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Optimization of fungal polygalacturonase synthesis by *Saccharomyces cerevisiae* in fed-batch culture

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Abstract

To define parameters for a technical fermentation process, the production of a fungal polygalacturonase by the yeast *Saccharomyces cerevisiae* was optimized in fed-batch culture. Synthesis of the secreted enzyme was markedly influenced by medium composition as well as by fermentation parameters. The kinetics of recombinant protein synthesis was growth-associated following an unstructured Monod kinetics under carbon-limited conditions. Productivity (as heterologous protein synthesized per time and biomass) did not remain constant during the fed-batch process and decreased after an initial peak, whereas the specific mRNA level remained high. Supplementing the medium with complex nutrients did not improve the production kinetics. However, an increased concentration of nitrogen (a C:N ratio of 6.5:1) resulted in a high level productivity that remained constant during the entire fed-batch process. While low nitrogen concentrations (C:N ratio of 14:1) were sufficient for optimal growth, higher nitrogen concentrations were required for maximum productivity. Using two control strains, it was shown that the recombinant plasmid per se had a negative effect on the growth rate (μ_{max}) in comparison to the wild-type strain, and that the synthesis of the recombinant protein moreover decreased the biomass yield (g cell dry weight per g sucrose). The high-level expression of the recombinant protein in the fed-batch process put metabolic stress on the cells which resulted in markedly reduced vitality. © 1997 Elsevier Science S.A.

Keywords: Saccharomyces; Recombinant; Fed-batch; Nitrogen; Metabolic stress

1. List of symbols

F	liquid feed rate to fermentor/l h^{-1}
P	PG concentration/U PG l^{-1}
S	sucrose concentration in the fermentor/g l^{-1}
S_0	sucrose concentration in the feeding solution/g l^{-1}
t	time/h
V	liquid volume in the fermentor during the fermentation/l
V_0	liquid volume at the start of the fermentation/l
X	cell concentration/g CDW l^{-1}
X_0	cell concentration at the start of the fermentation/g CDW l^{-1}
$Y_{X/S}$	yield factor for biomass per sucrose/g CDW per g sucrose
α	yield factor for PG per biomass/U PG per g CDW
μ	specific growth rate/h^{-1}

2. Introduction

Pectinases are widely used in the production of fruit and vegetable juices and for the processing of fruits and vegetables in the food industry [1]. A main constituent of pectinases is the debranching enzyme endo-polygalacturonase which is used in bulk quantities in the processing of carrots. We have previously reported the expression of the *Aspergillus niger* polygalacturonase (PG) in the yeast *Saccharomyces cerevisiae* [2]. This yeast is an attractive host for the production of food-grade recombinant enzymes (for a review see Ref. [3]). It neither contains nor produces toxins; it is not pathogenic and is a food organism which has been rated GRAS. It is also well suited for large-scale production [4]. The production of secreted proteins using *S. cerevisiae* is especially attractive as it does not secrete many homologous proteins, so that the recombinant protein is often the main constituent of the total secreted proteins [5]. Although yeast has a long history of use in large-scale fermentation processes, additional requirements have to be met for recombinant yeast processes [6]. These include the use of selective

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medium to ensure the maintenance of the recombinant plasmid and the optimization of the process with respect to cell and product yield. The production process must be able to yield large amounts of product while keeping the fermentation time as short as possible, and it should lead to complete utilization of the substrate at the same time, as the cost of raw material is the predominant manufacturing cost. In addition, the production strategy must take the regulation of the expression of the specific gene into account. This feature is determined by the promoter used.

In this paper, we report the kinetics of growth and product formation of a recombinant *S. cerevisiae* strain which produces extracellular *Aspergillus*-derived polygalacturonase (PG) in a fed-batch process. Special attention was paid to establishing a computer-controlled feeding pattern based on previously determined maximum growth rates and to the kinetics of PG productivity. Parameters were studied that determine the productivity in the fed-batch process and the effect of varied C/N ratios on the product yield was analysed. Furthermore, the growth characteristics of the host strain were compared with the production strain, and the plasmid stability and the vitality of the cells were analysed during the cultivation.

