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Potential of *Ophiostoma piceae* sterol esterase for biotechnologically relevant hydrolysis reactions

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The ascomycete *Ophiostoma piceae* produces a sterol esterase (OPE) with high affinity toward *p*-nitrophenol, glycerol, and sterol esters. Recently, this enzyme has been heterologously expressed in the methylotrophic yeast *Pichia pastoris* under the AOX1 methanol-inducible promoter (P_{AOX1}) using sorbitol as co-susbtrate, and the hydrolytic activity of the recombinant protein (OPE*) turned out to be improved from a kinetic point of view. In this study, we analyze the effects of sorbitol during the expression of OPE*, at first added as an additional carbon source, and methanol as inducer. The *O. piceae* enzyme was successfully used for PVAc hydrolysis, suggesting its potential applicability in recycled paper production to decrease stickies problems.

Keywords: sorbitol, methanol, *Pichia pastoris*, recombinant protein, polyvinyl acetate, stickies, recycled paper

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Introduction

The interest on biocatalysis, as an eco-friendly alternative to the traditional chemocatalysis, has grown significantly over the last decades. In most cases, its use is advantageous not only for allowing green processes, but also because enzymes can work efficiently under mild reaction conditions, displaying improved selectivity and specificity, and giving cleaner reactions as compared with chemical catalysts.

Esterases (EC 3.1) are defined for their ability to hydrolyze ester bonds and embrace, among others, lipases (EC 3.1.1.3) and sterol esterases (EC 3.1.1.13). The differences between both types of enzymes have been mainly based on the substrates they can transform and their mechanism of action, in which structural motifs have been studied. Lipases use primarily triglycerides or insoluble esters as substrates and catalyze the reactions at the organic phase-water interface, suffering an interfacial activation phenomenon which involves a structural domain called lid. Unlike the formers, sterol esterases hydrolyze easily sterol esters. In spite of this, the frontier between the two kinds of enzymes is not very clear, and several of them have been described showing the two activities.[1,2]

In general, these enzymes are 6.5 to 65 kDa proteins and many of them tend to aggregate giving pseudo-quaternary structures. All belong to the family of α/β hydrolases and share their main structural characteristics, having a highly conserved overall folding. The spatial arrangement of the loops that bear the catalytic triad, composed by the amino acids Ser (nucleophile), Asp/Glu, and His, is the best-conserved structural feature.[3] This reality contrasts with their different primary DNA sequences.

Most organisms synthesize esterases for their own metabolism, but those from microorganisms are the preferred source for biotechnological purposes. Some examples of these are the enzymes from the bacterium *Pseudomonas aeruginosa*,[4] the actinomycete *Streptomyces*,[5] the yeasts *Candida rugosa*,[1,6-8] *Candida antarctica*,[9] and *Geotrichum candidum*[10] or the filamentous fungi *Melanocarpus albomyces*[11] and *Trichoderma* sp AS59.[12]

Due to their versatility and broad substrate specificity, lipases and sterol esterases are extensively applied, either in hydrolysis or synthesis reactions, in a variety of fields including food, fats and oils,

health, chemicals, pharmaceuticals, cosmetics, and paper among others.[13]

It is clear that the use of enzymes is an attractive approach for many industrial processes but, in order to facilitate their implementation, the production of high levels of very stable biocatalysts, competitive in costs with chemical catalysts, is required. Some of these enzymes have been successfully expressed in heterologous hosts, optimizing their production yields and costs. Different expression systems, including bacteria, yeasts or filamentous fungi are available for this aim, but methylotrophic yeasts provide a great potential as biofactories, using methanol as their sole carbon source.[14] *P. pastoris* is probably the most exploited yeast for recombinant protein production[15,16] since this organism gives stable transformants through homologous recombination of the gene to be expressed, grows easily in minimal media and efficiently secretes heterologous proteins that carry the post-translational modifications of higher eukaryotes, namely protein folding, proteolytic processing, disulphide bond formation, and glycosylation.[17] In addition, the current bioprocesses designed for its cultivation in fermentors facilitate the scale-up to industrial level, yielding high amounts of protein.[16,18]

A sterol esterase from the saprophytic fungus *O. piceae* (OPE) was characterized[19] and expressed in *P. pastoris* at levels 7-fold higher than the native one.[20] This work, recently published, discloses that the improved kinetic parameters of the recombinant protein (OPE*) for hydrolysis reactions are due to the presence of 6–8 additional amino acid residues at the N-terminal end, resulting from the wrong processing of the α-mating factor pre-pro peptide and the cloning strategy. This modification alters hydrophobicity of the protein and causes relevant changes on its aggregation state, resulting in a mix of monomeric and dimeric forms instead of the big aggregates found for the native enzyme. Then, OPE* shows an increased solubility which, in turn, affects positively its hydrolytic efficiency.

