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Research Paper

Development of an improved variant of GH51 α-L-arabinofuranosidase from *Pleurotus ostreatus* by directed evolution

Simona Giacobbe[1], Florence Vincent[2] and Vincenza Faraco[1]

[1] Department of Chemical Sciences, University of Naples "Federico II", Complesso Universitario Monte S. Angelo, via Cintia, 4 80126 Naples, Italy
[2] CNRS, UMR7257, Aix-Marseille Universite, 163 Avenue de Luminy, Case 932, 13288 Marseille cedex 09, France

In this study, the α-L-arabinofuranosidase from *Pleurotus ostreatus* was subjected to directed evolution by expressing a library of around 7000 randomly mutated variants by error prone Polymerase Chain Reaction. High-throughput screening of the library for the most active variants was performed by assaying activity towards p-nitrophenyl α-L-arabinofuranoside, and a variant with higher activity than the wild type was selected, purified and characterised. It exhibited a k_{cat} of $7.3 \times 10^3 \pm 0.3$ min^{-1}, around 3-fold higher than that of the wild type ($2.2 \times 10^3 \pm 0.2$ min^{-1}), and a K_M (0.54 ± 0.06 mM) 30% lower than that of the wild type (0.70 ± 0.05 mM) towards this substrate. The mutant also showed improved catalytic properties towards pNP-β-D-glucopyranoside (k_{cat} of 50.85 ± 0.21 min^{-1} versus 11.0 ± 0.6 min^{-1}) and it was shown able to hydrolyse larch arabinogalactan which is not recognised by the wild type. The mutant was also more active than the wild type towards arabinoxylan and was able to hydrolyse arabinan, which was not transformed by the wild type. The ability of rPoAbf F435Y/Y446F to hydrolyse these insoluble substrates expands its potential for application also to hemicelluloses, which in some types of pretreatment are recovered in solid fractions.

Introduction

The high cost of enzymes is one of the major economic bottlenecks for implementation of economically feasible lignocellulosic biorefineries [1]. A finely planned cocktail of enzymes, consisting not only of cellulases but also of xylanases, is required to achieve the highest yields of fermentable sugars from the polysaccharide components of lignocellulosic biomass. In particular, α-L-arabinofuranosidase has been detected as one of the crucial components for a more complete hydrolysis of the complex hemicellulolse matrix in pretreated biomass [2–4]. α-L-Arabinofuranosidases work synergistically with other enzymes, such as hemicellulases and pectinases, for the complete degradation of hemicelluloses and pectins, respectively [5–8]. In fact, Gao *et al.* [2] demonstrated that in the absence of specific activities, such as β-xylosidase, α-arabinofuranosidase and α-glucuronidase, it was not possible to achieve high xylose hydrolysis yields.

In a previous study, we reported on the identification of a new α-L-arabinofuranosidase produced by the fungus *Pleurotus ostreatus* (PoAbf) and the cloning and sequencing of the corresponding gene and cDNA [9]. Heterologous recombinant expression of PoAbf was carried out in the yeast *Pichia pastoris* and the recombinant enzyme (rPoAbf) was purified and characterised. rPoAbf is highly specific for α-L-arabinofuranosyl linkages and shows a robust enzymatic activity at pH 5 ($t_{1/2}$ = 51 days). This led us to adopt this hemicellulase as a scaffold for directed evolution experiments to improve its catalytic properties.

In this study, a library of around 7000 random mutants of rPoAbf was generated by error prone PCR of *poabf* cDNA and recombinant expression of the mutants in *Saccharomyces cerevisiae*. Screening for extracellular enzymatic activity towards p-nitrophenyl α-L-arabinofuranoside led to the selection of an improved enzyme variant. This mutant was overproduced by recombinant expression in *Pichia pastoris*, purified and characterised showing an improvement of its catalytic properties in comparison to the wild type.

Corresponding author: Faraco, V. (vfaraco@unina.it)

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Materials and methods

Strains and culture media

The *Escherichia coli* strain used in DNA manipulations and conditions for its growth are reported in [9]. The *S. cerevisiae* strain and pSAL4 plasmid used for heterologous expression of the random mutants' library were kindly donated by Dr. T. Tron (CNRS UMR 6517, Marseille, France) and are reported in [10]. *S. cerevisiae* was grown on selective medium (SD) as described [10]. To overproduce the selected random mutant, the 'Easy Select Pichia Expression kit' (Invitrogen, Carlsbad, CA, USA) was used for expression in *P. pastoris* strain X33 with the vector pPICZB following manufacturer's instructions and using the growth conditions reported [9].

