Heterologous protein production by yeast in aerated fed-batch cultures: relevance of the host strain viability

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The auxotrofic *S. cerevisiae* BY4741, carrying the fusion *PIR4-IL1β*, and able to secrete the human interleukin-1β into the culture medium, has been employed in an aerated bioreactor working as a fed-batch. Notwithstanding proper formulation of the culture medium, the performance of the host strain BY4741 [Pir4-IL1β] in fed-batch was not satisfactory: biomass density was far from that expected, glucose and ethanol accumulated during the runs. To test if the oxidative stress was responsible for the observed behaviour, the mutant BY4741 Δyca1, deleted for the *YCA1* gene, coding for a caspase-like protein involved in yeast apoptosis, was transformed with the expression cassette containing the fusion *PIR4-IL1β*, and assayed in the same aerated fed-batch system. The different performances exhibited by both BY4741[Pir4-IL1β] and BY4741 Δyca1 [Pir4-IL1β], together with the study of death kinetics during operation runs, evidenced the relevance of strain viability in the operative conditions employed.

1. Introduction

Saccharomyces cerevisiae has been recognized as the most useful eukaryotic microorganism for heterologous protein production since 1978, when its genetic transformation was established (Hinnen et al., 1978).

S. cerevisiae is a glucose-sensitive yeast and displays aerobic alcoholic fermentation on glucose (Alexander and Jeffries, 1990). The regulation of glucose metabolism in yeast has a major implication in the production of heterologous proteins, since a complete oxidative metabolism of the carbon source should be maintained during the process to achieve high cell densities with high yields of recombinant product (Mendoza et al., 1994). In this concern, fed-batch technique, which permits substrate limitation, offers a tool for both metabolic control of sugar overflow metabolism and oxygen consumption rate, avoiding engineering limitations with respect to cooling and oxygen transfer (Enfors, 2001).

In the present paper, the auxotrofic *S. cerevisiae* BY4741, carrying the fusion *PIR4-IL1β,* has been employed as host strain to produce human interleukin-1β (IL-1β) in an aerated bioreactor working as a fed-batch. This strain already revealed capable to express and secrete interleukin-1β as a growth-linked product into the medium of shake-flask cultures (Romano F. et al., 2007).

To achieve high biomass yield and productivity, an exponentially increasing feed was initially applied to the bioreactor; then, the feed was switched to a constant value,

to get over the oxygen transfer rate limitation in the reactor. In the operative conditions employed, the behaviour of the recombinant strain was far from that expected.

Considering that fed-batch operations were carried out under vigorous and continuous aeration, it has been hypothesized that oxidative stress could play a significant role in determining the observed behaviour. To test this hypothesis, the mutant BY4741 Δyca1, deleted for the *YCA1* gene, coding for a caspase-like protein involved in yeast apoptosis (Madeo et al., 2002; Mazzoni et al., 2005) was transformed with the expression vector containing the fusion *PIR4-IL1β*, and assayed in the same aerated fed-batch system.

The performances exhibited by both the recombinant strains, BY4741 [Pir4-IL1β] and BY4741 Δyca1 [Pir4-IL1β], were compared and cell death kinetics during operation runs examined.

2. Materials and Methods

2.1 Strains

S. cerevisiae strain BY4741 *(MATα, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1)* (EUROSCARF collection, Heidelberg, Germany), transformed with a YEplac vector containing the gene fusion *PIR4-IL1β* (Romano F. et al., 2007), and indicated as BY4741 [Pir4-IL1β] was used in this work.

The same expression vector was employed to transform the deletion mutant *S. cerevisiae* BY4741 Δyca1 (*MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, yor197w::kanMX4)* (Madeo et al., 2002). Yeast transformants were selected for rail prototrophy on SD medium (0,67% YNB, 2% glucose and required auxotrophy at the concentration of 10 μg ml^{-1}).

2.2 Fed-batch cultures

Fed-batch cultures were perfomed in a 2.5 l stirred Bioflo 110 (New Brunswick, Sc.). Cultivation started with a batch phase using 1 l of defined mineral (DM) medium, prepared according to Verduyn et al. (1992), containing 20 g glucose and supplemented with vitamins, trace elements and bactocasaminoacids (BectonDickinson & Co., Sparks, MD 21152 USA) (Romano F. et al., 2007). 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.

When glucose was completely depleted, the feeding was started with a solution containing 50% w/v glucose, other than vitamins, trace elements, iron sulphate and bactocasaminoacids (Romano F. et al., 2007).

