Methanol addition after glucose depletion improves rPOXA 1B production under the *pGap* in *P. pastoris* X33: breaking the habit

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Abstract

The purpose of this study was to demonstrate that methanol addition after glucose depletion has a positive effect on improving rPOXA 1B production under the control of *pGap* in *P. pastoris*. Four different culture media (A, B, C and D) were used to culture *P. pastoris* X33/*pGapZaA-LaccPost-Stop* (clone 1), containing a previously optimized *POXA 1B* synthetic gene coding for *P. ostreatus* laccase, which after glucose depletion was supplemented or not with methanol. Enzyme activity in culture media without methanol (A, B, C and D) was influenced by media components, presenting activity of 1254.30 ± 182.44 , 1373.70 ± 182.44 , 1343.50 ± 40.30 and 8771.61 ± 218.79 U L⁻¹, respectively. In contrast, the same culture media (A, B, C and D) with methanol addition 24 h after glucose depletion attained activity of 4280.43 ± 148.82 , 3339.02 ± 64.36 , 3569.39 ± 68.38 and $14,868.06 \pm 461.58$ U L⁻¹ at 192 h, respectively, representing an increase of approximately 3.9-, 2.4-, 3.3- and 1.6-fold compared with culture media without methanol. Methanol supplementation had a greater impact on volumetric enzyme activity in comparison with biomass production. We demonstrated what was theoretically and biochemically expected: recombinant protein production under *pGap* control by methanol supplementation after glucose depletion was successful, as a feasible laboratory production strategy of sequential carbon source addition, breaking the habit of utilizing *pGap* with glucose.

Keywords *pGap* · *pAOX1* · *P. pastoris* X33 · rPOXA 1B · Laccase · Glucose · Methanol

1 Introduction

Laccases (p-diphenol: dioxygen oxidoreductases EC 1.10.3.2) are multi-copper oxidoreductase enzymes [4] capable of catalysing the reduction of molecular oxygen (O₂) to water (H₂O) [41, 55]. For industrial applications, the most advantageous laccases are those with a high redox potential [35], owing to the increased range of transformable substrates by the enzyme. The successful application of laccases in industry are derived from fungi, where considerable yields have been achieved [4]. However, fungi have certain limitations, as they

demand high oxygen availability and require long cultivation times because of important changes in the rheology of the medium due to mycelial growth [1, 8, 43, 49]. These limitations have led to the heterologous expression of laccases in hosts such as *Saccharomyces cerevisiae* [39], *Yarrowia lipolytica* [33], *Pichia pastoris* [12, 56] and *Kluyveromyces lactis* [21]. Nevertheless, *P. pastoris* remains an established system for the successful expression of recombinant proteins, mainly for biopharmaceuticals and enzymes for industrial or environmental application [14, 37]. It is also a widely used host for fungal laccase expression. The reasons for the use

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of *P. pastoris* are its high level of expression, the ability to use different carbon sources [51, 52], the advantage of growing in high cell density cultures and its capacity to produce large amounts of recombinant protein [4]. Additional advantages include its positive response to different fermentation strategies [25] and ability for high extracellular expression of recombinant proteins [74]. Lastly, *P. pastoris* is designated as generally recognized as safe (GRAS) by regulatory authorities [2].

For over three decades, diverse promoters have been used to express different types of recombinant proteins in *P. pastoris*, with *pAOX1* and *pGap* among those most frequently adopted [3, 14, 15, 17, 22, 36, 38, 48, 56, 67]. Methanol induces *pAOX1* (tightly regulated carbon source-dependent repression/induction promoter), favouring the expression of proteins under the control of the promoter. *pAOX1* is repressed by glucose and glycerol; even the absence of these carbon sources does not represent a significant transcription of *AOX1* (alcohol oxidase, E.C. 1.1.3.13), demonstrating that methanol is necessary to induce high levels of transcription [13]. Culturing *P. pastoris* in media containing methanol as the carbon source requires peroxisome biogenesis to detoxify the cell from H_2O_2 generated during methanol metabolism [3, 52, 61, 64, 65, 70]. Methanol is catabolized into dihydroxyacetone (DHA) and glyceraldehyde phosphate (GAP). DHA exits the peroxisome to be phosphorylated in the cytosol and is converted into dihydroxyacetone-phosphate (DHAP) and later to glyceraldehyde-3-phosphate (GA3P) that feeds the glycolytic pathways. In general, this is the mechanism by which *P. pastoris* processes methanol. Moreover, favoured by the methanol detoxification pathway, this is how it induces recombinant protein expression controlled by the *pAOX1* promoter [32, 52, 63].

