

ORIGINAL PAPER

Influence of trace elements supplementation on the production of recombinant frutalin by *Pichia pastoris* KM71H in fed-batch process

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Frutalin, a galactose-specific lectin used to detect specific tumour markers, is a protein with low expression level in breadfruit. In the present study, fed-batch fermentation in a stirred tank bioreactor was used as a strategy to enhance protein production by a recombinant *Pichia pastoris* KM71H. By using this process, the production of recombinant frutalin was 4-fold higher than the value obtained in shaker flasks batch assays. Supplementation of the fermentation medium with trace elements (*Pichia* trace minerals, PTM) was also evaluated in order to stimulate production of the recombinant protein. The addition of PTM to the minimum medium afforded a recombinant protein production of 13.4 mg L⁻¹, which was 2.5-fold higher than that achieved from the culture medium without PTM supplementation. These results are significant as the development of strategies to improve the production of recombinant frutalin may broaden its application in cancer diagnosis.

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Introduction

Lectins are proteins found in all life forms ranging from viruses, fungi, and bacteria, to animals and plants (Lannoo et al., 2007). These proteins are able to bind reversibly to mono- or oligosaccharides (Peumans et al., 1998) and are related to biological recognition phenomena, mainly cell recognition, interaction, and adhesion (Peumans & Van Damme, 1998). Frutalin is a homotetrameric α -D-galactose lectin belonging to the jacalin family, isolated from *Artocarpus incisa*. Functionally, this lectin is a potent lymphocyte stimulator (Rahman et al., 2002) capable of inducing chemotaxis and the rearrangement of actin cytoskeleton in human neutrophils, and it has been used successfully as a cancer diagnostic tool (Brando-Lima et

al., 2005). However, the use of frutalin isolated from its natural sources presents several disadvantages such as heterogeneity of the sample due to the presence of lectin isoforms, which may lead to variability in the results (Brando-Lima et al., 2006).

The expression of lectin in a heterologous system is an interesting way to overcome this problem, which affords protein samples with high purity. The methylotrophic yeast *Pichia pastoris* is a highly effective and versatile system for the expression of heterologous proteins (Macauley-Patrick et al., 2005) and has been successfully used for the expression of lectin from lower organisms such as *Aleuria aurantia* (Amano et al., 2003) and *Pleurotus cornucopiae* (Sumisa et al., 2004), as well as from plants such as *Phaseolus vulgaris* agglutinin (PHA), *Galanthus nivalis* agglutinin (GNA)

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(Raemaekers et al., 1999), and *Canavalia brasiliensis* (ConBr) (Bezerra et al., 2006). Synthetic frutalin was also cloned and expressed in *P. pastoris* under batch conditions (Oliveira et al., 2008).

The production of recombinant protein by fed-batch fermentation is considered more advantageous than the production by batch cultivations, since the toxic effect of the methanol induction is reduced, the production of protein without biomass gain is increased, and it is possible to express extracellular protein with a low quantity of homologous proteins (Taherzadeh et al., 2002). In addition, supplementation of the medium with trace elements (*Pichia* trace minerals, PTM) during the fed-batch process is reported to increase the production of heterologous proteins (Boze et al., 2001). Given the above, the present work sought to evaluate a fed-batch fermentation process with PTM supplementation as a strategy to increase the production of synthetic frutalin by *P. pastoris* KM71H.

Experimental

Yeast culture media

Previously constructed *Pichia pastoris* KM71H/pPICZ α A/recombinant frutalin (Oliveira et al., 2008) was grown in YPD (1 % yeast extract, 2 % peptone, and 2 % dextrose) or BMGH (0.1 M potassium phosphate, 4×10^{-5} % biotin, 1.34 % yeast nitrogen base (YNB), and 1 % glycerol; pH 6.0) media. The solution of trace elements (PTM) was composed of 1.0 g L^{-1} $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g L^{-1} $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01 g L^{-1} H_3BO_3 , 0.01 g L^{-1} $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.07 g L^{-1} $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.01 g L^{-1} $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. All the solutions were autoclaved at 121°C for 20 min, or sterilised by filtration through $0.22 \mu\text{m}$ filters (Millipore[®], France) prior to use.

All chemicals were purchased from Sigma–Aldrich (USA) or from Merck (Germany) and used without further purification.

Fed-batch fermentation

Fermentation assays were carried out in fed-batch mode in a 1.6 L stirred tank bioreactor (Autoclavable Benchtop Fermenter Type R'ALF, Bioengineering AG, Switzerland) containing 1 L of fermentation medium (working volume). The vessel was jacketed and the water temperature was maintained at 30°C . During the fermentations, the pH was maintained at 5.0 by using a 1.0 M NaOH solution, and 100 μL of anti-foaming agent (Antifoam A, Sigma–Aldrich) was added to the culture medium. The dissolved oxygen (DO) level was maintained at over 30 % of air saturation by a cascaded control of agitation ($320\text{--}550 \text{ min}^{-1}$) and aeration rate ($3\text{--}5 \text{ L min}^{-1}$). The temperature, DO, pH, agitation, aeration, anti-

foam, and carbon source were monitored throughout the fermentation period.

