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Heterologous protein production in *Zygosaccharomyces bailii*: physiological effects and fermentative strategies

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Abstract

The optimisation and scale-up of a specific protein production process have to take into account cultivation conditions as well as cell physiology of growth and the influence of foreign protein expression on host cell metabolism. The ability of *Zygosaccharomyces bailii* to tolerate high sugar concentrations as well as high temperatures and acidic environments renders this ''non-conventional'' yeast suitable for the development of biotechnological processes like heterologous protein production. This work addresses the production of human interleukin-1β by a recombinant *Z. bailii* strain. We found that the heterologous protein production causes some modifications of the *Z. bailii* carbon metabolism, leading to a reduced biomass yield. The other important factor is the dependence of the recombinant IL-1β production/secretion on the growth rate. Among the cultivation strategies studied, the most appropriate in terms of production and productivity was the fed-batch mode.

Keywords: *Zygosaccharomyces bailii*; Heterologous protein; Human interleukin-1β; Fermentation

1. Introduction

One of the most important goals in the production of proteins by recombinant organisms is to maximize the productivity of the desired protein. The understanding of the physiology of the cell and of the optimal operating conditions are essential to fulfil this goal. To obtain high production of a recombinant protein a cellular host should possess some properties which include a rapid growth and the availability of a stable and strong expression system. Today the hosts for recombinant biopharmaceutical productions are mainly *Escherichia coli*, yeasts and mammalian cells. Yeasts possess specific advantages over bacterial systems, as they provide a more suitable environment for post-translational modifications (protein folding, assembly, etc.). Notwithstanding various successful applications of *Saccharomyces cerevisiae*, specific disadvantages in the use of this organism are also encountered, as hyperglycosylation and a low secretion efficiency. A complicating factor in the cultivation of *S. cerevisiae* on sugars is its strong tendency to produce ethanol also in aerobic conditions, the so-called ''Crabtree-effect''. This leads to the need of fed-batch cultivation, which has to be operated at low specific growth rate to prevent reduction in biomass yield [1,2]. ''Non-conventional'' yeasts, such as *Kluyveromyces lactis*, *Yarrowia lipolytica* and methylotrophic yeasts as *Hansenula polymorpha* and *Pichia pastoris* have gained increasing interest as attractive hosts for the production of recombinant proteins [3–7]. *Zygosaccharomyces bailii* has been

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taken into account for biotechnological processes due to its ability to tolerate high sugar concentrations as well as high temperatures and acidic environments [8–10]. These features can be very useful for industrial fermentations because they can simplify the process, improving their economical value. The results so far obtained have been encouraging to further develop this yeast as a new host for heterologous protein production and as a cell factory for industrial productions [11,12].

The optimisation of recombinant protein production depends not exclusively on the molecular strategies employed. The physiology of growth as well as the effects of the expression of a foreign protein on host cell metabolism are both important in the development of the process. It has been shown that many parameters, such as the specific growth rate, the carbon source, oxygen supply, pH, temperature, can strongly modulate the production of the heterologous protein, because of the deep changes that they trigger in the physiological state of the cell. The expression of recombinant genes has an effect on the host organism, known as the "biosynthetic burden" [13,14], which negatively affects cell growth. Since productivity is correlated to biomass level, the optimisation of the production is then strictly depending on the physiology of growth and this is in turn closely related to the choice of the more suitable fermentation process to be used. So far, *Z. bailii* is known as a fructophilic yeast and it has been reported that *Z. bailii* is able to grow anaerobically in complex media [15,16]. Aerobic alcoholic fermentation has been observed in this yeast to be strictly dependent on the carbon source utilized, the Crabtree-effect being displayed on glucose only at a dilution rate close to the maximum specific growth rate [17].

In this work, we show how physiological and cultivation conditions influence the production of heterologous human interleukin-1β in *Z. bailii*. The aim of the present study was to set the optimal conditions of growth and to determine the parameters for the development of a fed-batch process to obtain a higher biomass and protein production.

