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Fed-Batch Production of Endoglucanase with a Recombinant Industrial Strain of the Yeast *Saccharomyces Cerevisiae*

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The *Saccharomyces cerevisiae* Y306 strain was used as a host for the production of a heterologous endoglucanase coded by *celA* from *Paenibacillus barcinonensis*. The endoglucanase (EG-CelA) was expressed in *S. cerevisiae* using the cell-wall protein Pir4 as fusion partner to determine the secretion of the enzyme into the culture medium.The recombinant *S. cerevisiae* Y306 was cultivated in aerobic fed-batch culture in order to maximize the heterologous enzyme production while avoiding the occurrence of pyruvate overflow during glucose metabolism. Aiming at this, an exponential feeding policy was adopted to achieve high cell density cultivation (HCDC) at a constant specific growth rate of 0.16 h^{-1}.The experimental results demonstrated that EG-CelA was efficiently secreted into the culture medium along the entire course of the fed-batch process as a growth linked product being expressed under the Pir4 promoter. The final titer in 2 L broth culture resulted 2.48 g, an amount in accordance with the best productions of cellulolytic enzymes, reported by other authors.Further, a simple unstructured, non segregated mathematical model was employed to highlight that in the HCDC system developed, the microbial mass grew following the set up profile along the entire time-course of process.

1. Introduction

Microbial cellulases have shown many potential industrial applications in several sectors such as pulp and paper, textile, laundry, food and feed industry, brewing, and many others.(Kuhad et al., 2011) Nevertheless, due to the fast depletion of fossil fuel resources, the fundamental application of these cellulolytic enzymes remains their use in the efficient and cost-effective hydrolysis of renewable lignocellulosic biomass for the production of biofuels and bio-based chemicals.Lignocellulosic biomass is a complex and heterogeneous substrate primarily composed by cellulose, hemicellulose, and lignin. After aqueous pretreatment (Wyman, 2013), the vegetal biomass is hydrolyzed with mixtures of three different types of enzymes that hydrolyze the β-1,4-glycosidic bonds, cellobiohydrolases (CBHs, EC 3.2.1.91), endo-β-1,4-glucanases (EGs, EC 3.2.1.4) and β-glucosidases (BGLs EC 3.2.1.21) into fermentable sugars (Lynd et al., 2005).These three hydrolytic enzymes act synergistically, meaning that the hydrolysis rate of a combination of them exceeds the sum of the rates of the single enzymes (Nidetzky et al., 1994).The best strategy to produce a growth related product like in the case of the EG-CelA (Blanco et al., 1998) object of this work, especially when dealing with glucose sensitive microorganism, being *S cerevisiae* one of them, where pyruvate overflow during glucose metabolism must be avoided, is the employment of a stirred tank fermenter operating in fed-batch mode to obtain an HCDC (Walker, 1998). In fact each fraction of the limiting substrate that will not be channelled into the respiratory route will be wasted as a result of the transformation of part of the employed carbon source into undesired byproducts (mainly ethanol in the case of *S. cerevisiae*).In this work, a recombinant Endoglucanase coded by *celA* (GenBank Access No. Y12512) from *Paenibacillus barcinonensis* was produced, in aerated fed-batch fermenter by employing, as host, an auxotrophic *Saccharomyces cerevisiae* strain, i.e., the industrial Y306 strain (auxotroph for the trypto-

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phan). The host strain was engineered by means of an original expression strategy (Andrès et al., 2005, Paciello et al., 2010) consisting in the gene fusion between the heterologous gene and that of the yeast cell-wall mannoprotein Pir4, with the consequent production of a fusion protein, namely Pir4-CelA (Mormeneo et al., 2012).The recombinant *S. cerevisiae* Y306 was cultivated in aerobic fed-batch culture in order to maximize the heterologous enzyme production while avoiding the occurrence of pyruvate overflow during glucose metabolism. Aiming at this, an exponential feeding policy was adopted to achieve high cell density cultivation (HCDC) at a constant specific growth rate of 0.16 h^{-1}.The vigorous growth of the *S. cerevisiae*Y306 [Pir4-CelA] strain during the entire course of the aerobic fed-batch cultivation and the consequent accumulation of the growth-linked heterologous endoglucanase, efficiently secreted into the culture medium, were successively described by means of a simple unstructured, non segregated mathematical model.