The constitutive *pGap* promoter is a strong promoter that controls the expression of NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12), an enzyme that participates in both glycolytic and gluconeogenesis pathways [42, 52, 69, 76]. However, the strength of *pGap* depends on the carbon source.

In 1997, a study conducted by Waterham et al. [69] demonstrated higher β -lactamase (E.C. 3.5.2.6) expression using the *pGap* promoter (*pGap-bla*) under glucose in comparison with the *pAOX1* (*pAOX1-bla*) promoter under methanol induction. Waterham et al. [69] also established remarkable expression of *pGap-bla* depending on the carbon source: 100% expression under glucose > 73% under glycerol > 45% under oleic acid > 36% under methanol [69]. This demonstrated that methanol stimulates *pGap*, thereby increasing the recombinant enzyme production under the control of this promoter. Furthermore, *pGap* and *pAOX1* efficiencies have been previously addressed [7, 69, 76]; however, other authors have obtained contradictory

results [9, 57, 66], suggesting that many other uncontrolled factors can interfere with recombinant protein expression, such as the nature of the protein and intrinsic characteristics [10].

Successful strategies have been used to improve recombinant protein expression under the control of *pAOX1* (*Mut^s* phenotype, slow methanol utilization) and/or the *pGap* promoter. For example, integration of several gene copies within *P. pastoris*' genome [7], culture with mixed-carbon sources, such as glucose/methanol [44], glycerol/methanol [30, 73] or sorbitol/methanol [31, 53] under *pAOX1* expression control. Additional strategies include the combined use of *pGap* and *pAOX1* within the same strain [71] to induce *pAOX1* after glucose depletion, and a strategy also used to sequentially express and recover the cloned protein separately (extra- and intracellular), [16, 71, 72]. In addition, the co-expression of chaperones and the engineering of glycosylation and secretory pathways have been implemented as a strategy to maximize the production of recombinants [37, 46] and regulate glycosylation patterns of pharmaceutical proteins [28, 29].

Our objective was to assay the effect of methanol addition after glucose depletion on recombinant laccase production controlled by *pGap* from *Pleurotus ostreatus* expressed in *P. pastoris*, hereinafter referred to as rPOXA 1B [56], for which different culture media were used. This equates to searching for a feasible laboratory production strategy of sequential carbon source addition, breaking the habit of using glucose to stimulate *pGap* and methanol to induce *pAOX1*.

2 Materials and methods

2.1 Strain

P. pastoris X33 containing expression vector *pGapZaA-LaccPost-Stop* (clone 1) with previously optimized *POXA 1B* synthetic gene coding for *Pleurotus ostreatus* laccase was used. This strain was previously preserved in YPG media (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) supplemented with 20% (w/v) glycerol and stored at -80°C [56].

2.2 Inoculum preparation

Vials obtained from *P. pastoris* X33/*pGapZaA-LaccPost-Stop* (clone 1) Master Cell Bank (MCB) were used [50]. Once thawed, 5 mL sterile YPG media supplemented with $100\ \mu\text{g mL}^{-1}$ Zeocin was inoculated and incubated at 30°C

overnight at 180 rpm. Five-hundred-milliliter Erlenmeyer flasks containing 100 mL fresh YPG-Z (effective work volume, EWV) were inoculated and cultured for 12 h under the same conditions.