2. Materials and methods

2.1. Yeast strains and media

Zygosaccharomyces bailii strain ATCC 36947 was transformed by the lithium acetate method with the centromeric plasmid pZ_3 which contains, as selective marker, the gene for the resistance to G418, or with pZ_3K1IL-1β, containing the human IL-1β gene functionally fused with the pre-sequence of the Kl killer toxin α subunit of the *K. lactis* signal sequence [11]. The utilised plasmid exploits the *S. cerevisiae* ARS/CEN sequences, resulting in *Z. bailii* transformants with no significant clonal differences: it can be speculated that also in *Z. bailii* the plasmid is maintained in one or two copies per cell, even if it was not experimentally tested. From the frozen culture (stored at −80 °C in 15% glycerol), cells were precultured at 30 °C on Verduyn medium [18], modified by adding ammonium sulfate at a final concentration of 7 $g\,l^{-1}$ and vitamins and trace elements three times more concentrated. G418 (GIBCO, Invitrogen, Auckland, NZ) was added to the growth medium at 400 $\mu g\,ml^{-1}$ in order to maintain the selective pressure on the cells.

2.2. Batch cultivations

Batch cultivations were performed in a Biostat-Q-system (B-Braun, Melsungen, Germany). A constant working volume of 0.8 l was maintained. An airflow of 1 $l\,min^{-1}$ and a stirrer speed of 1500 rpm maintained a dissolved-oxygen concentration above 30% of air saturation. The culture pH was maintained at 5.00 by automatic addition of 2-M KOH. The cell biomass from the preinoculum was washed and used to inoculate batch cultures on defined mineral medium, prepared as reported here above and containing the appropriate carbon source (glucose 7 $g\,l^{-1}$). Batch experiments were performed in duplicate.

For the high-glucose batch experiments we inoculated 1.5 l of the same synthetic medium but containing glucose at a concentration of 250 $g\,l^{-1}$.

2.3. Chemostat cultivations

Chemostat cultivations were performed as described for batch cultures. When the carbon source was exhausted in the batch phase, the continuous culture was started. All chemostat cultivations were repeated at least three times. The steady states were obtained by increasing the dilution rate from $D = 0.1\ h^{-1}$ to $D = 0.29\ h^{-1}$. Close to the D_{crit} the dilution rate was gradually increased by 0.02 h^{-1}. "Steady state" is defined as the situation in which at least five volume changes passed since the continuous culture started and biomass concentration, oxygen consumption, glucose and ethanol concentration in the medium kept constant over two volume changes. All steady states below the critical dilution rate (at which ethanol production starts) were repeated after further five volume changes. Above the critical dilution rate steady states were repeated after 10 residence times further.

At appropriate intervals, samples were withdrawn from the culture for analysis.

2.4. Fed-batch cultivations

High-density cultures were performed in a 2-l stirred tank (B-Braun Biostat B) system. Cultivation started

with a batch phase using 1 l of synthetic medium (Verduyn, unmodified) containing glucose (20 g l^{-1}) and G418 (400 mg l^{-1}). When glucose was completely depleted, an exponential feeding was started with a medium [19] containing glucose 500 g l^{-1} and G418 (400 mg l^{-1}). The addition of ammonium hydroxide (10% v/v) accounted for the nitrogen source and for pH control. Operative conditions were pH 5.00, aeration 2 l min^{-1} (pO_2 > 30%).

2.5. Analysis of extracellular metabolites and determination of dry weight

Samples were quickly withdrawn from batch, fed-batch and chemostat cultures at steady state. The concentrations of glucose, glycerol, ethanol and acetate in supernatants were determined with R-biopharm (Roche) enzymatic kits (716251, 148270, 176290, 148261, respectively). Pyruvate was detected in assay mixtures containing 100 mM potassium phosphate (pH 7.6), 0.3 mM NADH, 1.5 mM EDTA; the concentration of pyruvate was calculated from the decrease in extinction at 340 nm after the addition of 2 U of lactate dehydrogenase (Roche). All samples were analysed in triplicate and the values of standard deviation obtained varied between 1% and 2%.

For dry-weight determinations washed culture samples were filtered on a 0.45-μm glass microfiber GF/A filter (Millipore) and dried for 24 h at 85 °C. Parallel samples varied about 3–5%.

2.6. Western-blot analysis

Intracellular proteins were extracted from cells as reported [11]. Both cell extracts and the supernatants were analysed by SDS–PAGE on 12% polyacrylamide gels. All gels were made in triplicate and then blotted on nitrocellulose (0.45 μm pore size); the membranes were probed with IgM antibodies (1:500 S. cruz 548 in blocking buffer) directed against the hIL-1β protein. After extensive washing, the membranes were treated with a secondary anti-rabbit antibody (1:10000, from Jackson Immuno Research, Philadephia, PA, USA, cod. 711-036-152) and ECL staining (Amersham) and exposed.

