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Efficient secretion of inulin fructotransferase in *Pichia pastoris* using the formaldehyde dehydrogenase 1 promoter

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Abstract Inulin fructotransferase (IFTase) has received considerable attention due to its ability to catalyse inulin hydrolysis to difructose anhydride (DFA III), a natural low-calorie functional sweetener. In the present study, for the first time, we describe the expression of IFTase in *Pichia pastoris* under the control of the formaldehyde dehydrogenase 1 promoter (*PFLD1*). Using this system, we achieved efficient secretion with four substrate fed-batch strategies in a 3-L fermenter. The co-feeding induction strategy with methylamine hydrochloride and methanol achieved the maximum extracellular IFTase activity of 62.72 U mL^{-1}, which was 3.2-fold higher than that obtained with the wild-type strain. In addition to methanol, carbon sources such as glucose and glycerol could also be utilised by *PFLD1*-controlled *P. pastoris* for IFTase production using methylamine hydrochloride induction. However, we found that glycerol and glucose should be strictly controlled at low concentrations of 0.5–1.5 % (v/v) and 1–1.5 % (w/v), respectively. The use of glycerol and glucose demonstrated that *P. pastoris* was also attractive for IFTase production via methanol-free cultivation strategies. This study may provide the basis for the industrial use of this recombinant IFTase for the production of DFA III.

Keywords Difructose anhydride · Extracellular expression · Formaldehyde dehydrogenase promoter · Inulin fructotransferase · *Pichia pastoris*

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Introduction

Difructose anhydride (DFA III) is a cyclic disaccharide consisting of two fructose units. DFA III is a non-reducing sugar with half the sweetness and one-fifteenth the calories of sucrose [19]. It increases the absorption of calcium at low doses in both the small and large intestines of rats [34] and humans [32]. Inulin fructosyltransferase (DFA III-forming IFTase, EC 4.2.2.18), a member of the glycoside hydrolase family 91, catalyses the formation of DFA III from inulin using a β-2,1-fructans depolymerisation reaction and is considered to be the most promising enzyme for the production of DFA III [18]. Since 1997, many IFTase genes have been expressed as active enzymes in *Escherichia coli* [29]. Most of the recombinant IFTases exhibited relatively high levels of intracellular expression, but the extracellular enzyme activity of these IFTases was very low [13, 14, 20, 21]. The secretory production of proteins has several advantages, such as few contaminating proteins, no space limitation for the accumulation of the protein, simple downstream processing steps and little harm to the host cells [7]; thus, the efficient secretion of IFTase would be a significant step towards the industrial production of DFA III.

The *Pichia pastoris* expression system is being successfully used for the production of various recombinant heterologous proteins [1–3, 24]. A key advantage of using *P. pastoris* as a host system is that it combines the ability to efficiently secrete heterologous proteins with a unique capacity to grow in minimal medium at high cell densities with low levels of endogenous protein secretion [3]. The tightly regulated alcohol oxidase 1 promoter (*PAOX1*) is commonly used for heterologous protein expression in this system. It is highly and strictly induced during the growth of the yeast on methanol but is tightly repressed by most

common carbon sources such as glucose, glycerol and ethanol [24]. However, there are some concerns regarding the use of methanol as a carbon source for industrial production due to its potential as a fire hazard and its toxicity. Therefore, promoters that are not induced by methanol are more attractive for the expression of proteins. The constitutive strong promoter from glyceraldehyde-3-P dehydrogenase (*PGAP*) can achieve methanol-free cultivation and is readily available for heterologous expression of proteins in *P. pastoris* [11, 22]. However, strong constitutive promoters are not a good choice for the over-expression of proteins that are toxic to yeast [3].

Formaldehyde dehydrogenase promoter (*PFLD1*) is another important promoter for the efficient expression of foreign genes in *P. pastoris* [7, 26, 27]. It can achieve expression levels of the target protein that are similar to those obtained with methanol induction by *PAOX1* [6]. This *PFLD1*-controlled *P. pastoris* expression model is not only strongly induced with methanol as the sole carbon source (and ammonium sulphate as a nitrogen source), but it can also achieve high expression of heterologous proteins with certain methylated amines as the sole nitrogen sources, such as methylamine hydrochloride or methylamine [31]. In contrast to the *AOX1* genes, *FLD* expression could be induced by various nitrogen sources such as methylamine and methylamine hydrochloride and was highly dependent on carbon source which was different preference by *P. pastoris* for proteins expression [15, 26, 38]. In addition to generally utilised sorbitol [8, 27, 28], glucose and glycerol also have the potential to be used as carbon source in this *PFLD1*-controlled system [8, 28, 38]. Although, in some cases, cells grown in these carbon sources produce low levels of detectable active enzymes, Hartner et al. [15] concluded that using glucose as a carbon source did not impede the expression of heterologous proteins, and the absence of heterologous protein expression was due to a lack of induction rather than to strong repression. In addition, it was reported that the production of target proteins can be largely enhanced by methanol/methylamine co-induction strategy or sorbitol/methanol co-feeding induction strategy [9, 28]. Therefore, this system offers flexible nitrogen and carbon sources for *P. pastoris* to express high levels of recombinant proteins with an efficient inducer and allows for the selection of appropriate fermentation conditions for the industrial production of enzymes. Moreover, these features make *PFLD1* an attractive alternative to *PAOX1* for its application in methanol-free cultivation strategies [6, 27, 38]. However, there have been no reports regarding the expression of IFTase in *P. pastoris* under the control of *PFLD1*.