2.3 Culture media and conditions

For recombinant strain culture, four culture media were evaluated on an Erlenmeyer shake-flask scale (Table 1) and were cultured at 30 °C, 180 rpm, with or without methanol supplementation after glucose depletion. Culture media A, B and C were based on previously published sequential statistical design and improved processes [5]. Culture media D was based on a sequential statistical design and improved process, where nitrogen and carbon sources were substituted to generate a low-cost culture media for rPOXA 1B production [6] (Table 1). All these media were assayed in triplicate for 0.5% methanol (v/v) addition [26, 27], every 24 h, starting at 24 h. In general, samples were collected at 0, 12, 24 and every 24 h up to 168 h (for media A, B, C and D without methanol), and up to 192 h (for media A, B, C and D with methanol).

Samples were centrifuged at $5867 \times g$, 17 ± 2 °C for 15 min and assayed for residual glucose concentration (g L^{-1}) or residual reducing sugar concentration (g L^{-1}), [40], enzyme activity (U L^{-1}), [5, 47, 62], extracellular protein concentration (mg mL^{-1}), [19], specific enzyme activity (U mg^{-1}) and C/N ratio [54]. Every sample was collected before methanol addition.

2.4 Dry biomass determination (g L^{-1})

The dry biomass concentration was calculated for media A, B, C and D with methanol using a calibration curve as follows [34] (Eq. 1).

$$X = 1.1726 \times OD_{\lambda 600 \text{ nm}} \quad R = 0.9870 \quad (1)$$

where $X = \text{g L}^{-1}$ dry biomass, and $OD_{\lambda 600 \text{ nm}}$ = optical density at 600 nm. Subsequently, to obtain $\mu_{(X)}$ (h^{-1}) and dt (h), X (g L^{-1}) was transformed into $\text{Ln}_{(X/X_0)}$ [20].

2.5 Extracellular protein quantification (mg mL^{-1})

Residual protein for each sample was determined by direct absorbance quantification at 280 nm, using a NanoDrop UV-Vis spectrophotometer (Thermo Scientific) [19].

2.6 Laccase activity quantification (U L^{-1})

To determine laccase activity, two different buffers, 60 mM sodium acetate buffer and 0.1 M citrate buffer, were used.

In the first assay, laccase activity (U L^{-1}) was monitored by a change in absorbance at 436 nm ($\epsilon_{436} = 29.300 \text{ M}^{-1} \text{ cm}^{-1}$) due to ABTS [2, 20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] oxidation in a 60 mM sodium acetate buffer (pH 4.5 ± 0.2). An 800-micro-liter sample was added to 100 μL of 600 mM sodium acetate buffer, and 100 μL of 0.5 mM ABTS at room temperature (25 °C). Green radical formation was evaluated spectrophotometrically for 1 min. Blank solution contained 800 μL distilled water, 100 μL of 600 mM sodium acetate buffer solution and 100 μL of 0.5 mM ABTS [62]

Table 1 Conditions and component description of the four culture media used in the study

Description and theoretical composition	Culture media			
	A	B	C	D
Erlenmeyer total capacity (mL)	500			
Inoculum with $9.1 \pm 0.0005 \text{ g L}^{-1}$ biomass (% v/v)	10			
CuSO_4 (g L^{-1}), (rPOXA 1B enzyme cofactor)	0.16			
Chloramphenicol (g L^{-1}), (to avoid bacterial contamination)	0.10			
Media volume (mL), EWV	150	300		
Glucose (g L^{-1})	10			–
Glucose USP (g L^{-1})	–	–	–	20
Peptone (g L^{-1})	20	10	20	–
Soy protein isolate (g L^{-1})	–	–	–	40
Yeast extract (g L^{-1})	10	10	15	–
Malt extract (g L^{-1})	–	–	–	11.74
$(\text{NH}_4)_2\text{SO}_4$ (g L^{-1})	2.64	0.66	2.64	4.91
(C/N) ₀ ratio	3.4 ± 0.04	4.4 ± 0.03	3.5 ± 0.03	6.7 ± 0.02
References	[5]			[6]

(C/N)₀ ratio is the carbon/nitrogen ratio (at the beginning, before strain inoculation)

(Eq. 2). This technique was used only for culture media A, B and C with or without methanol.

$$U \text{ mL}^{-1} = \frac{(\Delta E \times V_t)}{(\epsilon \times d \times V_s)} \quad (2)$$

where ΔE corresponds to the difference between final and initial absorbance after 1 min of reaction, V_t refers to the total reaction volume (mL), ϵ is ABTS molar extinction coefficient ($\text{M}^{-1} \text{cm}^{-1}$), d is the length of the cuvette in cm and V_s is the volume of sample (mL) contained in the reaction.

