Biochemical and Biophysical Research Communications 414 (2011) 604–611

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Effect of auxotrophies on yeast performance in aerated fed-batch reactor

Carmine Landi [a], Lucia Paciello [a], Elisabetta de Alteriis [b], Luca Brambilla [c], Palma Parascandola [a,*]

[a] Dept. Ingegneria Industriale, Università di Salerno, Via Ponte Don Melillo, 84084 Fisciano, Salerno, Italy
[b] Dept. Biologia Strutturale e Funzionale, Università degli Studi di Napoli "Federico II", Via Cinthia, 80100 Napoli, Italy
[c] Dept. Biotecnologie e Bioscienze, Università Milano-Bicocca, Piazza della Scienza, 20126 Milano, Italy

ARTICLE INFO

*Article history:*
Received 22 September 2011
Available online 2 October 2011

*Keywords:*
Auxotrophic strains
Yeast
Fed-batch reactor
Modeling
Maintenance

ABSTRACT

A systematic investigation on the effects of auxotrophies on the performance of yeast in aerated fed-batch reactor was carried out. Six isogenic strains from the CEN.PK family of *Saccharomyces cerevisiae*, one prototroph and five auxotrophs, were grown in aerated fed-batch reactor using the same operative conditions and a proper nutritional supplementation. The performance of the strains, in terms of final biomass decreased with increasing the number of auxotrophies. Auxotrophy for leucine exerted a profound negative effect on the performance of the strains. Accumulation of reactive oxygen species (ROS) in the cells of the strain carrying four auxotrophies and its significant viability loss, were indicative of an oxidative stress response induced by exposure of cells to the environmental conditions. The mathematical model was fundamental to highlight how the carbon flux, depending on the number and type of auxotrophies, was diverted towards the production of increasingly large quantities of energy for maintenance.



## 1. Introduction

Yeasts are known as prototrophic microorganisms, however, a great number of auxotrophic strains have been developed and extensively used [1]. Auxotrophic strains of the yeast *Saccharomyces cerevisiae* are important systems for genetic experiments and are commonly used as hosts for heterologous protein production because they ensure the maintenance of plasmids with selectable markers [2–4]. The importance of the production of heterologous proteins with yeast is confirmed by the presence on the market of recombinant products that are used in many areas of human activity from that of food (α-galactosidase, glucoamylases, chymosin) [5] to that of medical therapy (hormones, vaccines, etc.) [6].

The cultural system mainly employed in the production of recombinant proteins with glucose-sensitive yeasts [7] is the aerated fed-batch reactor. Notwithstanding the extensive use of auxotrophic yeast strains in recombinant protein production, so far little attention has been paid to problems that arise from their cultivation in aerated fed-batch cultures.

Recently, we have described the performance in aerated fed-batch reactor of an auxotrophic commonly used yeast strain, *S. cerevisiae* BY4741 (*MATa*, *ura3Δ0*, *leu2Δ0*, *met15Δ0*, *his 3Δ1*) which was engineered for the expression and secretion of human interleukin-1β [8]. The recombinant BY4741 did not achieve a high-cell density even in the presence of a defined mineral medium containing a proper auxotrophy-amino acid supplementation [9] because it ceased to grow early unlike the parental prototrophic S288C strain, which proliferated strongly and for a long time under the same culture conditions.

In the present work, six isogenic haploid strains belonging to the CEN.PK family, one prototroph and five auxotrophs, have been considered to make a systematic investigation on the effect of auxotrophies on their growth in aerated fed-batch reactor, starting from the hypothesis of a relationship between the BY4741 unsatisfactory performance and its high number of auxotrophies. This was also done in the perspective to select the auxotrophic host that would provide the best performance in the reactor used for production. The strains belonging to the CEN.PK family are considered as reference strains in both basic and applied research on yeast [10]. Four of the strains were characterized by an increasing (up to four) number of auxotrophies, two of them differed only for the type of auxotrophy (uracil or leucine). In the aerated fed-batch reactor glucose was used as limiting substrate, in the presence of proper nutritional supplementation [11]. The feeding strategy consisted in an exponentially increasing feed covering the entire run so yeast strains grew with a constant specific growth rate. The auxotrophic yeast strains were compared on the basis of total biomass, biomass yield on sugar and volumetric biomass productivity, the most important quantitative design parameters considered in the industrial cultivation of *S. cerevisiae* [12].

* Corresponding author. Fax: +39 89 964057.
*E-mail address:* pparascandola@unisa.it (P. Parascandola).

