Journal of Biotechnology 141 (2009) 26–30

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Expression of an alkalo-tolerant fungal xylanase enhanced by directed evolution in *Pichia pastoris* and *Escherichia coli*

Nokuthula Peace Mchunu*, Suren Singh, Kugen Permaul

Department of Biotechnology and Food Technology, Durban University of Technology, 41/43 Centenary Road, P.O. Box 1334, Durban 4001, South Africa

ARTICLE INFO

Article history:
Received 7 December 2008
Received in revised form 20 February 2009
Accepted 26 February 2009

Keywords:
Xylanase
Directed evolution
Yeast expression
Pichia pastoris

ABSTRACT

The alkaline stability of the xylanase from *Thermomyces lanuginosus* was further improved by directed evolution using error-prone PCR mutagenesis. Positive clones were selected by their ability to produce zones of clearing on pH 9 and 12 xylan agar plates. Variant NC38 was able to withstand harsh alkaline conditions retaining 84% activity after exposure at pH 10 for 90 min at 60 °C, while the parent enzyme had 22% activity after 60 min. The alkaline stable variant NC38 was cloned into pBGP1 under the control *GAP* promoter and pET22b(+) for expression in *Pichia pastoris* and *Escherichia coli* BL21, respectively. Best extracellular expression of the recombinant xylanase was observed in *P. pastoris* (261.7 ± 0.61 U ml^{-1}) whereas intracellular activity was observed in *E. coli* (47.9 ± 0.28 U ml^{-1}) was low. Total activity obtained in *P. pastoris* was 545-fold higher than *E. coli*. The mutated alkaline stable xylanase from *P. pastoris* was secreted into the culture medium with little or no contamination by host proteins, which favours the application of this enzyme in the pulp and paper industry.

1. Introduction

Xylan represents more than 20–40% of plant biomass. Xylan is a complex molecule composed of β-1,4 linked xylose chains with branches containing arabinose and 4-*O*-methylgluconic acid. Although xylan requires the action of several enzymes for its complete degradation, β-1,4 endoxylanases play a major role in degradation of xylan by catalyzing the random hydrolysis of β-1,4 xylosidic linkages in xylan. Due to this ability, xylanases have potential application in pulp and paper industry (Buchert et al., 1994; Bajpai, 1999). Xylanases have also been added to animal feed to improve digestibility and better feed utilization, and used in baking and brewing industries to improve the quality of bread and to clarify juice (Biely, 1985).

Pre-treatment of paper pulp with xylanases (bio-bleaching) can enhance the efficiency of lignin extraction and so reduce the amount of chlorine that is required (Gomes et al., 1993). Because kraft bleaching employs high temperatures and alkaline conditions, the xylanases used for this application should be thermophilic and alkaline stable (Buchert et al., 1994; Bajpai, 1999). Potential usefulness of this enzyme in industry has spurred considerable research efforts towards producing more thermophilic and alkalophilic xylanases by screening for naturally-occurring xylanases. A second approach is to explore genetic engineering for producing a thermoalkalophilic xylanase. Researchers have used different approaches to improve proteins. 'Rational design' involves precise preconceived amino acid sequence changes, which are based on the knowledge of the protein structure, function and mechanism. This was demonstrated by production of an alkalophilic xylanase variant from *Neocallimastix patriciarum* (Chen et al., 2001) and improving the thermostability of a fungal peroxidase (Cherry et al., 1999). However, the relationship between structure and function is rarely available, making random mutagenesis an attractive option (Chen, 2001). By borrowing from the evolutionary algorithm of mutation and natural selection, directed evolution techniques have been developed which have allowed the generation of enzymes with greatly enhanced characteristics (Kuchner and Arnold, 1997).

Most of these genetic manipulations are done in *Escherichia coli*, which is ideal for over-expression of recombinant proteins, while *Pichia pastoris* is able to express and secrete large amounts of heterologous proteins. This coupled with the ability to perform complex posttranslational modifications and the ability to grow to high densities, makes this methylotrophic yeast a powerful tool for expression and production of recombinant proteins (Cereghino and Cregg, 2000).