2. Materials and method

2.1 Gene fusion strategy and expression of EG-CelA in *S. cerevisiae* Y306

The *S. cerevisiae* strain Y306 (10a12-13X38b4), a diploid strain, auxotroph for tryptophan, was kindly provided by Dr. F. Randez-Gil (IATA, CSIC, Valencia). *S. cerevisiae* was transformed with the shuttle vector pIA1 (Andrés et al., 2005), derived from the multicopy plasmid YEplac 195 (Gietz and Sugino,1988). pIA1 carries the complete sequence of PIR4 gene, including its regulatory sequences.The gene fusion between *PIR4* and EG-*celA* consisted of the insertion of the coding sequence of EG-*celA*, in the BglII and SalI sites of *PIR4*(Andrès et al., 2005 and Paciello et al., 2010). The insertion of EG-*celA* caused the loss of 365-bp of the carboxy-terminus of the *PIR4* open reading frame (ORF YJL158C; www.yeastgenome.org) that contains four cysteine residues at fixed positions, which, considering the extractability of some PIR-CWPs by reducing agents (Moukadiri and Zueco, 2001), should be responsible for cell wall retention. The loss of this region allows the fusion protein to be secreted into the culture medium. Transformation of the host strain and expression of endoglucanase activity was confirmed on YPD plates containing 0.5 % of carboxymethyl cellulose (CMC) as substrate (Strauss et al., 2001), subsequently flooded with a solution of Congo Red at 0.1 % (Teather and Wood, 1982), by determining the presence of haloes around the colonies as a result of the degradation of the substrate.

2.2 Shake-flask cultures

S. cerevisiae Y306 [Pir4-CelA] cells were grown in 500 mL Erlenmeyer flasks (0.2 initial OD_{590}) by medium inoculation with a suitable aliquot of a frozen stock cultures kept at -80 °C in 12.5% v/v glycerol. Shake-flask contained 100 mL of a defined mineral medium at pH 5.0 (Verduyn et al., 1992) with 2 % w/v initial α-D glucose, supplemented with 1% w/v casamino acids (BD Bacto™ Casamino Acids, Becton Dickinson & Co., Sparks, MD 21152 USA). Pre-cultures and cultures were incubated at 30 °C at 220 rpm (Stuart Scientific S150 Orbital Incubator for the time needed to obtain the desired quantity of viable microbial mass. Specific growth rate of *S. cerevisiae* Y306 [Pir4-CelA] was calculated from optical density measurements at 590 nm.

2.3 Fed-Batch culture

Aerobic fed-batch high cell density cultivation of *S. cerevisiae*Y306 [Pir4-CelA] strain was performed in a 2.0 L working volume stirred fermenter, Bioflo 110 (New Brunswick Scientific), at 30 °C. The bioreactor initially contained 1.0 L of a medium having the same composition of the shake-flask cultures. The fermenter was inoculated with an adequate aliquot of an exponential pre-culture, to give an initial $O.D._{590}$ of 0.04. After a 15 h batch phase, which allowed glucose in the medium to be completely consumed, fed phase of 26 hours, started by applying an exponentially increasing feed to allow the cells to proliferate at a constant specific growth rate (μ_0 = 16 h^{-1}), lower than the critical one (Enfors, 2001). The feeding solution (0.9 L) contained glucose (50 % w/v), salts, trace elements, glutamic acid and vitamins. Final salt concentrations per liter were: KH_2PO_4, 15.67 g, KCl, 5.00 g, $MgSO_4 \cdot 7H_2O$, 5.80 g, $CaCl_2 \cdot 2H_2O$, 1.37 g, NaCl, 0.40 g, $FeSO_4 \cdot 7H_2O$, 250 mg. The final glutamic acid, trace and vitamin concentrations per liter were calculated according to Paciello et al. (2010). Oxygen was supplied by air sparging (DOT set up at 30 % air saturation). The culture pH was maintained at 5.0 by automatic addition of 2 N KOH during batch phase and 10 % v/v NH_4OH during exponential phase. The foam level in the fermenter was controlled by the automatic addition of the antifoam B (dil. 1:10) (Sigma Aldrich).

