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PRODUCTION AND CHARACTERIZATION OF *ORPINOMYCES* MUTANT XYLANASES WITH IMPROVED TEMPERATURE AND pH STABILITIES

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ABSTRACT – The error-prone PCR technique has been widely used in order to obtain thermostable enzymes more suitable for industrial conditions. The *Orpinomyces xyn*A mutant library allowed the selection of four thermostable mutants (M1-M4). Molecular dynamics (MD) predicted an N-terminal tail as being a destabilizing structural region and allowed further enhancing of the mutant xylanases thermostability. Thus, removal of the 27 amino acid residues enabled an increase in the enzyme half-life values ($t_{1/2}$). However, besides the improved thermostability, the large enzyme production and high catalytic performance are also relevant for the biotechnological application of enzymes. During the mutant enzymes production in *E. coli*, the IPTG induction protocol allowed high expression levels of soluble and active xylanases. The mutant xylanases without the 27 amino acid residues showed improved thermostability and the shorter versions of M2 and M4 (named as SM2 and SM4) also presented a good performance in more extreme pH conditions.

1. INTRODUCTION

Xylanases are widely applied in the feed, paper and pulp, foodstuff and energy industries. Nowadays, due to the use of elevated temperatures in industrial processes, more effort is being put into the development of strategies to improve enzyme thermal stability. Among random mutagenesis methods, the error-prone PCR has been very successful (Wong *et al.*, 2004).

The native *Orpinomyces* GH11 xylanase has been most efficient at pH 5.0-5.5 and 50-55 °C (Li *et al.*, 1997). In a previous work, error-prone PCR was used to improve the thermostability of this xylanase (Trevizano *et al.*, 2012). Four mutant xylanases (M1-M4) displayed higher thermostability compared to the wild type (WT) XynA. However, these enzymes expressed in *E. coli* showed low activity, from 0.78 to 38.8 $U.mg^{-1}$ (Trevizano *et al.*, 2012), compared to the native XynA, which has a specific activity of 3,500 $U.mg^{-1}$ (Li *et al.*, 2007).

In order to combine stability and activity of these xylanases, both crucial parameters for industrial application, MD approaches were taken to evaluate tridimensional structures of mutant and wild type xylanases and to identify regions that could affect protein stability and

activity. The shorter versions of the xylanases were cloned in pET24b and expressed in *E.coli* BL21 (DE3) RIPL, a bacterial strain more appropriate for recombinant protein expression. Then, these enzymes were characterized evaluating kinetic and biochemical parameters.

2. METHODS

2.1. Xylanase Molecular Dynamics

The Swiss-Model modeling tools (http://swissmodel.expasy.org) were used to obtain a 3D model of WT and the mutant xylanases, and the structures were subjected to MD simulation. The complete xylanase sequences were simulated. Additionally, xylanase sequences without the first 27 residues were used. The simulations were performed with the GROMACS 4.0.7 package using the GROMOS 43A1 force field.

2.2. PCR Amplifications and Cloning

A pair of oligonucleotides, XF1 and XR1, was synthesized for performing the amplification of genes encoding the xylanases WT, M1, M2 and M4. XF1 and XR1 were used to amplify the region between amino acid residues 28 and 255. The pET24b and *E. coli* BL21(DE3) were used for the protein expression.

2.3. Xylanases Expression, Purification and Activity Determination

The *E. coli* BL21(DE3) cultures were induced using IPTG. The cell mass was sonicated and the intracellular xylanase preparations were centrifuged, where the supernatant was used as the crude enzyme extract. This enzyme fraction was loaded in a Q-Sepharose ion exchange column and the active fractions were analyzed by SDS-PAGE. Xylanase activity was determined from xylan hydrolysis by quantification of reducing sugar (Miller, 1959). The protein concentration was determined by Bradford (1976).

2.4. Biochemical and Kinetic Characterization

Influences of temperature and pH on xylanase activity were determined according to a standard assay, using several temperature and pH values, and thermal stability was also evaluated. The xylanase Michaelis-Menten constant (K_M) for birchwood xylan hydrolysis were calculated. Kcat and Kcat/K_M were also calculated to evaluate the catalytic efficiency of the enzymes.

3. RESULTS

A molecular dynamics protocol was applied as a strategy to further enhance the thermostability of mutant xylanases from *Orpinomyces*, previously obtained by directed evolution (Trevizano *et al.*, 2012). This analysis revealed that the N-terminal region of this xylanases is formed by β-sheets and it seems to interact weakly with the remaining global structure. Figure 1 shows the xylanase 3D structure emphasizing the N-terminal destabilizing region.

The MD simulations showed that the distance between the glutamate catalytic residues is basically the same for both structures, with and without the tail of 27 amino acid residues.

This result suggests that there are not considerable structural changes to the active site which probably ensures the catalytic performance of the enzymes. The radius of gyration suggests that the small enzyme has a more compact structure that could have implications on protein global stability. In order to improve the xylanase thermal stability, the 27 amino acid residues from the N-terminal region of the xylanases WT, M1, M2 and M4 were removed, generating smaller xylanases (SWT, SM1, SM2 and SM4).

