Production of a Sterol Esterase From *Ophiostoma* piceae in Batch and Fed-Batch Bioprocesses Using Different *Pichia pastoris* Phenotypes as Cell Factory

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DOI 10.1002/btpr.1939
Published online June 26, 2014 in Wiley Online Library (wileyonlinelibrary.com)

The potential biotechnological applications for the Ophiostoma piceae sterol esterase (OPE) are conditioned to the availability of high enzyme amounts at low prices. This enzyme is a versatile biocatalyst with different biotechnological applications. In this work a systematic study on its heterologous production in different Pichia pastoris strains and operational strategies is presented. The best results were obtained using an AOX1 *defective yeast strain in a fed-batch bioprocess using methanol as inducer substrate at a set point of 2.5 g* L^{-1} *and sorbitol as cosubstrate by means of a preprogramed exponential feeding rate at a* $\mu = 0.02$ h^{-1}*, reaching 30 U* mL^{-1} *of enzyme and a volumetric productivity of 403.5 U* L^{-1} h^{-1}*. These values are twofold higher than those obtained with a* Mut^{+} *phenotype using methanol a sole carbon source. OPE was the main protein secreted by the yeast, 55% for* Mut^{s} *versus 25% for* Mut^{+}*.* © 2014 American Institute of Chemical Engineers *Biotechnol. Prog.*, 30:1012–1020, 2014
Keywords: esterase, lipase, ascomycete, recombinant protein

Introduction

Microbial sterol esterases (EC 3.1.1.13), are hydrolases reported in bacteria, as *Pseudomonas aeruginosa*,[1] yeast, as *Candida rugosa*,[2] and filamentous fungi, as *Ophiostoma piceae*,[3] *Melanocarpus albomyces*,[4] *and Trichoderma* sp.[5] All of them act on esters of sterols and long-chain fatty acids but there is some controversy in their classification because the enzymes from *P. aeruginosa*, *C. rugosa*, *M. albomyces*, or *O. piceae*, are also able to hydrolyze typical lipase and carboxylesterase substrates.

The enzyme secreted by *O. piceae* is a robust biocatalyst able to hydrolyze pure or natural mixtures of sterol esters present in hardwood and softwood extractives, which are involved in the formation of undesirable deposits during pulp paper manufacture,[6] but it also is an efficient biocatalyst in synthesis reactions to produce phytosterol esters,[7] compounds which are recognized to decrease LDL cholesterol.[8]

Because of its higher biotechnological potential, the mature sequence of the enzyme has been successfully expressed in *Pichia pastoris,* yielding increased activity levels as compared to those obtained with the saprophytic fungus *O. piceae*.[9] Steady-state kinetic characterization of the recombinant enzyme from Erlenmeyer cultures, showed an improved catalytic efficiency to hydrolyze *p*-nitrophenol, glycerol, and cholesterol esters, as a consequence of its modified N-terminus because of the addition of various extra amino acids residues from pPIC9 vector, and the wrong processing during its expression in the yeast, which affected its aggregation behavior.[10] However, in order to increase the protein levels for industrial applications it is necessary to implement the enzyme production in bioreactors using different fed-batch operational strategies under controlled conditions to get a reproducible bioprocess.

The methylotrophic yeast *P. pastoris* have different phenotype strains regarding their ability to use methanol as carbon source. Mut^{+} strain, the most common phenotype, has both *AOX1* and *AOX2* alcohol oxidase genes available to metabolize this alcohol whereas Mut^{s} use it slowly, since *AOX1* gene is knocked out and *AOX2* is controlled by a much weaker promoter being responsible for 15% of the alcohol oxidase activity in the cell.[11] By this reason, the selection of the *P. pastoris* phenotype has important consequences on the fed-batch operational strategy selected. In this sense, bioprocesses with Mut^{s} phenotype are usually easier to scale up, comparing to Mut^{+} phenotype, because of its lower specific oxygen consumption rate and to the easy control of temperature during the bioprocess due to less heat generation.[12] Nevertheless, using methanol as the sole carbon source, Mut^{s} phenotype bioprocess results in long induction time with low growth rates. The use of mixed substrates can minimize these problems.[13]

Different fed-batch cultivations have been successfully developed with a mixed substrate strategy, mainly for Mut^{s} although also for Mut^{+}.[14,15] Glycerol is one of the most frequently used cosubstrate to obtain important levels of biomass but a rational process must be designed since at high concentration it can repress the expression of alcohol oxidase

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and subsequently of the product of interest.[12,16] As an alternative other cosubstrates, such as alanine, trehalose, mannitol, and sorbitol, jointly with methanol could be used.[17,18]

Between them, sorbitol is one of the most promise cosubstrate. The main advantage compared with glycerol is that an excess of sorbitol on culture broth not repress the *AOX1* promoter.[19] Inhibitory effect of sorbitol on cell growth has been not observed until a concentration of 50 g L^{-1}.[20] Hence the control of residual sorbitol concentration is less critical than with glycerol. Although sorbitol cell yields and maximum specific growth rate is lower than glycerol, a higher specific production rate are achieved in the production of sea raven antifreeze protein (srAFP)[21] and *Rhizopus oryzae* lipase.[22,23]

Different strategies have been developed for fed-batch cultures using sorbitol as cosubstrate increasing the final production compared to the use of methanol as sole carbon source. Fed-batch preprogramed exponential feeding rate.[24] Methanol addition rate programed by a control algorithm from on-line measurement of methanol to maintain a set-point of methanol and manual variable sorbitol addition rate or a preprogramed exponential feeding rate of sorbitol to control the specific growth rate at a constant value[25] and exponential feeding rate at different methanol-sorbitol ratio[20] are some examples.