In this addendum, we discuss the role of sorbitol and the effect of inducer concentration on OPE* production. We also describe the use of OPE and OPE* as catalysts of a reaction of potential biotechnological interest, the hydrolysis of the polyvinyl acetate (PVAc) homopolymer $(C_4H_6O_2)_n$, comparing their activities with that of commercial enzymes.

Inducible Expression of *O. piceae* Sterol Esterase

The *O. piceae* sterol esterase has been successfully expressed in *P. pastoris* under the control of the strong alcohol oxidase 1 promoter (P_{AOX1}).[20] This promoter is controlled by a repression/derepression and induction system where methanol acts as an inducer and other several carbon sources, such as glucose or glycerol, as repressors.[16] On the other hand, sorbitol has been described as a non-repressing carbon source during expression of recombinant proteins under the control of P_{AOX1}.[21] Several works report its use as a co-substrate during the yeast growth at bioreactor level, in order to balance the potential metabolic burden derived from overexpression of a recombinant protein which, besides, could trigger the unfolding protein response (UPR).[22] This response implies the induction of chaperones and foldases, and the action of the proteasome.[23]

Recently, we reported that the presence of sorbitol in YEP, a basal medium with yeast extract and peptone,[20] yielded 3-fold higher levels of esterase activity in methanol-induced cultures, compared with a similar medium without sorbitol. In this work, we describe the effect of this carbon source on heterologous expression of OPE in Erlenmeyer flasks, using the same basal medium in the presence or absence of 5 g/L methanol as inducer of P_{AOX1} and 10 g/L sorbitol. Four different formulations were assayed: (1) YEP medium, (2) this medium with methanol (YEP + I), (3) YEP medium with sorbitol (YEPS), and (4) YEPS with methanol (YEPS + I).

Figure 1A shows the esterase activity secreted in the four media, determined on 1.5 mM *p*-nitrophenyl butyrate (*p*NPB). As it was expected, the highest activity levels were achieved in cultures with sorbitol and methanol, reaching around 16 U/mL after 96 h of incubation. In the absence of sorbitol, the activity levels were about 2.4 U/mL, which is comparable to previously reported values using a similar medium.[20] Although no esterase production would be expected in absence of methanol, activities of 6 and 0.5 U/mL were detected respectively in YEPS and YEP non-induced media. The SDS-PAGE profiles of crude extracts obtained in the four assayed conditions (**Fig. 1B**) agree with these results, showing more intense OPE* bands in the media with higher esterase activity. As mentioned above, it is known that genes from the methanol utilization pathway (MUT pathway) are subjected to both carbon catabolite repression/derepression and induction by methanol, and the interaction between such mechanisms modulates the organism's response to a particular environment.[24] In this sense, *P. pastoris* expresses high levels of AOX1 when the alcohol is the sole carbon source in the medium, while no expression is observed in cells growing in glycerol or glucose, and only a relatively small derepression response (1–2%) is observed upon carbon starvation.[25] So, the low activity levels detected in non-induced cultures could be a consequence of the basal derepressed expression of the *AOX1* gene. However, it is noteworthy that the esterase activity reached in non-induced cultures with sorbitol (YEPS) was 2.4-fold higher than that obtained in YEP induced cultures. These results suggest that, in some way, sorbitol must promote heterologous expression of the enzyme. To the best of our knowledge, this is the first report of a quantitative estimation of the derepression effect of sorbitol on MUT pathway genes. Such results may reflect its role in the modulation of cellular stress, preventing a possible metabolic burden, and the activation of the UPR response. The role of sorbitol as molecular chaperone, favoring the expression of a soluble recombinant green fluorescent protein, has already been suggested.[26] This function could also contribute to explain the positive effect of sorbitol on recombinant sterol esterase production.