2.4 Biomass determination

Biomass was determined by optical density at 590 nm (O.D. 590) and dry weight determination. The calibration curve relating $O.D._{590}$ values to biomass density provided a correlation factor of 1.85$O.D._{590}$ per mg ml^{-1}.

2.5 Determination of the EG-CelA activity

EG-CelA activity was measured on carboxymethyl cellulose (CMC) sodium salt low viscosity (Sigma Aldrich) substrate mainly based on the procedure described by Mandels et al., (1976). In this method, EG-CelA activity was measured by determining reducing sugars released after 15 min of enzyme reaction with 0.5% CMC at pH 4.4 (acetate buffer) and 45°C. One unit (U) of EG-CelA activity was defined as the amount of enzyme that liberates 1 µmol of glucose per minute under assay conditions. Reducing sugar has been estimated by applying the Somogyi-Nelson method (Spiro, 1966).

3. Results and discussion

3.1 Performance of *S. cerevisiae Y306* [Pir4-CelA] growing at constant µ in fed-batch

To produce EG-CelA, *S. cerevisiae* Y306 [Pir4-CelA] was cultured in aerated fed-batch fermenter with a feeding profile set up to sustain the exponential growth of the yeast strain at a constant µ value of 0.16 h^{-1} to avoid the sugar concentration exceed a critical value above which over-flow metabolism significantly reduces biomass yield. As shown in Figure 1, the microbial mass produced during the fed-batch phase followed the set up profile over the entire time-course of cultivation. The final value reached when the glucose "reservoir" was exhausted (after 26 h), was substantially identical to that expected. In other words, Y306 was capable to keep a fully respiratory (oxidative) metabolism during the entire time-course of cultivation. In fact ethanol did not accumulate in the medium and no residual glucose was found in the culture medium.

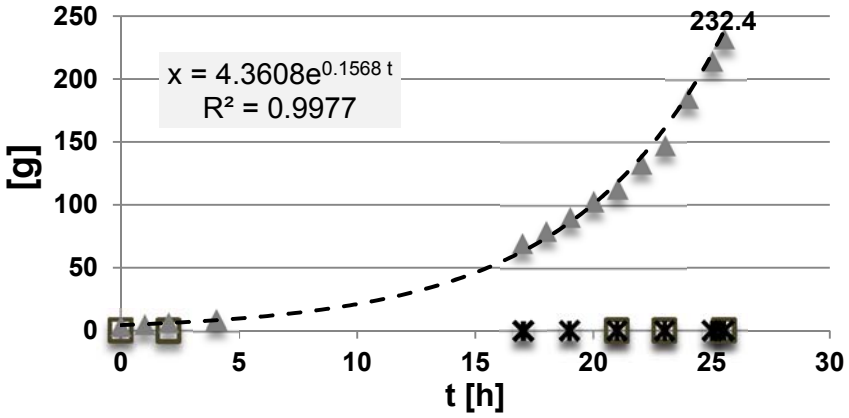

Figure 1. (full triangle) total microbial mass, (star) residual glucose and (empty rectangle) accumulated ethanol profiles during the entire course of the fed-batch cultivation

The recombinant enzyme concentration increased during the entire cultivation period reaching its maximum value at the end of the fed-batch run as shown in Figure 2.

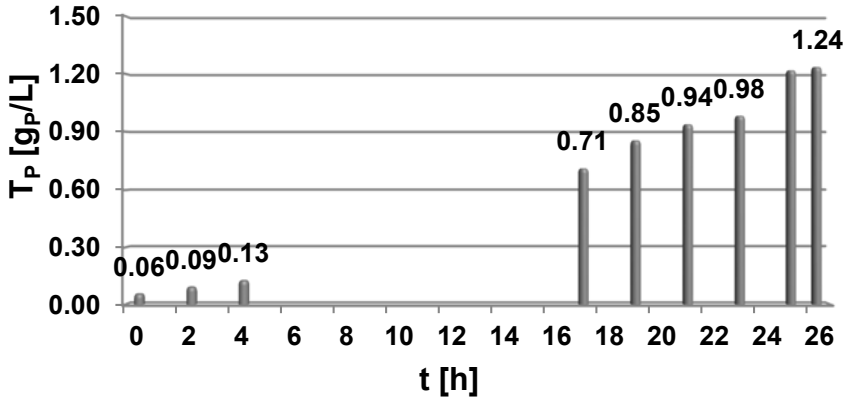

Figure 2. Profile of EG-CelA concentration during the entire time-course of the fed-batch cultivation