However, not always the use of sorbitol as cosubstrate, compared with the use of methanol as sole carbon source, increased the production of a beta-propeller phytase[26] or the productivity and quality of antibodies.[27]

In this work, we propose the bench scale production (1–5 L) of the recombinant sterol esterase from *O. piceae* in both Mut^+ and Mut^s *P. pastoris* strains as a first step to improve the biocatalyst yield for its use in different biotechnological applications. The production has been carried out operating both in batch and fed-batch bioprocesses and differences between strains used, in terms of production, productivities, and specific growth rate (μ), are discussed.

Materials and Methods

Chemicals

p-Nitrophenyl butyrate (*p*NPB) and biotin were purchased from Sigma. Yeast extract, peptone and agar were provided by Oxoid. Sorbitol and methanol were acquired from Panreac. Difco was the provider of yeast nitrogen base (YNB) without amino acids and ammonium sulfate. Other compounds were obtained from Panreac and Merck. All of them were of the purest grade.

Fungal strains and plasmids

P. pastoris Mut^+ phenotype derived of GS115 strain (*his*4 auxotrophy) and a Mut^s phenotype from the KM71 strain (*arg*4 *his*4 *aox*1Δ::*SARG4 AOX2*), both from Invitrogen™, were used as host strains for expressing the *O. piceae* mature sterol esterase sequence under the transcriptional control of the *AOX*1 promoter. The vectors pGEM®-T Easy (Promega) and pPIC9 (Invitrogen™) were used, in that order, for cloning and expressing the sequence.

Vectors construction, strains securing, and analysis of transformants

The mature *O. piceae* esterase sequence was cloned into pGEM®-T Easy vector, sequenced, and subcloned in pPIC9 vector, now called pPIC9 *O. piceae* sterol esterase (OPE), in frame with the coding sequence of the α-mating factor secretion signal peptide and downstream of the P_{AOX1} as was shown after sequencing. Genetically stable transformants were obtained after integration of the linearized vector in the genome of the yeast cells after their transformation.[9]

Five different Mut^s transformants were screened for sterol esterase production at small scale using BMG/BMM media (buffered minimal glycerol/buffered minimal methanol media).

In a first step to growth biomass, transformants were grown at 30°C and 230 rpm 24 h in 1 L Erlenmeyer flasks containing 100 mL of BMG medium (13.4 g L^{-1} YNB without amino acids, 4×10^{-4} g L^{-1} biotin, 100 mM potassium phosphate buffer, pH 6.0, and 10 g L^{-1} glycerol) until an OD_{600} of 20–60.

To induce protein expression, cells were harvested by centrifugation at 4,500 rpm and room temperature for 10 min, and resuspended in 60 mL of BMM medium (same composition that BMG but with 5 g L^{-1} MeOH instead of glycerol) in order to get an initial OD_{600} of 20–60. 10 mL of cells from every transformant were distributed in 50 mL Falcon tubes in order to obtain triplicates and maintained at 30°C and 230 rpm during 144 h. Pure methanol was added every 24 h to these media (final concentration 5 g·L^{-1}) for maintaining the induction and to counteract evaporation. An alternative screening was developed in BMM medium containing, furthermore, 5 g L^{-1} sorbitol as cosubstrate during the induction step, and in the same way than methanol, sorbitol was added daily at that concentration. The experiments were carried out in triplicate and esterase activity was assayed against *p*NPB.

Mut^+ transformants were previously screened in a complex medium and OPE1 selected as the best one.[9]

Inoculum preparation for fermentations

Preinocula for bioreactor cultures were grown at 30°C, 230 rpm for 48h in 1 L baffled Erlenmeyer flask containing 200 mL yeast extract Peptone Dextrose medium-YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} dextrose). All preinocula were obtained from a single colony grown in YPD agar (same composition than YPD plus 20 g L^{-1} agar). The culture was centrifuged 30 min at 8,000 rpm and 4°C, and the harvested cells were re-suspended in 200 mL sterile water and used to inoculate a 2-L Applikon batch or a 5-L Biostat B fed-batch bioreactor (Braun Biotech, Melsungen, Germany) equipped with air mass flow meters, so initial concentration of biomass was always equivalent to 1 g L^{-1} of dry cell weight (about 5.0 units of OD_{600}).

Batch fermentation culture medium and operational conditions

The medium routinely used for this kind of bioprocess contained 3.4 g L^{-1} YNB, 5 g L^{-1} $(NH_4)_2SO_4$, 10 g L^{-1} methanol, 0.1 mL L^{-1} antifoam 204 Sigma, 2 mL L^{-1} 0.02% (w/v) biotin and 5.0 mL L^{-1} PTM1 solution. The PTM1 salt trace solution contained 6 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 65 g $FeSO_4 \cdot 7H_2O$, 0.3 g biotin, and 5 mL of concentrated H_2SO_4 L^{-1}. 10 g L^{-1} sorbitol were added when a cofeeding strategy was used. Ammonium sulfate was autoclaved in the bioreactor as sorbitol, which

was added for some experiments. YNB, methanol and PTM1 solution were filter sterilized and added to the bioreactor at room temperature, and then pH was adjusted to 5.5 with 5 M KOH.

