However, no high level expression system is currently available for these enzymes. *S. lividans*, as a homologous expression system, is capable of very high yields of secreted protein [8]. Therefore, the project was undertaken to increase the thermostability of mesophilic *S. lividans* XlnA. This entailed the development of a screening system to select for variants of *S. lividans* XlnA with improved thermostability which is described in this report.

## Materials and methods

### *Construction Escherichia coli expression vector pIAF841*

The *E. coli* expression vector pIAF841 (Figure 1) was constructed from the *Hin*dIII/*Sac*I large fragment of pIAF 217 and WT-*xlnA*. The WT-*xlnA* gene (under control of the *lacZ* promoter) was engineered by polymerase chain reaction (PCR) to have the *E. coli* ribosomal binding site upstream of the starting methionine ATG. The WT-*xlnA* gene was amplified using the forward primer, 5′-GGGAAGCTTCAATTCTTGGAG-GAAAAAAGCATGCGCTCCTACGCCCTT-3′, which contains the *E. coli* sites for ribosomal binding, the *Hin*dIII, and a *Sph*I cloning site along with the reverse primer, 5′-ATATA-GAGCTCTCAGCCGTTGAGTGC-3′, which contains a stop codon following the codon of XlnA amino acid 302 and a *Sac*I cloning site. Following PCR, the amplified gene was verified to be correct by sequencing.

### *Mutagenesis*

Mutagenesis at the codon GAG, corresponding to position 49 of XlnA, was performed using the site-directed mutagenesis primer with overlap extension PCR [16]. The template used was the I49P-*xlnA* gene encoding the 302 amino acid catalytic domain (*Sph*I/*Sac*I fragment), cloned into the pTZ19U-based subcloning vector, pIAF 841. To minimize amplification associated errors, *Pfu*-Turbo polymerase (Stratagene, La Jolla, CA, USA) was used for all PCR amplifications. The PCR product was generated using the 5′-terminal primer (5′-GGCATGCGCTCC-TACGCCCTTCCCAGATCAGG-3′), which contains the *Sph*I restriction endonuclease cloning site, and the antisense primer, degenerate (bold characters) at position 49 (5′-CGGTTCGGT-GCCGTC(**G**/**C**)**NN**CCTCATCTCGTTCTCG-3′). The full-length *xlnA* gene was then amplified using the 5′ terminal primer and the 3′ terminal primer containing the *Sac*I restriction endonuclease cloning site and a stop codon following the codon of XlnA amino acid 302. The PCR products were purified using the QIAX II Gel Extraction Kit (Qiagen, Chatsworth, CA, USA) and the full-length gene, degenerate at codon position 49, digested with *Sph*I/*Sac*I restriction endonucleases and ligated back into the pIAF 841 vector. The ligated products were then transformed into *E. coli* strain XL1-Blue bacteria (Stratagene, La Jolla, CA, USA) and individual colonies

Correspondence: Dr C Dupont, Centre de Microbiologie et Biotechnologie, INRS-Institut Amand-Frappier, Laval, Québec, Canada HV7 1B7
[1]Present address: Dr. R Ebanks, National Research Council Canada, Institute for Marine Biosciences, Halifax, Nova Scotia, Canada, B3H 3Z1
Received 18 May 2000; accepted