The fusion product yield on biomass ($Y_{P/X}$) was kept nearly constant along the entire fermentation run as shown in Figure 3; indeed, the EG-CelA was efficiently secreted into the culture medium along the entire time-course of growth. This behaviour confirmed that the recombinant enzyme was a growth linked product.
A summary of the information obtained for the producer Y306 strain cultured in fed-batch reactor at constant μ has been reported in Table 1.

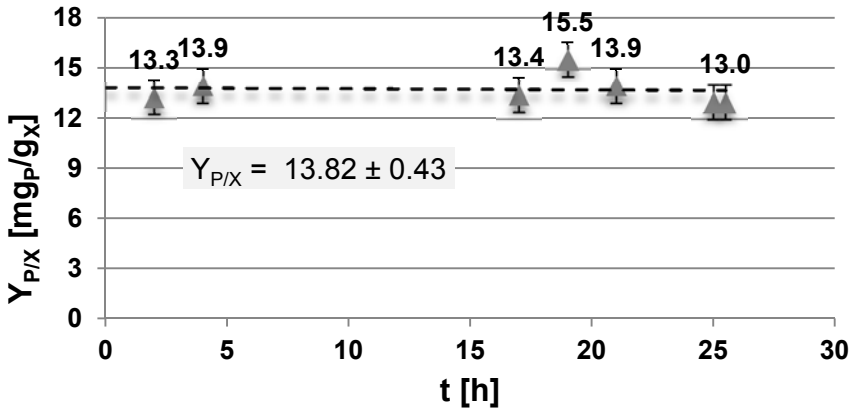

Figure 3. Profile of EG-CelA yield on biomass during the entire course of the fed-batch cultivation

Table 1. Final titer of recombinant enzyme (referred as product *P*) ($\boldsymbol{T_P}$), final product yield on microbial mass ($\boldsymbol{Y_{P/X}}$), product yield on glucose ($\boldsymbol{Y_{P/G}}$), final volumetric productivity ($\boldsymbol{Pr_V}$) values for Y306 [Pir4-CelA] tested in fed-batch reactor at constant specific μ.

***S. cerevisiae* Y306 [Pir4-CelA] - fed-batch cultivation - end fed-batch**	
$\boldsymbol{T_P}$ $[\boldsymbol{g_P L^{-1}}]$	1.24
$\boldsymbol{Y_{P/X}}$ $[\boldsymbol{mg_P g_X^{-1}}]$	13.0
$\boldsymbol{Y_{P/G}}$ $[\boldsymbol{mg_P g_G^{-1}}]$	6.6
$\boldsymbol{Pr_V}$ $[\boldsymbol{mg_P L^{-1} h^{-1}}]$	48.6

Viable count on agar plate performed both on non selective and selective medium highlighted a constant plasmid loss over time, amounting to an average of 15-20 % of the entire microbial population at the end of fermentation run.

3.2 Modeling of the fed-batch fermenter

A simple mathematical model was developed to described the high cell density fed-batch cultivation of *S. cerevisiae* Y306 [Pir4-CelA] strain on the basis of mass balances of some variables of interest such as microbial mass and growth limiting substrate (glucose).
The following simplified general mass balance equation was employed to describe the time-course of the arbitrary component with concentration *C* in the fermenter:

$$\frac{dC}{dt} = \frac{F_{(t)}}{V_{(t)}}(C_i - C) \mp (\mp r_C) \quad (1)$$

considering that, for a fed-batch fermenter with dilute solutions such as those generally used in bioprocessing, the overall mass balance on the volume of the culture medium is:

$$\frac{dV}{dt} = F_{(t)} \quad (2)$$

Equations (3), (4), (5), (6), (7) and (8) constitute the complete, analytic solution to the constant specific growth rate design problem:

$$X_{(t)}V_{(t)} = X_0 V_0 \exp(\mu_0 t) \quad (3)$$

$$b = \frac{1}{Y_{X/G} G_f} \quad (4)$$

$$F_0 = \mu_0 b X_0 V_0 \quad (5)$$

$$F_{(t)} = F_0 \exp(\mu_0 t) \quad (6)$$

$$\frac{V_{(t)}}{V_0} = 1 - bX_0 + bX_0 \exp(\mu_0 t) \quad (7)$$

$$\frac{X_{(t)}}{X_0} = \frac{\exp(\mu_0 t)}{1 - bX_0 + bX_0 \exp(\mu_0 t)} \quad (8)$$

