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ORIGINAL ARTICLE

Expression of human interleukin-1β in *Saccharomyces cerevisiae* using *PIR4* as fusion partner and production in aerated fed-batch reactor

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Abstract To circumvent cell wall retention commonly associated to *Saccharomyces cerevisiae* when used as a host for heterologous protein production, we have created a translational fusion of human interleukin-1β (IL-1β) to the Pir4 cell wall protein, so as to drive the secretion of the recombinant product to the growth medium. The auxotrophic *S. cerevisiae* BY4741 was used as host to express the Pir4-IL1β fusion protein. Once it was ascertained that the fusion protein was secreted to the culture medium and behaved as a growth-linked product, *S. cerevisiae* BY4741 [PIR4-IL1β] was cultured in an aerated fed-batch reactor to achieve high cell density and, consequently, high product concentration in the medium. Two cultivation media were employed, a rich complex and a defined mineral medium, the latter suitably supplemented with bacto-casamino acids as ACA (auxotrophy-complementing amino acid) source. The rich complex medium allowed a good performance of the producer strain only during batch growth, but was revealed to be inadequate for long-term fed-batch operations. The defined mineral medium ensured a better performance, even though not yet satisfactory in spite of a proper ACA supplementation. The behaviour of BY4741 was attributed to an intrinsic sensitivity of the producer strain to long-term aerated fed-batch operations.

Keywords Expression · Pir4 · Yeast · Fed-batch · Heterologous protein

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Introduction

One of the main drawbacks of *Saccharomyces cerevisiae* as a host system for heterologous protein production is represented by the cell wall which acts as a barrier that may limit the secretion of recombinant proteins (de Nobel and Barnett 1991), even when these proteins include a functional secretion signal and enter the secretory pathway (Porro et al. 2005).

To address this question, in this work, we have generated a yeast strain that expresses human interleukin-1β, using the signal peptide and pro domain of the Pir4 cell wall protein (Moukadiri et al. 1999) to drive its secretion to the culture medium. Pir4 has already been successfully used as a fusion partner for the targeting of other proteins of interest such as xylanase A from *Bacillus* sp. BP-7 (Andrés et al. 2005), the VP8* fragment from the rotavirus spike protein (Andrés et al. 2006) and lipase A from *Bacillus subtilis* (Mormeneo et al. 2008) either to the cell wall or to the culture medium.

The auxotrophic *S. cerevisiae* BY4741 (*MATa, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1*) strain, which is widely used for genetic experiments and molecular biology manipulations by the yeast research community (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/), has been used as host for interleukin-1β expression. Auxotrophic mutant and deletion strains having one or several auxotrophic requirements are the most commonly used tools in the development of recombinant strains due to the stable maintenance of expression vectors (Romanos et al. 1992; Hensing et al. 1995). Auxotrophic strains need to be grown in supplemented defined media; however, complex media can also be used if the percentage of the resulting plasmid loss is too low after many cell generations (Zhang et al. 2003).

The aim of this work was to obtain a yeast strain able to successfully secrete IL-1β to the culture medium as a Pir4 fusion protein, and test it under conditions that resemble a production process (aerated fed-batch reactor) in order to achieve high cell density and high product yield (Mendoza-Vega et al. 1994). In fact, sugar limitation accomplished by the fed-batch technique allows the oxidative metabolism of the carbon source to be maintained during the process, leading to higher yields of biomass and recombinant product (Mendoza-Vega et al. 1994; Enfors 2001).

Initially, to ascertain Pir4-IL1β expression, the recombinant BY4741 strain was cultured in shake flasks, and then the production of Pir4-IL1β was performed in an aerated fed-batch reactor in either rich complex or supplemented defined mineral media. Fed-batch cultivation of the producer strain was carried out with an initial phase of exponentially increasing feed of glucose which allowed the yeast to grow with a constant specific growth rate promoting oxidative metabolism. Special attention has been given to the composition of the feeding solution as regards the auxotrophy-complementing amino acid concentration. In fact, there is a high degree of variation in the literature on the optimal concentrations of the amino acids for which the organism is auxotrophic (Çakar et al. 1999).

The performance of the producer strain in fed-batch reactor has been evaluated in terms of total biomass and product obtained at the end of the process.

Materials and methods

Strains

Escherichia coli DH5α was used for the propagation of plasmids; it was grown in Luria broth supplemented with 100 μg of ampicillin per ml when necessary. The *S. cerevisiae* strain BY4741 (*MATa, ura3Δ0, leu2Δ0,met15Δ0, his3Δ1*) used in this study was obtained from the EUROSCARF collection (Heidelberg, Germany).

Yeast transformation was carried out by the lithium acetate method (Gietz and Sugino 1988). Plasmid DNA from *E. coli* was prepared using the QIAprep (Quiagen) kit and DNA fragments were purified from agarose gels using the kit QIAquick Gel Extraction (Qiagen).

