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A step forward in laccase exploitation: Recombinant production and evaluation of techno-economic feasibility of the process

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ABSTRACT

Protein heterologous production offers viable opportunities to tailor laccase properties to specific industrial needs. The high redox potential laccase POXA1b from *Pleurotus ostreatus* was chosen as case study of marketable enzyme, due to its desirable properties in terms of activity/stability profile, and already assessed applicability.

POXA1b was heterologously produced in *Pichia pastoris* by investigating the effect of inducible and constitutive expression systems on both the yield and the cost of its production. System performances were first assessed in shaken-flasks and then scaled-up in bioreactor. The production level obtained in the inducible system is 42 U/mL, while the activity value achieved with the constitutive one is 60 U/mL, the highest obtained in constitutive systems so far. The economic feasibility of recombinant laccase production was simulated, describing the case of an Italian small-medium enterprise. Two scenarios were evaluated: Scenario (I) production based on methanol inducible system; Scenario (II) production based on the constitutive system, fed with glycerol. At all the scales the glycerol-based fermentation is more economic than the methanol-based one. The price forecast for rPOXA1b production is 0.34 € k U^{-1} for glycerol-based process, and is very competitive with the current price of commercial laccase.

1. Introduction

Laccases (*p*-diphenol-dioxygen oxidoreductases; EC 1.10.3.2) are oxidative enzymes coupling the four single-electron oxidations of a broad spectrum of reducing substrates to the reduction of molecular oxygen (Giardina et al., 2010). The production of water as the only by-product makes laccases one of the "greenest" enzymes of the 21st century (Mate et al., 2010).

Properties of laccases are characterized by a considerable heterogeneity, especially with respect to their temperature optima (ranging from 25 °C to 80 °C) (Baldrian, 2006) and their substrate specificity (Dwivedi et al., 2011).

Despite having very similar structures, laccases exhibit a wide range of redox potential, from +0.430 to +0.800 V (*vs.* normal hydrogen electrode) (Cambria et al., 2012). The redox potential is a key parameter affecting substrate specificity since the higher is the laccase redox potential, the wider is the range of oxidised substrates. Fungal laccases display a higher redox potential respect to that of laccases from other sources, and find effective exploitations in several industrial applications (Pezzella et al., 2015).

Although laccases are supplied by several companies for diverse applications, several issues have still to be faced (Mate and Alcalde, 2016). In order to meet the increasing market demand for high redox potential laccases, interest in their heterologous expression in different hosts has been raising (Piscitelli et al., 2010; Piscitelli et al., 2013). The versatility and scaling-up possibilities of recombinant enzyme production opened up new commercial opportunities for their industrial uses. Furthermore, the availability of recombinant expression systems opened the possibility to design laccases with customized features through protein engineering (Mate and Alcalde, 2016).

The fungal laccase POXA1b (Giardina et al., 1999; Garzillo et al., 2001) from *Pleurotus ostreatus* is an important representative member of high redox potential laccases. POXA1b is an ideal candidate for the laccase market since it brings together several elements necessary for an effective laccase exploitation: i) stability and activity in a wide range of pHs (3–9) and temperatures (25°–65 °C); ii) high redox potential (+0.650 V) (Garzillo et al., 2001); iii) already assessed applicability in several fields; iv) high production level in heterologous hosts; v)

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availability of collection of evolved variants to be tailored to specific industrial needs. Indeed, POXA1b is the most thermostable isoenzyme amongst *P. ostreatus* laccases ($t_{1/2}$ at 60 °C = 3 h) also exhibiting a remarkable stability at alkaline pH ($t_{1/2}$ at pH 9 = 30 days) (Giardina et al., 1999). Such an attractive enzyme has encouraged a deep investigation on the optimization of its production (Piscitelli et al., 2005; Macellaro et al., 2014a), and on the molecular characterization of its variants (Miele et al., 2010a; Macellaro et al., 2014a; Giacobelli et al., 2017). Moreover, it has been successfully tested in different applications, such as in the bioremediation field (Miele et al., 2010b; Macellaro et al., 2014b), in fruit juice clarification (Lettera et al., 2016), and in dye synthesis (Pezzella et al., 2016).

Biopox s.r.l. is a small Italian biotech company involved in the design and development of green enzyme based processes having in its portfolio, among others, different oxidative enzymes. This work deepened the economic feasibility of POXA1b production and secretion in the yeast *P. pastoris* by investigating the effect of inducible (AOX) and constitutive (GAP) promoters on both the yield and the cost of its production at Biopox site.

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Expression vectors pPICZαB and pJGGαKR were purchased from Invitrogen (Carlsbad, CA, USA) and Biogrammatics, Ltd (Las Palmas Dr, Carlsbad, CA, USA), respectively. DNA restriction and modifying enzymes were supplied by Promega (Madison, WI, USA). Culture media were bought from BD Difco (Becton Drive, Franklin Lakes, NJ USA).

2.2. Strains and plasmids

Escherichia coli strain Top 10 was used in all DNA manipulation. *E. coli* was grown in Luria-Bertani (LB) medium (10 g/l bacto tryptone, 10 g/l NaCl, 5 g/l yeast extract). Selective medium was supplemented with 100 μg/ml of ampicillin antibiotic. LB low salt (10 g/l bacto tryptone, 5 g/l NaCl, 5 g/l yeast extract) was required for manipulation of pPICZαB and pJGGαKR (obtained from Biogrammatics) plasmids in *E. coli*.

The plasmid pJGGαKR is an extracellular expression vector, with *Saccharomyces cerevisiae* α mating factor leader sequence directing secretion. The constitutive GAP promoter drives the expression of the heterologous cDNA. The vector is integrative and it targets to AOX1 terminator locus. Resistance to antibiotic G418 is used as the selection marker.