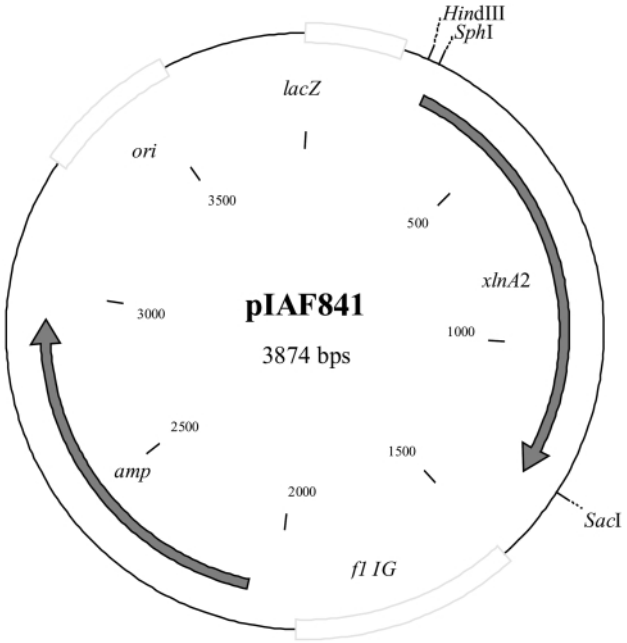


**Figure 1** Features of pIAF841 vector. Gene encoding *S. lividans* XlnA2 (*xlnA2*); gene encoding $\beta$-lactamase (*amp*); promotor *lacZ* (*lacZ*); *E. coli* origin of replication (*ori*); M13 phage origin of replication (*f1 IG*)

screened for XlnA thermostability. DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, CA, USA). The entire PCR-amplified region was sequenced to confirm the presence of the desired substitution, as well as the absence of additional mutations.

### *Thermostability screening assay*

The library of clones (randomized at codon 49) was ligated into pIAF 841 and transformed into *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA). The transformed *E. coli* was plated out onto 2X-TY plates containing 100 $\mu$g/ml ampicillin and 0.15% oat spelt xylan (Sigma Chemical, Oakville, Canada) coupled to Remazol Brilliant Blue (Sigma Chemical, Oakville, Canada) [2,10]. Xylanase activity was detected by the appearance of a clear zone around the colony. The colonies with xylanase activity were then picked onto master 2X-TY plates and into sterile Bio-Rad Titertubes in 96-well racks containing 0.5-ml liquid cultures (2X-TY broth containing 100 $\mu$g/ml ampicillin). The racks were covered with sterile Bio-Rad Titertube plugs (8-well strips) placed in a 37°C shaker and grown overnight. Cultures were used as source of xylanase for thermostability screening. Thermostability screening was carried out by transferring (using a multichannel pipetman) 50 $\mu$l of the *E. coli* cultures into duplicate 96-well Titertubes in racks with the bottom cut out to allow easy immersion in the water baths. Prior to starting the xylanase assay, 150 $\mu$l of 7 mg/ml birchwood xylan substrate in 50 mM citrate buffer, pH 6.0, was added, the tubes sealed with Titertube plugs, and incubated at 60°C and 70°C for 30 min. After the 30-min incubation, the racks were cooled in ice water and the released sugars were detected by addition of 500 $\mu$l of *p*-hydroxybenzoic acid hydrazide reagent [12]. The ratio of the xylanase activity at 70°C, divided by the activity at 60°C for the clones in comparison to that of WT-XlnA, was used as an indicator of increased thermostability.

### *Protein expression and purification*

For high-level protein expression, wild-type and mutant xylanase clones were subcloned (*Sph*I/*Sac*I) into the *Streptomyces* expression vector pIAF 20–109 [17] and transformed into *S. lividans* (strain IAF 10–164, xylanase- and cellulase-free) as described previously [9]. Four-hundred-milliliter cultures of wild-type and mutant strains were inoculated into shaker flasks which were incubated at 34°C, 250 rpm and grown for 72 h. After 72 h, the culture supernatants contained secreted xylanases which were 75–90% pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The cultures were then concentrated and fractionated by gel filtration chromatography on a Superdex HR75 beaded column (3×60 cm; Pharmacia, Baie d′Urfé, Canada) with 100 mM sodium phosphate, pH 7.0, as the eluant. The XlnA-containing fractions were collected, pooled, concentrated, dialyzed against acetate buffer, and freeze-dried. The protein concentration was determined using the modified Lowry assay [14], with bovine serum albumin (BSA; Bio-Rad, Mississauga, Canada) as the standard. Protein purity was determined by SDS-PAGE [11].

### *Enzyme assays and kinetic experiments*

Specific activity was determined by incubating 0.3 $\mu$g of enzyme for 10 min at 60°C with 4.5 mg/ml birchwood xylan (Sigma) in 50 mM citrate buffer, pH 6.0. The released reducing sugars were then detected using *p*-hydroxybenzoic acid hydrazide assay [12] adapted for microtiter plates.