The expression of hIL-1β was quantified using the EuroClone hIL-1β as a standard and the densitometric scanning program *ScionImage*™ (Frederick, MD, USA).

3. Results

3.1. Batch cultivations

Having previously observed that in *Z. bailii* the aerobic ethanol production on glucose is lower than on fructose [17], we performed batch cultivations of the recombinant *Z. bailii* strain, constitutively expressing the human IL-1β on glucose as carbon source. The main parameters of the cellular metabolism, the specific growth rate and the specific rate of glucose consumption and ethanol production were calculated and compared to the data obtained with the wild-type strain and with the strain transformed with the empty plasmid as controls (Table 1). The specific growth rates were similar for all the strains. The transformed strains showed a higher specific rate of glucose utilization than the wild type and a reduced rate of ethanol production. In addition to ethanol, other metabolites typically produced under a respiro-fermentative metabolism as glycerol and acetic acid were detected during the aerobic growth and were produced at a higher rate than in the wild type. The biomass yield was lower in the transformed than in the wild-type strain and the ethanol yields were similar. Nevertheless, the lower biomass yield of the transformed strains cannot be justified only by the levels of acetate and glycerol, and it is probably due to the production of some other, still unidentified, metabolites.

When glucose was depleted, ethanol, glycerol and acetic acid were utilized as carbon source.

In order to estimate the secretory capacity of the transformed strain *Z. bailii* [pZ_3Kl IL-1β] during the cultivation in batch, Western-blot analyses were performed on both supernatant and intracellular samples. The heterologous human IL-1β was almost totally secreted at the end of the cell growth, after 42 h (Fig. 1), in accordance with what was previously reported in shake-flask cultures on minimal medium [11].

To assess the biotechnological potential of *Z. bailii*, we exploited a batch cultivation at high glucose concentration (250 g l^{-1}). This strategy, very simple to pursue, usually causes a strong inhibition of the growth and it is not practiced. In this condition, however, the transformed *Z. bailii* strain was able to grow at $\mu = 0.18$ h^{-1} and to completely deplete the glucose in 44 h. In these experimental conditions oxygen became limiting

Table 1
Specific growth rate (μ), specific rate of glucose consumption and product formation (q), biomass and ethanol yield in aerobic batch cultures of *Z. bailii* wild-type, *Z. bailii* [pZ_3] and *Z. bailii* [pZ_3Kl IL-1β] on glucose

	Z. bailii wt	*Z. bailii* [pZ_3]	*Z. bailii* [pZ_3Kl IL-1β]
μ_{max} (h^{-1})	0.29	0.3	0.28
$q_{glucose}$ (mmol g^{-1} h^{-1})	4.6	6.34	13.81
$q_{ethanol}$ (mmol g^{-1} h^{-1})	1.5	1.07	0.7
$q_{acetate}$ (mmol g^{-1} h^{-1})	0.13	0.2	0.38
$q_{glycerol}$ (mmol g^{-1} h^{-1})	0.23	0.33	0.35
Biomas yield (g g^{-1})	0.43	0.32	0.22
Ethanol yield (g $g^{-1}_{glucose}$)	0.1	0.11	0.13

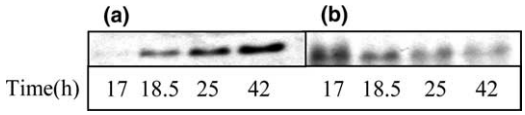

Fig. 1. Secretory capacity of *Z. bailii* [pZ$_3$Kl IL-1β] analyzed on samples picked up at various times during the batch experiments at 7 g l^{-1} of glucose: (a) protein secreted in the culture medium; (b) intracellular protein.

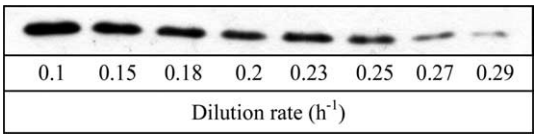

Fig. 2. Analysis of IL-1β secreted at dilution rates from 0.1 to 0.29 h^{-1} in chemostat cultivations. The same volume of supernatant was loaded for all samples.

along the growth, as indicated by a strong decrease of pO_2 values (below 10%). We impute the concomitant ethanol accumulation (till 12 g l^{-1}) to partial anaerobiosis, although we cannot exclude an implication of the Crabtree-effect in the phenomenon. In this condition the biomass yield value was 0.20 and levels of human IL-1β reached a value of about 2.8 mg l^{-1}.