Construction of the gene fusion between *PIR4* and *IL-1β*

The shuttle vector pIA1 (Andrés et al. 2005), derived from the multicopy plasmid YEplac 195 (Gietz and Sugino 1988), was used in this work. pIA1 carries the complete sequence of *PIR4* gene, including its regulatory sequences.

Construction of the gene fusion between *PIR4* and *IL-1β* consisted of the insertion of the coding sequence of *IL-1β* gene, in the *Bgl*II and *Sal*I sites of *PIR4*. For this, a 486-bp fragment of *IL-1β* was amplified by PCR using the following oligonucleotides: ILBGL primer, (5') AAA GCC *AGA TCT* TTG CAC CTG TAC GAT CAC TGC (3') and ILXHO primer, (5') CCT TTA *CTC GAG* GGA AGA CAC AAA TTG CATGG (3'), and plasmid pCXJ-kan1, carrying the mutant *IL-1β* allele ($Asn^7 \rightarrow Gln$), as template. In fact, to avoid iper-glycosylation in *S. cerevisiae* cells, we have used as cDNA a glycosylation-minus variant of the mature part of the structural gene of *IL-1β* obtained by site-directed mutagenesis of the canonical glycosylation site at Asn^7 (Fleer et al. 1991).

The oligonucleotides included the restriction sites for the enzymes *Bgl*II and *Xho*I (underlined nucleotides), which leaves overhangs compatible with the overhangs left by *Sal*I, and had been designed so that the *IL-1β* fragment could be inserted in-frame in *PIR4*. The PCR fragments amplified using Expand High Fidelity DNA polymerase (Roche) were subcloned in the pGEM-Teasy® vector (Promega), digested out with *Bgl*II, and *Xho*I, and inserted in the vector pIA1, previously digested by *Bgl*II and *Sal*I.

SDS-Polyacrylamide gels and Western blot analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% Polyacrylamide gels and transferred onto Hybond-C nitrocellulose membranes. The membranes were blocked overnight in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% non-fat milk. The blocked membranes were washed three times in TBST and incubated for 1 h in TBST containing primary antibody (Rabbit polyclonal IgG IL-1β; Santa Cruz Biotechnology) at a dilution of 1:3,000. After three washes in TBST, membranes were incubated for 20 min in TBST containing goat antirabbit IgG-peroxidase at a dilution of 1:10,000 and washed in TBST. Finally, antibody binding was visualized on X-ray film by using the ECL method (Amersham).

Cultivation media

For cultures in shake flasks and the bioreactor, two different media were employed, both containing 2% w/v glucose as carbon source: a rich complex medium with 1 and 2% w/v of yeast extract and bactopeptone (YP), respectively, and a defined mineral medium, prepared according to Verduyn et al. (1992), the latter containing vitamins and trace elements and made selective without uracil. The defined medium was supplemented with casamino acids (BD Bacto™ Casamino Acids; BectonDickinson, Sparks, MD, USA) as the auxotrophy-complementing amino acid (ACA) source and called SDMB (synthetic defined mineral bacto casamino acids) medium. The amount of casamino acids contained in SDMB medium employed in the shake flasks and the fermenter during batch phase was determined on the basis of the fermentative biomass yield on glucose (Hahn-Hägerdal et al. 2005) and the amino acidic composition of casamino acids (BectonDickinson, technical handbook).

Independently of the medium employed (rich or defined), in the fed-batch experiments, the feeding was carried out with 50% w/v glucose in the reservoir.

In the case of YP medium, the feeding solution also contained a four-fold concentrated YP solution (YPx4).

In the case of SDMB medium, the feeding solutions contained salts, trace elements, glutamic acid, vitamins, and the ACA source. Final salt concentrations per litre were: KH_2PO_4 15.70 g, KCl 5 g, $MgSO_4\ 7H_2O$, 5.83 g, $CaCl_2\ 2H_2O$, 1.20 g, NaCl 0.44 g, $FeSO_4 \cdot 7H_2O$ 250 mg. Final trace concentrations per litre were: $ZnSO_4 \cdot 7H_2O$ 50 mg, $CoCl_2 \cdot 6H_2O$ 2 mg, $CuSO_4 \cdot 5H_2O$ 40 mg, $MnCl_2 \cdot 4H_2O$ 50 mg, and final glutammic acid concentration was 1 g per litre. Filter-sterilized vitamins were added after heat sterilization. Final vitamin concentrations per litre were: biotin 4 mg, calcium-pantothenate 40 mg, nicotinammide 90 mg, myo-inositol 50 mg, thiamine HCl 100 mg, pyridoxine HCl 20 mg.

As regards the ACA concentration in the feeding solution, it was achieved using both casamino acids, up to 50 g l^{-1} and the essential individual amino acids (histidine, leucin, and methionine). The overall concentration of each auxotrophy-complementing amino acid was calculated from the equation developed for the initial feed rate (F_0) (see below), to give the amino acid concentration in the reservoir (A_R), taking into account the value of biomass yield for the given amino acid in aerobic conditions and considering an excess factor of ca. 25% (Pronk 2002).