Vectors

The EMBL Data Library accession number of the sequence of *P. ostreatus* arabinofuranosidase [9] reported in this paper is HE565356. The vector pSAL4-poabf used for recombinant expression of PoAbf in *S. cerevisiae* was prepared by cloning *poabf* cDNA in pSAL4 (in *Eco*RI/*Hind*III) under control of copper-inducible CUP1 promoter [10]. The vector pPICZ-abf used for recombinant expression of PoAbf in *P. pastoris* had been previously prepared cloning *poabf* cDNA in pPICZB (in *Eco*RI/*Xba*I) under control of methanol-inducible *AOX1* promoter [9]. It was used as template for site-directed mutagenesis experiments (below) to prepare the vector for recombinant expression of the selected random mutant PoAbf (F435Y/Y446F) in *P. pastoris*.

Random mutagenesis

Random mutagenesis of *poabf* cDNA was performed by Error Prone PCR (EP-PCR), using pSAL4-poabf as template and Gene Morph II Random Mutagenesis Kit (Agilent, La Jolla, CA). Primers, pSal4Fw (CCAACGCAATATGGATTGTCAG) and pSal4Rev (CAAGTGTAGCGGTCACGCTGCG) used in amplification experiments are complementary to the both ends of polylinker pSAL4 sequence. Cycling parameters were 30 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 2 min.

Construction of a mutant library

cDNAs resulting from EP-PCR were ligated in pSAL4 vector digested with *Eco*RI/*Hind*III restriction enzymes, exploiting homologous recombination expression system of *S. cerevisiae*. Yeast transformation and selections was performed following the protocols reported in [10].

Library screening

Agar plate assays on SD medium supplemented with the chromogenic substrate 4-methylumbelliferyl-a-L-arabinofuranoside (MUA) (Carbosynth, Compton, Berkshire, UK) were used to select transformants expressing α-L-arabinofuranosidase activity as described previously [9]. High-throughput screening was performed in 96-well plates, assaying α-L-arabinofuranosidase activity with p-nitrophenyl α-L-arabinofuranoside (pNPA) as substrate following the method reported in [9] with some modifications. The assay mixture contained 70 μL of the substrate solution (2 mM pNPA in 50 mM sodium phosphate buffer pH 6.5) and 70 μL of appropriately diluted enzyme solution. After incubation at 30 °C for 10 min, the reaction was stopped by the addition of 150 μL of 1.6 M Na_2CO_3. The liberated p-nitrophenol in the mixture was measured by spectrophotometer at 405 nm (ε = 17,000 M^{-1} cm^{-1}) after vigorous agitation. Cultures in shaken flasks were also performed for selected mutants as described [10]. Optical density and arabinofuranosidase activity were daily assayed.

DNA sequencing

Sequencing by dideoxy chain-termination method was performed by the Primm sequencing Service (Naples, Italy) using specific oligonucleotide primers.

Arabinofuranosidase activity assays

α-L-Arabinofuranosidase activity was measured spectrophotometrically with p-nitrophenyl α-L-arabinofuranoside (pNPA) (Gold Biotechnology, St Louis, MO, USA) as substrate as described in [9]. One unit of α-L-arabinofuranosidase activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per minute in the reaction mixture under these assay conditions.

Site-directed mutagenesis for recombinant expression of selected random mutant in P. pastoris

The pPICZ-abf containing the cDNA encoding for PoAbf was used for recombinant expression in *P. pastoris* as previously reported [9]. Site-directed mutagenesis was performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to introduce the desired mutations F435Y/Y446F, identified in the selected random mutants, and obtain the vector pPICZB-$abf_{F435Y/Y446F}$. The mutagenic primers for the mutant gene are the following (mutated nucleotides underlined and bold): Fw Y435F: CAGAACTCGTTCTTCT**T**CGATGATATGCCACGC; Fw F446Y: AATGGAGTCACCTTCT**A**CGAGGGTGAATATGC.

The mutated gene was confirmed by sequencing and the wild type and mutated proteins were overexpressed, purified and assayed for their activity on p-nitrophenyl α-L-arabinofuranoside (pNPA) (Gold Biotechnology, St Louis, MO, USA) as previously described [9].