Cells were cultured in a 2-L Applikon bioreactor under the following operational conditions: initial volume 1.5 L, stirring rate 600 rpm, temperature 30°C, pH controlled at 5.5 by adding 5 M KOH, dissolved oxygen above 30% air saturation, with an air flow rate of 0.7 vvm of compressed air. In order to decrease methanol stripping the condenser was connected to a 4°C refrigeration system during the experiments.

Fed-batch cultivation set-up and operational conditions

The basal salt synthetic medium (BSM) from Invitrogen was used for this kind of bioprocess: 26.7 mL L^{-1} H_3PO_4 85%, 1.17 g L^{-1} $CaSO_4{\cdot}2H_2O$, 18.2 g L^{-1} K_2SO_4, 14.9 g L^{-1} $MgSO_4{\cdot}7H_2O$, 4.13 g L^{-1} KOH 85%, 40 g L^{-1} glycerol, 5.0 mL L^{-1} PTM1 solution, 2 mL L^{-1} 0.02% (w/v) biotin, and 0.1 mL L^{-1} antifoam 204.

Cells were cultured in a 5-L Braun Biostat B bioreactor under the following operational conditions: initial volume 3.2 L, stirring rate 1,000 rpm, temperature 30°C, pH controlled at 5.5 by adding NH_4OH 30% (v/v) during the batch phase, and 5 M KOH during the transition (just before methanol addition) and induction phases, dissolved oxygen above 30% air saturation, with an air flow rate between 0.2 and 2.5 vvm of compressed air and 0.125 and 0.25 vvm of oxygen, when needed, depending on the phase and the strain. The condenser was refrigerated at 4°C to decrease methanol stripping and antifoam was added manually to control foaming.

The fermentation started with a 40-g L^{-1} glycerol batch phase to produce high biomass levels, followed by a transition phase, which have been reported to be beneficial for preparing yeast cells for the final induction phase.[28,29] During this phase, a preprogramed addition of glycerol and methanol was used for the Mut^+ strain,[28] while sorbitol and methanol were added separately to the bioreactor to final concentrations of 10 and 5 g L^{-1}, respectively, after glycerol depletion in Mut^s cultures.

Finally, when the substrates were consumed, the induction phase was started. For Mut^+ phenotype a 10-h preprogramed exponential methanol feeding rate at a specific growth rate set-point of 0.02 h^{-1} was implemented previously to a programed methanol addition rate by a predictive control algorithm coupled with a PI (proportional-integral) feedback controller previously developed to maintain a methanol set-point of 3 g L^{-1} by addition of a pure methanol solution with 5 mL L^{-1} PTM1 salts and 2 mL L^{-1} 0.02% (w/v) biotin.[30]

Induction phase for Mut^s strain started with a preprogramed exponential feeding rate of sorbitol, at a set-point of 0.02 h^{-1}, coupled to the a methanol addition rate, with the same control algorithm than Mut^+ at a set-point of 2.5 g L^{-1} and methanol feeding solution.[22] This sorbitol feeding solution contained 300 g L^{-1} sorbitol, 5 mL L^{-1} PTM1 solution and 2 mL L^{-1} biotin solution at 0.02% (w/v).

Methanol concentration was monitored on line using a methanol sensor (Raven Biotech, Vancouver, BC) immersed in the culture medium. Both carbon sources were added using MicroBU 2031 automatic microburettes (Crison Instruments, Barcelona, Spain). During the induction phase, 250 mL distilled water containing 55 g of NH_4Cl were added to the reactor when biomass reached 40–45 g L^{-1} in order to supply the nitrogen requirements of Mut^+ and Mut^s strains. Fermentations were finished when the pO_2 set-point could not be controlled and high biomass values were reached.

Biomass, proteins, and enzyme activity determination

Optical density of the samples was measured at 600 nm and correlated with dry cell weight throughout the expression: $DCW\ (g{\cdot}L^{-1}) = 0.212{\cdot}OD_{600}$. DCW was obtained as described.[31]

Protein concentration was determined by the Bradford microplate protocol (Pierce) using bovine serum albumin as standard (Pierce). Plates were read in a Microplate Reader 2001 (Whittaker Bioproducts) controlled by the WinRead 02.03 software (Anthos Labtec Instruments). New quadratic regression curves were used each time.

Esterase activity was routinely measured using 1.5 mM *p*NPB as substrate in 20 mM Tris-hydrochloric acid buffer, pH 7.2 at room temperature in a Cary-50 spectrophotometer. One unit of activity (1 U) is defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol ($\varepsilon_{410} = 15{,}200\ M^{-1}cm^{-1}$) per minute under the defined conditions.

Off-line determination of methanol, glycerol, and sorbitol

Off line methanol and glycerol determination was made by FID gas-chromatography in a HP 5890 gas chromatograph (Agilent technologies) using a HP-Innowax 30 m x 0.53 mm x 1.00 μm column (Agilent technologies) as previously reported.[32]

Sorbitol and methanol were determined by HPLC with a HP 1050 liquid chromatograph (Agilent technologies) using an Aminex HPX-87H ion-exchange column (Bio-Rad).