The fed-batch fermentation process consisted of three phases: (i) an initial glycerol batch to promote the cell growth; (ii) a glycerol fed-batch for the alcohol oxidase derepression and to obtain high cell density; (iii) an induction phase for expression of the recombinant frutalin. In the first step, *P. pastoris* KM71H was grown in a BMGH medium (1 L) over 24 h. After this period, the glycerol fed-batch phase started, being the feed medium (30 mL of 50 vol. % glycerol solution whether or not supplemented with 12 mL L^{-1} trace metal solution) fed at a flow-rate of $1.5 \text{ mL h}^{-1} \text{ L}^{-1}$ or $6.0 \text{ mL h}^{-1} \text{ L}^{-1}$. In the induction phase, methanol, whether or not supplemented with 12 mL L^{-1} of a trace metal solution, was added to the medium at a flow-rate of 6 mL min^{-1} . In this phase, the temperature was maintained at 28°C .

Assays in shaker flasks were carried out for expression on a small-scale. In these assays, a single colony of the microorganism was inoculated into 50 mL of BMGH medium and maintained at 30°C , 250 min^{-1} , for 24 h. Next, the cells were harvested by centrifugation ($3000g$, 4°C , 15 min) and re-suspended to an optical density (OD 600) of 1.0 in BMMH (0.1 M potassium phosphate, 4×10^{-5} % biotin, 13.4 % YNB, and 0.1 % methanol; pH 6.0) medium whether or not supplemented with 12 mL L^{-1} of a trace metal solution to induce expression. To overcome lack of oxygen, induction was carried out in 500 mL baffled flasks filled with 50 mL of culture medium and covered with two layers of sterile gauze. Every 24 h, methanol was added to the medium (to afford a final methanol concentration of 1.0 vol. %) to maintain induction. The induction phase occurred over 96 h, at 28°C .

Analytical methods

Cell concentration (dry mass per volume) was determined by measuring the optical density (OD) of the yeast culture samples at 600 nm, which was correlated to a calibration curve (dry mass \times OD). Glycerol and methanol concentrations were measured by high-performance liquid chromatography (HPLC) in a Jasco chromatograph (Japan) equipped with a refractive index detector and a Bio-Rad Aminex HPX-87H column ($300 \text{ mm} \times 7.8 \text{ mm}$) at 60°C , using 0.005 M sulphuric acid as eluent at a flow-rate of 0.7 mL min^{-1} . The total protein concentration in the samples was determined by the Bradford protein assay using bovine serum albumin (Sigma–Aldrich) as the concentration standard. The production of recombinant frutalin was analysed by SDS–PAGE using a 12 % gel stained with Coomassie brilliant blue (Sigma–Aldrich), as previously described (Laemmli, 1970). All the analytical determinations were performed in triplicate.

Recombinant protein purification

The supernatants from fed-batch fermentations were separated from the yeast cells by centrifugation (3000g, 4°C, 10 min). Salts were precipitated by the pH increase to 7.5 by adding 10 N NaOH, and removed by centrifugation (3000g, 4°C, 10 min). Next, the supernatants were filtered through 0.22 µm pore size filters, concentrated and washed with PBS buffer (8.00 g L⁻¹ NaCl, 0.20 g L⁻¹ KCl, 2.68 g L⁻¹ Na₂HPO₄ · 7H₂O, and 0.24 g L⁻¹ KH₂PO₄; pH 7.4) to a final volume of 1–1.5 mL in 10 kDa Amicon tubes (Millipore, France). The treated supernatants were analysed by SDS-PAGE and FPLC systems (Pharmacia Biotechnology, USA) using a Sephacryl S-100 HR column (HiPrep 16/60, Pharmacia Biotechnology), previously washed with distilled water and equilibrated with PBS buffer at a flow-rate of 1 mL min⁻¹. The purity of the frutalin was confirmed by SDS-PAGE followed by silver nitrate staining. The concentration of pure recombinant was determined by measuring the OD at 280 nm.

Haemagglutination assays

Samples of the recombinant frutalin were prepared by serial dilutions ($\varphi_r = 1 : 2$) from a starting ($\varphi_r = 1 : 1$) medium up to ($\varphi_r = 1 : 128$), and 100 µL was incubated with 100 µL of rabbit erythrocytes (2 vol. % in 0.15 M NaCl) at 37°C for 30 min (Oliveira et al., 2008). The degree of agglutination was visually monitored after the tubes had been left at 37°C for 30 min followed by another 30 min at ambient temperature. Samples yielding no visible agglutination activity after these incubation steps were regarded as negative. Native frutalin was used as a positive control, exhibiting agglutination activity.