P. pastoris transformation, cultivation and enzyme production

The recombinant plasmid pPICZB–abf and pPICZB-$abf_{F435Y/Y446F}$ were linearised by *Pme*I hydrolysis and introduced into *P. pastoris* strain GS115 by electroporation. The electroporation mixture was plated onto YPD–zeocin agar medium. After 72 h at 28°C, transformant colonies were screened for activity production on plates containing the chromogenic substrate MUA.

PoAbf purification

Proteins secreted by *P. pastoris* expressing rPoAbf and by *P. pastoris* expressing rPoAbf-F435Y/Y446F were purified following the protocol in [9].

Protein concentration determination

Protein concentration was determined by the Lowry method [11], using the BioRad Protein Assay (BioRad Laboratories S.r.l., Segrate, MI – Italy), with bovine serum albumin as standard.

Temperature optimum and temperature stability

The optimum temperature (among 30, 40, 50 and 60°C) and the thermo-resistance (at 40, 50 and 60°C) of the purified enzymes was determined as in [9].

Experiments were performed in duplicate and reported values are the average of three experiments.

pH optimum and pH stability

The optimum pH (among the pH values between 3.0 and 7.0) and pH stability (at pH 3–8 at 25°C) of the purified enzymes was determined as in [9]. Experiments were performed in duplicate and reported values are the average of three experiments.

Enzyme specificity assays

Activity of wild type and mutated rPoAbf was assayed against the substrates *p*NP-α-L-arabinofuranoside (Carbosynth, Berkshire, UK), *p*NP-β-D-xylopyranoside (Carbosynth, Berkshire, UK), *p*NP-α-D-glucopyranoside (Carbosynth, Berkshire, UK), *p*NP-β-D-glucopyranoside (Carbosynth, Berkshire, UK) and *o*NP-β-D-galactopyranoside (Carbosynth, Berkshire, UK) at concentrations in the range 0.1–6 mM in citrate phosphate buffer pH 5. Activity against the natural substrates CM-linear arabinan and larch arabinogalactan, and the arabinooligosaccharides 1,5-α-arabinotriose and 1,5-α-arabinohexaose (Megazyme International Ireland, Co., Wicklow, Ireland) was assayed as described in [9]. Activity against AZO-wheat arabinoxylan (Megazyme International Ireland, Co., Wicklow, Ireland) was assayed following supplier's instructions.

Endo-1,4-beta-D-xylanase and endo-1,5-α-L-arabinanase activity were determined against the insoluble substrates azurine-crosslinked (AZCL) wheat arabinoxylan (Megazyme International Ireland, Co., Wicklow, Ireland) and azurine-crosslinked (AZCL) arabinan (Debranched) (Megazyme International Ireland, Co., Wicklow, Ireland) respectively. 60 mU (measured on pNPA) of PoAbf wild type and PoAbf F435Y/Y446F were incubated with 1% (w/v) wheat arabinoxylan in 100 mM sodium phosphate buffer pH 6 or 1% (w/v) arabinan (Debranched) in 100 mM sodium acetate buffer pH 4.7 at 40°C for 5, 10 and 15 min. Reducing sugar content was measured by a modified Somogyi reducing sugar assay following the manufacturer's instructions. Reactions were terminated following the manufacturer's instructions and the absorbance was measured at 520 nm. One Unit of enzyme activity is the amount required to release one μmole of reducing sugar equivalents.

Experiments were performed in duplicate and reported values are the average of three experiments.

Determination of k_{cat} and K_M

For determination of the Michaelis-Menten constants K_M and k_{cat}, the activity assay was performed at substrate concentrations from 0.1 mM to 10 mM *p*NPA and from 0.1 mM to 16 mM *p*NP-β-D-glucopyranoside at pH 5, performing incubations of 10 min at 40°C. The experiments were performed in triplicate and reported values are the average of three experiments.