Development of the inoculum for the fed-batch reactor was made starting from the frozen culture (stored at −80°C in 12.5% v/v glycerol) and ensuring propagation in an Erlenmeyer flask (pre-culture) in the same medium used for the bioreactor.

To test plasmid stability during growth in Enlermeyer flasks, culture samples were withdrawn, properly diluted with 0.9% NaCl and used to inoculate agar plates containing either non-selective (YP + 2% glucose) or selective synthetic minimal medium SD (0.7% yeast nitrogen base without amino acids, 2% glucose, and amino acids as required). Colony-forming units on the two different media, after 5 days of incubation at 30°C, were evaluated and compared.

Fed-batch cultures

Fed-batch cultures were performed in a 2-l working volume stirred Bioflo 110 (New Brunswick Scientific). The bioreactor was inoculated with an adequate aliquot of a 18-h pre-culture, to give an initial OD_{590} of 0.200. The YP medium or the SDMB medium were employed for the batch phase.

Fed-batch culture with YP medium was performed in two steps. First, the reactor was allowed to proceed in a batch mode (1 l volume) overnight. The second step, (exponential feed phase) started when the glucose of batch phase was almost exhausted (15–16 h) before the ethanol produced by the glucose-sensitive yeast on the initial glucose charge was metabolized through the oxidative pathway. At that point, an exponentially increasing feed was applied, so that the biomass could increase with a constant specific growth rate selected below the critical value (that is 60% of the μ_{max} value), to avoid sugar overflow metabolism (Enfors 2001). In the case of fed-batch cultures carried out with SDBM medium, the exponential feed was switched after the second step described above, to a constant feed. Specific growth rates (μ) employed during the exponential feed phase with YP and SDMB were 0.14 and 0.16 h^{-1}, respectively.

The exponential profile of flow rate *F(t)* was obtained from the mass balance on limiting substrate, throughout the assumption of a quasi-steady state on the glucose balance (Enfors 2001), and calculated according to:

$$F = F_0 \cdot \exp(\mu \cdot t) \tag{1}$$

where F_0 was given by:

$$F_0 = \frac{\mu (XV)_0}{Y_{X/G} G_R} \tag{2}$$

X_0 and V_0 were biomass density and volume at the start of feeding, respectively; G_R was the concentration of the growth limiting glucose in the reservoir; $Y_{X/G}$ was the respiratory biomass yield (0.5 g biomass g^{-1} glucose) (van Dijken et al. 2000), and μ was the specific growth rate imposed.

During batch phase, oxygen was supplied by sparging the bioreactor with air at a flow of 1 vvm and the cascade system acted with the agitation speed automatically increasing or decreasing until the DOT set-point (30% air saturation) was reached. The culture pH was maintained at 5.00 by automatic addition of 2 N KOH. During exponential and constant feed phases, aeration was accomplished as described above, except for the air flow which was of 1.5 vvm and pH maintained by automatic addition of 10% v/v NH_4OH. The foam level in the bioreactor was controlled by the automatic addition of the antifoam Dow Corning 1510 (dilution 1:10).

Biomass determination

Biomass was determined by optical density measurements at 590 nm (OD_{590}) and dry weight determination. Unless otherwise stated, mg of biomass are always referred as dry weight. In the latter case, culture samples were washed twice, re-suspended with distilled water, and dried for 24 h at 105°C. Parallel samples varied about 3–5%. The calibration curve relating OD_{590} values to biomass concentration provided a correlation factor of 2.45 OD_{590} per mg ml^{-1}.

Analyses

Samples were quickly withdrawn from batch and fed-batch cultures, filtered on 0.45 μm GF/A filters (Millipore, Bedford, MA, USA) and filtrates analysed to determine residual glucose, ethanol and interleukin concentrations.

Residual glucose (mg ml^{-1}) in the medium was determined by GOD-Perid from R-Biopharm (Roche, Mannheim, Germany) or a method for reducing sugars (Nelson 1944). Ethanol production was measured with the enzymatic kit from R-Biopharm). Interleukin-1β was determined in quadruplicate by immuno-blot analysis in Bio-Dot® Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA) and quantified by densitometric analysis (MultiAnalyst; Bio-Rad) using the human EuroClone IL-1β as a standard, as previously reported (Romano et al. 2009).

All samples were analysed in triplicate and the values of standard deviation obtained varied between 1 and 2%.

Results

PIR4-IL1β gene fusion strategy and detection of Pir4-IL1β secreted to the growth medium by western-immunoblot

Pir4 consists of a signal peptide, processed at the endoplasmic reticulum, and a pro-peptide (Subunit I) that is processed at the Golgi by the Kex2p protease. The mature protein (Subunit II) includes a 19-amino acid repetitive domain and a very conserved carboxy-terminus that contains four cysteine residues at fixed positions. The structure of *PIR4*, as outlined above, and that of the *IL-1β* gene are shown in Fig. 1, together with a schematic representation of the fusion strategy used.