For the second assay, laccase activity (U L^{-1}) was monitored by a change in absorbance at 420 nm ($\epsilon_{420} = 36.000 \text{ M}^{-1} \text{cm}^{-1}$) due to ABTS oxidation in a 0.1 M citrate buffer (pH 3.0 ± 0.2). A two-microliter sample was added to 898 μL of 0.1 M citrate buffer and 100 μL of 2 mM ABTS at room temperature (25°C). Green radical formation was evaluated spectrophotometrically for 1 min. Blank solution contained 2 μL distilled water, 898 μL citrate buffer solution and 100 μL of 2 mM ABTS [5, 47] (Eq. 2). This technique was used only for culture media D with or without methanol.

For both assays, an enzyme activity unit is defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute. Specific activity was calculated as the quotient between enzymatic activity and total extracellular protein concentration, per minute (Eq. 3).

$$\text{Spec. Act.} = \frac{\text{Enz. Act.}}{\text{Prot. Conc.}} \quad (3)$$

where Enz. Act. is the enzyme activity expressed as U L^{-1} , Prot. Conc. is the extracellular protein concentration expressed as mg mL^{-1} .

Productivity as a function of enzyme biological activity, expressed as $\text{U L}^{-1} \text{h}^{-1}$ (Eq. 4), was calculated as follows:

$$P_{\text{Enz.}} = \frac{\text{Enz. Act.}}{\text{Time}} \quad (4)$$

2.7 Residual reducing sugar quantification (g L^{-1})

Total residual reducing sugar (glucose or glucose USP) concentrations were determined for each sample in triplicate. To this end, 3,5-dinitrosalicylic acid was used [40]. A standard curve was prepared with D-glucose ranging from 0.1 to 2 g L^{-1} ; values were calculated according to Eq. 5.

$$y = 0.6181x - 0.048; \quad R^2 = 0.9984 \quad (5)$$

2.8 Determination of total carbon (TC, g L^{-1}), total nitrogen (TN, g L^{-1}) and carbon/nitrogen (C/N) ratio

Both TC and TN concentrations were measured by using a total organic carbon (TOC) analyser (Shimadzu TOC-L). This device provides a unique method of catalytic combustion oxidation and NDIR, according to Standard Method 5310B [54]. Standard solutions (0.1 g L^{-1}) of each media component (separately) were prepared and then analysed in the TOC device. Once TC and TN concentrations were established, stoichiometric calculations were performed to determine the concentration for each culture media (g L^{-1}) and media (C/N) ratio. The C/N ratio of the kinetic behaviour (after strain inoculation) was assayed directly from the sample after biomass removal by centrifugation, following the previously mentioned conditions.

3 Results

In this study, different culture media (A, B, C and D) resulting from statistical processes of improvement, optimization and cost reduction were used [5, 6] to test the expression of the laccase POXA 1B from *Pleurotus ostreatus* expressed in *P. pastoris* under the control of the constitutive promoter *pGap*. Glucose was used as the primary carbon source, followed by the addition or not of methanol, after glucose depletion.

P. pastoris X33/*pGapZaA-LaccPost-Stop* (clone 1) kinetic behaviour grown in different media with or without methanol addition after glucose depletion is illustrated in Fig. 1, where it is possible to observe kinetic behaviour changes when methanol was added at 24 h of culture.

3.1 Culture media without methanol

For culture media without methanol (A, B, C and D), glucose or glucose USP concentration was depleted approximately at 12 or 24 h of culture (Fig. 1), suggesting that 24 h was an adequate time to initiate methanol addition [52].

Culture media A, B and C had similar initial glucose concentrations. They differed in their yeast extract, peptone and $(\text{NH}_4)_2\text{SO}_4$ concentrations (Table 1). Table 2 and Fig. 1 show the observed volumetric enzyme activity and specific activity for culture media A, B, C and D at 168 h of culture.