Methanol concentration is critical to get high levels of recombinant proteins in *P. pastoris* strains using P_{AOX1}. The optimization of this parameter is of special interest, since it must be added daily to maintain the induction and counteract its evaporation. Two concentrations of methanol (5 and 10 g/L) in YEPS medium were assayed. In general, the inducer concentration was positively correlated with

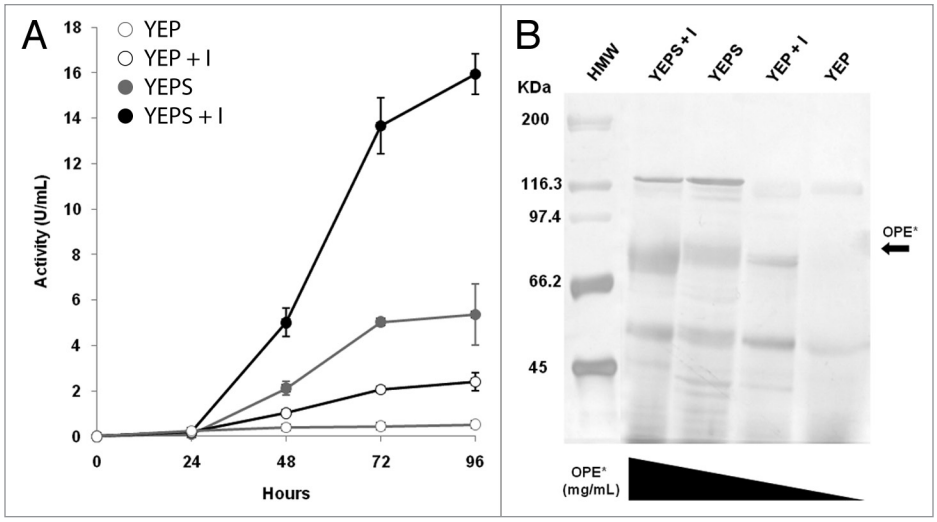

Figure 1. Influence of sorbitol on P_{AOX1} repression/derepression. (**A**) Activity levels detected in 25 mL cultures with YEP, YEP + I, YEPS and YEPS + I in 250 mL Erlenmeyer flasks at 28°C and 250 rpm. Error bars represent standard deviation of different experiments. (**B**) SDS-PAGE silver stained gel of the extracellular crude extracts.

biomass and total protein. However, no significant differences were found in the activity levels secreted at both methanol concentrations (**Fig. 2**) although some inhibitory effect appeared at 10 g/L as reported by Kobayashi and coworkers.[27] Taking into account these results, concentrations lower than 5 g/L methanol should be tested to avoid inhibition and reduce the overall cost of the process during production of recombinant sterol esterase.

Enzymatic Hydrolysis of PVAc

Production of recycled paper has greatly increased throughout the last decades. One of the unsolved problems from these industries comes from the deposition of tacky mixtures of debris from adhesives, coating binders, ink residues, deinking chemicals, and wood derivatives. Such deposits, usually known as "stickies," are formed by a broad range of compounds which include high and low molecular weight organic compounds, natural and synthetic polymers, and inorganic chemicals. The presence of these deposits on pulps and water process systems is detrimental for this industry, affecting process efficiency and quality of the final product, resulting in severe economic loses. Polyvinyl acetate (PVAc) is a common component of many adhesives or glues, and constitutes one of the most problematic compounds from stickies.[28]

PVAc and its corresponding alcohol (PVA) are polymers which exhibit an all carbon–carbon single bond backbone and also a 1,3-diol structure.[29] A linear PVAc homopolymer with a molecular weight of 12,800 (Sigma) was used as substrate for both *O. piceae* enzymes and three commercial esterase/lipase cocktails: Buzyme® 2517 and 2518, which are respectively the low and high-concentration version of the same enzyme preparation (personal communication from Buckman), and Optimyze® 530. All commercial cocktails were kindly supplied by Buckman. Unlike other works[30] where hydrolysis was performed at high temperatures, in organic solvent, and catalyzed by lipases devoted to other biotechnological applications (like Novozym 435 and *Candida rugosa* lipase), here reactions were developed at 28°C, in an aqueous dispersion of the polymer and using enzymes especially commercialized to control stickies. In fact, the use of Optimyze® for this purpose was patented by Buckman as it is advertised in the web page of the company (http://www.buckman.com), and received the 2004 "Greener Reaction Conditions Award" from the Environmental Protection Agency (US). PVAc is known to undergo hydrolysis by side-chain breakage,[31] in other words, enzymatic hydrolysis of the polymer consists of a deacetylation reaction, yielding acetic acid and polyvinyl alcohol (PVA) which is more hydrosoluble than PVAc.[32] The acetic acid release can be followed up by titrimetry and used to measure enzymatic activity, while PVA can be detected by MALDI-TOF mass spectrometry or FTIR spectroscopy.