The construction consisted of inserting all the coding sequence of the *IL-1β* gene into the naturally occurring restriction sites *Bgl*II and *Sal*I of the coding sequence of *PIR4*. For this, the *IL-1β* sequence was amplified using plasmid pCXJ-kan1 (Fleer et al. 1991) as template and oligonucleotides, which included in their 5' ends restriction sites compatible with the *Bgl*II and *Sal*I sites in *PIR4*, and which had been designed to fit in-frame in the corresponding sites in the ORF of *PIR4*. Then, the amplified *IL-1β* coding sequence was subcloned in the *Bgl*II–*Sal*I sites of *PIR4*, with the loss of the carboxy-terminal fragment of Subunit II of *PIR4* that contains three of the four highly conserved cysteine residues that are responsible for cell wall retention through disulphide bridges (Moukadiri and Zueco 2001).

The resulting construction was then transformed in *S. cerevisiae* BY4741. The recombinant strain so obtained, named *S. cerevisiae* BY4741 [PIR4-IL1β], was tested for the expression and secretion of Pir4-IL1β fusion protein to the growth medium. For this, supernatants from either recombinant strain or untransformed BY4741 culture collected at 48 h were concentrated, fractionated by SDS-PAGE, and probed by western immunoblot, using an antibody specific for IL-1β. The results (Fig. 2) showed that the antibody only recognised specific bands in the growth medium supernatant from the recombinant strain, which confirmed that the gene fusion was expressed and the resulting protein was secreted into the growth medium. The main band recognised by the antibody in the concentrated growth medium from the BY4741 [PIR4-IL1β] has a size of around 28 kDa, instead of the 17.4 kDa of the native IL-1β. However, this difference may be accounted for by the 38 amino acids which remain of the

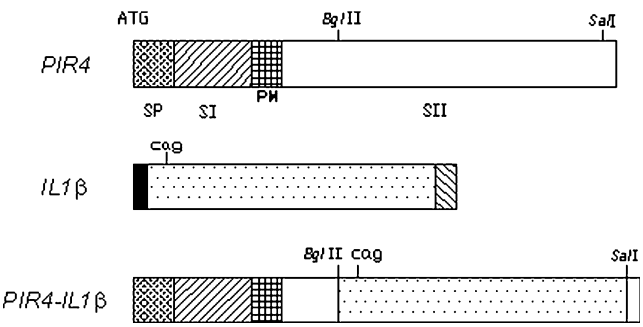

Fig. 1 Schematic representation of the *IL-1β* and *PIR4* genes together with the *PIR4-IL1β* fusion. *SP* signal peptide, *SI* subunit I, *PM* Pir motive, *SII* subunit II, *cag* is the mutation site $Asn^{7} \rightarrow Gln$

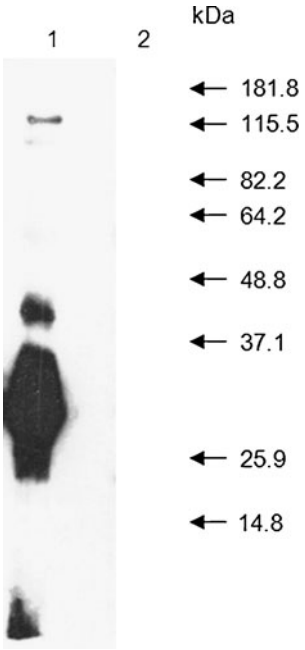

Fig. 2 Detection of Pir4-IL1β secreted to the growth medium by western-immunoblot using a specific antibody. *Lane 1* BY4741 [PIR4-IL1β] strain, *lane 2* untransformed BY4741 strain. Concentrated growth medium (YPD) after 48 h of growth. The equivalent of 0.5 μl was loaded per lane

subunit II of Pir4 in the fusion protein and, possibly, by O-glycosylation.

Growth of *S. cerevisiae* BY4741 [PIR4-IL1β] in shake flasks

Once it was confirmed that the gene fusion was expressed and that the Pir4-IL1β fusion was secreted into the growth medium, we proceeded to quantify the amount of fusion protein secreted and to study the growth characteristics of the producer strain. For this, the recombinant strain BY4741 [PIR4-IL1β] was grown in Erlenmeyer flasks, in a rich complex medium (YP) containing glucose as carbon source. The presence of the fusion protein was monitored in the supernatants over a 48 h incubation period and quantified by immunoblot, using an antibody specific for IL-1β, followed by densitometric analysis. The fusion protein was produced and secreted into the culture medium along the entire time course of growth (Fig. 3a).