It was reported that the *Arthrobacter aurescens* SK 8.001 IFTase was thermostable and could be a good candidate for the industrial production of DFA III [41]. We have previously described the construction of the recombinant pPIC9 K-IFTase expression vector with the mature IFTase gene under the control of the alcohol oxidase 1 promoter (*PAOX1*) [39]. In this work, the feasibility of the secretory expression of IFTase in *P. pastoris* under the control of *PFLD1* was investigated to achieve a promising alternative system to *E. coli*. Based on the studies of the effect of carbon and nitrogen sources on IFTase production in shake flasks, lab-scale fermentations were further carried out with various feeding-substrate strategies that might provide the basis for the industrial production of IFTase.

Methods

Strains, plasmid and media

The recombinant plasmid pPIC9K-IFTase, harbouring the matured IFTase gene from *Arthrobacter aurescens* SK 8.001 [40], was previously constructed. The *Escherichia coli* strain DH5α and the *P. pastoris* strain GS115 were purchased from Invitrogen (Carlsbad, CA, USA). MD solid medium: 1.34 g L^{-1} yeast nitrogen base (YNB), 4 mg L^{-1} biotin, 20 g L^{-1} glucose and 20 g L^{-1} agar. YPD solid medium: 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose and 20 g L^{-1} agar. Modified BMGY medium: 1.34 g L^{-1} YNB, 4 mg L^{-1} biotin, 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 0.1 M potassium phosphate buffer (pH 6.5) and carbon source of 0.5–2.5 % (v/v) methanol, 0.25–2 % (v/v) glycerol, or 0.5–2.5 % (w/v) glucose. Modified BMMY medium (pH 6.5): BMGY medium added nitrogen source of 0.5–3 % (w/v) methylamine hydrochloride or 0.1–2 % (v/v) ammonium sulphate.

Construction and transformation of *FLD1*-based expression vectors

The *PFLD1* fragment was amplified from the total *P. pastoris* GS115 DNA using the following primers: 5′-GCAGCGAGCTCGCATGCAGGAATCTCTGG-3′ (forward primer) and 5′-GGCCGGATCCTGTGAATATCAAGAATTGTATGAACAAGC-3′ (reverse primer). These primers introduced *Sac* I and *Bam*H I restriction sites at the 5′ and 3′ ends, respectively. The PCR products of the *PFLD1* fragment were digested with *Sac* I and *Bam*H I and ligated into the vector pPIC9K-IFTase that had been digested with the same restriction endonucleases to form the plasmid pPIC9K-FLD1-IFTase. Then, the recombinant expression vector pPIC9K-FLD1-IFTase was linearised with *Sal* I and then transformed into *P. pastoris* GS115 by electroporation using a Gene Pulser X-cell II under the parameters of 1,500 V, 200 Ω, 25 μF and 5 ms (Bio-Rad, Mississauga, ON, Canada).

Screening of transformants

Successful transformations (GS115-FLD1-IFTase) were preliminarily selected by growth on MD solid medium and further screened by growth on YPDS solid medium containing the antibiotic G418 with concentrations of 0.25, 1, 2 or 4 mg mL^{-1}. The transformants were confirmed by polymerase chain reaction (PCR).

Extracellular expression assays

P. pastoris GS115-FLD1-IFTase transformants were inoculated into 25 mL modified BMGY medium and incubated at 30 °C with shaking (200 rpm). When an optical density at 600 nm (OD_{600}) of 6 was obtained, the cells were harvested by centrifugation and resuspended to an OD_{600} of 1 in a 1-L shake flask containing 100 mL modified BMMY inducing medium (pH 6.5) with specific nitrogen source and carbon source. Every 24 h, a 1-mL sample was collected for biomass analysis and enzyme assay.

Enzyme assay

To assay IFTase activity, the IFTase-containing solution (0.2 mL) at a suitable dilution, distilled water (0.3 mL), 0.1 M citrate buffer (pH 6.0, 0.5 mL) and 2 % (w/v) inulin solution (1.0 mL) were mixed. The mixture was incubated at 60 °C for 15 min, and the reaction was halted by incubating it in boiling water for 10 min. The concentration of DFA III produced was determined by high-performance liquid chromatography (HPLC) using a water Sugur-PakTM 1 column (6.5 mm × 300 mm, USA) and a refractive index detector (Shodex RI101). The HPLC conditions and the definition of IFTase activity used are consistent with Hang [12].

