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Comparison of constitutive and inducible β-fructofuranosidase production by recombinant *Pichia pastoris* in fed-batch culture using defined and semi-defined media

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ABSTRACT

Short-chain fructooligosaccharides produced from sucrose by transfructosylation using β-fructofuranosidase (FFase), an industrially important enzyme, finds application in prebiotics, sweeteners and confectionary products. Using recombinant *Pichia pastoris*, the influence of replacing the commonly-used Invitrogen® medium with a semi-defined medium for FFase production under the control of the glyceraldehyde-3-phosphate dehydrogenase (GAP) and alcohol oxidase (AOX) promoters was investigated. Replacing the trace metals (PTM_1) solution with yeast extract resulted in a 54.3% decrease in FFase volumetric activity under control of the AOX promoter, suggesting a distinct requirement for trace metals for recombinant protein synthesis during methanol induction, given that the biomass yield on methanol decreased by only 10%. The same medium adjustment had no effect on enzyme production under GAP promoter control, although AOX promoter control resulted in double the FFase volumetric activity compared to glycerol-fed cultures. Decreasing basal salts by half did not affect the cultures, but alleviated precipitation during sterilisation. Optimisation of the glycerol feed rate and dissolved oxygen tension in DO-stat fed-batch fermentations using the semi-defined medium resulted in 17% increase in volutmetric activity of FFase expressed under the GAP promoter. This study highlighted the influence of carbon source and trace metals on heterologous protein production by *P. pastoris* using constitutive and inducible promoters.

1. Introduction

Short chain fructooligosaccharides (sc-FOS), comprising 1-nystose, 1-kestose and 1-fructofuranosyl-nystose are natural, low calorific sweeteners used in diabetic drug formulations, light jams, ice cream and confectionary products (Sangeetha et al., 2005). sc-FOS can be classified as important nutraceuticals produced from the hydrolysis-transferase action of β-fructofuranosidase (EC 3.2.1.26) whereby fructose monomers are added to sucrose molecules to yield fructooligomers of varying lengths (Chen et al, 2011; Maiorano et al., 2008). β-Fructofuranosidase (FFase) has been isolated and expressed in several bacteria and fungi, including *Aspergillus* spp. (Sangeetha et al., 2005). and *Bacillus macerans* (Fernandez et al., 2007) but the heterologous expression and production optimisation of this industrially important enzyme in the

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methylotrophic yeast *Pichia pastoris* was not previously reported.

P. pastoris is a versatile host successfully employed for heterologous protein production of diverse products, including vaccine sub-units and fully functional enzymes (Calik and Calik, 2012; Cos et al., 2006). Key success factors of using *P. pastoris* as expression host include high biomass yield from fed-batch culture (Cos et al., 2006), easily manipulated promoters inherent in this species (Vogl and Glieder, 2013) and a well-characterised genome (Creg, 1985). The yeast is non-fermentative and easily secretes most recombinant proteins through the *α-MF pre-pro* peptide sequence derived from *Saccharomyces cerevisiae* (Higgins, 2001), making it a suitable host for recombinant production of the FFase enzyme.

Fed-batch culture is essential to attain high volumetric biomass and hence, high product yields to ensure feasible production on commercial scale. During expression under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter (referred to as the GAP strain), batch fermentations commence with glycerol as carbon source (glycerol batch, GB), followed by a glycerol fed-batch phase (GFB) for maximum enzyme production. On the other hand, using the inducible AOX promoter (referred to as the AOX strain), the culture is initially grown in GB and GFB phases to high cell density before switching to methanol for induction of protein expression, the so-called methanol induction phase (MIP). Therefore, in the GAP strain, the GFB phase determines the overall product yield of the fermentation (Garcia-Ortega et al., 2013), whereas in the AOX strain, the GFB merely determines the pre-induction biomass concentration (Gao et al., 2012).