Also, residual glucose and ethanol concentrations during incubation were determined. *Saccharomyces cerevisiae* BY4741 [PIR4-IL1β] showed a diauxic growth (Fig. 3b), typical of a glucose-sensitive yeast. It grew rapidly on glucose in the first exponential growth phase, attaining a maximum specific growth rate (μ_{max}) of 0.45 h^{-1}. When the glucose was exhausted (10 h), growth was sustained by the ethanol previously produced. No plasmid loss from *S. cerevisiae* BY4741 [PIR4-IL1β] cells was observed after 72 h incubation, since the number of colony-forming units appearing on either selective or non-selective agar plates were the same.

When *S. cerevisiae* BY4741 [PIR4-IL1β] was cultured in Erlenmeyer flasks in a defined mineral medium, supplemented with casamino acids (SDMB) as ACA (auxotrophy-complementing amino acid) source and containing glucose, the μ_{max} value was lower than that determined in YP (0.39 vs 0.45 h^{-1}, respectively), reflecting that the rich complex medium contained biosynthetic precursors that can be directly channeled into anabolic pathways, thus saving the metabolic energy for biomass synthesis.

Moreover, μ_{max} values exhibited by *S. cerevisiae* BY4741 [PIR4-IL1β] grown in either YP or SDBM media were not significantly different with respect to those obtained with the untransformed strain cultured in the same conditions (data not shown), indicating the absence of a specific metabolic burden associated to the expression of the recombinant protein.

The high maximum specific growth rate encountered in shake flasks containing YP medium was considered promising for high cell density cultivation carried out in the aerated fed-batch reactor.

Growth of *S. cerevisiae* BY4741 [PIR4-IL1β] in an aerated fed-batch reactor

Saccharomyces cerevisiae BY4741 [PIR4-IL1β] was grown in an aerated fed-batch reactor in rich complex medium (YP) to set up IL-1β production.

In Fig. 4a, the time course of total biomass and product secreted into the medium during the exponential feeding phase has been reported. As expected, the fusion protein was produced along the entire time course of growth. Growth of the producer strain arrested after 26 h (Fig. 4a), when the total biomass achieved a value of 14 g (corresponding to a cell density of 11.3 g l^{-1}) very different from the value which should be achieved when using fed-batch culture (Mendoza-Vega et al. 1994). It is worth noticing that already during the first hours of exponential growth, the recombinant strain grew at a μ value of 0.11 h^{-1}, which deviated from the μ value (0.14 h^{-1}) imposed by the feeding profile (Fig. 4a, insert).

Glucose supplied to the reactor was fully taken inside the recombinant yeast cells during the first 13 h of exponential feeding, and consequently its residual amount in the medium was next to zero (Fig. 4b). Then, glucose began to accumulate in the medium, and concomitantly ethanol was produced (Fig. 4b), indicating the yeast metabolic shift towards fermentation.

Glucose which accumulated in the medium was consumed together with ethanol produced (Fig. 4b), without any net increase in biomass (Fig. 4a).

The low cellular density achieved, the accumulation of glucose in the medium, and the mismatch between the μ value imposed and that experimentally determined, suggested that yeast growth in the reactor might be limited by one or more nutrients.