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doi:10.1016/j.bbrc.2011.09.129

**Nomenclature**

| | |
|---|---|
| $E$ | ethanol concentration at time $t$ (g $l^{-1}$) |
| $E_0$ | ethanol concentration at the end of batch phase/beginning of fed-batch phase (g $l^{-1}$) |
| $F$ | glucose flow rate at time $t$ (l $h^{-1}$) |
| $F_0$ | glucose flow rate at time $t_0$ (l $h^{-1}$) |
| $G$ | residual glucose concentration at time $t$ (g $l^{-1}$) |
| $G_0$ | initial residual glucose concentration (g $l^{-1}$) |
| $G_R$ | glucose concentration in the reservoir (g $l^{-1}$) |
| $k_d$ | specific death rate ($h^{-1}$) |
| $(K_s)_E$ | ethanol saturation constant (g $l^{-1}$) |
| $\Gamma_i$ | decrease of the specific growth rate in the $i$th interval ($h^{-1}$) |
| $\Gamma_1$ | decrease of the specific growth rate for $0 \leqslant < t_1$ ($h^1$) |
| $\Gamma_2$ | decrease of the specific growth rate for $t \geqslant t_2$ ($h^{-1}$) |
| $\mu_{max}$ | maximum specific growth rate on glucose ($h^{-1}$) |
| $\mu_{Emax}$ | maximum specific growth rate on ethanol ($h^{-1}$) |
| $\mu_{EJ}$ | specific growth rate on ethanol in the $j$th interval ($h^{-1}$) |
| $\mu_{E1}$ | specific growth rate on ethanol for $0 \leqslant t < t_0$ ($h^{-1}$) |
| $\mu_{E2}$ | specific growth rate on ethanol for $t \geqslant t_0$ ($h^{-1}$) |
| $\mu_G$ | specific growth rate on glucose ($h^{-1}$) |
| $(q_{Ej})s$ | specific consumption rate of ethanol as substrate in the $j$th interval ($h^{-1}$) |
| $(q_{E1})s$ | specific consumption rate of ethanol as substrate for $0 \leqslant t < t_0$ ($h^{-1}$) |
| $(q_{E2})s$ | specific consumption rate of ethanol as substrate for $t \geqslant t_0$ ($h^{-1}$) |
| $(q_{Ek})p$ | specific production rate of ethanol, in the $k$th interval ($h^{-1}$) |
| $(q_{E1})p$ | specific production rate of ethanol between $0 \leqslant t < t_2$ ($h^{-1}$) |
| $(q_{E2})p$ | specific production rate of ethanol for $t \leqslant t_2$ ($h^{-1}$) |
| $q_{Gj}$ | specific glucose consumption rate for growth in the $j$th interval ($h^{-1}$) |
| $q_{G1}$ | specific glucose consumption rate for growth $0 \leqslant t < t_0$ ($h^{-1}$) |
| $q_{G2}$ | specific glucose consumption rate for growth for $t \geqslant t_0$ ($h^{-1}$) |
| $q_{ml}$ | specific glucose consumption rate for maintenance in the $l$th interval ($h^{-1}$) |
| $q_{m1}$ | specific glucose consumption rate for maintenance when $0 \leqslant t < t_1$ ($h^{-1}$) |
| $q_{m2}$ | specific glucose consumption rate for maintenance when $t^1 \leqslant t < t^3$ ($h^{-1}$) |
| $q_{m2}$ | specific glucose consumption rate for maintenance for $t \geqslant t_3$ ($h^{-1}$) |
| $q_s$ | specific consumption rate of an arbitrary substrate S ($h^{-1}$) |
| $r_y$ | volumetric reaction rate for production or consumption of component $y$ (g $l^{-1}$ $h^{-1}$) |
| $t$ | arbitrary time of run (h) |
| $t_0$ | time of fed-batch run corresponding to the complete ethanol consumption (h) |
| $t_1$ | time of fed-batch run at which the theoretical specific growth rate diminishes (h) |
| $t_2$ | time of fed-batch run when ethanol begins to be accumulated (h) |
| $t_3$ | time of fed-batch run when glucose begins to be accumulated (h) |
| $V$ | broth culture volume (l) |
| $X$ | biomass concentration at time $t$ (g $l^{-1}$) |
| $X_V$ | viable biomass concentration at time $t$ (g $l^{-1}$) |
| $X_{V0}$ | initial viable biomass concentration (g $l^{-1}$) |
| $y_i$ | concentration of the arbitrary component $y$ in the inlet (g $l^{-1}$) |
| $Y_{X/G}$ | biomass yield on glucose as substrate |
| $(Y_{X/E})_s$ | biomass yield on ethanol as substrate |

Aeration which is essential to promote respiratory metabolism in fed-batch systems, may contribute to cause oxidative stress through the action of the so-called reactive oxygen species (ROS) [13]. In this work, the presence of ROS was detected on cells from two strains (one bearing a single auxotrophy and the other one four auxotrophies), collected during the fed-batch runs.