A chemically-defined growth medium regularly used for growing *P. pastoris* was previously developed by Invitrogen Inc. (San Diego, CA, USA) and consists of basal salts, a *Pichia* trace metals (PTM_1) solution specifically formulated for *Pichia* cultures and a carbon source (Invitrogen Corporation, 2002). Salts precipitation, high ionic strength and unbalanced composition have been cited as major problems associated with this medium (Cos et al., 2006; Cereghino et al., 2002), which could negatively impact on productivity when using *Pichia* as recombinant production platform on industrial scale. Improving the medium for industrial heterologous protein production by *P. pastoris* would, therefore, significantly contribute to curb operating costs and enhance the economic attractiveness of using this yeast as expression host. Although much research was conducted on the effect of carbon substrate concentration in fed-batch cultures of this yeast, a distinct paucity remains in the literature dealing with the influence of the other components of the defined medium on recombinant protein production (Cos et al., 2006), specifically the effect of the PTM_1 solution on protein expression under the GAP and AOX promoters.

The purpose of this study was to investigate the effect of modifications to the commonly-used chemically-defined medium of *P. pastoris* on heterologous protein production under GAP and AOX promoter control. This was achieved by replacing PTM_1 solution with yeast extract and by decreasing the concentration of basal salts by a factor of 2. Finally, response surface methodology (RSM) was used to establish the optimal glycerol feed rate and DOT during the glycerol fed-batch (GFB) phase of the GAP strain grown in the semi-defined medium, given that glycerol would be preferable to methanol for the production of food, feed or pharmaceutical products.

2. Materials and methods

2.1. Strains and inoculum preparation

P. pastoris DSMZ 70382, was transformed with synthetic expression plasmids containing the *FopA* gene encoding the β-fructofuranosidase enzyme, obtained from DNA 2.0, CA, USA. Two phenotypes resulted with the *FopA* gene placed under the control of either the constitutive glyceraldehyde-3-phosphate dehydrogenase (pGAP) promoter or the methanol-inducible alcohol oxidase (pAOX) promoter (unpublished results), hereafter referred to as the GAP and AOX strains, respectively.

Stock cultures of the strains were stored at −80 °C in 1 ml aliquots containing 30% (w/v) glycerol as cryoprotectant. Both strains were routinely grown for 72 h at 30 °C on YPD agar plates, consisting out of (per litre): 10 g yeast extract, 20 g peptone, 20 g glucose and 13 g agar (Sigma–Aldrich, Kempton Park, South Africa). Colonies from agar cultures grown for 48 h were used to inoculate four 500 ml Erlenmeyer flasks, each containing 100 ml medium that consisted out of (per litre): 40 ml of a 1 mol/L solution of KH_2PO_4/K_2HPO_4 buffer, 13.4 g Yeast Nitrogen Base (YNB) without amino acids (BD Difco™, Sparks, MD, USA), 10 g $(NH_4)_2SO_4$ and 10 g glycerol (Sigma) and sterilised in an autoclave at 121 °C for 15 min. Shake flask cultures were grown at 30 °C on an orbital shaker (Yihder Technology Co. Ltd, Taipei, Taiwan) adjusted to 200 rpm for 24 h. The contents of the four flasks were used to inoculate 4 L medium in the bioreactor.

2.2. Growth medium

The GAP and AOX strains were grown in the complete defined medium (Medium 1) according to the Invitrogen® protocol (Xie et al., 2005; Invitrogen Corporation, 2002). The batch phase medium consisted of basal salts (BS) that contained (per litre): 26.7 ml of 85% (w/v) H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH, 40 g glycerol and 12 mL/L of *Pichia* trace elements (PTM_1) solution. One litre of the PTM_1 solution contains 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 ml 85% (w/w) H_2SO_4. The semi-defined medium (Medium 2) had the same BS composition as Medium 1, but was supplemented with 10 g/L yeast extract (YE), and the PTM_1 solution was excluded. In semi-defined Medium 3, the concentration of the BS was decreased by a factor of 2, and also supplemented with 10 g/L YE with the PTM_1 solution excluded. In the culture of the GAP strain, 50% (w/v) glycerol was used as the carbon source in all three media during the fed-batch phase, whereas 50% (w/v) glycerol and pure methanol were sequentially added to the reactor vessel during fed-batch cultures of the AOX strain. Foaming was controlled by adding 300 μL Antifoam 204 (Sigma) per litre to the initial fermentation broth.