Results and discussion

Construction and screening of mutant PoAbf libraries

To develop improved variants of *P. ostreatus* α-L-arabinofuranosidase PoAbf, it was subjected to directed evolution experiments. Random mutations were introduced into *poabf* cDNAs by EP-PCR. *S. cerevisiae* was chosen as the host for recombinant expression of the variants, being a more appropriate platform for directed evolution than *Pichia pastoris* [9], due to integrative phenomena taking place in the latter host. Two different PCR conditions were used to yield medium (MMF) and high (HMF) mutation frequency (4.5–9 mut/kbases and 9–16 mut/kbases, respectively). Mutated cDNAs were introduced into the expression vector pSAL4 using the homologous recombination system of *S. cerevisiae*. A total of 6400 transformants, 3300 and 3100 for MMF and HMF respectively, were obtained by *S. cerevisiae* transformation with pSAL4 vector and mutated cDNAs. The libraries were subjected to a selection designed to identify mutants exhibiting higher arabinofuranosidase activity than the wild type PoAbf. The first screening, performed on MUA-containing plates, to select only transformants expressing extracellular α-L-arabinofuranosidase activity, led to selection of 2100 mutants (1100 with MMF and 1000 with HMF), identified under UV light by the presence of fluorescence around the colonies after incubation at 28°C for 3 days. High-throughput screening of these active 2100 mutants in multiwell plates allowed the selection of 24 mutants, exhibiting at least 7-fold higher activity values towards pNPA than the wild type. Further screening was performed in shaken flasks monitoring time course of growth and activity production of the 24 clones, for 4 days (data not shown). Among the 24 mutants, four clones (H4B4, H4C4, H5C4, H13G9) exhibiting 2-fold higher activity values than the wild type at 48 h, were chosen for further analyses.

Characterisation of mutated and wild type PoAbf

Sequence analysis showed that all four selected clones have the same amino acid substitutions: F435Y/Y446F. A total of 509, 595, 592, and 562 nucleotide substitutions were identified in H4B4, H4C4, H5C4 and H13G9 respectively. In order to characterise the F435Y/Y446F mutant, it was expressed in *P. pastoris*, which had been shown to give higher rPoAbf expression levels than *S. cerevisiae* [9], and time course of production of arabinofuranosidase specific activity was investigated at 20 and 28°C in comparison to that of wild type (data not shown). These temperatures were chosen as typical for recombinant expression in *P. pastoris* [9]. It was found that the specific enzymatic activity of rPoAbfF 435Y/Y446F towards pNPA was two-fold higher than that of wild type at both 20 (36.8 ± 1.8 versus 16.7 ± 0.0 mU/OD) and 28°C (8.0 ± 0.3 versus 4.8 ± 0.4 mU/OD).

The recombinant PoAbf F435Y/Y446F was purified to apparent homogeneity (Fig. 1) and structurally and functionally characterised in comparison to the recombinant PoAbf wild type protein. The electrophoretic mobility of the mutant in a SDS-PAGE is similar to that of the wild type (Fig. 1). The catalytic properties of the mutants were determined towards the substrate pNPA and compared to those of the wild type. The mutant F435Y/Y446F showed a k_{cat} of $7.3 \times 10^3 \pm 0.3$ min^{-1}, around 3-fold higher than that of the wild type $2.2 \times 10^3 \pm 0.2$ min^{-1}. The K_M of rPoAbf F435Y/Y446F towards pNPA was lower (0.54 ± 0.06 mM) than that of the wild type (0.70 ± 0.05 mM).

In order to determine the enzymatic properties of the selected mutant rPoAbf F435Y/Y446F, a panel of experiments was performed to define the conditions and the field of its activity. The effect of pH and temperature on the activity of rPoAbf F435Y/Y446F towards pNPA (Fig. 2) was investigated in order to establish the optimal reaction conditions. Thermoresistance (Fig. 3) and pH resistance (Fig. 4) of the mutant were also determined and compared to the wild type, enzyme stability being a crucial property for applications.

FIGURE 1

SDS-PAGE profiling of the purified rPoAbf wild type and rPoAbf F435Y/Y446F expressed in *P. pastoris*. Lane 1: protein molecular weight marker; lane 2: purified rPoAbf; lane 3: purified rPoAbf F435Y/Y446F.