Fed-batch fermentation

A comparison study of fed-batch cultivation strategies using methylamine hydrochloride/ammonium sulphate as a nitrogen source and glycerol, glucose or methanol as carbon sources was conducted in a 3-L bioreactor (LiFlus GM BioTRON, Korea) with a working volume of 2 L. The inoculum was produced at 30 °C in a 1-L flask containing 200 mL YPD medium shaken at 250 rpm for 18 h. Then, 10 % (v/v) of the inoculum was inoculated into the 3-L fermenter containing a mineral medium with the following basal composition per litre: KH_2PO_4 12.0 g, $MgSO_4 \cdot 7H_2O$ 4.70 g, $CaCl_2 \cdot 2H_2O$ 0.36 g, 0.1 mL alkoxylated ester JG73 (LookChem, China), and 1 mL of PTM4 trace salts' solution ($CuSO_4 \cdot 5H_2O$, 2.0 g L^{-1}; NaI, 0.08 g L^{-1}; $MnSO_4 \cdot H_2O$, 3.0 g L^{-1}; $Na_2MoO_4 \cdot 2H_2O$, 0.2 g L^{-1}; H_3BO_3, 0.02 g L^{-1}; $CaSO_4 \cdot 2H_2O$, 0.5 g L^{-1}; $CoCl_2$, 0.5 g L^{-1}; $ZnCl_2$, 7 g L^{-1}; $FeSO_4 \cdot 7H_2O$, 22 g L^{-1}; biotin, 0.2 g L^{-1} and 1 mL L^{-1} concentrated H_2SO_4).

The pH of the medium was adjusted and maintained at 6.0 with the addition of 25 % ammonium hydroxide and 30 % phosphoric acid. The dissolved oxygen (DO) level was maintained above 30 % of air saturation. The temperature was controlled at 30 °C for the glycerol batch and fed-batch phase and then decreased to 22 °C at the beginning of the induction phase. When glycerol was depleted from the culture broth, the glycerol fed-batch phase was started. The fed-batch solution contained 500 g L^{-1} glycerol and 2 mL L^{-1} PTM4 stock solution that was pumped into the fermenter according to a predetermined protocol [23]. The methylamine hydrochloride and carbon source of glycerol or glucose feeding stock solution contained the following components: 400 g L^{-1} glycerol or 400 g L^{-1} glucose, 80 g L^{-1} methylamine hydrochloride and 2 mL L^{-1} PTM4 stock solution. The methanol and nitrogen source of hydrochloride or ammonium sulphate feeding stock solution contained 700 mL L^{-1} pure methanol, 2 mL L^{-1} PTM4 stock solution and 80 g L^{-1} methylamine hydrochloride or 80 g L^{-1} ammonium sulphate. During the induction stage, the mixed-feed rate of the feeding substrates was conducted as follows: the feed rate was set to 5 mL h^{-1} L^{-1} initial fermentation volume during the first 5 h and then increased to 7.5 mL h^{-1} L^{-1} initial fermentation volume; after 10–12 h of induction, the feed rate was increased to 10 mL h^{-1} L^{-1} initial fermentation volume and was maintained at this level throughout the remainder of the fermentation.

Results

Screening of multicopy strains

After transformation, the *P. pastoris* expression vectors were integrated into the host chromosome to maximise the stability of the expression strains, which contained varying copy numbers of heterologous gene [3]. The gene copy number can be roughly correlated to the level of G418 resistance and has been identified as a 'rate-limiting' step in the production of recombinant proteins from *P. pastoris* [5, 30, 33]. To investigate the effect of the IFTase gene (*ift*) copy number on the yield of IFTase, we compared the IFTase production of four *P. pastoris* strains with *PFLD1* that were selected from YPDS medium containing 0.25, 1, 2 and 4 mg mL^{-1} G418. As shown in Fig. 1, all of the transformants exhibited extracellular expression of active IFTase with the induction of 1 % methylamine hydrochloride (m/v). However, strains with different *ift* copy numbers showed little effect on their IFTase activity ($p > 0.05$). *P. pastoris* strains with resistance to 1 mg mL^{-1} G418 exhibited slightly higher IFTase activity than the other strains.

It was reported that the expression levels of recombinant proteins in *P. pastoris* could be greatly enhanced by utilising multicopy transformants [23, 33, 36]; however, the effect of copy number on recombinant protein expression levels is unpredictable in this system [35]. This could be because increasing the copy number of the expression cassettes did not exert a knock-on effect (a significant positive correlation between gene copy number and activity of foreign proteins) on transcription and translation, both of which may become rate-limiting due to a lack of resources, such as energy and precursors [24]. Similar results have been reported elsewhere [4, 16]. Therefore, the GS115-FLD1-IFTase strain with 1 mg mL^{-1} G418 resistance was selected for use in subsequent studies.