2.3. Fed-batch cultures

Fed-batch fermentations were carried out using a DO-stat strategy where the medium feed was controlled by the dissolved oxygen tension (DO) and where the medium feed pump was switched on when the DO crossed a user-defined threshold, which indicated a requirement for substrate (Lee et al., 2003). Fermentations were carried out in two 10.5 L BioFlo 110 bioreactors (Eppendorf- New Brunswick, Hamburg,

Germany) with 8 L working volume, each equipped with a polarographic DOT probe and a combination glass pH electrode (all Mettler Toledo, Sandton, South Africa). Throughout all cultivations the temperature, pH and aeration rate were maintained at 30 °C, pH 5.5 and 6 L/min of air flow, respectively. The pH was controlled by automatic titration of 7.1 mol/L NH_4OH.

The glycerol batch phase (GB) varied between 24 h and 27 h and glycerol depletion was identified as a spike in the DOT with a corresponding decrease in the agitation rate, since the stirrer speed was cascaded to the DOT. This signal was used to manually initiate the glycerol fed-batch (GFB) phase at a constant glycerol feed rate of 32 g/h (Invitrogen Corporation, 2002), which lasted 72 h in fermentations of the GAP strain. For the AOX strain, a 24-h GFB phase was followed by the methanol induction phase (MIP) at a constant feed rate of 0.525 ml/(min L) for the first 4 h, followed by 1.1 ml/(min L) for 44 h.

2.4. Optimisation of glycerol feed rate and DOT

The volumetric FFase activity for the GAP strain was optimised as a function of glycerol feed rate (A) and DOT (B) during the GFB phase using response surface methodology (RSM) with a two-factor central composite experimental design (CCD) as shown in Table 1. The CCD comprised 4 replicated centre points, 4 axial points and 4 star points distributed evenly in a sphere. Based on the results of Lee et al. (2003), who investigated the effects of substrate feed rate and DOT independently in fed-batch culture of *P. pastoris* expressing α-amylase, the input factors in the design were in the ranges of $8\ g/h \leq A \leq 50\ g/h$ and $5\% \leq B \leq 60\%$, where A represents the glycerol feed rate and B the dissolved oxygen tension. Thus, a total of 12 experiments were carried out with the volumetric FFase activity as the response variable. Design and analysis of the experiments were done using Design Expert® software (Stat-Ease Inc., Minneapolis, USA).

Table 1 – Central composite design for glycerol feed rate (A) and dissolved oxygen tension (B) with results of biomass concentration and volumetric FFase activity achieved in each experimental run of the GAP strain.

Run no.	Coded values		Experimental values		Response	
	X_A^*	x_B	A (g/h)	B (%)	X[a] (g/L)	U_f (U/ml)[b]
1	0	0	35	30	105.8	4738.67
2	0	1.5	35	60	104.6	4392.14
3	−1	1	15	50	74.2	4141.55
4	0	0	35	30	100.6	4535.33
5	1	1	55	50	95.4	4888.72
6	1	−1	55	10	88.1	4569.25
7	0	−1.25	35	5	90.7	4854.04
8	0	0	35	30	102.2	4896.28
9	−1.35	0	8	30	43.2	4121.01
10	1.4	0	63	30	63.6	4047.17
11	0	0	35	30	101.2	4956.65
12	−1	−1	15	10	67.8	3661.71

*Design values were altered to form a slightly skewed spherical space due to physical constraints in running the fermentation experiments, e.g. in Run 9, coded value of −1.4 gave a glycerol feed rate of 0 g/h, which is not feasible in the experiments.