3. Materials and methods

3.1. Strains, plasmids and media

The *Saccharomyces cerevisiae* host strain used was AH22 (MAT a *leu*2-3, *leu*2-112, *his*4-519, *can*1, cir$^+$). The PG-producing strain was AH22 carrying the recombinant plasmid pPG6 [2]. Plasmid YEpoE1 is analogous to pPG6, but devoid of the expression cassette. It was constructed from plasmid YEp13 [7] by *Xho*I and partial *Sal*I restriction. The 5 kb fragment containing the yeast LEU2 gene and the 2 μm-DNA replication origin was electroeluted from an agarose gel, self-ligated and transformed in strain AH22. *LEU*2 transformants were analysed for plasmid content and plasmid structure by Southern hybridization of cellular DNA.

The medium used for the fermentations was minimal medium WM VIII [2]. WM VIII contains per litre 50 g sucrose, 0.25 g $NH_4H_2PO_4$, 2.8 g NH_4Cl, 10 g sodium glutamate, 0.25 g $MgCl_2.6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 2 g KH_2PO_4, 0.55 g $MgSO_4 \cdot 7H_2O$, 75 mg *m*-inositol, 1.75 mg $ZnSO_4.7H_2O$, 0.5 mg $FeSO_4 \cdot 7H_2O$, 0.1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg $MnCl_2 \cdot 4H_2O$, 0.1 mg $Na_2MoO_4 \cdot 2H_2O$, 10 mg nicotinic acid, 25 mg pyridoxin–HCl, 10 mg thiamin–HCl, 2.5 mg biotin, and 50 mg calcium pantothenate. Amino acids were supplemented as required at 40 mg l^{-1} for shake-flask cultures and at 100 mg l^{-1} in fermentation media. Media used to check for plasmid stability were YE (0.5% yeast extract, 2% glucose) as a rich non-selective medium, YNB (0.67% yeast nitrogen base (Difco, Detroit, USA), 2% glucose) as a selective minimal medium and YNB supplemented with 40 mg l^{-1} leucine as a non-selective minimal medium.

3.2. Culture conditions

Strains were routinely grown at 28 °C in shake-flask cultures at 120 rpm. In order to obtain biomass for fed-batch fermentations, cells were first inoculated from glycerol cultures kept at −70 °C in 20 ml WMIII medium and grown overnight. 0.4 ml of the culture were subsequently inoculated in 400 ml WM VIII medium and grown for 3 days. Cells were collected and inoculated in 2 l WM VIII medium in a 5 l-Biostat E fermentor (Braun Diessel Biotech, Melsungen, Germany) and grown as a batch culture for 23 to 28 h. Cells were collected and used to inoculate the fed-batch fermentation.

3.3. Fermentor and fed-batch conditions

Batch and fed-batch fermentations were performed in a Biostat E fermentor (Braun Diessel, Melsungen, Germany) with a 5-l working volume equipped with temperature, pH, air flow rate, dissolved oxygen pressure and agitation controllers. Unless otherwise stated, fermentations were performed at pH 5.0 and 28 °C. The pH was controlled by automatic addition of 1 M H_2SO_4 and 1 M KOH. Dissolved oxygen was measured by an pO_2 electrode and was controlled by agitation (500–600 rpm) and air flow rate (1.4–2.0 Nl min^{-1}). The fermentation medium was WM VIII with 5% sucrose as carbon source. Fed-batch fermentations were started with 2 l of 2-fold concentrated salts, vitamins and trace elements and 18 g cell dry weight from the preceding batch culture. A sucrose solution was added to the fermentation broth via a peristaltic pump (B. Braun FE211) by using a computer-controlled feeding scheme to maintain a specific growth rate. This scheme incorporates the cell yield (g cell dry weight (CDW) per g sucrose) and the theoretical sucrose consumption at the selected growth rate. In fermentations with nitrogen supplementation, the nitrogen source was fed together with the sucrose solution.