Enzyme activities were expressed in international units (IU) where one unit represents the amount of enzyme releasing 1 $\mu$mol/min of reducing sugar using xylose as a standard. The $K_m$ values were determined by measuring the initial velocity of the enzymes at 60°C in 50 mM sodium citrate buffer, pH 6.0, using birchwood xylan at increasing concentrations from 0.04 to 4.5 mg/ml.

### *Thermal inactivation analysis*

Purified, wild-type I49A and I49C XlnA at 500 $\mu$g/ml protein were incubated in the absence of substrate at 60°C in 50 mM sodium citrate buffer, pH 6.0. At various times, samples were taken, put on ice, and the residual activity determined, as described. In all cases, the irreversible thermal inactivation of XlnA followed first-order kinetics.

### *Circular dichroism (CD) spectroscopy*

CD spectra were obtained using a Jasco J-710 spectropolarimeter interfaced with an IBM computer. Protein solutions of 300 $\mu$g/ml in 10 mM sodium phosphate buffer, pH 6.0, were analyzed at room temperature using a jacketed 0.05-cm quartz cell. Spectra were obtained by signal averaging of 10 spectra and at wavelengths ranging from 260 to 190 nm, at a scan rate of 100 nm/min. Thermal denaturation curves, as followed by CD, were obtained by heating the above protein solution from 45°C to 85°C (0.5°C/min) using a Neslab 110 programmable water bath. Ellipticity changes were followed at 210 nm at every 0.2°C. The denaturation CD data were then transformed into the fraction of unfolded protein, Fd, using the equation: $Fd=(X-X_n)/(X_d-X_n)$, where $X_n$ is the ellipticity value of the native protein, $X_d$ is the ellipticity value of the denatured protein and $X$ is the observed ellipticity [15]. $T_m$ is the

temperature at which 50% of the denatured fraction is observed and was obtained using described procedures [15].

## Results

### Test library

To develop a screening assay to identify mutant clones of *S. lividans* XlnA, which have increased resistance to thermal denaturation, a test library of mutant xylanase clones was used to evaluate if the screening assay could detect clones with increased thermotolerance. Previous unpublished work in this laboratory had identified a I49P mutation in *S. lividans* XlnA which resulted in a fourfold increase in thermostability. The test library was designed to explore if other amino acid substitutions, aside from proline, at position 49 of *S. lividans* XlnA lead to increased thermostability. The test library used an oligonucleotide which codes for all possible amino acids at position 49 of the protein as described in the Materials and Methods.

### Xylanase library screening

Two hundred eighty clones with xylanase activity (seen by plating *E. coli* transformed with the library onto 2X-TY agar plates with RBB-xylan substrate and grown at 37°C overnight) were screened for increased thermostability by comparing the ratio of residual activity at 70°C to that at 60°C. Cultures from *E. coli* transformed with either the blank pUC vector or pIAF 841 with WT-*xlnA* served as controls for the thermostability assay. All comparisons were based on the subtraction of the background absorbance (from the pUC control) from the absorbance of the WT-*xlnA* and the library clones at the respective temperatures. Initial experiments showed that the pUC control-transformed *E. coli* clones supernatants consistently showed background absorbance. Initial experiments also showed that in XlnA-transformed *E. coli*, more than 90% of the xylanase activity came from the cell pellet, which further suggests that the xylanase activity in the supernatant is the result of leaking or breakdown of the cells.

**Table 1** Residual xylanase activity of WT-XlnA and selected library clones along with sequencing results at codon 49

| Clone number | Assay | | | | Codon 49 | Amino acid 49 |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | | |
| WT | 37[a] | 74 | 41 | 55 | GAG[b] | Ile |
| 6 | 74 | 107 | – | – | CCC | Pro |
| 15 | 73 | 104 | – | – | CCC | Pro |
| 17 | 65 | 105 | – | – | CCC | Pro |
| 19 | – | – | 55 | 110 | TGC | Cys |
| 64 | – | – | 85 | 201 | CCA | Pro |
| 121 | – | – | 67 | 144 | GCC | Ala |
| 129 | – | – | 273 | 116 | CCG | Pro |
| 132 | – | – | 63 | 151 | TGC | Cys |
| 161 | – | – | 162 | 66 | CCC | Pro |
| 229 | – | – | 92 | 212 | GCC | Ala |
| 34[c] | – | 22 | – | 6 | TTG | Leu |
| 94[c] | – | 42 | – | – | CTC | Leu |

[a]Relative residual [(activity at 70°C/activity 60°C)×100].
[b]DNA sequence at codon position 49.
[c]Clones judged to be less stable than WT-XlnA.