The transformation of PVAc into PVA was visually observed as a decrease of the turbidity of the reaction (**Fig. 3A**). pH-stat assays, using 0.1 N NaOH as tritant, showed that both *O. piceae* enzymes and the commercial ones were able to deacetylate PVAc, though hydrolysis reactions passed slowly under the experimental conditions used. The *O. piceae* enzymes and Optimyze® 530 showed the highest activity levels (approximately 0.03 U/mg), while Buzyme® 2517 and Buzyme® 2518 had activities around 5 and 20-fold lower, respectively (**Fig. 3B**). Initially, all enzymes showed esterase activity under standard conditions[20] although only *O. piceae* enzymes and Optimyze® 530 retained it when 1% (v/v) of Genapol X-100 was added to the reaction. This could partly explain the low activity of Buzyme® 2517 and 2518 on PVAc during pH-stat assays in the presence of the surfactant, necessary for maintaining the polymer dispersed in agitation.

MALDI-TOF mass spectrometry and FTIR spectroscopy corroborated polymer deacetylation. In the first case, the spectra of PVAc before and after the treatment (without detergent) showed the typical

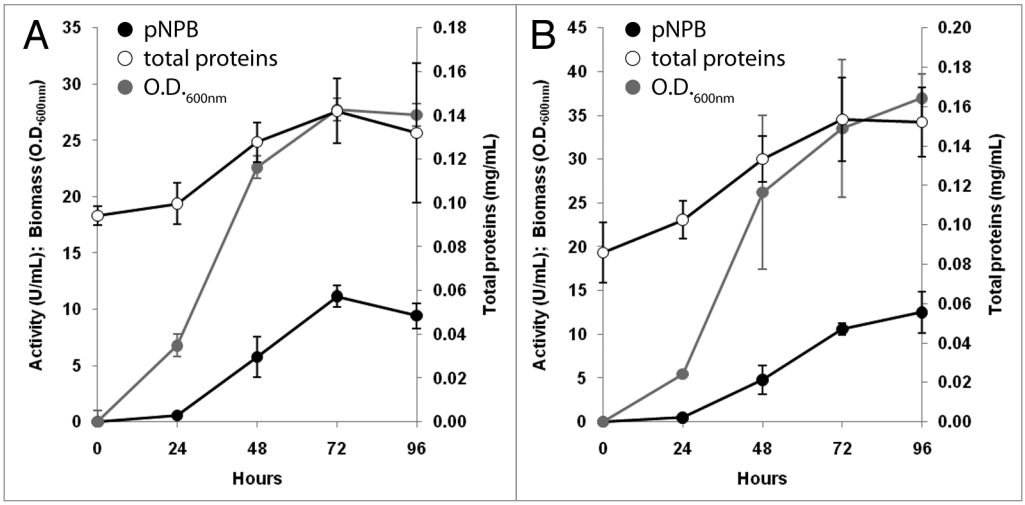

Figure 2. Influence of methanol concentration on heterologous expression of OPE* in YEPS. (**A**) 5 g/L and (**B**) 10 g/L methanol. Activity on *p*NPB, total proteins, and O.D.$_{600nm}$. Error bars represent standard deviation of three different experiments.