The design of the fed-batch fermenter by means of the above equations is based on the following assumptions:

- a feed rate $F_{(t)}$ so that $G = G_0 \approx 0$ and $\mu = \mu_0 = 0.16\ h^{-1}$ throughout the entire course of fermentation;
- the validity of the pseudo steady-state hypothesis (PSSH) $dG/dt \approx 0$;
- a single black box stoichiometry with constant $Y_{X/G}$.

The numerical values of the model parameters, obtained by fitting the experimental data by means of the equation (8), for the modelling of the producer Y306 strain cultured in fed-batch reactor at constant specific growth rate (μ_0) have been listed in Table 2.

Table 2. Parameter values for modeling fed-batch cultivation of Y306 [Pir4-CelA] at constant μ.

***S. cerevisiae* Y306 [Pir4-CelA] - fed-batch cultivation - mathematical model parameters**	
$\boldsymbol{Y_{X/G}}$ $[\boldsymbol{g_X g_G^{-1}}]$	0.479
$\boldsymbol{b}$ $[\boldsymbol{L g_G^{-1}}] Eq.(4)$	0.140
SSE[1]	0.03938
R-square	0.9936
RMSE[2]	0.06615

[1] **S**um **S**quared **E**rror (**SSE**); [2]**R**oot **M**ean **S**quare **E**rror (**RMSE**)

4. Conclusions

The performance in the bioreactor exhibited by the *S. cerevisiae* Y306 producer strain highlighted that, the biomass profile followed the set up path and that the recombinant enzyme was efficiently secreted into the culture medium along the entire time course of growth. Indeed EG-CelA was produced by the microbial host as a growth linked product. The simple unstructured, non segregated mathematical model employed to fit the experimental values of the microbial mass concentration further evidenced that in, the HCDC system developed, the microbial mass grew following almost perfectly the theoretical biomass profile.

To our knowledge the upper limit for the secretion capacity of extracellular recombinant cellulases has been fixed, recently, by Ilmén et al., (2011). In their experimental study they have been able to obtain, in HCDC system, a titer of secreted cellobiohydrolase (CHB) equal to 1.0 g L^{-1} exceeding any previous reports on cellulase production in *S. cerevisiae*. Regarding this work, employing as production platform the Y306 yeast cell factory, in a high cell density fermentation carried out with a constant exponential feeding strategy, a relevant final titer of 1.2 g L^{-1} of secreted endoglucanase (EG-CelA) was reached.

5. Acknowledgments

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6. List of symbols

μ specific dry microbial mass production rate/specific growth rate $[T^{-1}]$
μ_0 specific feed rate for the constant specific growth rate phase $[T^{-1}]$
F_0 initial volumetric feed rate $[L^3 T^{-1}]$

$F_{(t)}$ actual volumetric feed rate $[L^3T^{-1}]$
$(\mp r_C)$ volumetric rate of mass consumption/production $[ML^{-3}T^{-1}]$
C_0 initial arbitrary component concentration in the culture vessel $[ML^{-3}]$
$C_{(t)}$ actual arbitrary component concentration in the culture vessel $[ML^{-3}]$
G_0 limiting-glucose concentration in the culture vessel $[ML^{-3}]$
G_f limiting-substrate concentration in the feeding stream $[ML^{-3}]$
V_0 culture medium volume at the end of the batch phase $[L^3]$
$V_{(t)}$ actual culture medium volume $[L^3]$
X_0 dry microbial mass concentration in the culture vessel at the end of the batch phase $[ML^{-3}]$
$X_{(t)}$ actual dry microbial mass concentration in the culture vessel $[ML^{-3}]$
$Y_{X/G}$ microbial mass yield on the limiting substrate (glucose)
$Y_{P/X}$ recombinant enzyme yield on the microbial mass

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