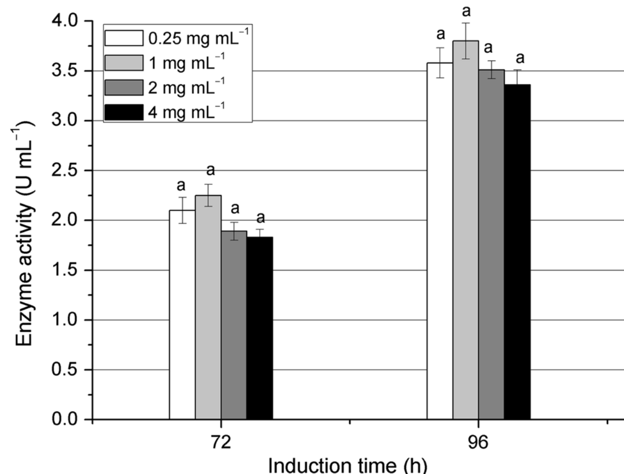

Fig. 1 Comparison of IFTase activity in GS115-FLD1-IFTase recombinants at 72 and 96 h. The strains were selected on 0.25, 1, 2, and 4 mg mL^{-1} G418 and were cultivated in shake flasks at 30 °C and 250 rpm with 1 % (v/v) methanol as a carbon source and 1 % (w/v) methylamine hydrochloride as an inducer. [a]Values in the same induction time that do not share the same superscript are not significantly different. Significant differences were defined at $p < 0.05$. Data were compared by ANOVA and Duncan's tests with SPSS statistics package software (Version 18.0). The *error bars* correspond to the standard deviation of three independent determinations

Effects of nitrogen sources on IFTase production

FLD1 was a key enzyme required for the methylated amine metabolism as nitrogen source, as well as in the methanol catabolism as a carbon source in methylotrophic yeasts [3, 10, 31]. FLD1 is involved in both the oxidation of formic acid in methanol metabolism and the metabolism of formaldehyde generated from primary amines that serve as the primary source of energy and protect cells against the toxic effects of formaldehyde [37]. To investigate the effects of nitrogen source on IFTase production in the *PFLD1*-controlled *P. pastoris*, the GS115-FLD1-IFTase strain was grown with different nitrogen sources using methanol as the carbon source. We observed that both methylamine hydrochloride and ammonium sulphate were the available nitrogen sources for IFTase production by the *PFLD1*-controlled *P. pastoris* (Fig. 2). *P. pastoris* cultivated in methylamine hydrochloride exhibited relatively higher IFTase activity (with maximum value of 5.21 U mL^{-1}) and cell density (with maximum OD_{600} value of 4.64) than when it was grown with a nitrogen source of ammonium sulphate (with maximum IFTase activity and OD_{600} of 4.25 U mL^{-1} and 3.99, respectively) after 96 h of induction. These results were in accordance with those reported for lipase expression in *PFLD1*-controlled *P. pastoris* strains under analogous culture conditions [28]. In this article, we further investigated the effect of nitrogen concentrations on IFTase production and proposed a nitrogen-controlled strategy for the efficient secretion of IFTase. As shown in Fig. 2a, whilst the

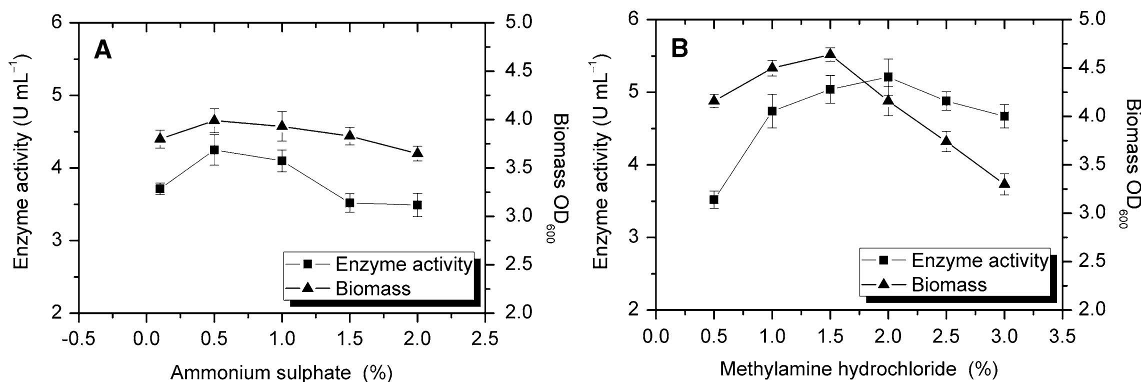

Fig. 2 Effects of different nitrogen sources on cell growth and extracellular IFTase activity in the *PFLD1*-controlled *P. pastoris* with 1 % (v/v) methanol as carbon source. **a** ammonium sulphate, **b** methylamine hydrochloride. Recombinant strains were induced for 96 h. The extracellular IFTase activity (*filled squares*) and OD_{600} (*filled triangles*) are shown. The *error bars* correspond to the standard deviation of three independent determinations