3.2. Chemostat cultivations

The production of heterologous proteins causes some effects on the yeast physiology and metabolism due to the energetic burden that the extra-synthesis entails. Chemostat cultivation is the preferred system for a most accurate analysis of cellular metabolism. Further, data obtained in chemostat cultivation can predict adequately the fermentative capacity in dynamic fed-batch cultures [20]. With this aim we performed glucose-limited continuous cultures of the recombinant *Z. bailii* [pZ$_3$Kl IL-1β] strain, in a range of dilution rates between $D = 0.1$ h^{-1} and $D = 0.29$ h^{-1}. In this range the genetic stability of the plasmid was over 90%, except for the $D = 0.29$ h^{-1} where it was 60%.

The analysis of the specific rate of glucose consumption (Table 2) showed that this parameter rose with the increase of dilution rate, as expected. The production of ethanol started at $D = 0.2$ h^{-1} and its specific rate of production maintained at a very low level until $D = 0.25$ h^{-1}. As a consequence, no deep variation of the biomass yield was observed till $D = 0.25$ h^{-1}. At higher dilution rates, both the specific rate of glucose consumption and the ethanol production rose, the latter causing a strong decrease of the biomass yield (about 50% of the value at $D = 0.25$ h^{-1}). Moreover, other metabolites as glycerol and acetate were detected at $D = 0.25$ h^{-1} too, with a specific rate of production of 0.512 and 0.192 mmol g^{-1} h^{-1}, respectively.

As both a high specific and a high volumetric productivity are main features for industrial processes, we performed Western-blot analysis to quantify the production of the recombinant protein at all the dilution rates (Fig. 2). The highest production/secretion was detected at $D = 0.1$ h^{-1}, while it decreased at the higher dilution rates. At $D = 0.1$ h^{-1} we calculated that the volumetric productivity was 0.0155 mg l^{-1} h^{-1} and the specific productivity was 0.005 mg g^{-1} h^{-1}. From $D = 0.1$ h^{-1} to $D = 0.25$ h^{-1} the values of the relative volumetric productivity kept very similar. Due to the lower biomass yield beyond $D = 0.25$ h^{-1}, we observed a strong decrease of volumetric productivity. The specific productivity decreased too, as a consequence of the reduced production/secretion.

3.3. Fed-batch cultivations

The results obtained with chemostat cultures were used to develop the most suitable fed-batch protocol. This technique is a common strategy in heterologous protein production because it allows to pursue the highest values of biomass concentration. The application of a simple exponential feeding allowed us to grow the transformed cells till high densities and to control the growth rates. The exponential feeding was started at the end of a batch phase, immediately after complete glucose depletion. The low amount of ethanol present was rapidly consumed and did not disturb the exponential growth. Starting from the chemostat data, we assayed some of the relevant growth rates from $\mu = 0.08$ h^{-1} to $\mu = 0.2$ h^{-1}. Cells reached good biomass concentrations at all the growth rates tested (Table 3), although the increasing oxygen demand was reflected

Table 2
Specific rate of glucose consumption and product formation (q) and biomass yield in aerobic glucose-limited continuous culture of *Z. bailii* [pZ$_3$Kl IL-1β] (in brackets data from *Z. bailii* wild-type strain [16])

	Dilution rate (h^{-1})							
	0.1	0.15	0.18	0.2	0.23	0.25	0.27	0.29
$q_{glucose}$ (mmol g^{-1} h^{-1})	1.16	1.86	2.57	2.64	3.02	3.11 (3.14)	6.59 (3.11)	7.04 (6.57)
$q_{ethanol}$ (mmol g^{-1} h^{-1})	0.009	0	0	0.018	0.058	0.027 (0.026)	7.34 (0.033)	5.58 (6.79)
Biomass yield (g g^{-1})	0.478	0.447	0.389	0.42	0.422	0.445 (0.441)	0.227 (0.482)	0.227 (0.256)

Table 3
Comparison among some of the relevant fermentative parameters for cells growing in fed-batch, high-glucose batch and chemostat

	μ (h^{-1})	Dry weight ($g\,l^{-1}$)	Biomass yield ($g\,g^{-1}$)	IL-1β ($mg\,l^{-1}$)	Specific productivity ($mg\,g^{-1}\,h^{-1}$)	Volumetric productivity ($mg\,l^{-1}\,h^{-1}$)
Fed-batch	0.08	76	0.25	40.5	0.049	1.152
	0.10	61	0.32	50.6	0.090	1.949
	0.20	42	0.20	6.10	0.029	0.318
High-glucose batch	0.18	51	0.2	2.84	0.009	0.065
Chemostat	0.10	3.4	0.48	0.155	0.005	0.016