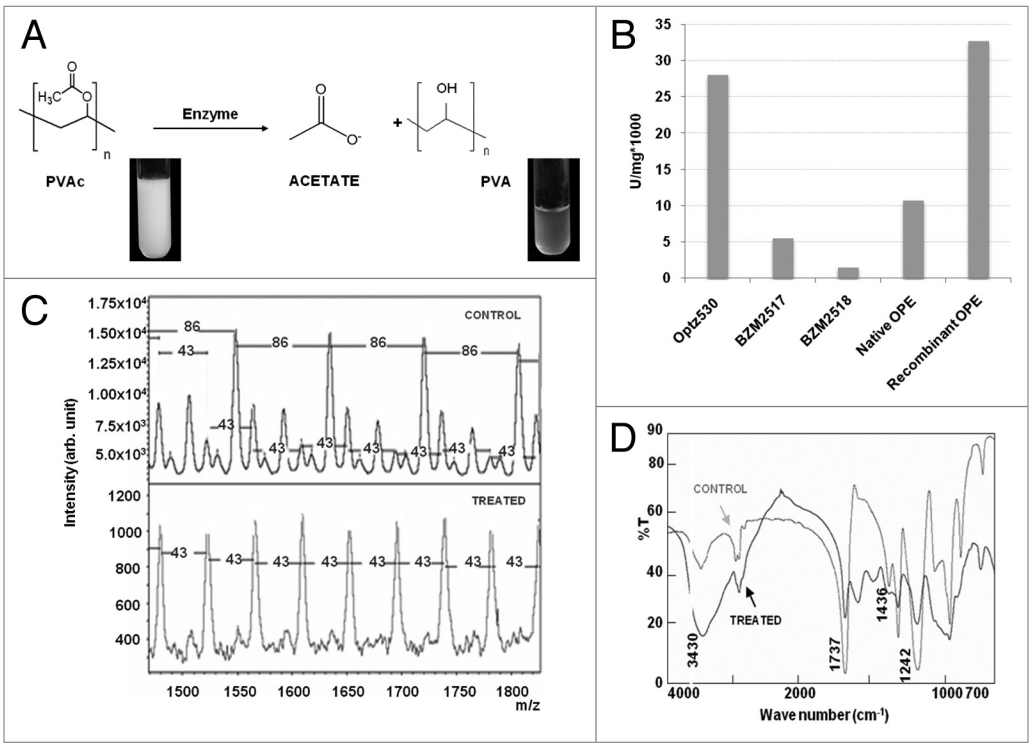

Figure 3. Enzymatic hydrolysis of PVAc. (**A**) Visual aspect of the reactions. (**B**) Activity assays in pH-stat: 0.3% (v/v) PVAc in 1 mM Tris-HCl pH 7.0 buffer with 0.15M NaCl and 1% (v/v) Genapol X-100, 40% agitation, 25°C. BZM (Buzyme®) and Optz (Optizyme®). (**C**) Detail of the MALDI-TOF spectra of untreated and treated PVAc sample with *O. piceae* sterol esterase showing the mass of the repeating unit in each polymer: 0.3% (v/v) PVAc dispersed in a methanol/water solution, 150 rpm in a water-bath at 28°C, 48 h or longer. (**D**) FTIR analysis of a control and an enzymatically treated dispersion of the polymer. Characteristic bands of PVAc and PVA are indicated.

polymeric distribution of this kind of compounds, with repeating units of 86 Da in untreated samples from PVAc which are transformed after the enzymatic treatment into 43 Da repeating units in PVA (**Fig. 3C**). FTIR analysis of enzymatically treated samples showed the decrease of the absorption bands around 1250, 1460, and 1740 cm^{-1}, characteristic of the ester linkage, while a new band, attributable to the hydroxyl group from PVA, appeared around 3350 cm^{-1} (**Fig. 3D**).

Conclusions

Sorbitol has turned out to be interesting to favor *P. pastoris* growth and to enhance heterologous expression of OPE* in presence or absence of methanol. Then, its

incorporation to the culture medium could be helpful for optimization of recombinant expression of any protein in the methylotrophic yeast, independently of its Mut phenotype. Regarding the effects of inducer concentration on expression, no significant differences have been observed using 5 and 10 g/L of methanol, although a slight inhibition of the specific esterase activity, referred to biomass concentration, has been found with 10 g/L. Then, lower methanol concentrations should be tested to optimize both expression and process costs.

The potential role of the *O. piceae* enzymes to decrease stickies deposition during recycled paper production has been demonstrated, based on the hydrolysis of PVAc. The conversion extent of this model compound into PVA was similar to that obtained with Optimyze® 530, the enzyme commercially available for this application, and much better than that attained with the other commercial lipases assayed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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