Primary stages of downstream processing of the cultivation broth

The culture broth was centrifuged 30 min at 8,000 rpm and 4°C to remove most of the biomass. The remaining biomass was eliminated by filtration through 0.45 μm cutoff filters (Whatman). Depending on the initial volume, the liquid medium was concentrated by ultrafiltration with a Filtron and/or Pellicon system (Millipore) using 10 kDa cutoff membranes. The concentrated liquid was frozen at −80°C and lyophilized if necessary.

Samples of concentrated crude enzymes were desalted by diafiltration using microtubes (PALL Corporation) and deglycosylated with Endo H (Roche).[10] sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the samples was performed in a Mini-protean III unit (Bio-Rad) using 7.5% running gels. Protein bands were visualized with Coomassie R-250 (Bio-Rad). Gels were densitometred using a GS-800™ densitometer (Bio-Rad) and analyzed with Quantity One 4.6 software (Bio-Rad).

Results and Discussion

Different Mut^s transformants were screened in minimal BMM medium with or without sorbitol. Enzyme production related to biomass was around 1.2-fold higher when the medium was supplemented with sorbitol after 96 h of incubation. The best one, in terms of sterol esterase production

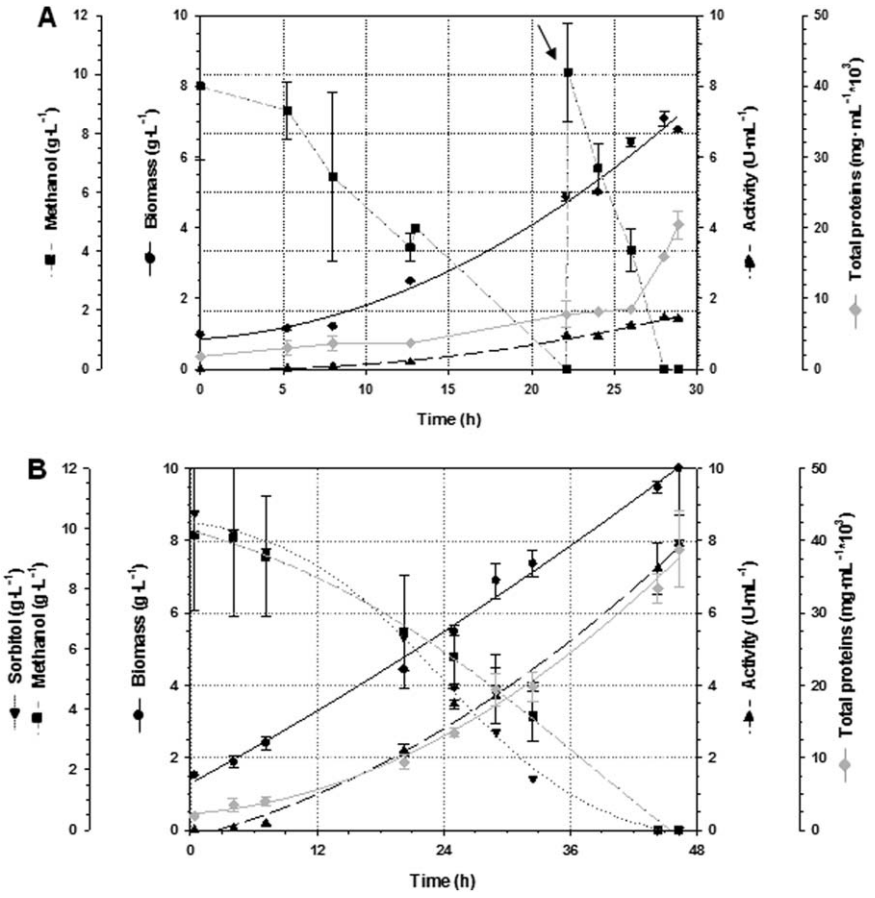

Figure 1. Batch bioprocess for Mut$^+$ (A) and Mut^s (B) strains expressing OPE*. Time evolution of activity, biomass, total protein, methanol and sorbitol concentrations in YNB medium. Methanol was used as inducer at 10 g L^{-1} for both strains and sorbitol as cosubstrate at the same concentration for Mut^s. Arrows indicate methanol addition during Mut$^+$ cultivation. Experimental data were statistically fitted using Sigma plot 11.0 software. Error bars correspond to standard deviation for those measurements.

in the supplemented medium, colony B, was selected for the bioprocess. A Mut$^+$ transformant was previously selected according to its activity levels in a complex medium.[9]

Batch cultures

The evolution of the main fermentation variables for both phenotypes in YNB medium is presented in Figure 1. With Mut$^+$ phenotype strain, once initial methanol is consuming, two more pulses of methanol were added after 13 h (to increase concentration in 1 g·L^{-1}) and 22 h (reaching 10 g·L^{-1}; Figure 1A). The levels of extracellular enzyme after 29 h using methanol as the sole carbon source were 1.4 U mL^{-1} (Table 1).