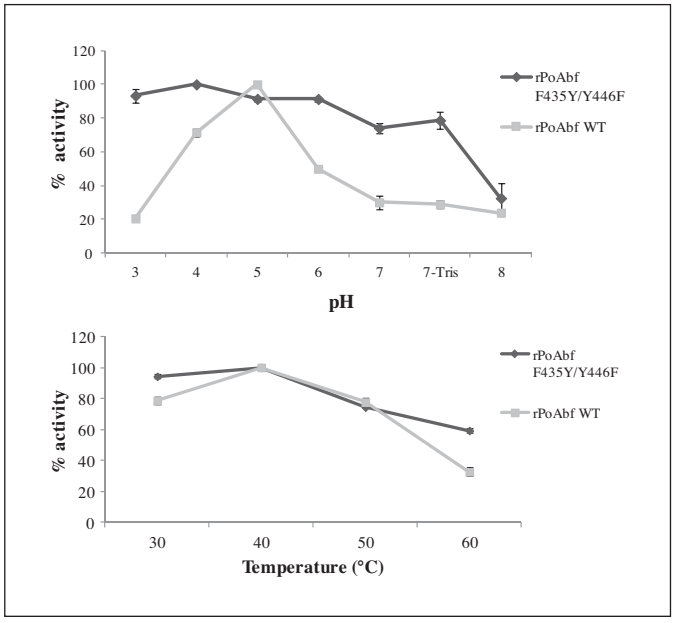

FIGURE 2

Effect of (a) pH and (b) temperature on activity of rPoAbf wild type (rPoAbf WT) and rPoAbfF435Y/Y446F towards pNPA. To determine the optimum pH of the purified enzyme, the substrate of the activity assay (2 mM pNPA) was dissolved in citrate phosphate buffers with pH values between 3.0 and 7.0 and the incubation (10 min) was performed at 40°C. To determine the optimum temperature of the purified enzyme, the substrate of the activity assay (2 mM pNPA) was dissolved in 50 mM sodium phosphate buffer pH 6.5 and the incubation (10 min) was performed at 30°C, 40°C, 50°C and 60°C. The percent activity reported in (a) and (b) was calculated as a ratio to the maximum activity at optimum pH (5) and temperature (40°C), respectively.

As regards the main significant improvements shown by the mutant, it exhibits a wide range of optimal pH values (3–6), differing from the wild type which shows an optimum at pH 5, and has 94% of maximum activity (exhibited like the wild type at 40°C) at 30°C. The selected mutant is therefore more versatile than the wild type, working over a larger range of operating conditions. Moreover, the mutant rPoAbf F435Y/Y446F was more thermoresistant than the wild type at all the tested temperatures (40, 50 and 60°C) and exhibited a pH resistance similar to, or higher than, that of the wild type. Thus, while the mutant F435Y/Y446F was selected for its higher activity towards pNPA, it also showed a higher resistance to temperature and pH than wild type. These observations further increase the applicative potential of the selected mutant.

To investigate the substrate specificity of the selected mutant, the hydrolytic ability of rPoAbf F435Y/Y446F was tested against a series of other nitrophenyl glycosides in comparison to the wild type, namely pNP-β-D-xylopyranoside, pNP-α-D-glucopyranoside, pNP-β-D-glucopyranoside and oNP-β-D-galactopyranoside, in order to assess whether the enzyme is able to hydrolyse other types of glycosidic bonds that are present in hemicellulose matrix. Among these compounds, only pNP-β-D-glucopyranoside was recognised by the mutant enzyme similarly to the wild type and a K_M of 3.55 ± 0.08 mM (wt: 3.76 ± 0.15 mM) and a k_{cat} of 50.8 ± 0.2 min^{-1} (wt: 11.0 ± 0.6 min^{-1}) were measured for this substrate.

Ability of the mutant to hydrolyse the arabinooligosaccharides 5-α-arabinotriose and 1,5-α-arabinohexaose were also analysed since these are intermediate products of hemicellulose hydrolysis, whose further hydrolysis is required during lignocellulose conversion to produce the fermentable pentose monosaccharides. The mutant was shown able to hydrolyse both the tested arabinooligosaccharides 1,5-α-arabinotriose and 1,5-α-arabinohexaose, with a slightly higher and similar activity than the wild type, respectively (Table 1).

rPoAbf F435Y/Y446F shows a similar activity to the wild type towards CM-linear arabinan. On the other hand, it is worth noting that rPoAbf F435Y/Y446F is able to hydrolyse larch arabinogalactan, which is not hydrolysed by the wild type as demonstrated by the assay (Table 1) in which less than the minimum detectable of arabinose (0.346 μg mL^{-1}) was released when performed with the wild type. Moreover, when the enzyme was incubated with the AZO-wheat arabinoxylan, it was shown to possess an endo-1,4-β-xylanase activity of 0.81 ± 0.03 U mL^{-1}, which was slightly higher to that of the wild type (0.63 ± 0.03 U mL^{-1}).