In parallel, a mathematical model was developed to describe the growth of CEN.PK strains in aerated fed-batch reactor. Modeling revealed to be an invaluable powerful tool to shed light on an emergent property of auxotrophic yeast strains when they grow in aerated fed-batch reactor.

## 2. Materials and methods

### 2.1. Strains

The strains used in this work belong to the CEN.PK family and were purchased by the laboratory of Biotechnology, University of Milano–Bicocca, except for *S. cerevisiae* CEN.PK2-1C bought at the EUROSCARF (www.uni-frankfurt.de/fb15/mikro/euroscarf).

The strains (with their genotypes) and the abbreviations used in this work are the following: CEN.PK113-5D (*MATa* ***ura3-52*** *HIS3 LEU2 TRP1 MAL2-8$^c$ SUC2), ura$^-$*;CEN.PK111-32D (*MATa URA3 HIS3* ***leu2-3,112*** *TRP1 MAL2-8$^c$ SUC2), leu$^-$*; CEN.PK102-3A (*MATa* ***ura3-52*** *HIS3* ***leu2-3,112*** *TRP1 MAL2-8$^c$ SUC2), ura$^-$ leu$^-$*; CEN.PK102-5B (*MATa* ***ura3-52 his3-Δ1 leu2-3,112*** *TRP1 MAL2-8$^c$ SUC2), ura$^-$his$^-$ leu$^-$*; CEN.PK2-1C (*MATa* ***ura3-52 his3-Δ1 leu2-3,112 trp1-289*** *MAL2-8$^c$ SUC2), ura$^-$ his$^-$ leu$^-$ trp$^-$*).

### 2.2. Fed-batch cultures

Fed-batch cultures were performed in a 2.5 l working volume of a stirred fermenter, Bioflo 110 (New Brunswick Scientific). For all the strains examined, the bioreactor initially contained 1 l of a medium prepared according to Verduyn et al. [14] and containing vitamins and trace elements. The medium was supplemented with auxotrophy-complementing amino acid source according to [9]. Uracil was directly added to 1 l batch volume, in an amount (1.65 g) capable to widely cover its request during fed-batch phase. The initial glucose concentration was 2% w/v. The bioreactor, so prepared, was inoculated with an adequate aliquot of an exponential pre-culture prepared in the same conditions above described except for uracil (15 mg $l^{-1}$) to give an initial $O.D._{590}$ of 0.04.

Fed-batch cultures was started when glucose in the batch was exhausted after 15 h, and before the ethanol produced by fermentation was fully breathed. Than, an exponentially increasing feed was applied to allow the biomass to proliferate with a constant specific growth rate.

The feeding solutions contained glucose (50% w/v), salts, trace elements, glutamic acid, vitamins, and the auxotrophy-complementing amino acids according to Paciello et al. [11]. The overall concentration of each auxotrophy-complementing amino acid was calculated according to Paciello et al. [11], taking into account the value of biomass yield for the given amino acid in aerobic conditions and considering an excess factor of *ca.* 25%.

Oxygen was supplied to the fed-batch reactor by air sparging. The cascade system acted with the agitation speed automatically

increasing or decreasing in the range 50–900 rpm, until the DOT set-point (30% air saturation) was reached. Air enrichment with oxygen was automatically accomplished when needed. The culture pH was maintained at 5.00 by automatic addition of 2 N KOH during batch phase, whereas ammonium supply and control of pH were ensured by automatic addition of 10% v/v $NH_4OH$ during exponential phase. The foam level in the bioreactor was controlled by the automatic addition of the antifoam B (Sigma Aldrich) (dil. 1:10).

### 2.3. Biomass and cell viability determinations

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 2.30 $O.D._{590}$ per mg $ml^{-1}$.

Cell viability during fed-batch runs was determined by viable count (in triplicate) on YPD agar plates incubated at 30 °C for 48 h. $k_d$ (the specific death rate constant) was evaluated as a first order kinetic rate constant by plotting the ratio CFU $ml^{-1}/O.D._{590}$ vs. time, where CFU corresponded to the colony forming units on the plates originated by viable cells, and $O.D._{590}$ corresponded to the total amount of cells in the medium.

### 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 analyzed to determine residual glucose and ethanol concentrations according to Paciello et al. [15].