In contrast, with Mut^s phenotype in the presence of around 10 g·L^{-1} methanol and sorbitol, 7.9 U mL^{-1} of enzyme were obtained at the end of the fermentation (Figure 1B), a value 5.6-fold higher than with Mut$^+$. Although the Mut$^+$ strain showed a higher specific growth rate than Mut^s strain, $Y_{P/X}$, volumetric and specific productivity values were much higher for Mut^s: 3.9-, 3.4-, and 2.4-fold higher respectively (Table 1). Similar behavior has been observed in the production of recombinant lipase from *R. oryzae* (ROL) since the use of sorbitol as cosubstrate in Mut^s phenotype strain improved production and secretion of the recombinant protein.[23]

No proteolysis was observed in both fermentations. However, at the end of the Mut$^+$ fermentation an important increase in total protein levels was detected, this phenomenon was not observed in Mut^s phenotype. Before these suddenly increase, the relation between activity units and mg of total protein was quite similar for both phenotypes along the fermentation (around 0.17 U μg^{-1}).

Our results agree with data previously reported describing that utilization of sorbitol as a second additional carbon source in Mut^s phenotype has the advantage of allowing the P_{AOX1} induction at the same time that cells show increased growth rates and an enhanced enzyme production comparing with the use of methanol as sole carbon source.[21,23]

Table 1. Comparison of Process Parameters of Different *P. pastoris* Phenotypes (Mut$^+$ and Mut^s) Expressing OPE* in Bioreactor Batch Cultures

	Mut$^+$	Mut^s
	YNB Medium	YNB Medium
Max. activity (U mL^{-1})	1.40	7.90
$Y_{X/S}$ (gX gsubstrate^{-1})	0.27	–
$Y_{P/X}$ (U gX^{-1})*	238.60	934.90
Volumetric productivity (U L^{-1} h^{-1})*	52.00	175.50
Specific productivity (U gX^{-1} h^{-1})*	8.50	20.80
μ_{max} (h^{-1})	0.09	0.03

Fermentations were carried out with 10 g L^{-1} methanol for both strains and 10 g L^{-1} sorbitol as cosubstrate for Mut^s strain. *The parameters were calculated at the maximal activity.

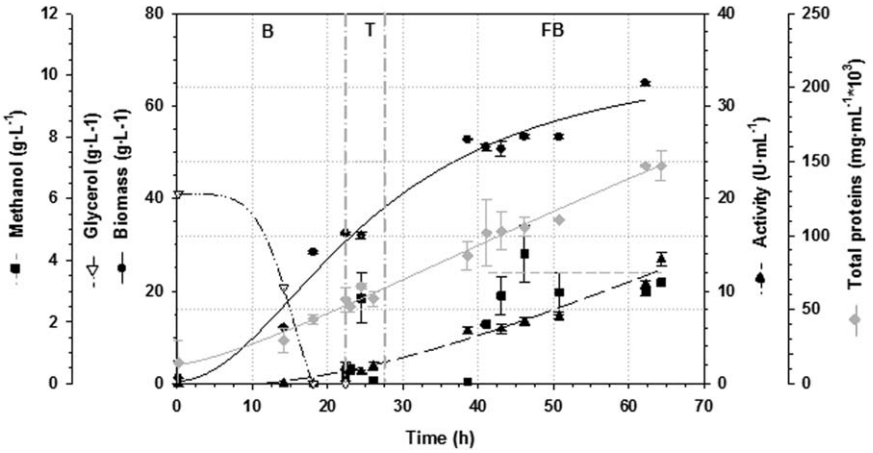

Figure 2. Fed-batch bioprocess for Mut$^+$ strain expressing OPE*. Time evolution of activity, biomass, total protein, glycerol and methanol concentrations in fed-batch culture at a methanol set point of 3.0 g L^{-1}. Batch phase (B), transition phase (T), and fed-batch phase (FB). Experimental data were statistically fitted using Sigma plot 11.0 software. Error bars correspond to standard deviation for those measurements.

Fed-batch cultures

Once the performance in batch cultures was determined, a fed-batch strategy was implemented for both phenotypes. Fed-batch strategies started with a batch culture using 40 g L^{-1} glycerol as the sole carbon source for both phenotypes, to reach high biomass concentrations in a short time.

After the transition phase (Materials and methods: Fed-batch cultivation set-up and operational conditions section), a fed-batch culture of Mut$^+$ phenotype was performed using methanol as the sole carbon and energy source (Figure 2). The first 10 h of induction were under methanol-limited conditions in order to get an easy adaptation of the yeast to the inducer substrate. After that, a predictive-PI control, previously developed for a Mut^s phenotype was adapted to a Mut$^+$ phenotype,[30] at a methanol concentration set-point of 3.0 g L^{-1}.

In fed-batch high cell density bioprocesses, methanol concentration have to be fixed to prevent growth rate-inhibitory effects in the cells.[33] In fact, it has been described that the specific growth rate exhibits a typical Monod kinetics at methanol concentrations up 3–5 g L^{-1}.[34] So we selected a methanol concentration set point of 3 g L^{-1}.