3.4. Analytical methods

Biomass was determined gravimetrically. Broth turbidity was measured photometrically (OD at 600 nm, using broth dilutions to obtain values of less than 1.0) and correlated to cell dry weight by a standard calibration curve. Ethanol concentration was determined by the alcohol dehydrogenase-NAD method [8]. Sucrose concentration was measured after enzymatic hydrolysis by determining the glucose concentration using the hexokinase, glucose-6-phosphate dehydrogenase-NADP method [9]. Polygalacturonase activity was determined using the reducing sugar assay as previously described [2]. The concentration of ammonium ions in the fermentation broth was determined using an ORION ammonia electrode (Fa. Colora, Lorch, Germany). The number of plasmid-carrying cells was determined by plating 100–200 cells on minimal medium (YNB) and supplemented minimal medium (YNB + leucine) or complete medium (YE), and

was calculated from the difference in the number of colonies growing on selective and non-selective plates.

3.5. Molecular methods

Methods for mRNA isolation and Northern blotting have been described [2]. The relative abundance of PG-specific mRNA was measured using a BioRad phosphoimager (GS-250 Molecular Imager System). The data were normalized to the ribosomal RNA content of the samples by densitometric scanning using NIH Image analysis software (version 1.54). Proteins were separated on SDS-polyacrylamide gels and stained with Coomassie blue. Stained gels were analysed using an imaging densitometer (Bio-Rad, Munich, Germany) to calculate relative PG concentrations. Total protein concentration was determined by the Bradford method using a UV standard test kit (Bio-Rad Protein Assay).

3.6. Fed-batch fermentation modeling (sucrose oxidation)

The following equations were used to describe the cell growth and the feed rate

$$\frac{d(XV)}{dt} = \mu XV \quad (1)$$

$$F = \frac{1}{Y_{X/S}S_0}\mu X_0 V_0 e^{\mu t} \quad (2)$$

The product balance for the fed-batch fermentation is calculated as

$$\frac{dP}{dt} = \mu X\alpha - \frac{F}{C}P \quad (3)$$

4. Results

4.1. Medium enrichment and growth parameters

In order to optimize the product yield from a recombinant yeast strain which expresses an *Aspergillus* polygalacturonase, we first analysed its growth parameters in batch culture (shake-flask culture or batch fermentations) and studied the effect of medium composition and culture parameters on product yield. While interpreting the results, it was especially important to consider simultaneously the effect of changes on plasmid stability, on recombinant product yield and on cell growth (growth rate and biomass yield). The standard medium used (WM VIII) had been developed for PG production [2] and has been been optimized with respect to salt, trace element and vitamin composition and concentrations. The growth temperature was maximal at 26 and 28 °C, showing a rapid decrease at temperatures higher than 32 °C. Plasmid stability was between 67% and 100% at or below 30 °C, and decreased markedly at higher temperatures. PG synthesis per cell was highest at 28 °C with only a minor decrease below and a drastic decrease above this temperature. Cultivation at different pH values (between 4 and 6) had little overall effect; however, a slight decrease in plasmid stability (from 85% at pH 5 to 69% at pH 6) and biomass yield was observed at pH 6. In contrast to observations made by other authors [10], histidine supplementation (which has to be added to account for the histidine auxotrophy of the host strain AH22) in concentrations between 10 and 100 mg l^{-1} did not influence plasmid stability nor did it affect biomass yield or productivity. Based on these results, standard fermentations were run at 28 °C, pH 5 using WM VIII medium with 100 mg l^{-1} histidine.

Different sources of amino acids (casein, peptone, yeast extract) were used as medium enrichment at a concentration of 0.5%. All of the supplements tested had a positive effect on biomass yield. In addition, yeast extract markedly influenced PG productivity: 0.4% yeast extract proved sufficient for a maximum increase in cell dry weight and PG productivity. Only the addition of a concentration as high as 2% gave a further increase in PG productivity. Interestingly, the addition of amino acids as yeast extract which renders the medium non-selective for plasmid-carrying cells did not result in increased plasmid loss.