methylamine hydrochloride concentration decreased from 1 to 0.5 %, the IFTase activity was drastically reduced whilst *P. pastoris* maintained a relatively high biomass. However, when the methylamine hydrochloride concentration was increased from 1.5 % (w/v) to 3 % (w/v), the biomass sharply decreased and gradually led to decreased IFTase activity. This result suggested that low concentrations of methylamine hydrochloride weakly induced the *ift* gene, whereas high concentrations of methylamine hydrochloride were conducive to IFTase expression but detrimental to cell growth. Thus, the concentration of methylamine hydrochloride is an important parameter that needs to be regulated for efficient IFTase expression. We concluded that *P. pastoris* at a high cell density might achieve high IFTase expression levels induced by relatively high 1.5 % (w/v) to 3 % (w/v) concentrations of methylamine hydrochloride.

Effect of carbon sources on IFTase production

As described above, FLD1 is involved in the detoxification of formaldehyde in methanol metabolism [25]. In principle, C1-amines are oxidised to carbon dioxide or assimilated into the biomass following the same pathways that are involved in methanol metabolism. However, yeast cannot use methylamine as a sole carbon and nitrogen source, and therefore a supplementary source of easily metabolised carbon must be provided for sustained growth [37]. To investigate the effect of carbon sources on cell growth and IFTase production in *PFLD1*-controlled *P. pastoris*, the recombinant strains were cultivated with different carbon sources using 1 % methylamine hydrochloride (w/v) as the inducer. Interestingly, as shown in Fig. 3, methanol, glycerol and glucose were all effective carbon sources for IFTase production in *PFLD1*-controlled *P. pastoris* with optimum concentrations of 2, 0.75 and 1 %, respectively. Glucose and glycerol were more suitable carbon sources for cell growth and resulted in 2.1-fold and 3.5-fold increased biomass than cells cultivated in methanol due to the high toxicity of methanol at low cell densities of *P. pastoris* [17]. It has reported that glycerol and glucose could be used as carbon sources for the expression of foreign proteins, and they were very suitable for cell growth in this *P. pastoris* system in conjunction with methylamine hydrochloride or methylamine induction. However, the lipolytic activities were very low [28, 38]. In this research, IFTase maintained relatively stable activity at low concentrations of glycerol (0.5–1.5 %) and glucose (1–1.5 %) but was negatively impacted at concentrations exceeding these ranges (Fig. 3b). To some extent, high concentrations of glucose and glycerol could result in continuous rapid cell growth and therefore reduce the expression of heterologous proteins (Fig. 3b, c). Determination of residual carbon sources also showed that the low concentrations of 0.25 % (v/v) glycerol and 0.5 % (w/v) glucose exhibited carbon source limitation, and high concentrations (2 and 2.5 %, respectively) exhibited distinctly carbon source excess (Data not shown). We demonstrated that concentrations of glycerol and glucose may greatly affect the IFTase production in *P. pastoris*. Hartner et al. [15] confirmed that the carbon source did not impede the expression of heterologous proteins with suitable induction. Therefore, in addition to methanol, glycerol and glucose could be efficient carbon sources for IFTase production but should be strictly controlled at low concentrations. In addition, it was reported that sorbitol is an available