The ability of the mutant to hydrolyse the insoluble substrates arabinoxylan and arabinan was also analysed to verify its applicability to hemicelluloses which in some kinds of pretreatments are recovered in solid fractions. Towards AZCL-Arabinoxylan, the mutant exhibited an activity of 3.0 ± 0.2 mU mL^{-1}, two-fold higher than that of the wild type, and it was also able to hydrolyse

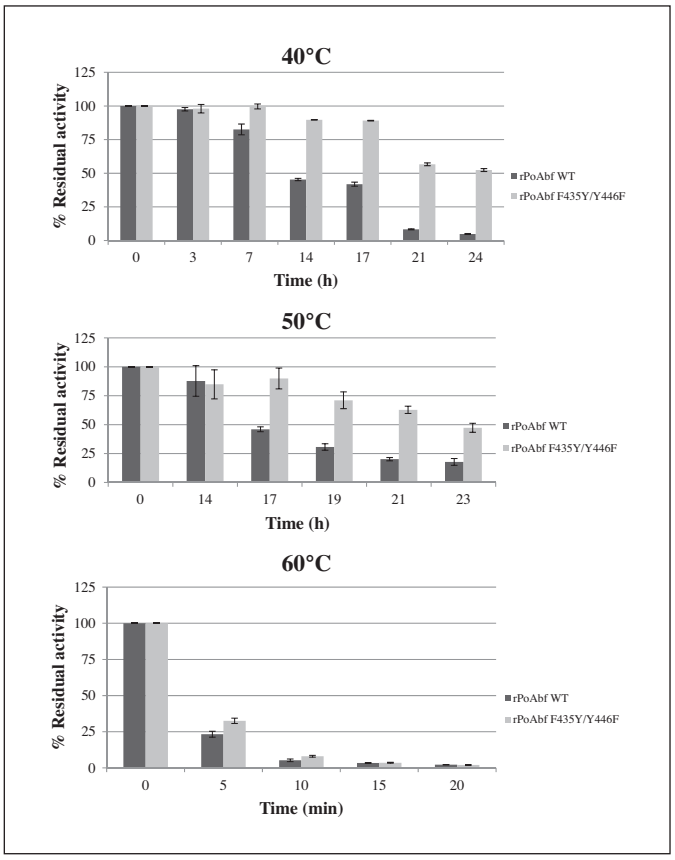

FIGURE 3

pH resistance of rPoAbf wild type (rPoAbf WT) and rPoAbf F435Y/Y446F. The pH resistance of the purified enzyme preparations was studied by dilution in citrate phosphate buffers, pH 3–8 and incubating at 25°C. At different times, samples were withdrawn and immediately assayed for residual α-arabinosidase activity performing incubation (10 min) at 40°C.

AZCL-Arabinan (debranched) showing an activity of 1.28 ± 0.05 mU mL^{-1}, whilst the wild type was unable to hydrolyse it, showing a negative (lower than the blank) absorbance. Hence, this work allowed isolation of an enzyme variant with higher arabinofuranosidase activity towards different substrates.

A 3D structure model of PoAbf was obtained by homology detection and structure prediction by HMM-HMM (Hidden Markov Models) comparison to localise the two mutations Y435F and F446Y (Fig. 5). Five 3D structures of GH51 family proteins are available: PDB: 1QW9 from *Geobacillus stearothermophilus* showing 8% sequence identity (id.) to PoAbf; PDB: 2C7F from *Clostridium thermocellum* (6% id.); PDB: 2VRQ from *Thermobacillus xylanilyticus* (14% id.); PDB: 2Y2W from *Bifidobacterium longum* (14% id.); and PDB: 3S2C from *Thermotoga petrophila* (14% id.). They have been used as templates to calculate a model for PoAbf, using HHpred and Modeller [12,13]. Although each template shares very low sequence identity with PoAbf, this enzyme is expected to adopt the same fold as the GH51 members, since all they belong to the same GH family, even though with distant homologies [14]. In the model, the key catalytic residues Glu acid/base and Glu nucleophile are conserved and superimpose well with the templates.

The structural model obtained for PoAbf suggests that the two mutations Y435F and F446Y should be located far from the active site and therefore no hypothesis regarding the role of these mutations on catalysis can be made. However, unlike the templates, the molecular surface of the model shows a channel starting from the bottom of the active site and crossing the protein. The wall of the channel creates a hydrophilic environment suggesting the presence of a water channel. Residue Y446 stands at the bottom of the central β-barrel and is close to the channel, such that the mutation of F446 into Y could impact on the water-relay inside this putative channel and would favour the release of water molecules in the active site.