The presence of reactive oxygen species (ROS) was detected with dihydrorhodamine 123 (DHR) (Sigma Aldrich) as follows. Cell samples were taken at defined time intervals during the run and diluted up to $O.D._{590}$ = 1.00, then DHR was added at 15 μg per ml of cell culture from a 1 mg $ml^{-1}$ stock solution in ethanol and samples were kept for 2 h at 30 °C. Then cells were washed twice in PBS and viewed with an Olympus BX61 epifluorescence microscope equipped with a rhodamine optical filter. The number of cells analyzed for each sample was set to 300.

### 2.5. Development of the mathematical model and exponential feeding profile

The mathematical model to describe the fed-batch cultures was developed on the basis of mass balances equations of variables of interest: biomass, growth limiting substrate (glucose) and ethanol. Total mass balance on the reaction volume was considered:

**Table 1**
Balance equations and kinetic models for the variables of interest.

| Variable | Balance equation | Kinetic model |
|---|---|---|
| Biomass | $\begin{cases} \frac{dX_v}{dt} = -\frac{F}{V}X_v + (\mu_G - \Gamma_i)\cdot X_v + \mu_{Ej}\cdot X_v - k_d \cdot X_v \\ t = 0 \quad X_v = X_{v0} \end{cases}$<br>$i = 1,2$<br>$j = 1,2$ | $\mu_G$ = constant $\forall t$<br>$k_d$ = constant $\forall t$<br>$\Gamma_1$ constant $0 \leqslant t < t_1$<br>$\Gamma_2$ constant $t \geqslant t_1$<br>$\mu_{E1} = \mu_{Emax}\cdot\frac{E}{(K_s)_E+E} \quad 0 \leqslant t < t_0$<br>$\mu_{E2} = 0 \qquad t \geqslant t_0$ |
| Ethanol | $\begin{cases} \frac{dE}{dt} = -\frac{F}{V}E - (q_{Ej})_s \cdot X_v + (q_{Ek})_p \cdot X_v \\ t = 0 \quad E = E_0 \end{cases}$<br>$i = 1,2$<br>$k = 1,2$ | $(q_{E1})_s = \mu_{E1}/(Y_{X/E})_s 0 \leq t < t_0$<br>$(q_{E2})_s = \mu_{E2}/(Y_{X/E})_s t \geq t_0$<br>$(q_{E1})_p = 0\ 0 \leq t < t_2$<br>$(q_{E2})_p$ = constant $0 \leq t > t_2$ |
| Residual glucose | $\begin{cases} \frac{dG}{dt} = \frac{F}{V}(G_R - G) - q_{Gj}\cdot X_v - q_{ml}\cdot X_v \\ t = 0 \quad G = 0 \end{cases}$<br>$j = 1,2$<br>$l = 1,2,3$ | $q_{G1} = f(t) 0 \leq t < t_0$<br>$q_{G2}$ = constant $t \geq t_0$<br>$q_{m1} = 0\ 0 \leq t < t_1$<br>$q_{m2} = f(t) t_1 \leq t < t_3$<br>$q_{m3}$ = constant $t \geq t_3$ |

**Table 2**
Parameter values for modeling of fed-batch reactors with *S. cerevisiae* CEN.PK strains.

| Parameters | CEN.PK prototroph | CEN.PK ***ura***$^-$ | CEN.PK ***leu***$^-$ | CEN.PK ***ura***$^-$***leu***$^-$***his***$^-$***trp***$^-$ |
|---|---|---|---|---|
| $\mu$ ($h^{-1}$) | 0.16 | 0.16 | 0.16 | 0.16 |
| $\Gamma_1$ ($h^{-1}$) | 0 | 0 | 0 | 0.06 |
| $\Gamma_2$ ($h^{-1}$) | 0.027 | 0.04 | 0.093 | 0.09 |
| $\mu_{Emax}$ ($h^{-1}$) | 0.085 | 0.085 | 0.085 | 0.085 |
| $(K_s)_E$ ($g\,l^{-1}$) | 0.10 | 0.10 | 0.10 | 0.10 |
| $k_d$ ($h^{-1}$) | 0 | 0 | 0.017 | 0.032 |
| $(Y_{X/E})_S$) | 0.68 | 0.68 | 0.68 | 0.68 |
| $q_{G1}$ ($h^{-1}$) | $0.35e^{(-0.08t)}$ | $0.28e^{(-0.079t)}$ | $0.35e^{(-0.062t)}$ | 0.35 |
| $q_{G2}$ ($h^{-1}$) | 0.22 | 0.18 | 0.24 | 0.35 |
| $(q_{E2})_P$ ($h^{-1}$) | – | 0.062 | 0.23 | 0.54 |
| $q_{m1}$ ($h^{-1}$) | 0 | 0 | 0 | 0 – 0.1 |
| $q_{m2}$ ($h^{-1}$) | $10^{-4}(t-t_1)^2$+0.0061$(t-t_1)$ | $2.10^{-4}(t-t_1)^2$+0.0077$(t-t_1)$ | 0.0034$(t-t_1)^2$+0.0138$(t-t_1)$ | 0.0061$(t-t_1)^2$0.039$(t-t_1)+0.1$ |
| $q_{m3}$ ($h^{-1}$) | – | – | 0.95 | 1.3 |
| $t_1$ (h) | 5.7 | 5.7 | 6.5 | 5.0 |
| $t_1$ (h) | 7.5 | 7.5 | 7.5 | 7.5 |
| $t_2$ (h) | – | 20 | 17.5 | 18.5 |
| $t_3$ (h) | – | – | 22.3 | 18.5 |