4.2. Kinetics of growth and PG synthesis in fed-batch culture

To study the kinetics of product formation with respect to cell growth, fed-batch fermentations were performed. The fermentation was started by adding sucrose to cells suspended in salt and mineral solution. Feeding was adjusted to allow immediate consumption of the sugar and to avoid strictly ethanol formation due to the Crabtree effect. The growth status of the cells was either monitored by exit gas analysis (RQ being lower or equal to 1) or by ethanol formation. The feeding rate was increased manually in correlation with the cell growth (monitored by OD measurement). The biomass increase and the product formation (as U PG) in these fed-batch fermentations showed a positive correlation. The data obtained were used to model the fed-batch fermentation to provide us with parameters for establishing a computer-based fermentation scheme and to calculate the kinetic parameters of the strain employed. To do these calculations, it was assumed that the kinetics were of the Monod type and that product (PG) formation was associated to growth. Using this approach, the results presented in Fig. 1 show that the kinetics of the modelling data and the data obtained in the fed-batch fermentation are identical. There is only a small deviation between them, the square error being 1.4×10^{-1} and standard deviation being 6.9×10^{-6}. The calculations provided us with the following parameters: the maximum specific growth rate (μ_{max}) was 0.0725 h^{-1}, the saturation constant (K_s) was 0.0059 g l^{-1}, the yield factor for PG was 12.3×10^6 (U PG per g CDW). The yield factor for the biomass was calculated to be 0.482 (g CDW per g sucrose).

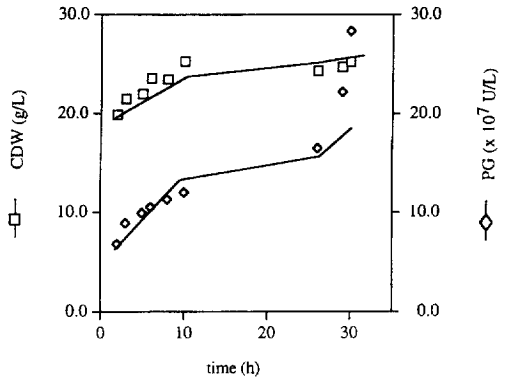

Fig. 1. Kinetics of cell growth and enzyme synthesis of strain *S. cerevisiae* AH22/pPG6 in fed-batch fermentation. Model simulation (solid lines) and experimental data of biomass concentration (CDW) and enzyme activity (PG) in the fed-batch process.

To improve the yield of heterologous enzyme in the fed-batch fermentation, a computer-controlled feeding scheme requiring the specification of the growth rate was used. The highest growth rate specific for the strain and the medium employed was experimentally determined by gradually increasing the sucrose feed and determining the onset of ethanol production. This marked the metabolic shift to aerobic fermentation (Crabtree effect) and was a clear indication that the maximal oxidative growth rate had been exceeded. The growth rate determined in this way proved to be higher than μ_{max} derived from the model. This may be due to the fact that a low rate of ethanol formation was tolerated in these fermentations (up to concentrations of 80 mg l^{-1}), while data for the modelling were based on a cultivation where the RQ values were kept strictly below 1.0. Following this scheme, the carbon source was fed exponentially resulting in a drastically reduced fermentation time in the final fed-batch process of 6 to 10 hours, versus 30 h in the manually controlled process and yielding a PG concentration and a biomass similar to the manually controlled fed-batch processes.

4.3. Optimization of productivity: correlation to the specific mRNA level

To analyse further parameters that govern product yield and growth in the fed-batch process, the productivity of the cells was measured as a time course during the process. These data, depicted in Fig. 2 as an example, show that the PG productivity, given as units produced per hour and g CDW, is not constant during the fermentation but is a sharp peak which then declines rapidly, while the sucrose feeding rate was kept constant.

The relative level of PG-specific mRNA during the fed-batch fermentation positively correlated with the PG productivity in that the increase in enzyme synthesis was preceeded by a 10- to 20-fold increase in PG-mRNA. The mRNA analyses showed, furthermore, that, in contrast to the sharp and immediate decrease seen for the PG productivity, the PG-specific mRNA level remains high as long as a sucrose signal is present and the *ADH1* promoter is induced. The PG-specific