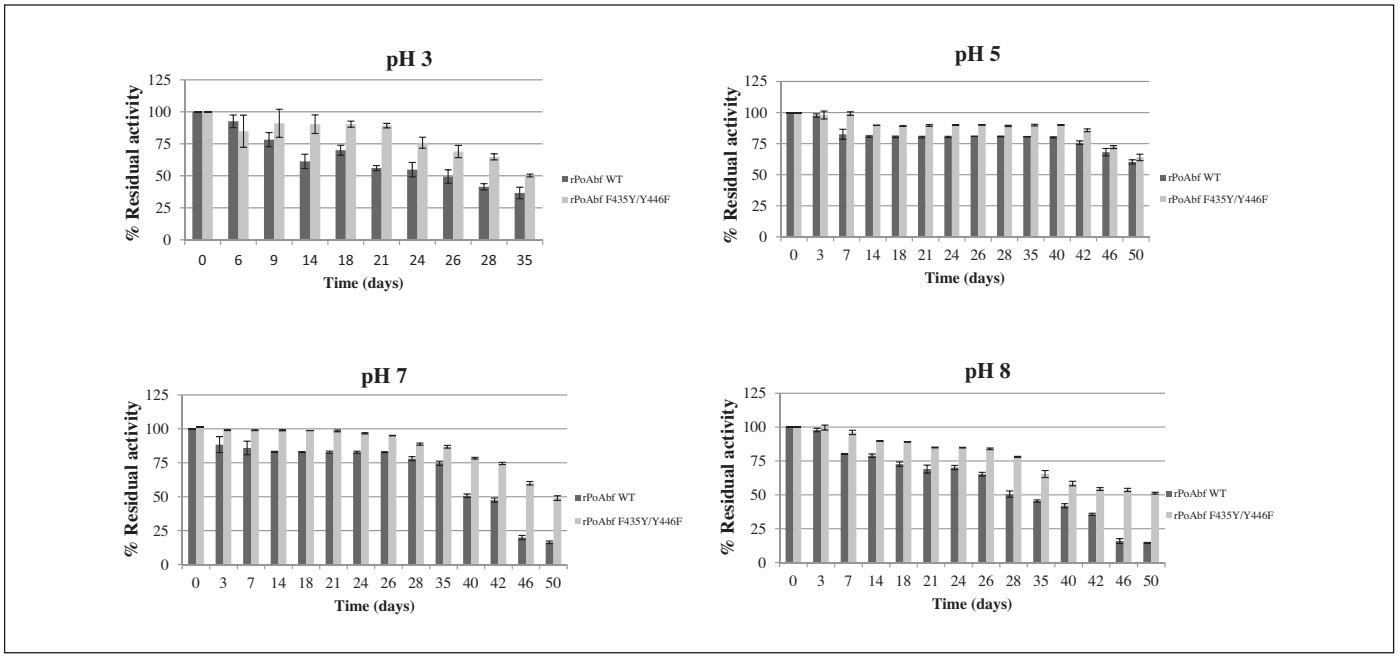

FIGURE 4

Thermo-resistance of rPoAbf wild type (rPoAbf WT) and rPoAbf F435Y/Y446F. The temperature resistance was studied by incubating the purified enzyme preparations in 50 mM citrate phosphate buffer pH 5.0, at 40°C, 50°C and 60°C. The samples withdrawn were assayed for residual α-arabinosidase activity performing incubation (10 min) at 40°C.

TABLE 1

Arabinose liberation from natural substrates and arabinooligosaccharides by rPoAbf wild-type and rPoAbf F435Y/Y446F. Activity against the natural substrates CM-linear arabinan and larch arabinogalactan, and the arabinooligosaccharides 1,5-α-arabinotriose and 1,5-α-arabinohexaose (Megazyme International Ireland, Co., Wicklow, Ireland) was assayed measuring the liberation of arabinose as equivalent of galactose, by means of the D-galactose/lactose kit (Megazyme International Ireland, Co., Wicklow, Ireland), following the manufacturer's instructions. 600 mU (measured on pNPA) of PoAbf were incubated with 0.2% arabinans in 100 mM sodium acetate buffer pH 4.6, at 37°C for 72 h (final volume, 300 μL). Arabinooligosaccharides were dissolved in 100 mM sodium acetate buffer pH 4.6 and incubated with 7 mU (measured on pNPA) of PoAbf at 37°C for 1 h (final volume 300 μL)