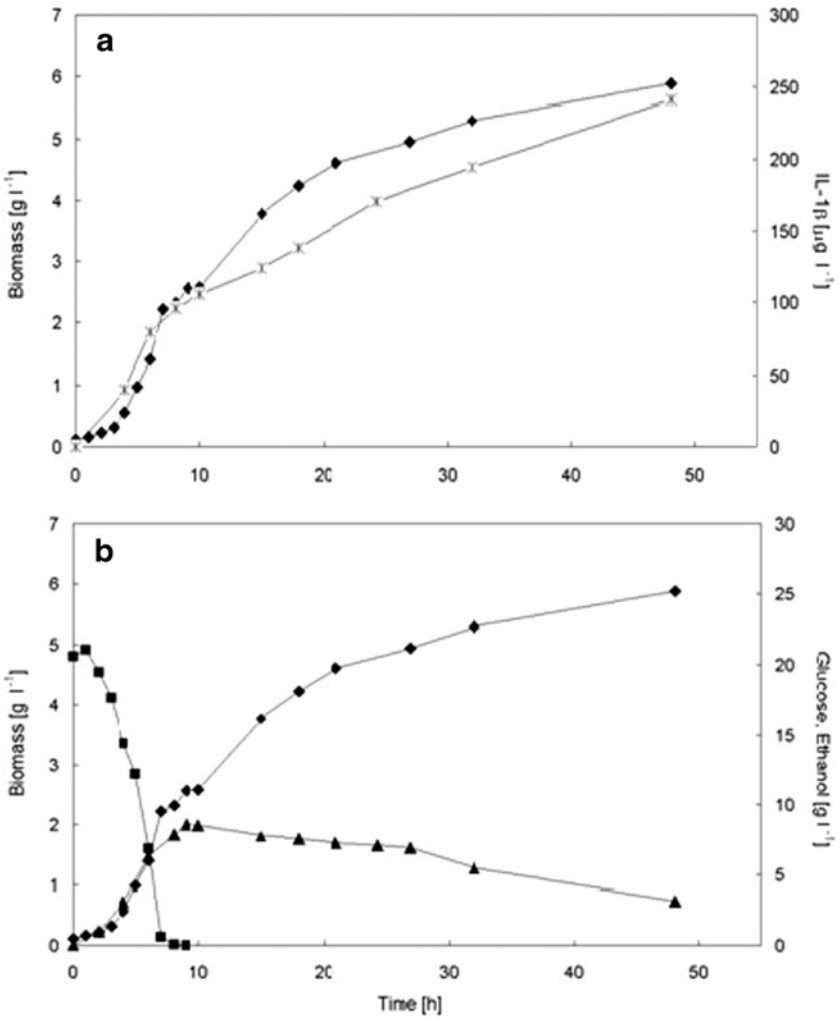

Fig. 3 Growth of *S. cerevisiae* BY4741 [PIR4-IL1β] in Erlenmeyer flasks. **a** Time course of biomass (rhombus) and Pir4-IL1β (star). **b** Biomass (*rhombus*), residual glucose (*squares*), and ethanol (*triangles*)

Therefore, a defined medium (SDMB, see "Materials and methods") containing vitamins and trace elements and properly supplemented with an auxotrophy-complementing amino acid (ACA) source was used for cultivation instead of YP. The time course of total biomass and product during the fed-batch run are reported in Fig. 5a. Total biomass significantly enhanced (50 g corresponding to a cell density of 30 g l^{-1}). Moreover, during the first 7 h of exponential feeding (Fig. 5a, insert), the yeast was able to grow with the specific growth rate imposed (0.16 h^{-1}). In the time interval considered, biomass yield (0.5 g biomass g^{-1} glucose) was indicative of a fully respiratory metabolism. However, yeast growth progressively decreased during the run and arrested after 29 h of feeding (Fig. 5a), i.e. 1 h before feeding interruption (Fig. 5b).

The product curve followed the growth curve (Fig. 5a), but the amount of product obtained was only doubled with respect to that obtained with YP (Fig. 4a). It seems reasonable to suppose that the ethanol produced in a great amount during the run (up to 19.5% v/v; Fig. 5b) would have a denaturing effect on the heterologous protein, thus lowering the amount of product detectable by immunoblot.

During the run carried out with SDMB medium and a proper ACA concentration in the feeding, glucose supplied to the reactor was actively taken up by the cells until 29 h, so that the residual glucose was null. At that point, glucose began to accumulate (Fig. 5b), even though to a lesser extent than in the case of YP medium. Glucose consumption was coupled with a massive ethanol production (Fig. 5b) indicating that the metabolism of the producer strain was also in this case unavoidably shifted towards fermentation.

As in the case of YP medium, glucose and ethanol accumulated and were consumed without any increase in biomass (Fig. 5a).

The same unexpected behaviour, i.e. the shift to fermentative metabolism after 7 h of cultivation and then the arrest of growth, was also observed in the case of the untrasformed BY4741 strain, which achieved a final total biomass of 49 g when cultivated under the same operative conditions as the recombinant producer strain.