Figure 2 shows the evolution of biomass, enzyme activity, total protein, glycerol and methanol concentrations during the bioprocess. The maximum biomass level was 64.9 g L^{-1} after 60 h. At this point, pure oxygen at 0.5 vvm and 1,000 rpm were not enough to maintain dissolved oxygen values higher than 20% and fermentation was stopped. On the other hand, the use of an external cooler was necessary to circulate refrigerated water through the jacket to maintain the temperature at 30°C due to the high heat of combustion of methanol (−727 kJ C-mol^{-1}), which generates considerable heat during the growth.[34]

Once the control was activated (40 h) methanol concentration was maintained around the set point and the specific growth rate reached a constant value of 0.035 h^{-1}. This value was 2.6-fold lower than the μ_{max} observed on batch mode using YNB medium. The use of Invitrogen medium but also the fed-batch strategy could be the cause of this lower value. Similar behavior was observed in ROL production using the same media and strategy, although the observed specific growth rate at this methanol set point was a little bit higher (0.046 h^{-1}), indicating the influence of the target heterologous protein on the specific growth rate of *P. pastoris*.[36]

Table 2. Comparison of Process Parameters of Different *P. pastoris* Phenotypes Expressing OPE* in Bioreactor Fed-Batch Cultures at Methanol Set-Point on 3 g L^{-1} and 2.5 g L^{-1} for Mut$^+$ and Mut^s Strain, Respectively

	Mut$^+$	Mut^s
	BSM Medium	BSM Medium
Max. activity (U mL^{-1})	13.40	29.40
$Y_{P/X}$ (U gX^{-1})	206.40	410.80
Volumetric productivity (U L^{-1} h^{-1})	208.20	403.50
Specific productivity (U gX^{-1} h^{-1})	3.20	5.60
μ_{mean} (h^{-1})	0.035	0.018

In Mut^s strain sorbitol was used as cosubstrate during transition and induction phases.

Despite this problem, although $Y_{P/X}$ was quite similar, the maximal activity obtained using such strategy was increased 9.6-fold and the volumetric productivity was 4-fold higher compared with the batch bioprocess (Table 2).

Comparing with batch fermentation, the profile of total extracellular protein was similar to growth and OPE production, a suddenly increase in total protein levels was not observed along the fermentation. However, the relation between activity units and mg of total protein was twofold lower (around 0.089 U μg^{-1}). It could be indicating a high endogenous *P. pastoris* protein production in this operational strategy with this defined medium. Further research is necessary to clarify this point.

Due to the operational problems with Mut$^+$ phenotype, high oxygen consumption rate and generated heat at lab bioreactor scale, and the excellent performance observed with Mut^s phenotype batch cultures using sorbitol as cosubstrate, a fed-batch culture on mixed substrates with Mut^s strain was performed.

Mixed substrate strategy was performed by a 2.5-g L^{-1} methanol set-point value applying a similar control algorithm c than in Mut$^+$ phenotype, and a preprogramed exponential feeding rate of sorbitol to reach a specific growth rate of 0.02 h^{-1}, as it was previously developed for ROL production.[22] Figure 3 shows the evolution of biomass, enzyme

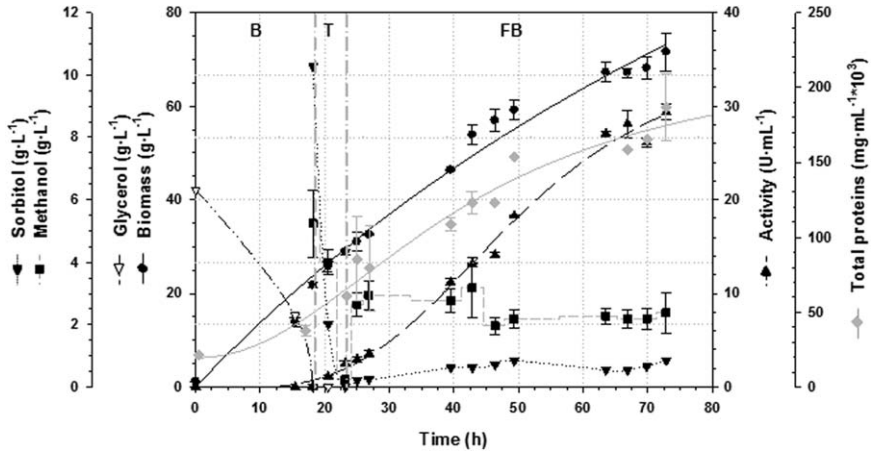

Figure 3. Fed-batch bioprocess for Mut^s strain expressing OPE*. Time evolution of activity, biomass, total proteins, glycerol, sorbitol and methanol concentrations in a fed-batch culture using sorbitol as limiting cosubstrate, at a methanol set point of 2.5 g L^{-1} Abbreviations: Batch phase (B), transition phase (T), and fed-batch phase (FB). Experimental data were statistically fitted using Sigma plot 11.0 software. Error bars correspond to standard deviation for those measurements.

activity, total protein, glycerol, sorbitol and methanol concentrations during the bioprocess. No temperature problems were observed with Mut^s phenotype. However it was necessary to supplement the air with oxygen, although dissolved oxygen levels were always higher than 20% along the fermentation. Assuming that the enthalpy of combustion of sorbitol is close to that of mannitol (-507.8 KJ C-mol^{-1}), which represents 69% of enthalpy of combustion of methanol,[12] and taking into account the low specific consumption rate of methanol of this phenotype, less oxygen consumption and heat generation was demanded. Under these conditions, the contribution of methanol consumption to growth was minimal being sorbitol the main carbon source responsible of *P. pastoris* growth. Thus, the sorbitol preprogramed exponential feeding rate was well implemented because the average specific growth rate reached along the bioprocess (0.018 h^{-1}) was a little bit lower than the set-point selected (0.02 h^{-1}).