The exponential profile of flow rate *F(t)* was obtained from the mass balance on limiting substrate (Enfors, 2001) and calculated according to:

$$F = F_0 \cdot \exp(\mu \cdot t) \qquad (1)$$

where F_0 was given by:

$$F_0 = \frac{\mu \cdot (X_0 \cdot V_0)}{S_R \cdot (Y_{X/S})} \qquad (2),$$

X_0 and V_0 were biomass density and volume at the start of feeding, respectively; S_R was the concentration of the growth limiting substrate in the reservoir; $Y_{x,s}$ was the respiratory biomass yield; and μ was the specific growth rate of the producer strain. The exponential feeding was built up to ensure a constant μ of 0,16 h^{-1}, below the maximum (μ_{max}= 0,39 h^{-1}), which was evaluated in a preliminary experiment carried out in Erlenmeyer flask containing DM medium (Romano et al., 2007).

After the exponentially increasing flow rate, feed was switched to a constant value. During fed-batch runs, aeration was accomplished as described above in the case of the batch phase, except for the air flow which was of 1.5 vvm and pH maintained by automatic addition of 10% v/v NH_4OH. Dow Corning 1510 (dil. 1:10) was used as antifoam. The bioreactor was inoculated with an adequate aliquot of a 24 h pre-culture, to give an initial $O.D._{590}$ of 0.2 in the bioreactor.

All the fed-batch cultures were carried out in triplicate.

2.3. Biomass determination

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

Cell viability during fed-batch runs was determined by viable count on supplemented DM agar plates, incubated at 30 °C for 48 h.

2.4. Analyses

Samples were quickly withdrawn from 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-1β concentrations. Residual glucose in the medium was determined by GOD-Perid from R-Biopharm (Roche, Mannheim, Germany). Ethanol production was measured with the enzymatic kit (176290) from R-Biopharm (Roche, Mannheim, Germany). Interleukin-1β was determined 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 (Romano V. et al., 2007). All samples were analysed in triplicate and the values of standard deviation obtained varied between 3-5%.

3. Results

3.1 Growth of *S. cerevisiae* BY4741 [Pir4-IL1β] in aerated fed-batch culture

Saccharomyces cerevisiae BY4741 [Pir4-IL1β] was cultured in the aerated bioreactor, employing DM medium and glucose as carbon source. After a batch phase of 16 h, exponential feed was started and maintained for 21 h, then the feed was switched to a constant value for 9 h.

In Fig. 1a, total biomass of the producer strain and total amount of the fusion protein secreted into the medium during the fed-batch phase, are reported. The recombinant product accumulated along the entire time course of growth, with a yield ($Y_{p,x}$) of 387 ng mg^{-1}. After 20-22 h of feeding, no further increase in biomass and, consequently, in product, was observed.

Yeast metabolism was fully oxidative in the first 8-10 h of the exponential feeding, being residual glucose in the medium absent and ethanol concentration at the same level achieved at the end of the batch phase (Fig. 1b). Then, the behaviour of the recombinant yeast was no longer consistent with the feeding profile applied: accumulation of glucose in the medium and concomitant ethanol production were observed (Fig. 1b). Apparently, glucose up-take of yeast cells was impaired and/or the amount of viable cell in the bioreactor diminished. The resulting excess glucose determined the shift of yeast metabolism towards fermentation.