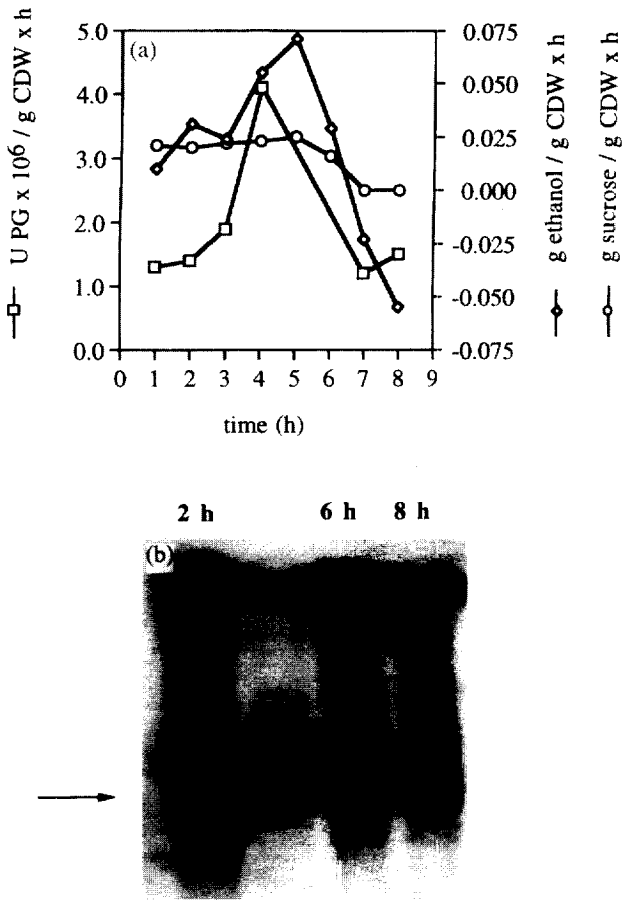

Fig. 2. Polygalacturonase production kinetics of strain AH22/pPG6 and PG-specific mRNA during a fed-batch culture. (a): Course of PG productivity, ethanol formation and sucrose consumption. (b): Northern blot of total RNA isolated at time points indicated and hybridization with PG-gene as probe. The arrow indicates the position of the PG-specific transcript.

mRNA was observed at an induction level of 23 times the background signal at time point 2 of the fermentation presented in Fig. 2, and was as high as 25-fold at time point 6 where the corresponding PG productivity decreased to nearly 0.

4.4. Nitrogen feeding and productivity

To understand why the cells did not continue to synthesize polygalacturonase in spite of the abundance of its transcript, the medium composition and the influence of different supplements were checked. In the first approach, yeast extract, which had considerably increased the PG synthesis in shake flask cultures (see above), was fed to a final concentration of 0.4% in a fed-batch cultivation. Its effect on growth, biomass yield and PG productivity was analysed (Table 1). The maximum (oxidative) growth rate that does not yet induce the Crabtree effect was higher in the medium supplemented with yeast extract than in the basic medium; the overall PG yield and the maximal productivity were markedly increased. The productivity was not, however, constantly high during the fermentation but decreased drastically after an initial

Table 1
The effect of medium enrichment on the growth rate, the PG yield, the productivity and the biomass yield of strain AH22/pPG6 in fed-batch fermentations using different growth media

Medium	μ_{max}/ h^{-1} [a]	PG yield/ U per g CDW	Highest productivity/ U h^{-1} g^{-1}	Biomass yield/ g CDW per g sucrose
WM VIII	0.127	1.2×10^7	1.3×10^6	0.50
WM VIII + 0.4% yeast extract	0.152	2.0×10^7	3.2×10^6	0.62 [b]
WM VIII + 10.8 g urea/L	0.116	2.2×10^7	3.0×10^6	0.53

[a] μ_{max} is calculated according to the Monod equation, and is defined as the highest growth rate that allows growth without ethanol formation.
[b] The carbon content of yeast extract was not considered in this calculation.

peak, although the feeding was continued at increased rates (in parallel with the carbon source). This sharp decrease of productivity is similar to the kinetics observed for the fed-batch cultivation using the standard medium (see Fig. 2) and implies that yeast extract has a positive effect on the biomass yield, whereas it acts adversely on recombinant product formation.

The amount of nitrogen fed was altered as a second parameter. Adding urea at a concentration of 10.8 g l^{-1} gives a similar increase in overall PG yield and maximal PG productivity as for the yeast extract supplementation (Table 1). The maximum (oxidative) growth rate was reduced when compared to the basic medium. Thus the feeding of nitrogen acts predominantly on the recombinant product formation. Interestingly, the course of productivity differs from the profiles observed for the standard medium and the yeast-extract-supplemented medium. Product formation does not stop after an initial peak but continues as long as the carbon source is available.