[a] On dry cell weight basis.

[b] Volumetric activity is based on the total volume of fermentation broth.

2.5. Analyses

One unit of FFase enzyme activity (U) was defined as the amount of enzyme required to produce 1 μmol of glucose per minute from a 100 g/L sucrose solution incubated at 40 °C and pH 5.5 (Hidaka, 1988). Enzymatic activity was determined by dissolving 13.3 g of sucrose in 100 ml citrate-phosphate buffer consisting of 42.4 ml of 0.1 M citric acid and 57.6 ml of 0.2 M Na_2HPO_4 (all Sigma). Of this solution, 0.75 ml was added to 0.25 ml culture supernatant and incubated in a water bath at 40 °C. After 60 min, 61 μl of 35% (w/v) perchloric acid was added to stop the reaction. The solution was centrifuged and the clear liquid analysed with high performance liquid chromatography (HPLC) to determine the concentrations of glucose, 1-kestose and 1-nystose produced during the reaction.

The concentrations of glucose, 1-kestose and 1-nystose were determined by an HPLC equipped with Xbridge™ Amide column with dimensions of 4.6 × 250 mm and 3.5 μm particle size (Waters Corporation, Milford, MA, USA). The mobile phases used for elution were 0.0125% (w/v) ammonium hydroxide in water and 0.0125% ammonium hydroxide in 90% (v/v) acetonitrile at a flow rate of 0.7 ml/min. Peaks were detected by an evaporative light-scattering detector (Varian 380-LC, Varian Inc., CA, USA). External calibration curves based on commercially-available standards (Sigma) were used to quantify the sugars in each sample.

The biomass concentration was determined gravimetrically by centrifuging 5 ml sample of fermentation broth in an oven-dried, pre-weighed tube at 8000 rpm for 5 min. The pellet was washed twice with deionized water and dried at 105 °C in an oven to a constant mass. The mean of duplicate measurements was reported.

3. Results and discussion

3.1. Medium development

Two modifications were made to the conventional chemically-defined medium (referred to as Medium 1) for *P. pastoris* cultivation, namely (i) substituting the PTM_1 solution with 10 g/L yeast extract (Medium 2), and (ii) reducing the concentration of basal salts by half in the presence of 10 g/L yeast extract (Medium 3), also in the absence of PTM_1. The biomass concentrations, as well as the volumetric FFase activities by both the GAP and AOX strains in the three media are compared in Fig. 1.

No significant difference in the volumetric FFase activity and biomass concentration was apparent in cultures of the GAP strain when the PTM_1 solution in Medium 1 was replaced with yeast extract (Medium 2, Fig. 1a). However, a dramatic decrease in volumetric FFase activity of 54% was evident for the AOX strain when PTM_1 was replaced with yeast extract (Medium 2, Fig. 1b), and equalled that recorded for the GAP strain (Fig. 1a). On the other hand, the biomass yield on methanol during the MIP phase (AOX strain) remained similar at 0.11, 0.098 and 0.094 $g_{biomass}/g_{MeOH}$ for Media 1, 2 and 3, respectively. Therefore, whereas the specific FFase activity expressed in terms of dry cell weight (DCW) decreased by more than 47%, from 92.56 U/g_{DCW} to 48.98 U/g_{DCW} when PTM_1 was replaced with yeast extract, the 13% decrease in biomass concentration, corresponding to an approximate 10% decrease in biomass yield ($g_{biomass}/g_{MeOH}$) appeared small by