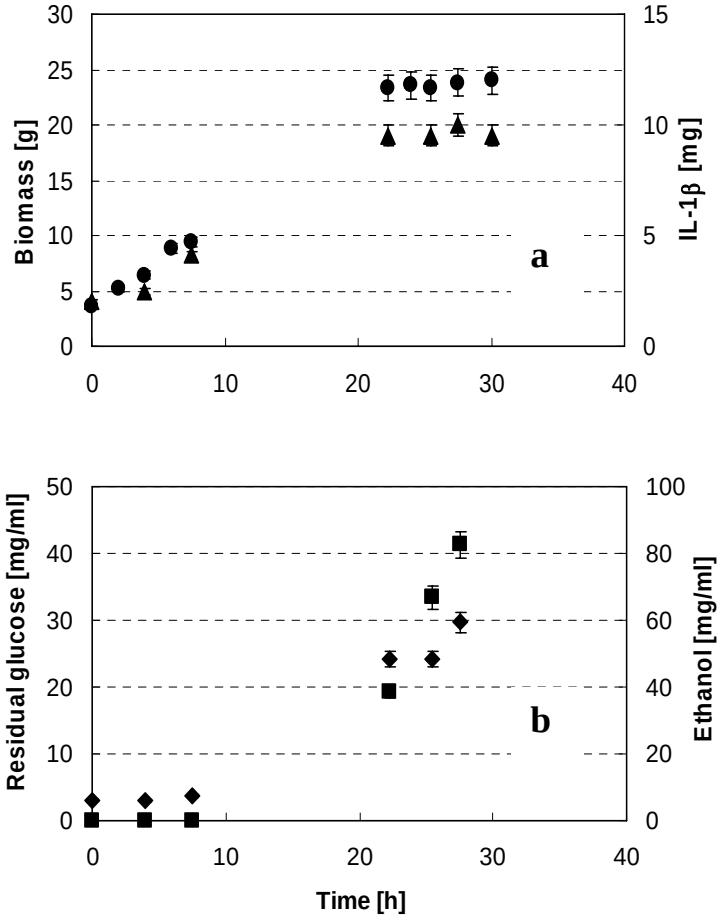

Fig. 1. Fed-batch run with BY4741 [Pir4-IL1β]: total biomass (●) and total amount of recombinant protein (▲) (**a**); residual glucose (■) and ethanol (♦) in the medium (**b**).

In the same operative conditions, a baker's yeast strain, isolated from a commercial sample, was able to display a fully respiratory metabolism along the entire fed-batch run (data not shown), achieving a final biomass concentration of 77 mg ml^{-1}, significantly higher than that obtained with BY4741 [Pir4-IL1β] (14,7 mg ml^{-1}, considering that the final volume of the broth-culture was 1.7 l) (Fig. 1a).

3.2. Comparison between BY4741 [Pir4-IL1β] and BY4741 Δyca1 [Pir4-IL1β] in aerated fed-batch cultures

Considering that fed-batch operations were carried out under continuous aeration, it was supposed that oxidative stress could affect strain viability and, consequently, explain the behaviour of the recombinant yeast. To test this hypothesis, the mutant BY4741 Δyca [Pir4-IL1β] was assayed in the same operative conditions previously employed for BY4741 [Pir4-IL1β].

Total biomass and residual glucose of BY4741 Δyca [Pir4-IL1β] monitored along the fed-batch run, are reported in Fig. 2, and compared to the corresponding data obtained with the original strain. Growth of the deletion mutant prolonged for further 10 hours with respect to that of BY4741 [Pir4-IL1β] (Fig. 2, top panel). As a consequence, total final biomass was 34 *vs*. 25 g of the original strain. Accordingly, a higher amount of recombinant protein was obtained, since the product yield ($Y_{p,x}$) remained the same (data not shown). Also in the case of the deletion mutant, glucose accumulated in the medium (Fig. 2, bottom panel), even though to a lesser extent.

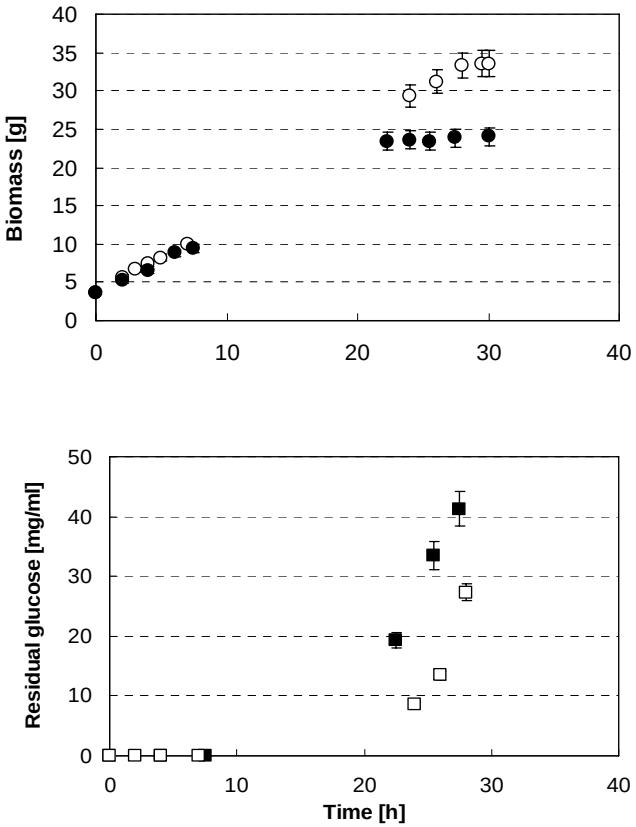

Fig. 2. Performance in fed-batch of the two recombinant strains: total biomass (●) and residual glucose (■) of BY4741 [Pir4-IL1β]; total biomass (○) and residual glucose (□) of BY4741 Δyca [Pir4-IL1β].