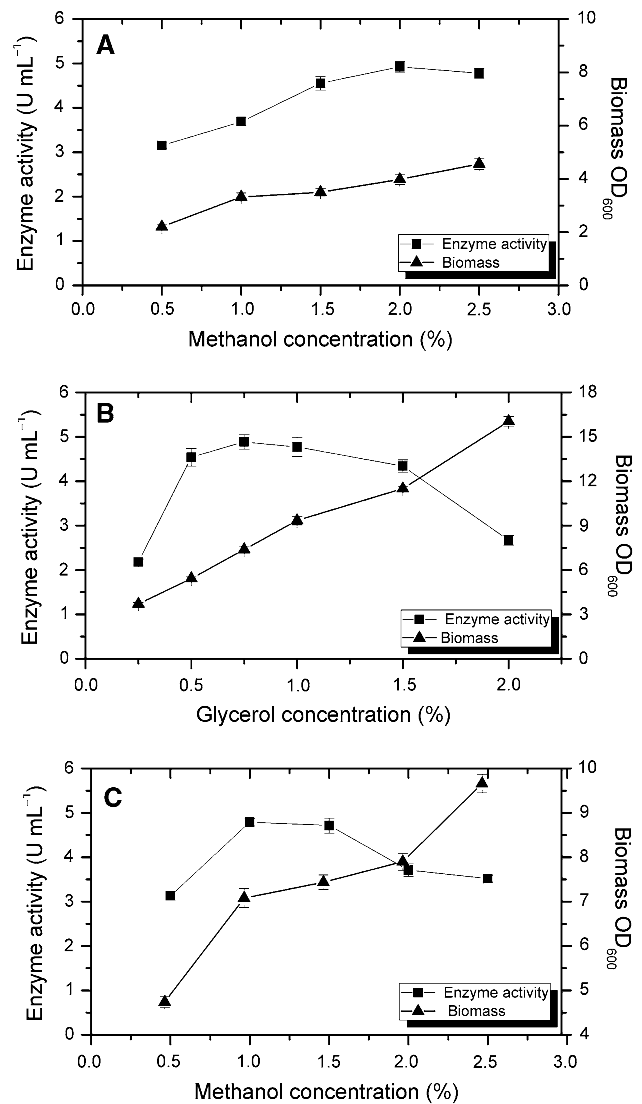

Fig. 3 Effects of different carbon sources on cell growth and extracellular IFTase activity in the *PFLD1*-controlled *P. pastoris* with 1 % methylamine hydrochloride (w/v) as nitrogen source. **a** methanol, **b** glycerol, **c** glucose. Recombinant strains were induced for 96 h. The extracellular IFTase activity (*filled squares*) and OD_{600} (*filled triangles*) are shown. The *error bars* correspond to the standard deviation of three independent determinations

carbon source for the expression of lipase in this system [8, 26]; however, it was slightly utilised for IFTase production (data not shown), presumably owing to the lack of induction of *ift* transcription by sorbitol in *P. pastoris* [42].

Characterisation of *PFLD1*-controlled *P. pastoris* in shake flasks

Based on the effect of nitrogen and carbon sources on the IFTase production by the recombinant strains, *P. pastoris* was cultivated in shake flasks in the following media with the optimised concentrations described above: 2 % (w/v) methylamine hydrochloride/0.75 % (v/v) glycerol (MA-Gly), 2 % (w/v) methylamine hydrochloride/1 % (w/v) glucose (MA-Glu), 2 % (w/v) methylamine hydrochloride/2 % (v/v) methanol (MA-Met) and 0.5 % (w/v) ammonium sulphate/2 % (v/v) methanol (NH4-Met). As shown in Fig. 4, the GS115-FLD1-IFTase strain exhibited a relatively high IFTase production in the MA-Gly and MA-Glu media, which was consistent with cell growth throughout the cultivation phase. In contrast, relatively low IFTase activity and biomass in methanol-induced cultivation were observed when the strain was grown in methylamine hydrochloride or ammonium sulphate medium. In particular, even under optimised conditions of carbon and nitrogen sources, cell growth and IFTase enzyme activity were only slightly improved in any medium. It has been previously reported that the concentration of secreted proteins in the medium is roughly proportional to the concentration of cells in culture, thus the low biomass in the shake flask cultivation may be responsible for this result [3].

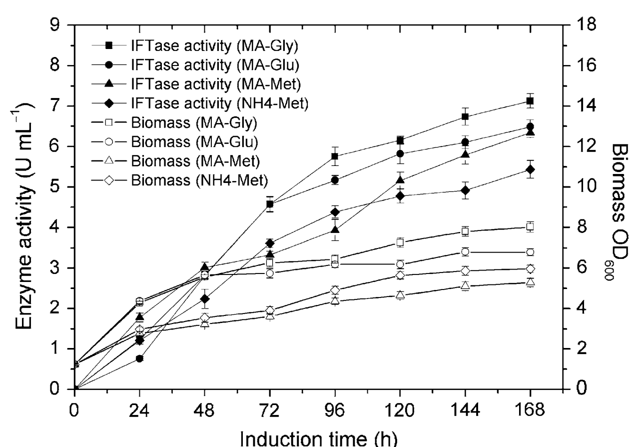

Fig. 4 Characterisation of the *PFLD1*-controlled *P. pastoris* with different carbon and nitrogen sources in shake flasks. The extracellular IFTase activity in MA-Gly (*filled squares*), extracellular IFTase activity in MA-Glu (*filled circles*), extracellular IFTase activity in MA-Met (*filled triangles*), extracellular IFTase activity in NH4-Met (*filled diamonds*), OD_{600} in MA-Gly (*open squares*), OD_{600} in MA-Glu (*open circles*), OD_{600} in MA-Met (*open triangles*), and OD_{600} in NH4-Met (*open diamonds*) are shown. The concentrations of MA, Gly, Glu, Met, and NH4 were 2, 0.75, 1, 2, and 0.5 %, respectively. The culture conditions were 50 mL/250 mL medium at 30 °C and 250 rpm. The *error bars* correspond to the standard deviation of three independent determinations

Comparison of IFTase production with different feeding substrates in fed-batch bioreactor cultivations