For A and B media without methanol, maximum volumetric productivity was obtained at 72 h of culture (12.28 and $10.81 \text{ U L}^{-1} \text{h}^{-1}$) and decreased to 7.47 and $8.18 \text{ U L}^{-1} \text{h}^{-1}$ at 168 h, respectively (Fig. 1). For culture media C, no sample was collected at 72 h; nonetheless, volumetric productivity at 168 h was $8.0 \text{ U L}^{-1} \text{h}^{-1}$.

Fig. 1 Growth kinetic of *P. pastoris* X33/pGapZaA-LaccPost-Stop (clone 1) cultured in different media (A, B, C and D) with or without methanol addition after glucose depletion (media A, B and C) or

residual reducing sugar (media D) depletion. In media with methanol, the red arrow indicates time of initial methanol addition, and the blue arrow indicates the last methanol supplementation

Higher volumetric enzyme activity and specific activity at 168 h of culture were observed in media D in comparison with media A, B and C (Table 2, Fig. 1). Yet, higher volumetric productivity ($105.97 \text{ U L}^{-1} \text{ h}^{-1}$) was obtained at 12 h of culture and decreased to $52.21 \text{ U L}^{-1} \text{ h}^{-1}$ at 168 h of culture.

3.2 Culture media with methanol

Glucose or glucose USP concentration depletion exhibited similar trends (at approximately 12 or 24 h of culture) for all culture media (A, B, C and D with methanol), thus

Table 2 Volumetric (U L^{-1}) a specific (U mg^{-1}) activity at 168 and 192 h of culture of different media with or without methanol supplementation, respectively

Culture media	Volumetric activity	
	$\text{U L}^{-1} \pm \text{SD}$ (168 h)	$\text{U L}^{-1} \pm \text{SD}$ (192 h)
A	1254.30 ± 182.44	
B	1373.70 ± 182.44	
C	1343.50 ± 40.30	
D	8771.61 ± 218.79	
A with methanol		4280.43 ± 148.82
B with methanol		3339.02 ± 64.36
C with methanol		3569.39 ± 68.38
D with methanol		$14,868.06 \pm 461.58$
Culture media	Specific activity	
	$\text{U mg}^{-1} \pm \text{SD}$ (168 h)	$\text{U mg}^{-1} \pm \text{SD}$ (192 h)
A	40.87 ± 7.15	
B	57.05 ± 7.15	
C	31.64 ± 1.22	
D	971.92 ± 15.21	
A with methanol		160.38 ± 2.90
B with methanol		135.95 ± 4.30
C with methanol		105.56 ± 2.66
D with methanol		1597.6 ± 76.28

confirming the decision to start methanol supplementation at 24 h.

The C/N ratio increased slightly at the beginning (0 h), as a result of the inoculation effect (Table 1, Fig. 1A, B, C, D with methanol), as follows: For media A with methanol from 3.4 ± 0.04 to 3.7 ± 0.5 ; for media B with methanol from 4.4 ± 0.03 to 4.6 ± 0.39 ; media C with methanol from 3.5 ± 0.003 to 3.7 ± 0.3 ; and media D with methanol from 6.7 ± 0.02 to 7.1 ± 1.2 . Subsequently, the C/N ratio decreased from 0 to 24 h, but a modest increase from 24 h up to 120 h (end of methanol addition) was observed. Lastly, a final decrease at 192 h of culture was recognized to conclude at 3.43 ± 0.3 , 6.03 ± 0.13 , 4.35 ± 0.41 and 9.42 ± 0.27 , for media A, B, C and D, respectively (Fig. 1A, B, C, D).

For culture media A, B, C and D with methanol at 192 h of culture, volumetric enzyme activity increased (Table 2), representing an approximate 3.4-, 2.4-, 2.7- and 1.7-fold increase compared with media without methanol, respectively. Specific activity also increased (Table 2), exhibiting an approximate 3.9-, 2.4-, 3.3- and 1.6-fold increase (Fig. 1) compared with the same media without methanol.