The influence of nitrogen feeding on product formation was analysed in more detail. To avoid any simultaneous effect of catabolite repression on the PG synthesis and to prevent a decreased vitality as observed at high growth rates (see below), the culture was kept at oxidative respiratory conditions by applying a constant low growth rate of 0.09. While the basic medium (WM VIII) has a carbon to nitrogen ratio of 14:1, a variation towards an increased nitrogen concentration with final ratios of 6.5:1 and 4:1 (C/N) was applied by feeding ammonium chloride or urea. As depicted in Fig. 3, PG productivity increases to a peak value during the first 6 hours of the fermentation and decreases steeply in the next three hours when the standard medium is used; supplementing with nitrogen results in an optimized productivity profile and a high-level productivity during the entire fermentation. This consequently yields a high overall product yield. However, increasing nitrogen feeding had no influence on the biomass yield (data not shown); this shows that the basic medium has a well balanced nitrogen content with respect to cell growth. However, an inhibitory effect of ammonium ions on the biomass production similar to that described by Ref. [11] can be observed at the highest ammonium chloride concentrations applied. The nitrogen content of the yeast dry mass, calculated from the concentration of ammonium ions

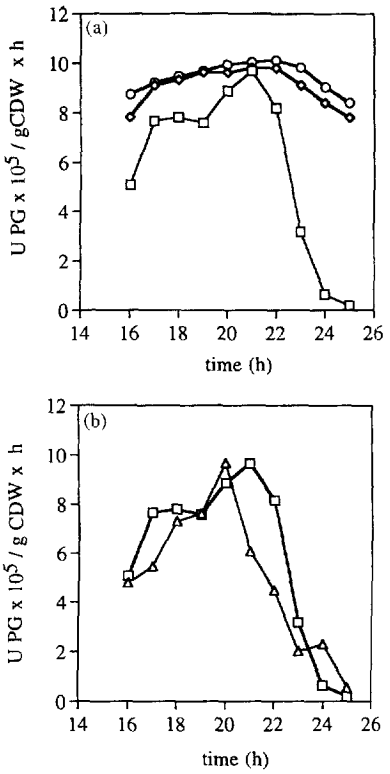

Fig. 3. Polygalacturonase production kinetics in fed-batch cultures of strain AH22/pPG6 in WM VIII medium supplemented with carbon and nitrogen at different ratios. A. Nitrogen as ammonium chloride was fed with the sucrose solution at C/N ratios of 4:1 (squares), 6.5:1 (rhombi) and 14:1 (circles). B. Nitrogen as ammonium chloride was exponentially fed at a C/N ratio of 4:1 (squares) or supplied at the start of the fermentation to the identical final concentration (triangles).

supplied and present in the cell-free supernatant at the end of the fermentation, was less than 6% in the standard medium, and was 8.5% when the C/N ratio was kept at 6.5:1. Values of 11% nitrogen in the biomass were obtained when the C/N ratio of 4:1 was applied but there was no further increase in recombinant protein productivity under these conditions (Fig. 3A). The continuous feeding of the nitrogen source was compared to supplying the total nitrogen at the start of the fed-batch. Feeding the nitrogen source resulted in a higher overall product (PG) yield than under conditions of low nitrogen supplementation (Fig. 3B) while no difference was observed in growth kinetics and biomass yield.

Table 2
The effect of plasmid content and PG production on the maximum growth rate and the biomass yield of the production strains and the control strains in fed-batch fermentations

Strain	μ_{max}/ h^{-1} [a]	Biomass yield/ g CDW per g sucrose
AH22	0.152	0.54
AH22/pPG6	0.127	0.50
AH22/YEpoE1	0.127	0.53

[a] See footnote a in Table 1.