Because biomass was the limiting factor for IFTase expression by *PFLD1*-controlled *P. pastoris*, a fed-batch fermentation strategy was utilised to study the potential expression capacity of the GS115-FLD1-IFTase strain fed with different carbon and nitrogen substrates. As shown in Table 1, the activity of recombinant IFTase obtained with fermenters was 4.6- to 9.9-fold higher than that achieved from shaken flasks because the cell density and the extent of aeration were higher. As shown in Fig. 5b, when methanol was added as inducer, the recombinant IFTase activity dramatically increased regardless of whether methylamine hydrochloride or ammonium sulphate was used as the nitrogen source, and the maximum IFTase activity of 62.72 and 53.38 U mL^{-1} occurred at 100 and 112 h with relatively high biomass (OD_{600} of 112.4 and 168.1 corresponding with dry cell weight of 16.9 and 22.9 g L^{-1}, respectively), respectively. Notably, methanol–methylamine hydrochloride co-feeding induction greatly enhanced the IFTase production by the *PFLD1*-controlled *P. pastoris* with a high cell density strategy, which was 9.9-fold higher than the

Table 1 Comparison of IFTase activity secreted by *PFLD1*-controlled *P. pastoris* with different carbon and nitrogen sources in shake flasks and 3 L fermenter

Nitrogen sources	Carbon sources	IFTase activity (U mL^{-1})		Specific activity (U mL^{-1} OD_{600}^{-1})	Productivity (U mL^{-1} h^{-1})	Total protein Yield (g L^{-1})
		Shake flasks[d]	3 L fermenter[e]			
Methylamine hydrochloride	Glycerol	6.49 ± 0.13[b, c]	30.13 ± 1.06[a]	0.12	0.28	1.37
Methylamine hydrochloride	Glucose	7.22 ± 0.10[c]	51.68 ± 1.77[b]	0.24	0.48	1.92
Methylamine hydrochloride	Methanol	6.33 ± 0.17[b]	62.72 ± 1.60[c]	0.57	0.63	2.25
Ammonium sulphate	Methanol	5.42 ± 0.31[a]	53.38 ± 1.84[b]	0.33	0.49	2.01

[a, b, c] Values in the same cultivation levels that do not share the same superscript are significantly different

[d, e] The ratio of IFTase activity secreted by *P. pastoris* in 3 L fermenter and in shake flasks or that of wild-type strain

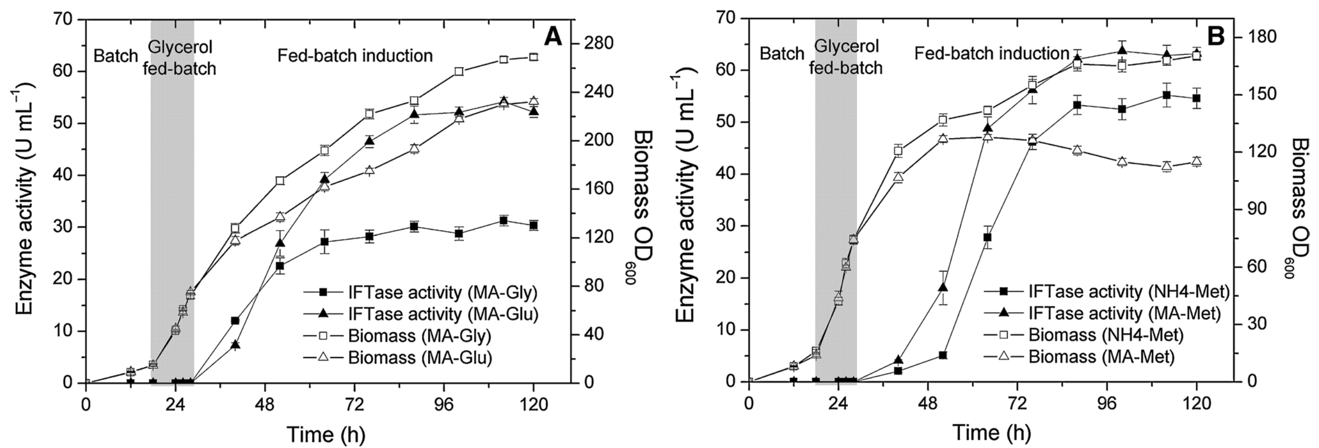

Fig. 5 Time-course profile of recombinant IFTase production with different carbon and nitrogen sources in 3 L fermenter. **a** Methylamine hydrochloride as an inducer. The extracellular IFTase activity in MA-Gly (*filled squares*), extracellular IFTase activity in MA-Glu (*filled triangles*), OD_{600} in MA-Gly (*open squares*) and OD_{600} in MA-Glu (*open triangles*) are shown. **b** Methanol as an inducer. The extracellular IFTase activity in NH4-Met (*filled squares*), extracellular IFTase activity in MA-Met (*filled triangles*), OD_{600} in NH4-Met (*open squares*) and OD_{600} in MA-Met (*open triangles*) are shown. One unit of OD_{600} corresponds with 0.14 g L^{-1} dry cell weight. "Fed-batch induction" represents the induction stage of methylamine hydrochloride and (or) methanol, feeding with carbon source (methanol, glycerol or glucose) and nitrogen source (methylamine hydrochloride or ammonium sulphate). The *error bars* correspond to the standard deviation of three independent determinations