Volumetric productivity behaviour depended on culture media. During the methanol addition period (between 24 and 120 h) for A and C media, its value increased up to 192 h with $22.29 \text{ U L}^{-1} \text{ h}^{-1}$ and $18.59 \text{ U L}^{-1} \text{ h}^{-1}$, respectively. In contrast, for media B at 120 h, a maximum value was observed with $17.61 \text{ U L}^{-1} \text{ h}^{-1}$. Lastly, at 192 h, a negligible decrease

was detected ending with $17.39 \text{ U L}^{-1} \text{ h}^{-1}$. Furthermore, for media D, the highest volumetric productivity was observed at 48 h with $94.0 \text{ U L}^{-1} \text{ h}^{-1}$, and a gradual decrease was recognized until reaching $77.4 \text{ U L}^{-1} \text{ h}^{-1}$ at 192 h (Fig. 1A, B, C).

Biomass growth in culture media A, B, C and D with methanol increased, depending on glucose or glucose USP consumption up to 24 h. As expected, a growth slope change was displayed after sugar depletion, which extended from 24 to 192 h including the methanol supplementation period (Fig. 1A, B, C, D). Specific growth rate ($\mu_x \text{ h}^{-1}$) and doubling time (dt h) were similar for all the different culture media assayed with methanol. On the other hand, maximum biomass expressed as g L^{-1} (Table 3) (at the end of culture) oscillated between 12 and 23 g L^{-1} .

Table 3 Growth slope final biomass and kinetic parameters after glucose depletion, during methanol supplementation

Culture media (with methanol)	$\mu_x (\text{h}^{-1})$	dt (h)	Biomass ($\text{g L}^{-1} \pm \text{SD}$) 192 h
A	0.003	208	15.85 ± 0.68
B	0.004	155	12.28 ± 0.06
C	0.005	148	12.22 ± 0.33
D	0.003	225	18.84 ± 0.16

4 Discussion

In this study, we conducted assays to demonstrate the positive effect of methanol supplementation after glucose depletion to produce rPOXA 1B laccase under the control of the *pGap* promoter in a *P. pastoris* culture. Different culture media previously devised by statistical designs were used [5, 6]. As previously suggested, statistical designs are more efficient than the traditional one factor at time of optimization process [11]. The selection of rich culture media instead of a minimal one responds to minimal culture media limitations. In general, minimal media contains an unbalanced composition, favours salt precipitation, with high ionic strength and insufficient nutrient supply. Moreover, it promotes precipitation of phosphorus compounds, generates osmotic stress that can lead to induction of protein unfolding and favours the formation of reactive oxygen species (ROS), among others, as has been summarized by Burgard et al. [11].

On the other hand, laccase substrate specificity varies with pH, among other factors; therefore, to assay laccase activity from different species, several buffers [47, 62] and substrates [55] have been utilized. In this study, only one substrate (ABTS) employing two different buffers (acetate or citrate) was used. The intention was that after glucose depletion, methanol addition would be the main factor increasing enzyme activity, regardless of the influence the buffer might have on volumetric enzyme activity detection, in addition to the affinity of the enzyme for the substrate. Hence, for culture media D (with or without methanol), enzyme activity was assayed in citrate buffer. Although enzyme activity is higher when using the citrate buffer at pH 3.0 compared with acetate buffer at pH 4.5, media D produced higher enzymatic activity compared to the other studied media, even when enzyme activity was evaluated using acetate buffer at pH 4.5 (data not shown).

4.1 Culture media without methanol

P. pastoris is a methylotrophic yeast which, when grown in glucose, glycerol or ethanol, promotes peroxisomal active degradation through autophagy (mediated by proteolysis), repressing Mut pathway genes expression, such AOX [23, 24, 52, 68, 75].

In this study we used glucose as the primary carbon source, because rPOXA 1B is under the *pGap* control [56]. Additionally, yeast generally have a higher preference for glucose as a substrate, resulting in a rapid activation of the glucose transport signal towards the biosynthesis and cellular regulation machinery, repressing the genes involved in gluconeogenesis, respiration, transport, the glyoxylate

cycle and metabolism of alternative carbon substrates [60].