For sake of comparison also the data referring to the best chemostat production are reported.

in lower biomass yields at the highest growth rates. The exit from the exponential phase of growth was always marked by appearance of ethanol; at this point we registered an increase in intracellular levels of human IL-1β (data not shown), quickly followed by the interruption of the production. Although glucose and ethanol levels remained very low during the course of the exponential growth, biomass yields were similar to those measured in batch cultures, and far from the chemostat values registered for similar growth rates. Also in this case, like in batch cultures, this behaviour, which is quite different from that observed in *S. cerevisiae*, cannot be justified only by the levels of acetate and glycerol (data not shown), and is probably due to the production of some other still unidentified metabolites.

As reported in Table 3, data obtained with chemostat cultivations were confirmed, with the best productive parameters, both volumetric and specific productivity, reached at a $\mu = 0.1\ h^{-1}$.

4. Discussion

In order to maximize the productivity of biomass of a "Crabtree-positive" yeast, the cultivation must be operated close to the critical dilution rate (D_{crit}), just before the start of the aerobic ethanol production. This is of great importance in heterologous protein production too, since the productivity of the desired protein and biomass could be closely related to each other. The specific growth rate and the sugar concentration can be controlled via the supply of carbohydrate feedstock by the use of fed-batch protocols. We previously have shown that *Z. bailii* is a "Crabtree-positive" yeast, but the aerobic ethanol production in *Z. bailii* is lower than in *S. cerevisiae* [17].

In *S. cerevisiae* it has been shown that the expression of heterologous protein negatively affects cell growth by lowering growth rate and biomass yield [14,21]. In *Z. bailii* we showed that the stress caused by the synthesis of human IL-1β, as well as the synthesis of aminoglycoside phosphotransferase conferring G418 resistance in the control strain, affect the biomass yield, indicating a decreased efficiency to convert nutrients in cellular components. This fact also causes a higher rate of glucose consumption. Moreover, the higher flux in glycerol and acetate production (NADH re-oxidation and NADH/NADPH formation) could reflect also a higher flux in protein metabolism for the transformed strains than for the wild type, in order to cope with the synthesis of extra-proteins. Transformed strains of *Z. bailii* appear then to be able to maintain the same growth rate as the wild-type strain by increasing fluxes in some metabolic pathways. As a consequence of the increased glycolytic flux, the transformed strain in continuous culture showed a lower critical dilution rate (D_{crit}) than the wild-type strain and this in turn produced an earlier decrease in biomass yield (Table 2). This fact has a great impact in terms of biomass productivity, which as a result is lower in the transformed strain ($0.061\ g\,g^{-1}\,h^{-1}$ compared with the value of $0.130\ g\,g^{-1}\,h^{-1}$ of the wild-type strain at $D = 0.27\ h^{-1}$), and thus in terms of IL-1β productivity.

The second important factor is the dependence of the recombinant IL-1β production/secretion on growth rate. Our results indicate a decreased production/secretion of the heterologous protein associated with the increase of the growth rate. In this respect, data obtained from chemostat experiments are valuable to assess the best cultural parameters for fed-batch growths. However, the specific dynamics of different heterologous proteins appear to modulate these characteristics. For example, specific productivity values show a huge difference due to difference in dynamics of product accumulation and secretion in the medium between chemostat and fed-batch cultures. For both techniques, the growth rate of $0.1\ h^{-1}$ appears to be particularly favourable for the production, as it is characterized by the best specific and volumetric productivity values.

All these data suggested the use of the fed-batch strategy to optimize the production of biomass and the overall productivity of this heterologous protein. The higher biomass concentration reached in a few hours results in better production and productivity values for all the fed-batch cultures tested, when compared to the chemostat performances at the same growth rates. Finally, the possibility to grow *Z. bailii* to high density with a simple batch strategy (high-glucose batch) has been tested. Although the levels of human IL-1β obtained in this way remain far lower than the best value obtained in fed-batch processes, they are similar to that reported using *S. cerevisiae*

transformed with a multicopy expression vector [22]. *K. lactis* has been reported to produce a higher level of human IL-1β with the use of a multicopy expression system [23]. The exploitation of plasmids based on the sequences of the 2-μm-like endogenous *Z. bailii* plasmid, currently under development in our laboratory, could improve the production from *Z. bailii* hosts [12,24].

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