**Table 2** Properties of wild-type and I49A/C mutant *S. lividans* XlnA

| XlnA | Specific activity (IU/mg protein) | $K_m$ (mg substrate/ml) | Half-life at 60°C[a] (min) | $T_m$[b] (°C) |
|---|---|---|---|---|
| WT | 286 | 0.45 | 23 | 67.2 |
| I49A | 204 | 0.36 | 62 | 69.4 |
| I49C | 178 | 0.30 | 169 | 68.1 |

[a]The half-lives of WT-XlnA and I49A/C mutant enzymes were determined from the first-order enzymatic inactivation rate constants at 60°C.
[b]Data from CD.

Of the 280 clones tested, all those which showed increased thermostability with respect to wild-type XlnA were retested in a second confirmatory assay. Ten clones, which were judged to be thermally more stable than the WT, and two that were not were then chosen and plasmid isolated and the insert DNA sequenced. Table 1 shows the ratio of the residual activity at 70°C to that at 60°C for WT-*xlnA* and these clones. The ratio of the activity at 70°C to that at 60°C for WT-XlnA and that of the mutant clones showed that the residual activity varied up to two- to threefold between assays. However, clones which showed increased thermostability, with respect to WT-XlnA, showed increased stability, irrespective of the variability between the different assays. Sequencing of the putative thermostable clones showed that 60% (6/10) encoded proline at position 49. The thermostability screening assay also selected I49C and I49A clones twice.

### Characterization of the I49A and I49C clones

The I49A and I49C clones were chosen for further kinetic and thermostability characterization. The I49A/C mutants were cloned

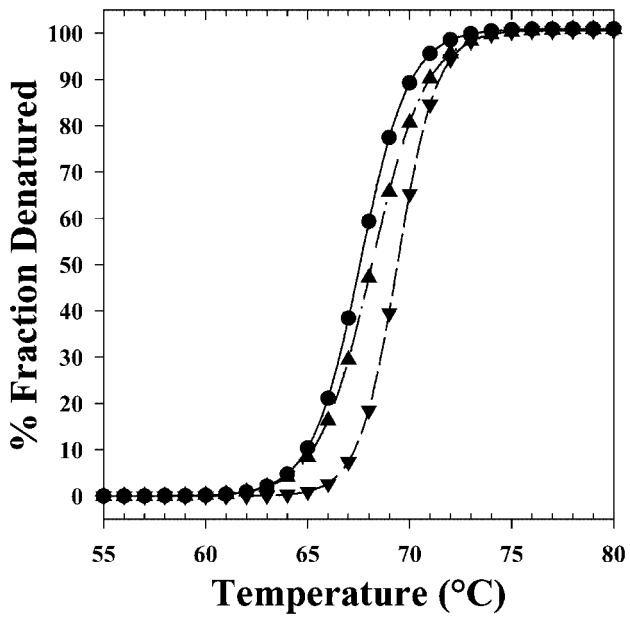


**Figure 2** Normalized thermal unfolding profiles of wild-type *S. lividans* XlnA (●), I49A (▲), and I49C (▼) mutants as monitored by CD spectroscopy. The data shown were all acquired with 300 μg/ml protein samples in 50 mM phosphate buffer, pH 6.0, from 45°C to 85°C at a heating rate of 0.5°C/min.