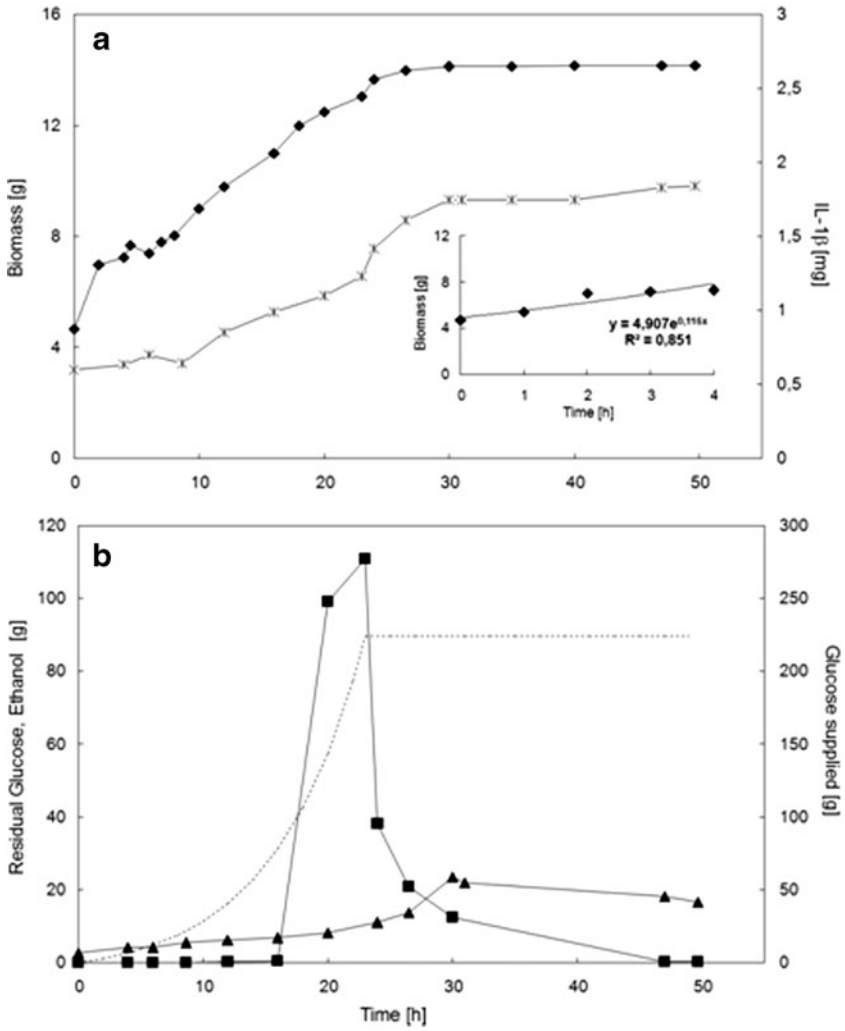

Fig. 4 Growth of *S. cerevisiae* BY4741 [PIR4-IL1β] in the aerated fed-batch reactor using YP medium. The exponential feeding profile (duration, 23 h) was designed to allow the producer strain to grow with a specific growth rate (μ) of 0.14 h^{-1}. **a** Time course of biomass (*full symbols*) and Pir4-IL1β (*empty symbols*). In the *insert* the interpolation of the experimental data regarding the biomass during the first 4 h of exponential feeding. **b** Time course of residual glucose (*squares*) and ethanol produced (*triangles*). The *dashed line* is the profile of glucose fed to the reactor. During the run, the feeding was maintained for 23 h, being marked only by an exponentially increasing feeding

Discussion

In this work, we have shown the use of a Pir4-based expression system in the BY4741 strain of *S. cerevisiae* that drives the secretion of human IL-1β to the growth medium. The *PIR4-IL1β* gene fusion involves the substitution of the region containing three of the four conserved cysteine residues of Pir4 by IL-1β, so that the fusion protein is no longer retained in the cell wall but released into the growth medium. Also, to avoid the potential metabolic burden factor derived from the use of strong promoters in *S. cerevisiae* (Da Silva and Bailey 1991; Görgens et al. 2001), the native constitutive *PIR4* promoter was used.

The main IL-1β polypeptide secreted by the BY4741 strain shows a size of 28 kDa, higher than the 17.4 kDa of native IL-1β. This difference may be accounted for by O-glycosylation, the presence of 38 amino acids of subunit II of Pir4 or even changes in mobility associated to the formation of intra-molecular disulphide bridges between the two cysteine residues in positions 8 and 71 of IL-1β, which form a disulphide bridge when the protein is expressed in *S. cerevisiae* (Casagli et al. 1989; Pucci et al. 1990), or between these two cysteines and the cysteine residue from Pir4 that constitutes the carboxy-terminus of the fusion protein.

Pir4-IL1β behaved as a growth-linked product, since it was expressed under the constitutive promoter of the *PIR4* gene. As a consequence, optimisation of growth of the producer strain would lead to a high product concentration in the medium, which is the primary target in a bioprocess. Yeast growth in the fed-batch reactor significantly improved when the rich complex medium was replaced by a defined mineral medium containing vitamins, trace elements and supplemented with casamino acids as amino acid source. The rich complex medium allowed the recombinant *S. cerevisiae* BY4741 to grow vigorously only during batch growth (shake flasks or batch phase in the bioreactor), whilst it was inadequate for long-term operation. In