4.5. Influence of the recombinant plasmid and the heterologous protein production on cell yield and growth rate

The synthesis of high amounts of a heterologous product by recombinant cells can be expected to put a metabolic burden on the cell. In addition, it has been reported that the presence of a recombinant plasmid per se results in altered growth characteristics of the host strain [12]. To analyse the effect of both these parameters on the strain used in this work, fed-batch fermentations were performed with two control strains, i.e. the host strain not carrying a recombinant plasmid, and a recombinant control strain not possessing the *ADH*I promoter or the expression cassette on the recombinant plasmid. Data obtained are summarized in Table 2 and show that the two recombinant strains (AH22/pPG6 and AH22/YEpoE1) exhibit a reduced growth rate compared to the host strain (AH22). The recombinant product (PG) does not, however, exert an additional negative effect on growth rate. The biomass yield on sucrose, on the other hand, is not influenced by the presence of a recombinant plasmid per se but is only reduced when the recombinant protein is produced (strain AH22/pPG6). This shows that the recombinant plasmid is no metabolic burden by itself; physiological consequences can be detected, however, as soon as additional energy is required to synthesize a plasmid-encoded product.

4.6. Effect of overexpression of the recombinant protein on cell vitality

The lower growth rates observed in fed-batch cultivations of the recombinant strains cannot only be caused by a metabolic burden but may also be the result of mitotic plasmid instability. A loss of the plasmid means the loss of the selectable marker *LEU2* and would hamper growth in the minimal medium used. We therefore analysed plasmid stability during the cultivation by plating cells on selective (leucine-free) and non-selective minimal medium. We observed that the number of plasmid-carrying cells of the PG-producing strain (AH22/pPG6) did not decrease during scale-up or in a fed-batch cultivation, but was between 90 and 100% and did not drop below 80% even at the end of the fed-batch cultivation. This was also true for the recombinant control strain (AH22/YEpoE1). When we, however, substituted the non-selective minimal medium with a non-selective complete (yeast extract) medium, an increasing number of cells were detected which were no longer able to form colonies on minimal medium, though still carrying the recombinant plasmid (Table 3). This suggests a lack of vitality that becomes more and more pronounced with cultivation time and it correlates with an increased growth rate (μ), coupled to ethanol formation, in the fed-batch process. This decrease in vitality was not observed in the host strain (AH22) or the recombinant non-producing control strain.

5. Discussion

We established a fed-batch process for the production of a secreted recombinant protein in yeast. The growth and production kinetics were model-simulated. They follow a simple unstructured Monod kinetics and the product synthesis is growth-associated. This agrees with kinetics one would expect for the expression of a protein from a constitutive promoter [6]. The modelling data were used to establish a computer-controlled sucrose-feeding program which maintains growth at the maximum aerobic growth rate. This rate is strain-specific and it depends on the medium composition. The rate observed in this system is comparable to aerobic growth rates reported in the literature for recombinant yeasts [13,14]. These are, in general, lower than the aerobic growth rates of 0.2 to 0.25 of industrially used strains [15]. The feeding policy applied yielded up to 160 mg of recombinant PG per litre within 8 h of fermentation time, i.e. 20 mg PG per hour and liter. This falls in the range of yields described for high productivity systems in the literature; Alberghina and co-workers [16] reported productivities of 33 mg β-galactosidase per hour and litre in a high-cell-density fermentation using an elaborated feeding scheme based on

Table 3
Plasmid stability in strain AH22/pPG6 (% of cells on MM/MM + leu) and vitality of the cells on minimal medium (% of cells growing on YE/MM + leu) at different stages of the cultivation in minimal medium WM VIII. The fed-batch fermentation was performed at high growth rates; μ was 0.15 at 24 h

Strain	Sample	Plasmid-carrying cells/ %MM/MM + leu	Cells able to grow on MM/ %YE/MM + leu
AH22/pPG6	Shake-flask culture	100	97
AH22/pPG6	Batch fermentation	96	88
AH22/pPG6	Fed-batch fermentation 17 h	99	75
AH22/pPG6	Fed-batch fermentation 24 h	80	66

galactose induction. Productivities given as mg product per hour and g biomass were compared by Hensing and co-workers [6] and were found to be between 0.03 and 1.2 for different recombinant *S. cerevisiae* strains that synthesize intracellular products. Using this definition of productivity, we obtained a yield of 0.26 mg PG per hour and g biomass, which is comparable to those fed-batch processes. In addition, a comparison to the fungal host for PG is possible based on these productivity calculations. The PG gene was overexpressed in *Aspergillus nidulans* [17] and was produced at 1 g per litre medium in a 46 h fermentation. This is equal to a productivity of 22 mg PG per hour and litre which is similar to the yields in recombinant yeast reported here.