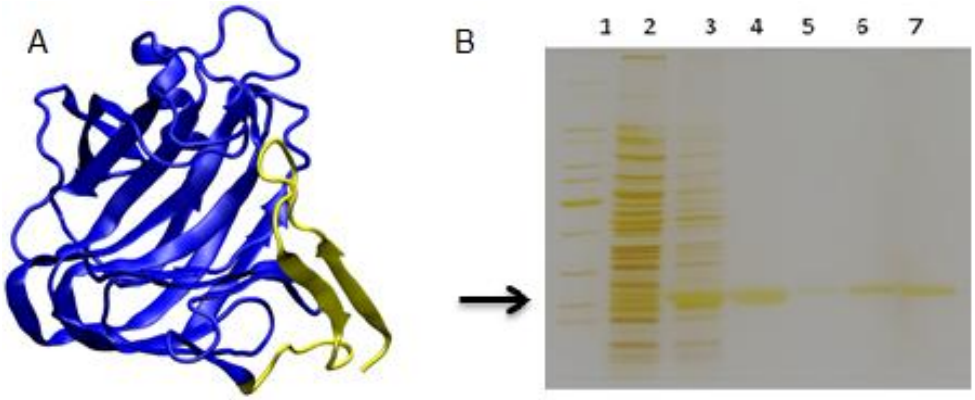

Figure 1: A - Structure of the xylanase catalytic domain. The β-sheets that could affect the xylanase stability in the N-terminal region are shown in yellow. B - SDS-PAGE 12,5 %. 1- Molecular weight marker, 2- Uninduced crude extract, 3- Induced crude extract, 4-7 purified SWT, SM1, SM2 e SM4, respectively. The arrow indicates the band related to the 25 kDa xylanase.

Each selected xylanase showed different mutations. The substitutions introduced by error-prone PCR in each xylanase were: Q13R, D28G, N53S, H148R, E158G in SM1, V108A, A199T in SM2, and T135A in SM4. Specific activity values (pH 6.5; 40 °C) of the enzymes SWT, SM1, SM2 and SM4 produced by *E. coli* cultivated in the presence of the IPTG were 1,991.1, 1,178.2, 3,393.7 and 963.7 $U.mg^{-1}$, respectively.

Mutant xylanases without the 27 amino acid residues presented higher half-life ($t_{1/2}$) values at 60 °C compared to that of the SWT (Table 1). Figure 2 shows the effects of temperature and pH on xylanase activity. It is interesting to emphasize that SM2 and SM4 are active at alkaline pH conditions. $Kcat/K_M$ is a parameter that evaluates the catalytic efficiency of the enzymes, indicating that they exhibited similar performances.

Table 1 - Enzyme half-life at 60 °C ($t_{½}$) and kinetic properties of xylanases against birchwood xylan (*thermostability evaluated at 65 ºC)

Enzymes	$t_{1/2}$ (hours)	K_M ($mg.mL^{-1}$)	Kcat (min^{-1})	$Kcat/K_M$ ($mL.min^{-1}.mg^{-1}$)
SWT	0.29	0.94	6.6×10^5	7.0×10^5
SM1	42.50*	0.16	4.8×10^5	3.0×10^6
SM2	5.47	0.90	5.0×10^5	5.5×10^5
SM4	0.56	1.20	3.3×10^5	2.8×10^5

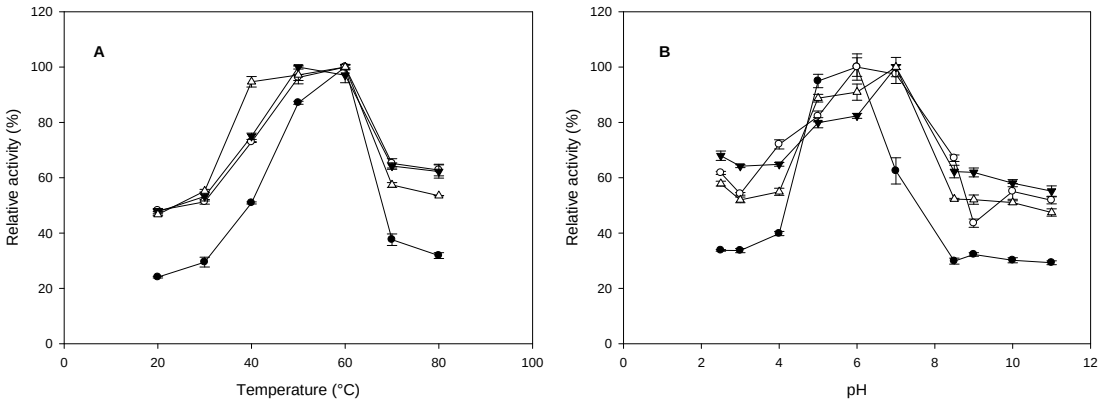

Figure 2: A - Effect of temperature on xylanase activity at pH 6.5, B - Effect of pH on xylanase activity at 40 °C. SM1 (●), SM2 (○), SM4 (▼), SWT (Δ).

4. CONCLUSIONS

The molecular dynamics simulations allowed studies from the structural changes responsible for the increased thermostability of xylanases. The induction protocol allowed for production of xylanases presenting solubility and high levels of activity without the tail of 27 amino acid residues.

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