The amount of residual sorbitol in the culture medium was lower than 1 g L^{-1} and did not affect recombinant protein production and cell growth since it is far from 50 g L^{-1}, the value reported as the inhibitory growth concentration.[20] This suggests that the presence of residual amounts of sorbitol during the induction phase is less critical than the presence of glycerol and supposes an important advantage comparing with other possible cosubstrates. Although the specific growth rate growing on glycerol is higher than on sorbitol, and consequently higher productivity should be obtained, it has been reported that programing a glycerol exponential feeding rate higher than the 0.03 h^{-1}, drastically reduced extracellular heterologous protein levels.[12]

On the other hand, methanol concentration was kept always below the set-point of 2.5 g L^{-1}, with oscillations lower than 0.6 g L^{-1}, showing the good performance of the implemented control algorithm. Mut^s phenotype showed a better methanol control performance than Mut$^+$ due to its lower specific methanol consumption rate.[37]

At the end of the bioprocess, 29.4 U mL^{-1} of enzyme activity were obtained, 3.7-fold higher compared with the results from batch bioprocess. Also volumetric productivity was 2.3-fold higher. However, $Y_{P/X}$ and specific productivity were 2.2- and 3.7-fold lower, probably because in the fed-batch bioprocess a less rich in nutrients synthetic defined media was used (Table 2).

Although the specific growth rate was lower for the Mut^s phenotype, maximal activity, $Y_{P/X}$, volumetric and specific productivities were higher than in Mut$^+$ phenotype (Table 2). Thus, the advantages of use sorbitol as cosubstrate in batch cultures were corroborated in the fed-batch strategy.

The results suggest that sterol esterase secretion is favored in Mut^s strain, according to data reported for ROL expressed in *P. pastoris*.[38] In order to understand this circumstance, it should be emphasized that the production of a recombinant protein represents an increased energetic demand for the host cell to face up synthesis, folding, post-translational modifications and secretion.[39] So, as was previously reported,[23] it is feasible to consider that overexpression of the recombinant enzyme provokes lower stress levels in the Mut^s strain because of its own physiology and the cofeeding strategy with sorbitol.

In view of these results, the Mut^s phenotype can be considered as the best strain for sterol esterase production, also from an operational and economic point of view.

Concentrated culture broth samples obtained at the end of both fed-batch fermentations were analyzed by SDS-PAGE (Figure 4). The same protein profile was shown for both strains. OPE* was confirmed to be one of the main proteins secreted by the yeast (around 25 and 55% for samples from Mut$^+$ and Mut^s, respectively, according to densitometry gel analysis). Thus, Mut^s produces twofold more OPE* than Mut$^+$. This relation agrees with the ratio of activity level gathered in Table 2. As previously reported,[9] OPE* had a molecular weight about 76 kDa, and around 60 kDa after deglycosylation, although a great heterogeneity can be seen due to posttranslational processing. In any case, after downstream processing, protein profiles for both strains were similar and recombinant OPE represented the major protein secreted by the yeast (Figure 4). This result has a great significance since downstream processing can constitute up to 50% of the total manufacturing cost.[40]

According to the densitometry analysis of the gel, a total amount of pure protein from the fed-batch cultures (5 L) of 177 and 319 mg was estimated from concentrated crudes of Mut$^+$ and Mut^s fed-batches, respectively.

Figure 5A presents a comparison among activity levels reached in Erlenmeyer, batch and fed-batch bioprocesses.

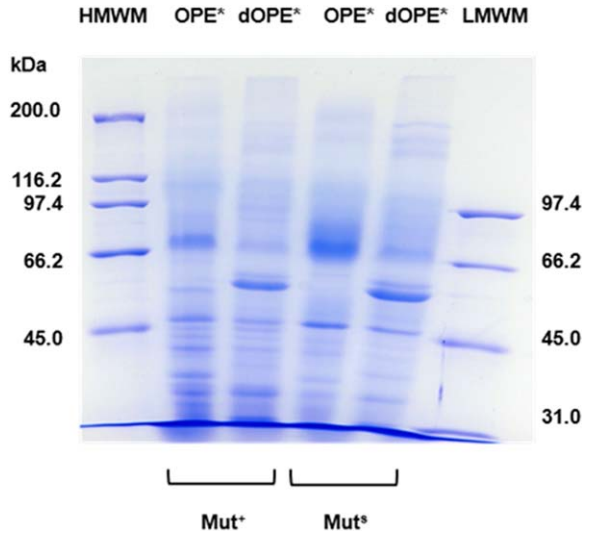

Figure 4. Protein analysis by SDS-PAGE after downstream processing. 30 μg crude protein samples obtained after the downstream processing were desalted and analyzed by gel electrophoresis. dOPE*: deglycosylated OPE*. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