Substrate	Amount of released sugar (μg mL^{-1})	
	rPoAbf wild-type	rPoAbf F435Y/Y446F
Larch arabinogalactan	0	205.4 ± 13.8
CM-linear arabinan	240 ± 8	288.7 ± 4.6
Arabinotriose	112.7 ± 2.9	111 ± 4
Arabinohexaose	107.4 ± 8	132.06 ± 18.61

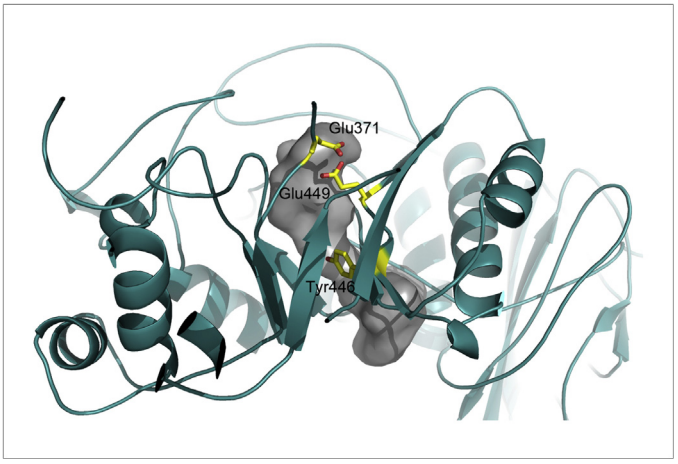

FIGURE 5

Cartoon and surface representation of the model of PoAbf. The conserved catalytic residues Glu371 and 449, as well as Tyr446, are shown in yellow. The channel crossing the modeled protein is represented with a blue arrow. The penta saccharide bound to the homolog from *Thermobacillus xylanilyticus* has been fitted into the active site of PoAbF and indicates the entrance of the channel.

Summary

A library of 6400 random mutants of the enzyme α-L-arabinofuranosidase from *Pleurotus ostreatus* was generated by error prone PCR of the *poabf* cDNA and expression of mutated cDNAs in *S. cerevisiae*. 24 variants exhibiting higher activity than the wild type enzyme towards p-nitrophenyl α-L-arabinofuranoside (pNPA) in multiwell microplates were selected and further analysed for production of extracellular α-L-arabinofuranosidase activity in shaken flasks. Four of the more active mutants were selected and their analysis showed the presence of the mutations F435Y/Y446F in all the variants. Characterisation of the mutant rPoAbf F435Y/Y446F over-expressed in *P. pastoris* showed a k_{cat} towards pNPA around 3-fold higher than that of the wild type. Moreover, analysis of the effect of pH and temperature on activity of rPoAbf F435Y/Y446F towards pNPA showed that it exhibits a wide range of optimal pH values (pH 3–6) differing from the wild type that shows an optimum pH of pH 5 and thus revealing more versatile than the parental enzyme. Furthermore, the hydrolytic abilities of rPoAbf F435Y/Y446F were shown to be improved towards other substrates. In fact, rPoAbf F435Y/Y446F shows a k_{cat} towards pNP-β-D-glucopyranoside around 5-fold higher than that of the wild type, and it is able to hydrolyse larch arabinogalactan which is not recognised by the wild type. The mutant was also more active than the wild type towards AZCL-arabinoxylan and was able to hydrolyse AZCL-arabinan (debranched) which was not transformed by the wild type.

Based on these results, the mutant rPoAbf F435Y/Y446F could be used as a template for new directed evolution rounds aimed at improving its ability to hydrolyse these substrates and increase its potential of application. Moreover, while the mutant rPoAbf F435Y/Y446F was selected for its higher activity, it showed also a higher resistance to temperature and pH than the wild type. Thus, the potential of *in vitro* evolution of the α-L-arabinofuranosidase PoAbf as a strategy to achieve improved variants of this hemicellulase was demonstrated. Conservative mutations were identified in the selected mutants indicating their importance for the evolutionary selection. To the best of our knowledge, this is the first manuscript of directed evolution of a α-L-arabinofuranosidase giving selection of enzyme variants with improved hydrolytic activity, the only other recently published work being aimed at improved transglycosidase activity [15].

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