The *P. pastoris* strain used for heterologous expression was BG-10 (BioGrammatics Ltd). *P. pastoris* strain was propagated in YPDS medium (10 g/l yeast extract; 20 g/l bacto tryptone; 20 g/l glucose; 182.2 g/l sorbitol).

2.3. Vectors construction and transformation

A gene coding for POXA1b from *P. ostreatus* (GenBank AJ005018) was synthesized and optimized according to *P. pastoris* codon usage (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The gene was properly designed in order to allow its cloning into the expression plasmids. The gene product was: i) restricted with *Eco*RI-*Xba*I for cloning into pPICZαB under the control of AOX1 promoter to get the recombinant plasmid pPICZαB-POXA1b and ii) hydrolyzed with *Bsa*I and ligated into the corresponding site of the pJGGαkR vector under the control of the constitutive GAP promoter, yielding the recombinant plasmid pJGG-POXA1b. Both recombinant plasmids were linearized by *Bsi*WI and transformed into *P. pastoris* BG10 by electroporation, as already reported by Piscitelli et al. (Piscitelli et al., 2017).

The cell suspensions were spread on YPDS medium supplemented with 100 μg/ml Zeocin or 900 μg/ml G418 respectively for pPICZαB-POXA1b and pJGG-POXA1b transformations, and incubated for 3–7 days at 28 °C until colony formation. The colonies were then streaked on solid MM medium (13 g/l yeast nitrogen base with ammonium sulfate without amminoacids, 4×10^{-4} g/l biotin, 0.5% methanol, 0.6 mM $CuSO_4$, 100 μg/ml Zeocin) for pPICZαB-POXA1b and MD medium (13 g/l yeast nitrogen base with ammonium sulfate without amminoacids; 4×10^{-4} g/l biotin; 20 g/l glucose, 0.6 mM $CuSO_4$, and 900 μg ml^{-1} G418) for pJGG-POXA1b. Both media were supplemented with the laccase chromogenic substrate ABTS (0.2 mM), to verify laccase expression. Plates were incubated upside down for 4 days at 28 °C and checked for the development of green colour. Positive clones were inoculated in liquid media (BMGY for pJGG-POXA1b bearing clones and BMMY for pPICZαB-POXA1b ones), incubated at 28 °C for 8 days on a rotary shaker (250 rpm), and daily assayed for cell density and secreted laccase activity. Media composition was as follows: 13 g/l yeast nitrogen base with ammonium sulfate without amminoacids, 10 g/l yeast extract; 20 g/l peptone, 100 mM potassium phosphate, pH 6.0; 4×10^{-4} g/l biotin, 1% glucose (in BMGY) or 0.5% methanol (in BMMY). The best producing clones for each transformation, namely AOX system (among the pPICZαB-POXA1b bearing clones) and GAP system (among the pJGG-POXA1b recombinant clones) were selected and used for the recombinant expression.

2.4. Recombinant laccase production in shaken flask

The two selected recombinant clones were pre-inoculated in 50 ml BMGY medium and grown overnight at 28 °C on a rotary shaker (250 rpm). The pre-cultures were then diluted into 250 ml in 1 l flask of BMMY in the case of AOX system, and in BMGY in the case of GAP system to get a starting OD_{600} value of 1.0. 1% glycerol or glucose were tested as alternative carbon sources in BMGY. Cells were grown for 9 days on a rotary shaker (250 rpm) at 28 °C. 1.5% methanol was daily added to the culture of AOX system to induce protein expression. The supernatant was daily recovered and assayed for laccase production.

2.5. Laccase assay

Laccase activity was assayed at 25 °C by monitoring the oxidation of ABTS at 420 nm ($\varepsilon_{420} = 36 \times 10^3\ M^{-1}\ cm^{-1}$). The assay mixture contained 2 mM ABTS in 0.1 M sodium citrate buffer (pH 3.0).

Laccase activity towards 2,6-dimethoxyphenol (DMP) was assayed in a mixture containing 1 mM DMP in the McIlvaine's citrate-phosphate buffer adjusted to pH 5.0. Oxidation of DMP was followed by an absorbance increase at 477 nm ($\varepsilon_{477} = 14.8 \times 10^3\ M^{-1}\ cm^{-1}$).

K_m values were estimated using the software GraphPad Prism (GraphPad Software, La Jolla, California- USA http://www.graphpad.com) on a wide range of substrate concentrations (0.05 − 3 mM). Enzyme activity was expressed in international units (IU).

2.6. Recombinant laccase production in fed batch fermentation

Fed-batch fermentations of the constitutive GAP system were performed with an Applikon mini-bioreactor with a final working volume of 2 l. The media were as follows. PTM_1 trace salts stock solution contained, per liter: 6.0 g $CuSO_4{\cdot}5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4{\cdot}H_2O$, 0.2 g $Na_2MoO_4{\cdot}2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4{\cdot}7H_2O$, 0.2 g biotin and 5.0 ml H_2SO_4 (95–98%). Batch medium contained, per liter: 23.7 ml H_3PO_4 (85%), 0.6 g $CaSO_4{\cdot}2H_2O$, 9.5 g K_2SO_4, 7.8 g $MgSO_4{\cdot}7H_2O$, 2.6 g KOH, 40 g glycerol,0.5 ml Antifoam, 4.4 ml PTM_1 trace salts stock solution and 0.6 mM $CuSO_4{\cdot}5H_2O$. The feed solution contained, per liter: 550 ml glycerol or 550 g of glucose and 12 ml PTM_1 trace salts stock solution. The dissolved oxygen was controlled at DO ≈30% with a stirrer speed of 600–1200 rpm. Aeration rate was 100 l x h^{-1} air. The temperatures