Results and discussion

Production of recombinant frutalin by fed-batch fermentation

Recombinant *P. pastoris* KM71H was grown in a minimal medium containing glycerol. After exhaustion of the glycerol from the medium, frutalin production was induced by the addition of 0.8 % methanol, which also acted as a carbon source for *P. pastoris* during the fermentation (Cereghino & Cregg, 2000). By using this fed-batch process, a biomass production of 122.3 g L⁻¹ and a final concentration of pure frutalin of 5.23 mg L⁻¹ were obtained. This frutalin concentration is higher than that achieved in Erlenmeyer flasks (2.1 mg L⁻¹). In effect, the levels of protein obtained in shaker flasks experiments are generally lower than those obtained in bioreactors, because the cell density is lower and the aeration is often limited (Daly & Hearn, 2005). However, the values ob-

tained in the present work are lower than others reported for recombinant lectins expressed in *P. pastoris* in shaker flasks (fruiting-body lectins of *Pleurotus cornucopiae* (60 mg L⁻¹) (Iijima et al., 2003) and *N. tabacum* lectin (6 mg L⁻¹) (Lannoo et al., 2007)) and in fed-batch experiments (*Amaryllidaceae* snowdrop agglutinin (80.0 mg L⁻¹) (Baumgartner et al., 2003) and *Phaseolus vulgaris* phytohemagglutinin E-form (100 mg L⁻¹) (Baumgartner et al., 2002)), suggesting that the system used in the present work was not efficient enough to produce high levels of frutalin.

The recombinant frutalin produced exhibited an SDS-PAGE migration pattern very similar to that of the native frutalin (Fig. 1b). The upper band in the SDS-PAGE gel indicates that the recombinant frutalin has been glycosylated by *P. pastoris* KM71H. As expected, the recombinant frutalin did not present haemagglutinating activity towards rabbit erythrocytes. This result has been attributed to the glycosylation pattern of *P. pastoris* and has also been observed for another fruiting-body lectin (Iijima et al., 2003).

Influence of PTM supplementation on frutalin production

During the phase to obtain high cell density, the cells may experience a limitation of basal salts, which may prevent use of the carbon source. As a result, some studies report the need for nutrients supplementation during the feed and induction phases of the fed-batch process (Hang et al., 2009; Arnau et al., 2010; Khongto et al., 2011). PTM supplementation satisfies the nutritional requirements of the cells during the nutrients starvation period (Xiao et al., 2006). In the present study, assays in shaker flasks and in the fed-batch bioreactor were performed seeking to verify the influence of the medium supplementation with PTM on frutalin production. As can be seen in Fig. 1a, cell growth was similar in the media supplemented with or without PTM, so differences were not observed in the specific growth rate (μ , Table 1). Although supplementation of the medium with PTM has been reported to overcome the basal salts limitation by yeast cell, and to reverberate in biomass production (Chauhan et al., 1999; Hang et al., 2009; Arnau et al., 2010), it had no significant influence on the cell growth of *P. pastoris* KM71H (present study). Similar results for the fermentation medium supplementation with trace elements on cell growth have been reported (Chauhan et al., 1999).

The addition of PTM (12 mL L⁻¹) during the methanol feed resulted in an increase in the levels of recombinant frutalin compared with the levels obtained by induction in the absence of supplementation (Table 1). By supplementing the cultivation medium with PTM, *P. pastoris* was able to produce 13.4 mg L⁻¹ of recombinant frutalin, which is 2.5-fold higher than that obtained in the absence

Table 1. Biomass production, recombinant frutalin concentration and specific growth rate (μ) obtained during fed-batch fermentation with *Pichia pastoris* KM71H in stirred tank bioreactor

Induction conditions	Dry mass	μ^a	Pure recombinant frutalin
	g L^{-1}	h^{-1}	mg L^{-1}
PTM supplementation	124.1	0.060	13.40
Without PTM supplementation	122.3	0.059	5.23

a) Calculated by linear regression of specific growth rate observed during glycerol feeding.