In general, when *P. pastoris* is grown in glucose, intracellular cAMP levels are low, because glucose inhibits adenyl cyclase (E.C.4.6.1.1) activity. When glucose is depleted, cAMP concentration rapidly increases, forming a complex with “cAMP receptor protein”, which may act on the pAOX1 promoter, inducing the use of other carbon sources [18, 51].

Results obtained with media A, B and C (with glucose and without methanol) at 168 h of culture suggest that a slightly higher media (C/N)₀ ratio and a lower oxygen transfer (volume of medium/volume of shake flask ratio 3:5) surface area (Table 1) discretely favoured enzyme activity ($1373.70 \pm 182.44 \text{ U L}^{-1}$) in culture media B, presenting a 1.095-fold increase in comparison to culture A and 1.022-fold increase in comparison to culture C (Fig. 1).

Culture media D (without methanol) had the following modifications in comparison to other media: glucose was substituted for a two-fold increase in glucose USP concentration (compared to media A, B and C without methanol). Additionally, peptone and yeast extract were both substituted by isolate soy protein [6], resulting in a media (C/N)₀ ratio of 6.7 ± 0.02 (Table 1). This modification represented a 7.0-, 6.4- and 6.5-fold increase in volumetric enzyme activity compared with culture A, B and C, respectively (Media D volumetric enzyme activity $8771.61 \pm 218.79 \text{ U L}^{-1}$). These data suggest that laccase activity is favoured with a higher C/N ratio when compared with media A, B and C. This phenomenon has been observed for native laccases, upon native fungus wood colonization, where laccases are produced in greater quantity as a result of C/N ratio increase. Therefore, in our study, an increase in the C/N ratio possibly mimics “nature’s own C/N ratio environment” [45].

4.2 Culture media with methanol

When *P. pastoris* is grown in methanol, as mentioned previously, peroxisome biogenesis occurs to detoxify methanol; however, peroxisomes are also involved in nitrogen source assimilation, mainly (NH₄)₂SO₄. Nevertheless, in this study, soy protein isolate and malt extract were included in the culture media as organic nitrogen sources, (Table 1) by using amine oxidases [58, 59, 77]. In this regard, other authors have demonstrated that upregulation of nitrogen-associated genes is greater upon methanol supplementation [11], possibly explaining why media D with methanol further increased enzyme activity in comparison with other assayed culture media, due to greater nitrogen source concentrations (Table 1).

Methanol addition every 24 h resulted in important enhancements for all cultures (A, B, C and D), with

augmentations ranging from a 1.7- to 3.4-fold increase, compared with similar cultures without methanol (Fig. 1). On the other hand, a slight increase in the C/N ratio during the methanol addition period was supported by the increase in methanol concentration, coinciding with growth slope observed changes after glucose depletion (Fig. 1A, B, C and D with methanol).

At 168 h, culture media A, B and C without methanol had very similar enzyme activity results. For the same culture media with methanol, an even greater increase was observed, with similar values among media (Table 2, Fig. 1). In relation to enzyme activity, medium D with and without methanol had very similar behaviour between 0 and 72 h; however, after 72 h, medium D with methanol showed a considerably higher increase in enzyme activity (Fig. 1). When comparing the tendency of the enzymatic activity (U L^{-1}) between the media with and without methanol at 168 h (Fig. 1), it was observed enzymatic activity was much higher in the cultures with methanol.

Biomass production only doubled at the end of culture (A, B, C and D with methanol), depending on culture media composition (Table 3). In contrast, depending on culture media composition, volumetric enzyme activity (U L^{-1}) increased during methanol supplementation, ranging between 3339.02 ± 64.36 and $14,868.06 \pm 461.58 \text{ U L}^{-1}$ at 192 h, suggesting methanol addition was less critical for cell growth (Table 3), as has been previously investigated [7]. However, its main importance is based on the fact that methanol metabolism generates formaldehyde, which is transferred to xylulose-5-phosphate (Xu5P), generating G3P and DHA. Xu5P is regenerated in the Xu5P cycle (located in the peroxisomes), involving reactions of the pentose phosphate pathway [46]. Collectively, methanol supply stimulates peroxisome biogenesis, activating the Mut pathway, Xu5P cycle and glycolytic and pentose phosphate pathways [46, 52], thus activating both native and putative *pGap* promoters, important for NAD-dependent glyceraldehyde-3-phosphate dehydrogenase and in this case, rPOXA 1B production, respectively.