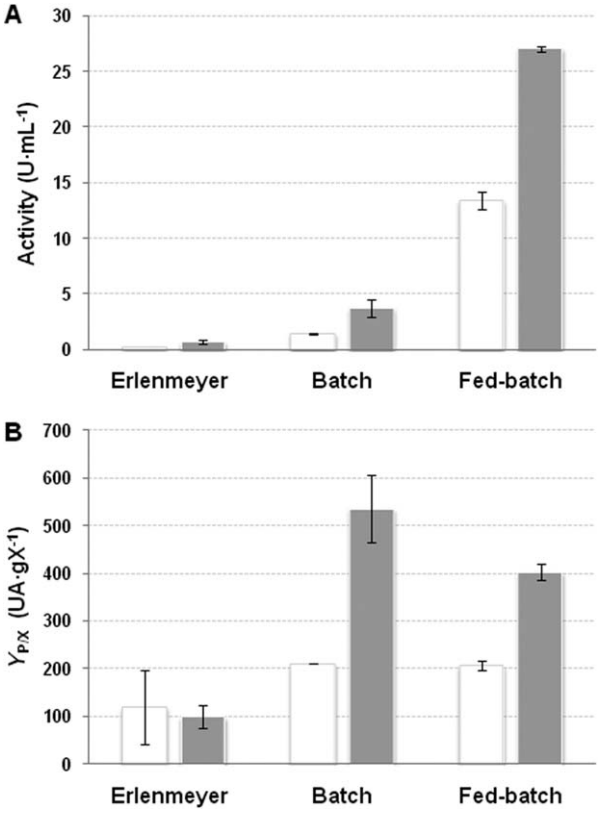

Figure 5. Overview of the scale-up of OPE* production. Production expressed as maximal esterase activity (A) and $Y_{P/X}$ (B). Comparison was made in similar culture medium and production time. In all cases sorbitol was used as cosubstrate for the Mut^s strain. Mut$^+$ (white columns); Mut^s (gray columns). Error bars correspond to standard deviation.

The comparison for the three strategies, in terms of $Y_{P/X}$, is shown in Figure 5B. Clearly, the non controlled fermentation conditions from Erlenmeyers influence negatively the $Y_{P/X}$ ratio comparing with the other strategies. This negative effect is more important for Mut^s phenotype. It could be interesting taking these results into account when performing clone selection since, from our experience, not always the best clones obtained in Erlenmeyer cultures are the best at bioreactor scale (data not shown). The implementation of robust microreactors for clone screening in *P. pastoris* under *PAOX1* promoter (using methanol as substrate) should drastically reduce time and consumables.[41]

Comparing the $Y_{P/X}$ obtained in batch and fed-batch fermentation for Mut$^+$ phenotype, no difference was observed although fermentation media was different. However, for Mut^s $Y_{P/X}$ in batch fermentation was higher than in fed-batch. Probably, the use of a rich medium could compensate the low methanol consumption rate due to the deletion of the *AOX1* gene, responsible for the 85% of alcohol oxidase production.[11] Nevertheless, in terms of total production, the fed-batch strategy is the best operational strategy. The use of a rich medium was not tested in fed-batch strategy in order to use a defined and cheaper medium which is interesting for industrial applications.

Finally, it is worthy to mention that process analytical technology allowed the monitoring of the production of a recombinant protein in a reproducible way, decreasing economic costs and production time which is mandatory from an industrial point of view. In this sense, the control of parameters such as substrate feeding rate and inducer (methanol) has been key for a high-level and reproducible production of the OPE at bioreactor scale.

This recombinant sterol esterase has been tested in the hydrolysis of polyvinyl acetate (PVAc), one of the most problematic compounds from stickies.[42] The higher hydrolysis efficiency reported for the recombinant sterol esterase on PVAc, comparing with the native one, agrees with its improved catalytic properties on triglycerides and sterol esters because of its modified N-terminus, which allowed changes in its aggregation behavior, affecting positively its hydrolysis efficiency.[10]

Conclusions

The present work demonstrates that high-level production of the recombinant sterol esterase from *O. piceae* has been possible through batch and fed-batch bioprocesses, using two different strains of *P. pastoris* differing in their ability to metabolize methanol (Mut$^+$ and Mut^s), grown in minimal synthetic media.

Mut^s phenotype shows better performance in terms of $Y_{P/X}$, volumetric and specific activity than Mut$^+$ but also in terms of more friendly operational conditions with lower high oxygen consumption and heat generation than Mut$^+$. So OPE* expression seems to be favored in this defective strain probably due to both its own genetic background and the use of sorbitol as cosubstrate. The use of sorbitol as cosubstrate allowed not only the increase in the specific growth rate of Mut^s phenotype growing on methanol as sole carbon source, but also higher secretion levels of heterologous product. The higher hydrolysis efficiency of the recombinant sterol esterase makes this enzyme more adequate than the native one for its application in aqueous solutions.

Acknowledgments

V. Barba Cedillo thanks the financial support for student mobility in the framework of FPU program (2005–2009). This research has been financed by the Spanish projects

BIO2009-08446, BIO2012-36372, CTQ2010-15131, 2009-SGR-281 and the Reference Network in Biotechnology (XRB) (Generalitat de Catalunya). Authors thank A. Prieto from Centro de Investigaciones Biológicas, Madrid, Spain, for her help and comments.

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Manuscript received Apr. 7, 2014, and revision received May 17, 2014.