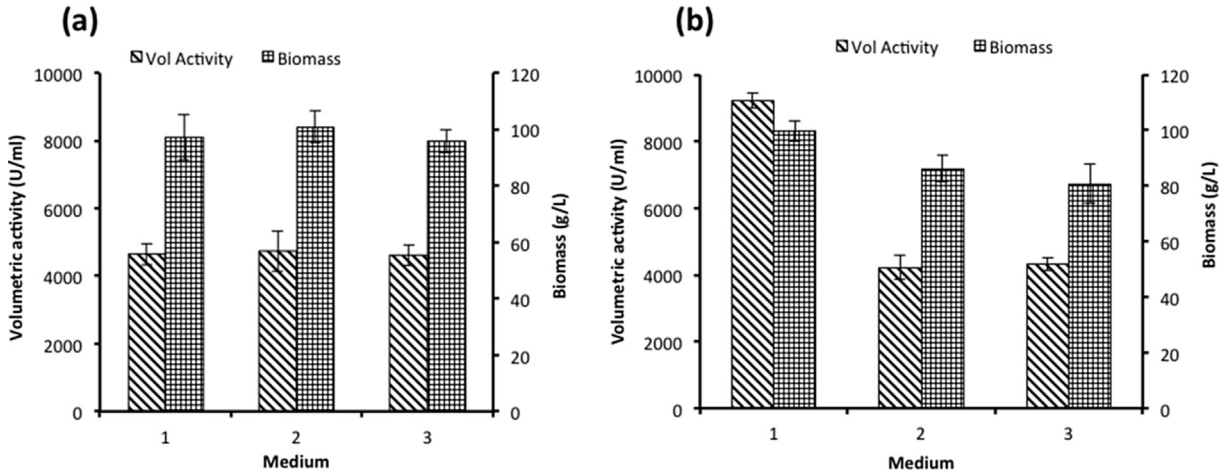

Fig. 1 – Volumetric FFase activity and biomass concentration from fed-batch culture of GAP (a) and AOX (b) strains of *P. pastoris* grown on different media. Medium 1 (control experiment) consisted of the chemically-defined medium by Invitrogen® for *Pichia* cultivations; in Medium 2 the trace elements were replaced by 10 g/L yeast extract, whereas the concentration of basal salts remained unaltered; in Medium 3 the concentration of the basal salts solution was halved and supplemented with 10 g/L yeast extract, whereas trace elements were omitted. Error bars show the standard deviation from the mean determined for triplicate experiments.

comparison. These differences pointed to a distinct requirement for trace elements during heterologous protein production, whereas the effect of trace metals omission on biomass synthesis was much less pronounced. The impact of PTM_1 solution on heterologous protein production by *P. pastoris* has not been described extensively in the literature (Cos et al., 2006), although Wanderley et al. (2013) reported a 60% decrease in the concentration of frutalin expressed in *P. pastoris* KM71H under control of the AOX promoter when PTM_1 solution was eliminated from the growth medium. However, in their study no substitute nutrient source was offered to the culture, such as yeast extract used in the present work.

The results suggest that (i) micronutrients are critical for heterologous protein production under AOX promoter control when using *P. pastoris* as expression host, (ii) in the absence of PTM_1, available trace metals seems to be sequestered for biomass biosynthesis leading to lower levels of enzyme production, and (iii) this difference might be related to the carbon source used during fermentation. Although the exact nature of the interaction between methanol and trace elements during recombinant protein synthesis in *P. pastoris* is not yet clear, Hartner and Glieder (2006) proposed a complex metabolic route for methanol assimilation involving several metal ions acting as enzyme co-factors, which could substantiate the requirement for trace elements for efficient methanol utilisation for biomass synthesis. On the other hand, glycerol has a higher carbon content than methanol and is metabolised through the EMP pathway (Sola et al., 2004) where yeast extract supplementation proved sufficient for FFase production under control of the GAP promoter. Although FFase volumetric activities of the latter strain were lower than that recorded for the AOX strain (Fig. 1), the cellular biosynthetic mechanisms could apparently sustain foreign protein production, in spite of omitting PTM_1. At the molecular level, the effect of these two carbon sources on the nutritional requirements and metabolic behaviour of *P. pastoris* for recombinant protein production remains largely unexplored (Ghosalker et al., 2008; Cos et al., 2006). Our future research includes elucidation of trace element requirements, possibly using continuous culture, and material balances in fed-batch culture.