into the *Streptomyces* expression vector pIAF 20–109 and the resulting protein was purified to homogeneity. Table 2 shows the specific activities as well as the kinetic and thermostability properties of WT-XlnA and that of the XlnA-I49A and XlnA-I49C. Both the I49A and I49C mutants have specific activities only slightly lower than that of WT-XlnA. Under the conditions used, the specific activity should be a measure of the apparent $k_{cat}$, since a saturating concentration of substrate was used. The $K_m$ values of the mutants XlnA were also similar to that of WT-XlnA. The data suggest that the I49A and I49C mutations had minimal effects on the binding affinity to the substrate. With respect to enzymatic thermostability, the I49A and I49C mutant XlnA had half-lives at 60°C three and seven times longer than WT-XlnA. The thermostability of wild-type and mutants was also quantified as temperature shifts in the protein thermal denaturation curves by CD. The extent of denaturation was determined by measuring the change in ellipticity at 210 nm of the CD spectra as a function of temperature (Figure 2). The calculated midpoints of the thermal transitions ($T_m$) are also depicted in Table 2.

## Discussion

In this study, an assay was developed to screen for xylanase mutants with increased thermostability. The assay was tested on a xylanase mutant library with random mutations at position codon 49 in the gene encoding *S. lividans* XlnA. Since this assay selects for clones with increased thermostability, and I49P is significantly more stable than WT-XlnA, an enrichment in proline at position 49 in the isolated clones was expected. Sequencing of the clones with improved thermostability showed that 60% had proline at position 49. One trend seen in proteins isolated from thermophilic organisms is an increase in the proline content. Typically, the proline content is 40–50% higher in thermophilic proteins than in their mesophilic counterparts [4,19]. Conversely, the number of prolines in psychrophilic proteins is typically lower than that of their homologous mesophilic counterparts [1,7]. Substitution of prolines in mesophilic proteins has been shown to lead to increases in thermostability [13,19]. Clones with alanine and cysteine at position 49 (both at 20% prevalence) were also isolated using the assay. The fact, that only proline, alanine, and cysteine at position 49 were selected for in the assay and that clones not selected had sequences other than these three amino acids, suggests that only these contribute to thermostability at position 49 *S. lividans* XlnA. However, only 12 clones were sequenced, so it is possible that other substitutions at position 49 would contribute to thermostability. These results show that at position 49 of XlnA, other amino acids aside from proline can contribute to thermostabilization of the molecule.

Purification and characterization of the I49A and I49C mutant xylanase proteins showed similar kinetic properties to WT-XlnA, but with a 2.8- and a 7-fold improvement of thermostability (half-life) over that of WT-XlnA, respectively. This improved enzymatic thermostability was confirmed by following the thermal denaturation by circular dichroism, which showed a 2.2°C and 0.9°C rise in the temperature at which 50% of the protein is denatured for I49A and I49C, respectively. However, the increase in the backbone unfolding temperature at which we have 50% unfolded ($T_m$) is contrary to what was expected. I49A has a 2.2°C increase in the $T_m$ with a threefold increase in enzymatic stability at 60°C, whereas I49C has only a 0.9°C increase in $T_m$ with a sevenfold increase in enzymatic stability at 60°C. This suggests that the backbone unfolding temperature is not linearly related to the enzymatic thermostability. The possibility exists that the free cysteine at position 49 could enable the formation of dimers of the I49C mutant since this position is on the surface of the protein. However, non-denaturating PAGE and gel filtration chromatography showed no evidence of dimers or higher-order polymers of I49C (data not shown).

The xylanase thermostability screening assay used an *E. coli* expression system as the source of the crude enzyme. The assay is capable of selecting for clones with increased thermostability. The number of potential clones screened per day can significantly increase by screening at only one temperature, in this case, screening at a temperature which totally inactivates the wild-type enzyme in, e.g., 30 min, and searching for clones which have residual enzymatic activity. This screening assay is, therefore, suitable for screening of small- to medium-sized libraries of up to 5000–20,000 clones.

## Acknowledgements

This work is supported by a grant from the Natural Science and Engineering Research Council of Canada and by a INRS post-doctoral fellowship to R.E.