shake flask cultivation and 3.2-fold greater than the activity of the wild-type strain. This result was consistent with the above nitrogen source studies in shake flasks. Moreover, compared with other substrate feeding strategies, cells cultivated in this co-feeding induction medium exhibited higher IFTase specific activity, productivity and total protein yields (Table 1). These results were in accordance with those reported for YlLIP2 production in the same expression system under analogous culture conditions, which confirmed the presumption that methanol and methylamine hydrochloride exhibited a synergistic effect on enzyme production [38]. Wang et al. [38] also reported that the YlLIP2 expression in *PFLD1*-controlled *P. pastoris* showed higher levels than those obtained in a *PAOX1*-controlled system, regardless of the inducers (methanol, methylamine hydrochloride or a mixture of both) used in high cell density fed-batch cultures. However, in terms of IFTase production, *PAOX1*-controlled expression system exhibited higher values of productivity and yield, but the specificity activity of IFTase was not as high as in PFLD1 system (Data not shown). These results suggested that further optimisation studies were needed to improve IFTase production in *PFLD1*-controlled system, as well as to determine the optimum mixed ratio of methanol and methylamine hydrochloride.

When methylamine hydrochloride was used as inducer, IFTase production was greatly dependent on the use of glucose or glycerol as the carbon source (Fig. 5a). As a methanol-free cultivation strategy in *P. pastoris*, glucose was a suitable carbon source for IFTase expression by *PFLD1*-controlled *P. pastoris*, and the IFTase activity was 2.6-fold higher than that obtained with the wild-type strain (Table 1). The use of glycerol as a carbon source clearly generated a low IFTase production ratio of fermentation to shake flasks (Table 1) but yielded an extremely high final biomass compared with cells grown on other carbon sources (Fig. 5). After induction of 36 h, IFTase activity increased very slowly, whereas cells were sustained rapid growth for 36 h. Wang et al. [38] reported that compared with sorbitol, glucose and methanol, glycerol were the optimum carbon sources for cell growth with induction of methylamine, whereas lipase production was very low. Using the same carbon and nitrogen substrate, Resina et al. [28] also detected low levels of lipolytic activity in the culture supernatant at the end of the cultivation, only when glycerol concentration became limiting (with batch cultivations). It demonstrated that glycerol concentration could be an important factor for the production of enzymes, which was also consistent with our results of glycerol concentration effect in shake flasks. It was also presumed that foreign proteins may also have an effect on the utilisation of carbon sources in this expression system. This phenomenon is most likely related to a physiological condition of nitrogen limitation that cells experience when growing in the presence of methylamine hydrochloride as the sole nitrogen source [28].

Furthermore, using glycerol or glucose carbon source feeding strategies, the production of IFTase is still lower than when using methanol as a feeding carbon source; this observation further indicated that the co-induction with carbon and nitrogen sources for *PFLD1*-controlled *P. pastoris* has a significant induction role in the expression of foreign proteins.

Overall, it was demonstrated that the four feeding substrates could be used for the efficient secretion of IFTase in high cell density fed-batch fermentation strategies with *PFLD1*-controlled *P. pastoris*. The expression of IFTase in *PFLD1*-controlled *P. pastoris* was dependent on the nitrogen sources or inducers and the carbon sources used for cell growth. The extracellular IFTase activity achieved with co-induction was higher than the values when either methanol or methylamine hydrochloride was used as the sole inducer. The same behaviour was also reported for lipase production by Wang et al. [38] and Resina et al. [28], and these observations confirmed that methanol and methylamine have a synergistic effect on foreign protein production in this co-induction fermentation protocol.

Discussion

In this report, we describe how *PFLD1*-controlled IFTase expression and secretion was achieved in *P. pastoris* for the first time. Using a fed-batch fermentation strategy, efficient and regulated recombinant IFTase was induced by methanol or methylamine hydrochloride. With four feeding-substrate strategies, the recombinant IFTase activity was 1.5- to 3.2-fold higher than that obtained with the wild-type strain.

The IFTase production by the GS115-FLD1-IFTase strain was slightly affected by the *ift* copy and was closely related to cell growth during the induction phase. It was significantly affected by carbon sources (and its concentrations) or inducers. Co-induction with methanol and methylamine hydrochloride resulted in higher IFTase activity compared to induction by a single inducer at high cell density, but the opposite result was obtained when the *P. pastoris* biomass was at low levels (for example in shake flask cultivations). In particular, this *PFLD1*-controlled *P. pastoris* system was especially attractive as offering a methanol-free cultivation strategy and flexible nitrogen and carbon sources for IFTase extracellular expression in *P. pastoris*, which might provide the basis for the expression of other heterologous proteins in this system. These results indicated that the *PFLD1*-controlled *P. pastoris* is a promising alternative to the *E. coli* system for the industrial production of IFTase and DFA III. Further studies are needed to achieve higher IFTase production by optimising the fermentation conditions for industrial applications and to understand the regulation mechanisms of *PFLD1* for IFTase expression with different carbon and nitrogen sources at varying concentrations.

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