Decreasing the concentration of basal salts in the presence of yeast extract had little effect on the volumetric activity of FFase, irrespective of medium and carbon source used (Fig. 1). In fact, given that basal salts assist in maintaining isoosmotic pressure, provide pH buffering and supply sulphates and phosphates (Kampen, 2007), the greatest benefit derived from decreasing these medium constituents was decreased salt precipitation during sterilisation, as observed in Medium 1 in the present study. Therefore, the concentration of BS components in the chemically defined Invitrogen medium may be overestimated. The sulphates concentration in Medium 1 is substantially greater compared to defined media used for growth of *S. cerevisiae*, for example (Kampen, 2007). Brady et al. (2001) decreased the concentration of sulphates to 25% of the concentrations in Medium 1 and observed no difference in concentration of *Plasmodium falciparum* merozoite protein-I expressed in *P. pastoris*. Therefore, in large scale production of FFase and other recombinant proteins using the *P. pastoris* expression system, the concentration of basal salts could be decreased by at least 50% to achieve the same production levels as in Medium 1.

3.2. *Optimisation of fed-batch cultures of the GAP strain*

Whereas the AOX system clearly outperformed the GAP system in terms of FFase production, improvement of heterologous protein production using glycerol instead of methanol remains desirable. Methanol as carbon source during microbial fermentation poses several challenges, including high flammability, high volatility and strict specifications for methanol levels provided to the end-user (Waterham et al., 1997; Richter, 2014). Optimisation of the GFB phase of fed-batch culture to improve volumetric FFase activity by the GAP strain was therefore worth investigating, to assess the extent by which enzyme production could be enhanced.

The results from the central composite design (CCD) where volumetric FFase activity (U_f) was optimised through variation of the glycerol feed rate (A) and DOT (B) during the fed-batch phase of the GAP strain using medium 3 are given in Table 1 and plotted in Fig. 2. The data was fitted using quadratic