The composition of the medium has proven to be of utmost importance. The enrichment of the growth medium with yeast extract or peptone has, in some cases, been found to have a positive effect on product yield [12,18]. Our data confirm these findings. However, in addition to influencing the overall productivity, the medium composition has a marked impact on the productivity profile during the fed-batch fermentation. Both the standard fermentation medium and the medium supplemented with yeast extract give profiles with a drastic decrease in productivity towards the end of the fermentation. In addition, supplementing with yeast extract increase the biomass yield, probably as a consequence of undefined carbon components of the supplement.

Conversely, the variation of the carbon-to-nitrogen ratio proved to have a major influence on product yield and in particular on the productivity profile during the fed-batch process. Increasing nitrogen feeding (as urea or ammonium chloride) to establish a C/N ratio of 6.5:1, resulted in a constant and high level productivity in the course of the fed-batch culture. This increase in nitrogen did not influence the biomass yield, which was already maximal at the C/N ratio of 14:1 as present in the basic medium. A calculation of the nitrogen content of the yeast biomass based on the amount of nitrogen in the feeding solution and the concentration of ammonia present in the culture broth at the end of the fermentation (data not shown) shows a nitrogen content of about 6% of the dry biomass in the basic medium (WM VIII) and 8.5% and 11% in the media of 6.5:1 and 4:1 C/N ratios, respectively. This implies that the level of product yield is influenced by the nitrogen feeding at concentrations where no influence on the growth of the strain is discernable and that a high-level productivity requires a markedly higher nitrogen input than is required for obtaining maximal growth rates.

Furthermore, we could show that the presence of the product-specific messenger RNA is a suitable reporter for detecting limitations in product formation during a fed-batch process. The abundance of mRNA correctly indicated a bottleneck due to nitrogen limitation in our study. The determination of specific mRNA excludes any regulations that may act on the transcriptional level and it might be of general use as an early indicator for the presence of unbalanced growth conditions.

The product (PG) itself does not seem to exert a harmful effect on the growth of the host cells. This can be concluded from the fact that there is no difference in the growth rate between the PG-producing and the non-producing strain, AH22/pPG6 and AH22/YEpoE1, in the fed-batch fermentation. The vitality of the cells, measured as growth on non-selective minimal medium, is, however, drastically reduced at the end of a fed-batch process run at a high growth rate. This might be due to an exhaustion of the intracellular pools of metabolites (e.g. amino acids, vitamins) and has to be studied further. A reduced cell viability of recombinant yeast cells overproducing hirudin in a fed-batch process has also been reported by Mendoza-Vega and coworkers [11]. The synthesis of the recombinant proteins and the replication of the recombinant plasmids is generally assumed to put a burden on the host cells which manifests itself in reduced growth rates and decreased biomass yields [19,20]. To differentiate between the effect exerted through the overproduction of the heterologous protein and the presence of the recombinant plasmid, we studied two control strains; the untransformed host strain and the transformed, PG-non-producing strain. Interestingly, the recombinant plasmid alone (in strain AH22/YEpoE1) exerted a negative effect on the maximum growth rate when compared to the host strain (AH22), whereas only the overproduction of the recombinant protein (in strain AH22/pPG6) decreased the biomass yield.

In summary, we determined kinetic parameters describing growth and product formation in the fed-batch process and could establish the following data important for a technical process: the nitrogen-to-carbon ratio must be carefully controlled and kept at a value exceeding that for optimal biomass yield to achieve a constant high-level product synthesis; nitrogen can be supplied as ammonium ions or as urea, the latter being preferable for the technical process as it is the cheaper nitrogen source; the product-specific mRNA is a useful and sensitive monitor for medium imbalances; and impairment of cell vitality, which occurs as an answer to stress conditons such as high growth rate and ethanol formation, should be avoided in a high productivity process.

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