Cell viability of both BY4741 [Pir4-IL1β] and BY4741 Δyca [Pir4-IL1β] was monitored during the respective fed-batch runs by viable count of colony forming units (c.f.u.) and expressed as the ratio c.f.u./$O.D._{590}$, where $O.D._{590}$ corresponded to the total biomass (viable and dead cells) present in the bioreactor. Data obtained are reported in Fig. 3. Viability of both the strains diminished during the runs, though in the case of the deletion mutant the value of the specific death rate constant was lower than that of the original strain (0,011 *vs.* 0,026 h^{-1}, respectively). Apparently, the deletion in the *YCA1* gene increased host viability, in the operative conditions examined.

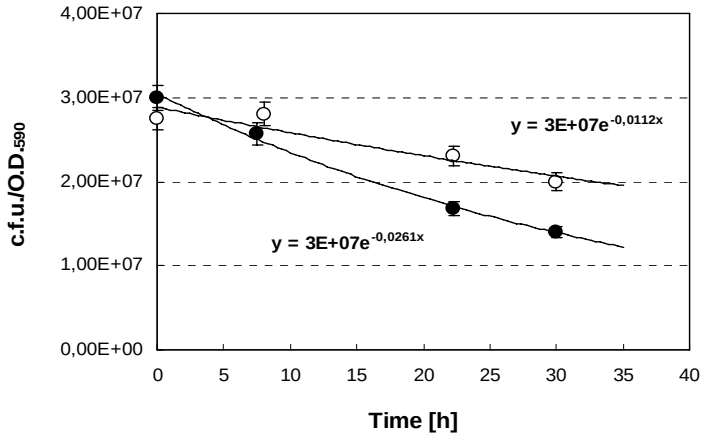

Fig. 3. Death kinetics of the two recombinant strains**,** BY4741 [Pir4-IL1β] (●) and BY4741 Δyca [Pir4-IL1β] (○) during fed-batch runs.

4. Discussion

In heterologous protein production by *S. cerevisiae* strains, auxotrophic host systems (*i.e*. strains with multiple auxotrophic mutations, such as *leu2*, *ura3*, *his3*, *trp1*, etc.), derived from industrial yeast strains, are widely used to ensure maintenance of expression vectors with selectable markers (Çakar et al., 1999). These systems are usually isogenic and haploid, differently from industrial strains which are usually diploid or polyploid and prototrophic.

Heterologous protein production with *S. cerevisiae* strains is mostly performed in bioreactors working in a fed-batch mode to avoid engineering limitations and exert metabolic control by sugar limitation. The latter promotes oxidative metabolism in the host with high biomass and product yields.

Notwithstanding proper formulation of the culture medium, the recombinant BY4741 [Pir4-IL1β], grown in fed-batch reactor, is able to sustain a fully oxidative metabolism only half-way through the exponential feeding, then the carbon source begins to accumulate and ethanol is produced until cell growth definitively stops. As a matter of fact, BY4741 cells precociously begin to die during the run, as shown by cell death kinetics.

This unexpected behaviour cannot be ascribed to the so-called "metabolic burden", consequent to the introduction of the foreign DNA (Görgens et al., 2001), since it is observed also in the case of the wild type BY4741 (data not shown).

Deletion of *YCA1* gene undoubtedly improves host viability. This supports the hypothesis that oxidative stress, arising during the continuous and vigorous aeration in the bioreactor, is highly probable and consistently leads to apoptotic scenarios. Apoptosis or programmed cell death (PCD) is now recognized to occur also in yeasts (Madeo et al., 1999).

Under oxidative stress, yeast cells would produce reactive oxygen species (ROS), which would induce *YCA1* gene to produce the protein caspase, involved in the regulated process of PCD (Madeo et al., 2002).

However, the performance of the deletion mutant as well, is far from that of an industrial strain. Apparently, other environmental stresses, deriving from process conditions (e.g. shear stress) are exerted on the yeast cells and result more severe for auxotrofic strains with respect to prototrophic ones and, mostly, haploid *versus* diploid. Indeed, *S. cerevisiae* BY4741, a haploid strain mutagenized to introduce many auxotrophies, could be intrinsically weaker than prototrophic yeasts.

The results obtained show that host viability is a relevant strain feature which has to be carefully evaluated in the set up of a bioprocess. However, taking into consideration that auxotrophic strains continue to be both useful tools acting on genes involved in the sensitivity to different stress and longevity, and convenient platforms in the field of heterologous protein production (Pronk, 2002), new metabolic and bioprocess strategies should be developed for a better their exploitation.

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