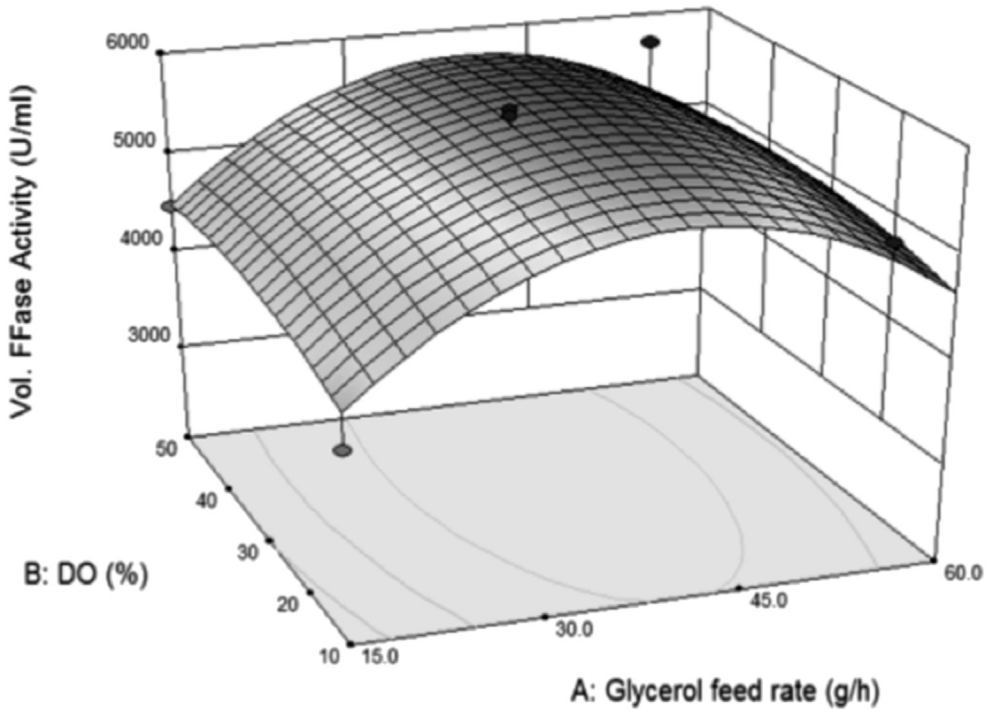

Fig. 2 – Response surface plots of volumetric activity (U_f) of β-fructofuranosidase as a function of glycerol feed rate (A) and dissolved oxygen tension (B) during the glycerol fed-batch phase of GAP cultures grown in Medium 3.

regression of U_f as a function of glycerol feed rate (A) and DOT (B) and the resulting model is given in Equation (1).

$$U_f = 2838.8 + 80.9A + 22.8B - 0.1AB - 0.965A^2 - 0.292B^2 \quad (1)$$

The analysis of variance (ANOVA) in Table 2 shows that DOT had less influence (p-value = 0.0582, Table 2) on volumetric activity of FFase compared to feed rate (p-value = 0.0016). Moreover, there was a strong interaction (p-value of the AB term, Table 2) between the two input factors (DOT and Glycerol) in determining the final enzymatic yield of the process. Therefore a clear optimum for both the feed rate and the DOT could be defined from the surface plot.

The optimal glycerol feed rate and DOT during the fed-batch phase based on the surface plot were 40.3 g/h and 32.23%, respectively, with U_f of 5387.83 U/ml. This volumetric activity was about 17% higher than before optimisation (Fig. 1a, Medium 3). However, the FFase activity for the GAP strain cultivated under these optimum conditions remained about 40% less than that for the AOX strain cultivated in Medium 1 (Fig. 1a). This difference in expression levels between the GAP and AOX promoters observed in the current work has also been reported (Waterham et al., 1997; Zhang et al., 2009; Potvin et al., 2010), where the tight regulation of the alcohol oxidase gene (and hence the AOX promoter) by methanol often results in better expression levels than in the constitutive GAP promoter (Hartner and Glieder, 2006; Kim et al., 2013). Therefore, other optimisation methods at the molecular level, such as increasing the gene copy number and codon optimisation may improve production of FFase under control of the GAP promoter.

Table 2 – ANOVA for regression analysis of volumetric FFase activity for the GAP strain. A = glycerol feed rate, B = dissolved oxygen tension.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-value	p-value
Model	4218.08	5	843.62	35.38	0.0002
A	700.60	1	700.60	29.38	0.0016
B	130.05	1	130.05	5.45	0.0582
AB	0.20	1	0.20	0.00849	0.9296
A^2	3387.07	1	3387.07	142.04	<0.0001
B^2	1.53	1	1.53	0.064	0.8086
Lack of fit	126.80	3	42.27	7.79	0.0628

4. Conclusions

The AOX promoter-MIP fed-batch system remains superior for heterologous FFase production compared to the GAP promoter-GFB system. Furthermore, trace metals proved a critical requirement for heterologous protein production using the AOX system, although this requirement appeared less critical for biomass synthesis. This finding suggests that the higher effectiveness of protein production under methanol consumption imposes a burden on the cellular biosynthetic functions, requiring exogenous co-factor supplementation, evidently not required under GAP promoter control. Therefore, the data confirms that the chemically-defined medium based on the formulation of Invitrogen remains the medium of choice for recombinant enzyme production when using the AOX as promoter with methanol induction. On the other hand, should heterologous protein production under GAP promoter control be the preferred choice, the PTM_1 solution could be substituted with yeast extract, together with a decrease in the basal salts concentration by a factor of at least 2, without compromising the enzyme production levels. The lower cost of a semi-defined medium should be played off against lower process yields. Further study on the identity and function of these trace elements is required to fully understand how enzyme expression under the AOX promoter control can be optimised.

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