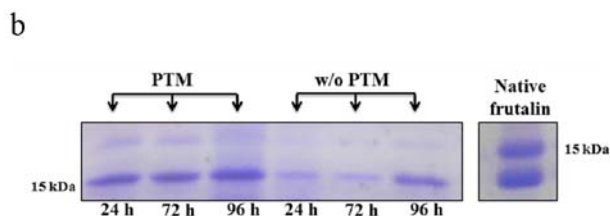
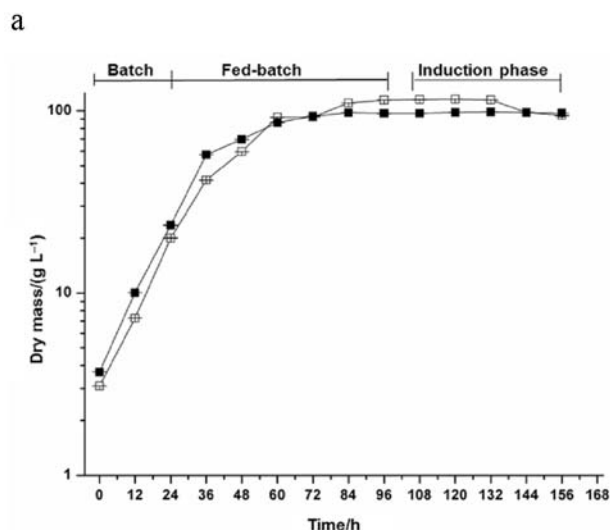


Fig. 1. Fed-batch process for production of recombinant frutalin by *Pichia pastoris* KM71H. Comparative profile of yeast growth in medium supplemented with PTM (\square) or without PTM (\blacksquare), and time course of batch, fed-batch and induction phases (a), recombinant frutalin migration in SDS-PAGE gel followed by Coomassie brilliant blue staining at 24 h, 72 h, and 96 h after onset of growth, and influence of PTM supplementation on recombinant frutalin production (b).

of PTM. In shaker-flasks, the frutalin production achieved 3.3 mg L^{-1} , representing a 1.5-fold increase over the non-supplemented assays. Some studies also reported an increase in the production of other recombinant proteins by *P. pastoris*, including hepatitis B virus surface antigen (HBsAg), phytase, angiotensin, and alpha-amylase, after supplementation of the medium with trace elements during the fed-batch process (Chauhan et al., 1999; Lee et al., 2003; Chen et al., 2004; Xie et al., 2005).

The cultivation of *P. pastoris* on defined media is associated with a high rate of protease expression, es-

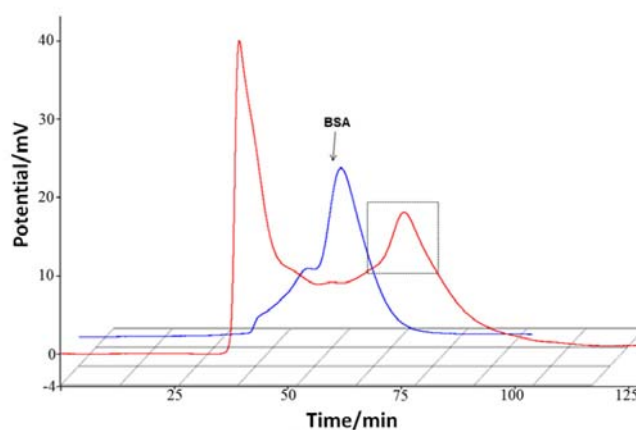


Fig. 2. Elution profile of fed-batch process supernatant on Sephacryl® S-100HR column.

pecially in high cell density cultures. In defined media, the nitrogen depletion results in an increase in the protease activity, which can be avoided by increasing the initial nitrogen concentration or by supplementing the medium with it. Nutrient starvation leads to autophagic cell degradation and lyses, leading to the release of vacuolar proteases (Todde et al., 2009). The use of PTM increases the protein production by increasing the cell viability as well as by improving the final protein concentration due to the strong decrease in the proteolytic degradation of recombinant proteins (Chauhan et al., 1999; Boze et al., 2001; Xiao et al., 2006; Ghosalkar et al., 2008; Roepcke et al., 2011). Moreover, the stability of the recombinant protein is further enhanced by the addition of PTM to the culture medium, possibly by acting as alternative and competing substrates for one or more proteases. These supplements can also repress protease induction caused by nutrients limitation (Macauley-Patrick et al., 2005).

Analysis by SDS-PAGE revealed the recombinant frutalin produced by *P. pastoris* KM71H after 24 h, 72 h, and 96 h of methanol induction (Fig. 1b). As can be seen, the production of recombinant frutalin increased with the increase in induction time, both in the presence and in the absence of PTM. Furthermore, the production is higher in the presence of PTM, showing the positive effect of the supplementation on the production of recombinant frutalin. This also corrobo-

rates the levels of recombinant frutalin production referred to above. The size-exclusion chromatogram in Fig. 2 shows the elution profile of the recombinant frutalin during the purification process. The SDS-PAGE analysis followed by silver nitrate staining confirmed the purity of the recombinant frutalin.

Conclusions

Supplementation of the fermentation medium with PTM during the fed-batch process was demonstrated to be an important and significant strategy to increase the production of recombinant frutalin by *Pichia pastoris* KM71H. This finding is of great interest since increased production of recombinant frutalin may extend its application in cancer diagnosis and therapy.

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