In this paper, we report the use of the error-prone PCR technique to enhance the alkaline stability of the xylanase from the thermophilic filamentous fungus, *Thermomyces lanuginosus* DSM 5826. Many of the research studies previously reported focused on the improvement of thermostability of xylanases, whereas improvement of alkaline stability has received scant attention. Furthermore, *P. pastoris* was shown to be an appropriate host for high level expression of this xylanase.

* Corresponding author. Tel.: +27 31 373 5370; fax: +27 31 373 5351.
E-mail address: nokuthula@dut.ac.za (N.P. Mchunu).

0168-1656/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2009.02.021

2. Materials and methods

2.1. Growth and maintenance of cultures

E. coli D2 mutant was used as the starting point for error-prone PCR (Stephens et al., 2007; Schlacher et al., 1996). Luria-Bertani (LB) medium was used to grow *E. coli* cultures (XL1 Blue and BL21) in broth and agar plates at 37 °C. Ampicillin (100 μg ml^{-1}) was added to the medium to maintain the plasmids. The STET method, a variation of the boiling lysis method of Sambrook et al. (1989), was used to isolate plasmid DNA, with minor modifications to the original protocol. The plasmids pET22b(+) for *E. coli* BL21 strain (Novogen) and pBGP1 (Lee et al., 2005) for *P. pastoris* GS511 (Invitrogen) were used for expression. *P. pastoris* cultures were maintained on YPD medium (10 g l^{-1} yeast extract, 20 g l^{-1}, peptone and 20 g l^{-1}) containing 100 μg ml^{-1} of zeocin for plasmid maintenance.

2.2. Error-prone PCR, cloning and transformation

The xylanase gene was amplified and mutated by varying the concentration of dNTPs, $MgCl_2$ and addition of $MnCl_2$ during PCR (Chen et al., 2001). The PCR conditions that were used were as follows: 95 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min. The PCR reactions were carried out using a PCR Genius (Techne) thermal cycler for a total of 30 cycles. Vector pBSK and PCR products were restricted with endonucleases EcoRI and XhoI to create compatible sticky ends for ligation. DNA was then purified using the GFX PCR DNA and GEL Band Purification Kit (Amersham Biosciences). The purified gene was then ligated to pBSK. Ligated DNA was added to $CaCl_2$ competent *E. coli* cells, followed by heat shocking the cells. The cells were then incubated at 37 °C for 1 h in a shaking incubator. Aliquots were plated out on to Remazol Brilliant Blue-xylan LB medium containing 100 μg ml^{-1}of ampicillin and incubated at 37 °C overnight. Positive xylanase-producing colonies were selected by identification of a zone of hydrolysis around the colonies (Beguin, 1982).

2.3. Screening for mutants that produce pH tolerant xylanases

Positive mutants were detected by their ability to produce a zone of hydrolysis on acid and alkaline RBB-xylan plates. Potential mutants were replica plated on 0.1% xylan plates made with different buffers at pH 5 (citrate), pH 9 (Tris–HCl) and pH 12 (glycine–NaOH) at 50 °C overnight. Those clones that exhibited larger zones than the control were further tested at pH 4–12. The mutants were grown overnight in LB broth which was followed by enzyme extraction using a breaking buffer (6.8 g l^{-1} KH_2PO_4, 0.61 g l^{-1} $MgCl_2{\cdot}6H_2O$, 0.77 g l^{-1} DTT, 0.37 g l^{-1} EDTA – pH 6.8 followed by addition of PMSF after autoclaving) and sonication using the following conditions: 1 min pulsing followed by incubation on ice for 1 min for 3 cycles with frequency of 20 kHz. After extraction, the enzyme was diluted buffers ranging from pH 3 to 9 and incubated at 50 °C for 30 min, then assayed for residual activity (Chen et al., 2001; Bailey et al., 1992). Long term stability of the enzyme at alkaline pH and thermostabilty was tested by incubating the enzyme at 60 °C at pH 10, for 90 min (Bailey et al., 1992).