P. pastoris combined with use of *pGap* and *pAOX1* has been previously reported [71, 76]. As a case in point, the human granulocyte-macrophage colony-stimulating factor (hGM-CSF) gene was inserted into the pPIC9 K and pGapZA vectors. Both vectors were transformed into GS115 host strain to constitutively produce hGM-CSF under the *pGap* promoter and induce hGM-CSF production by methanol addition after glucose depletion. Other authors have studied recombinant protein sequential expression and their separate recovery (intra- or extracellular) from a *Pichia pastoris* cultivation [72]. In the aforementioned study, β -galactosidase (β -gal) was constitutively and intracellularly expressed under the *pGap* promoter control, and hGM-CSF was secreted by *pAOX1*

methanol induction, after the medium with glucose (yeast extract, peptone, dextrose) was replaced with a medium with methanol (yeast extract, peptone, methanol), respectively. It is important to delineate that in our research when glucose was depleted, it was not necessary to replace the media to induce native *pAOX*; methanol addition followed immediately, although the medium had traces of glucose.

Azoun and Kallel [7] studied by a transcriptomic approach in *P. pastoris* the effect a carbon source would have on rabies virus glycoprotein (RABV-G) production. Their results demonstrated the transcription levels of genes involved in central metabolism were upregulated when glucose was used as a carbon source (*pGap*-RABV-G), while the transcription levels of anti-oxidative genes were lower. When methanol was used (*pAOX1*-RABV-G), the metabolism was redirected towards the dissimilar pathway by positive regulation of formaldehyde dehydrogenase (E.C. 1.2.1.1) and negative regulation of enzymes involved in central metabolism. These observations supported glucose use in *P. pastoris* to improve RABV-G expression [7]. Their results agree with the same effect of glucose had in our work.

Burgard et al. [11] evaluated the expression of porcine pro-carboxypeptidase B (CPB) under the control of *pGap* in *P. pastoris*. In their work, after glycerol depletion, the methanol pulse tests showed strong induction of gene regulation (significant up- and down-regulation) 1 h after the methanol feed was initiated [11]. These results correlated with the restructure of the yeast's metabolism towards methanol use. Their findings pointed out that the regulation of peroxisomal genes was positive during the addition of methanol, which agrees with our findings, however, using glucose instead of glycerol.

5 Conclusion

In the present study, once glucose was depleted, methanol addition directly stimulated native *pAOX1* and *pAOX2*, increasing the production of alcohol oxidase used by the cell to detoxify methanol in the peroxisomes [51, 52]. On the other hand, as a consequence of detoxification, the G3P generated stimulated native and putative *pGap*, where the putative promoter controlled rPOXA 1B expression.

Our proposal could be an interesting strategy, whose results were theoretically and biochemically expected and previously assayed by using glycerol instead of glucose as a primary carbon source on the expression of the CPB gene, under the *pGap* promoter. However, it is important to note that methanol addition is not an environmentally friendly option, since the enzyme used is impure (for

environmental purposes) because it may contain traces of methanol. Nonetheless, this strategy could be useful if the production of the pure enzyme is required for non-environmental applications, or if enzyme production is carried out at a small scale for research purposes. In addition, our proposal breaks the habit of strictly using glucose (or glycerol) for the expression of recombinant proteins under the control of the *pGap*, as is customary.

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Author's contributions RAP-P: Conceived and designed the experiments, analysed the data and wrote the manuscript. LDA-L, EDM-Á and CMR-H: Performed the experiments and analysed the data. AMP-R, BEQ-H, AP-F: Analysed the data and critically reviewed the manuscript. LDA-L, EDM-Á: Wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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