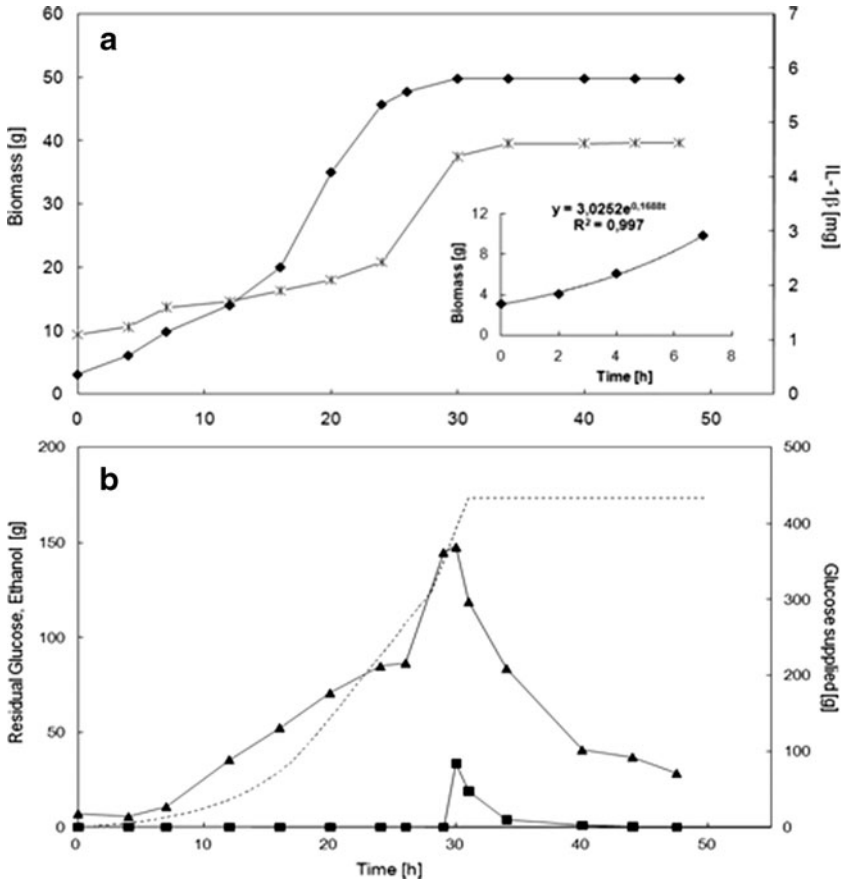

Fig. 5 Growth of *S. cerevisiae* BY4741 [PIR4-IL1β] in aerated fed-batch reactor, with SDMB medium. Feeding profile was designed to allow the producer strain to grow with a specific growth rate (μ) of 0.16 h^{-1}. **a** Time course of biomass (*rhombus*) and product (*star*) secreted into the medium; in the *insert* the interpolation of the experimental data regarding the biomass during the first 7 h of exponential feeding. **b** Time course of residual glucose (*squares*) and ethanol produced (*triangles*). The *dashed line* is the profile of glucose fed to the reactor. During the run, the feeding was maintained for 30 h, being marked by an initial exponentially increasing feeding of 22 h followed by a constant feeding of 6–8 h

contrast, the supplemented defined mineral medium ensured the availability of all required components during the fed-batch run.

For auxotrophic strains, such as BY4741, some compounds (e.g. histidine, leucin, methionin, uracil) are essential nutrients and the cultivation media have to be supplemented with them to allow growth. Auxotrophy-complementing compounds are often added at concentrations which are not critical for qualitative tests or strain maintenance, but can result in growth limitation when quantitative parameters such as biomass yields are determined in liquid media (Pronk 2002). For this reason, the concentration of ACA source in the feeding solution was calculated from the equation developed for the initial feed rate (F_0) (see "Materials and methods), taking into account the value of biomass yield for the given amino acid, in aerobic growth conditions and considering an excess factor of ca. 25%, to avoid "hidden" nutrient limitation. An excess factor of 25% is recommended, since auxotrophic strains may display a requirement of supplements even higher than that estimated from biomass yield and biomass composition, due to metabolic interferences and/or environmental conditions (Pronk 2002). Supplementation with a proper amount of auxotrophy-complementing amino acids led yeast cells to a final cell density threefold higher than that obtained with rich-complex medium (YP) but still far from the typical values reported for high cell density yeast cultures (80-100 g l^{-1}) (van Dijken et al. 2000). In fact, the producer strain, as well as the untransformed BY4741 strain, was unable to display a fully oxidative metabolism for a long period, since a full respiratory capacity was maintained only up to half-way through the exponential feeding phase, then a mixed respiro-fermentative metabolism progressively took place. A few hours before the end of glucose supply, growth unavoidably arrested, glucose accumulated in the medium and the ethanol produced during the run probably exerted a denaturing effect on the product.

The results obtained suggest that the auxotrophic BY4741 strain was sensitive to long-term aerated operations. Though the reasons for this sensitivity still remain to be elucidated, it is worth noting, as reported in the literature (Brachmann et al. 1998; Bitterman et al. 2003), that the presence of auxotrophies (mutations by deletion) may give 'collateral' effects on one or more adjacent genes that can determine unexpected behaviour. In our case, respiratory capacity was displayed only for few hours during exponentially increasing feed-phase of the fed-batch cultivation.

The shift from respiratory to fermentative metabolism for both the transformed and untrasformed strain during fed-batch cultivation was unavoidable and occurred even when the μ value imposed by the feeding profile was reduced up to 0.10 h^{-1} (data not shown).

Though some studies have shown that the effect of excessive auxotrophic markers in transformed strains often result in overconsumption of the required metabolite and decreased growth (vanDusen et al. 1997; Chopra et al. 1999; Shiba et al. 2001), very scarce information is available on the problems encountered during cultivations in fed-batch reactors. Problems related to the use of auxotrophic strains do not seem to be confined to yeast: it has been reported that auxotrophic strains of *E. coli* in fed-batch culture do not achieve high cell densities (Riesenberg 1991).

These considerations lead to the claim that a microbial strain should be tested under fed-batch conditions which resemble those of a production process before being chosen as host for heterologous protein production, because its behaviour may differ considerably from that observed during preliminary test cultures.

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