## References

1 Aghajari N, G Feller, C Gerday and R Haser. 1998. Structures of the psychrophilic *Alteromonas haloplanctis* alpha-amylase give insights into cold adaptation at a molecular level. *Structure* 6: 1503–1516.
2 Biely P, D Mislovicova and R Toman. 1985. Sensitive detection of endo-1,4-β-glucanases and endo-1,4-β-xylanases in gels. *Anal Biochem* 144: 147–151.
3 Coutinho PM and Henrissat B. 1999. Carbohydrate-active Enzymes server at URL: http//afmb.cnrs-mrs.fr/~pedro/CAZY/db.html.
4 Delboni LF, SC Mande, F Rentier-Delrue, V Mainford, S Turley, FM Vellieux, JA Martial and WG Hol. 1995. Crystal structure of recombinant triosephosphate isomerase from *Bacillus stearothermophilus*. An analysis of the potential thermostability factors in six isomerases with known three-dimensional structures points to the importance of hydrophobic interactions. *Protein Sci* 4: 2594–2604.
5 Derewenda U, L Swenson, R Green, Y Wei, R Morosoli, F Shareck, D Kluepfel and ZS Derewenda. 1994. Crystal structure at 2.6 Å resolution of the *Streptomyces lividans* xylanase A, a member of the F family of β-1,4-D-glycanase. *J Biol Chem* 269: 20811–20814.
6 Dupont C, M Roberge, F Shareck, R Morosoli and D Kluepfel. 1998. Substrate-binding domains of glycanases from *Streptomyces lividans*: characterization of a new family of xylan-binding domains. *Biochem J* 330: 41–45.
7 Feller G and C Gerday. 1997. Psychrophilic enzymes: molecular basis of cold adaptation. *Cell Mol Life Sci* 53: 830–841.
8 Gilbert M, R Morosoli, F Shareck and D Kluepfel. 1995. Production and secretion of proteins by *Streptomyces*. *Crit Rev Boitechnol* 15: 13–39.
9 Hurtubise Y, F Shareck, D Kluepfel and R Morosoli. 1995. A cellulase/xylanase-negative mutant of *Streptomyces lividans* 1326 defective in cellobiose and xylobiose uptake is mutated in a gene encoding a protein homologous to ATP-binding proteins. *Mol Microbiol* 17: 367–377.
10 Kluepfel D. 1988. Screening of prokaryotes for cellulose- and hemicellulose-degrading enzymes. *Methods Enzymol* 160: 180–186.
11 Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227: 680–685.
12 Lever M, TA Walmsley, R Visser and S Ryde. 1984. Optimal conditions for 4-hydroxybenzoyl and 2-furoylhydrazine as reagents for the determination of carbohydrates, including ketosamines. *Anal Biochem* 139: 205–211.

13 Li Y, PJ Reilly and C Ford. 1997. Effect of introducing proline residues on the stability of *Aspergillus awamori*. *Protein Eng* 10: 1199–1204.

14 Lowry OH, NJ Rosebrough, AL Farr and RJ Randall. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275.

15 Pace CN, BA Shirley and JA Thompson. 1989. Measuring the conformational stability of a protein. In: Creighton TE (Ed.), Protein Structure: A Practical Approach. IRL Press, Oxford, UK, pp. 311–330.

16 Sarkar G and SS Sommer. 1990. The "megaprimer" method of site-directed mutagenesis. *BioTechniques* 8: 404–407.

17 Sulzenbacher G, F Shareck, R Morosoli, C Dupont and GJ Davies. 1997. The *Streptomyces lividans* family 12 endoglucanase: construction of the catalytic core, expression, and X-ray crystal structure at 1.75 Å resolution. *Biochemistry* 36: 16032–16039.

18 Viikari L, M Ranua, A Kantelinen, M Linko and J Sundquist. 1986. Bleaching with enzymes. Biotechnology in the pulp and paper industry. Proc. 3rd Int. Conf., Stockholm, Sweden, pp. 67–69.

19 Watanabe K, K Chishiro, K Kitamura and Y Suzuki. 1991. Proline residues responsible for thermostability occur with high frequency in the loop regions of an extremely thermostable oligo-1,6-glucosidase from *Bacillus thermoglucosidacius* KP1006. *J Biol Chem* 266: 24287–24294.