2.4. Construction of expression clones and transformation

The xylanase gene variant showing alkaline stability (NC38) was amplified using cycling conditions similar to error-prone PCR. Plasmids pET22b(+), pBGP1 and PCR products were restricted with endonucleases EcoRI and XhoI. DNA was then purified from agarose gels followed by ligation. Ligated DNA was added to $CaCl_2$ competent *E. coli* cells, followed by heat shocking the cells. Electrocompetent *P. pastoris* cells were transformed in 0.2 cm cuvettes using preset electroporation conditions (1.5 kV, 200 Ω, 25 μF). Aliquots were plated out onto RBB-xylan LB medium with ampicillin (100 μg ml^{-1}) for *E. coli* and RBB-xylan YPD medium with zeocin (100 μg ml^{-1}) for *P. pastoris*. Transformants were identified by a zone of hydrolysis around the colonies.

2.5. Xylanase expression and extraction

Single colonies were used to inoculate 20 ml YPD medium with zeocin for *P. pastoris* and LB with ampicillin for *E. coli*. Subsequently, cultures were inoculated into 200 ml liquid media for xylanase production. Expression in *P. pastoris* was terminated after 60 h and in *E. coli* after 8 h, which included 4 h of induction with 1 mM IPTG. Enzyme extraction in *E. coli* was carried out using Bugbuster Protein Extraction Reagent (Merck Biosciences). This was followed by centrifugation at 10,000 × *g* for 30 min at 4 °C. The supernatant containing the enzyme was stored at 4 °C for further studies.

2.6. SDS-PAGE and zymogram analysis

The protein samples were analysed using SDS-PAGE (12.5% running gel and 5% stacking gel (Laemmli, 1970). The gel was allowed to run for 3 h at 85 V, followed by staining with Coomassie Brilliant Blue R250. To perform zymogram analysis, the procedure for SDS-PAGE was followed except that the gel was not stained. The proteins were renaturated in a solution containing 1% Triton X-100 and 20 mM Tris–HCl, pH 7 for 30 min and washed in 20 mM Tris–HCl solution for 1 h. The gel was then sandwiched with an agarose gel containing 0.3% xylan and incubated for 1 h at 50 °C. This was followed by staining with 1% Congo Red.

3. Results and discussion

3.1. Screening for alkaline stable mutants

In this study, directed evolution was employed in an attempt to enhance the alkaline tolerance of a xylanase from *T. lanuginosus* DSM 5826. This protein expressed by the wild type strain has high activity and a optimum pH of 6.5 (Singh et al., 2000). One round of error-prone PCR of the *xynA* gene yielded the xylanase variant D2 having high activity but poor thermostability (Stephens et al., 2007). D2 variant was used for further error-prone PCR and 35 mutants were obtained which exhibited a larger zone of hydrolysis than the parent strain on xylan plates at pH 5, 9 and 12. Two variants from *E. coli*, NC38 and NC39 had a larger zones than the other 35 mutants, were selected for pH profiling. Surprisingly, the pH optima of the xylanases expressed by the two mutant genes was pH 5 compared to pH 6 for the parent enzyme, D2, but both variants maintained greater activity in the alkaline pH range, exhibiting 43% of its maximum activity at pH 9 (Fig. 1).

3.2. Alkaline stability

The NC38 xylanase was able to withstand harsh alkaline conditions, retaining 95% of its activity at pH 10 after 45 min and 84% after 90 min at 60 °C. In contrast, the parent lost 70% of its maximal activity after 45 min and only 22% enzyme activity was retained after 1 h (Fig. 2). On the other hand, the NC39 xylanase was less alkalo-tolerant than NC38 but more tolerant than the parent. In some studies, a high stability and high activity have been negatively correlated (Stephens et al., 2007). The explanation most commonly offered for the trade-off between high catalytic activity and stability is that during natural evolution, enzyme structure has adjusted to optimise the balance between rigidity (for stability) and flexibility (for activity) at their physiological relevant temperature (Arnold