$$\frac{dV}{dt} = F.$$

To describe the change with time of the arbitrary component with concentration $y$ in the reactor, the following simplified general mass balance equation was used:

$$\frac{dy}{dt} = \frac{F}{V}(y_i - y) \pm r_y. \quad (1)$$

The differential equations obtained for fed-batch culture were solved only numerically by the Eulero's method [16].

The model (Table 1), considers that the glucose flux is split into two metabolic fluxes, i.e. $q_G$ and $q_m$witch are the specific glucose consumption rates for growth and maintenance, respectively. Ethanol was employed either as substrate (fully oxidative metabolism) or product (fermentation).

Mathematical model parameters (Table 2), such as specific growth rate ($\mu$), specific death rate ($k_d$) and yield coefficients ($Y$), were determined from experimental data. Biomass yield on ethanol ($Y_{X/E}$) was obtained from the literature [17]. All the other parameters were obtained by fitting.

Only the viable biomass was considered in the mathematical model. Its density $X_v$ (g $l^{-1}$), was calculated by the Eq. (2) according to [18]

$$X_{V_{(t)}} = X_V \cdot \left(\text{CFU ml}^{-1}\right)_t / \left(\text{CFU ml}^{-1}\right)_{t=0}. \quad (2)$$

The exponential profile of flow rate $F(t)$:

$$F_{(t)} = F_0 e^{\mu_G t} = \frac{\mu_G (XV)_0 e^{\mu_G t}}{G \cdot Y_{X/G}} \quad (3)$$

was obtained from the mass balance on limiting substrate, throughout the assumption of a quasi-steady-state on limiting glucose [16].

## 3. Results

### 3.1. Effect of the type of auxotrophy on yeast performance in aerated fed-batch reactor

To assess the influence of the auxotrophic genotype of CEN.PK yeast strains on their performance in fed-batch reactor, two strains bearing a single auxotrophy,*S. cerevisiae* CENPK *ura*$^-$ and *S. cerevisiae* CEN.PK *leu*$^-$, respectively, have been considered and cultivated at a $\mu$ value of 0.16 $h^{-1}$. This value was selected considering that $\mu_{max}$ of all the auxotrophic strains considered was the same, i.e. 0.50 ± 0.02 $h^{-1}$ (data not shown) and $\mu_{critical}$(when ethanol begins to be produced with low biomass yield) assessable around 60% of the $\mu_{max}$ [19].

Total biomass produced by CEN.PK *ura*$^-$ (189 g) (Fig. 1A) was approximately threefold higher than that achieved by CEN.PK *leu*$^-$(Fig. 1A) and lower than that of the prototrophic strain (245 g) (data not shown). As regards glucose supplied, it was completely consumed in CEN.PK *ura*$^-$ since residual glucose (Fig. 1B) was zero, whereas it accumulated in CEN.PK *leu*$^-$, towards the end of the run. At that time, both strains produced ethanol (Fig. 1C), indicating that metabolism was not fully oxidative. After feeding interruption (25 h), both glucose and ethanol accumulated were consumed (Fig. 1B and C).

### 3.2. The performance of S. cerevisiae CEN.PK strains in aerated fed-batch reactor is inversely related to the number of auxotrophies

To evaluate the effect of the number of auxotrophies on the performance of *S. cerevisiae* CEN.PK strains in aerated fed-batch reactor, three strains, characterized by an increasing number of auxotrophies, CEN.PK *ura*$^-$*leu*$^-$, CEN.PK *ura*$^-$*leu*$^-$*his*$^-$, and CEN.PK *ura*$^-$*leu*$^-$*his*$^-$*trp*$^-$, were taken into consideration.

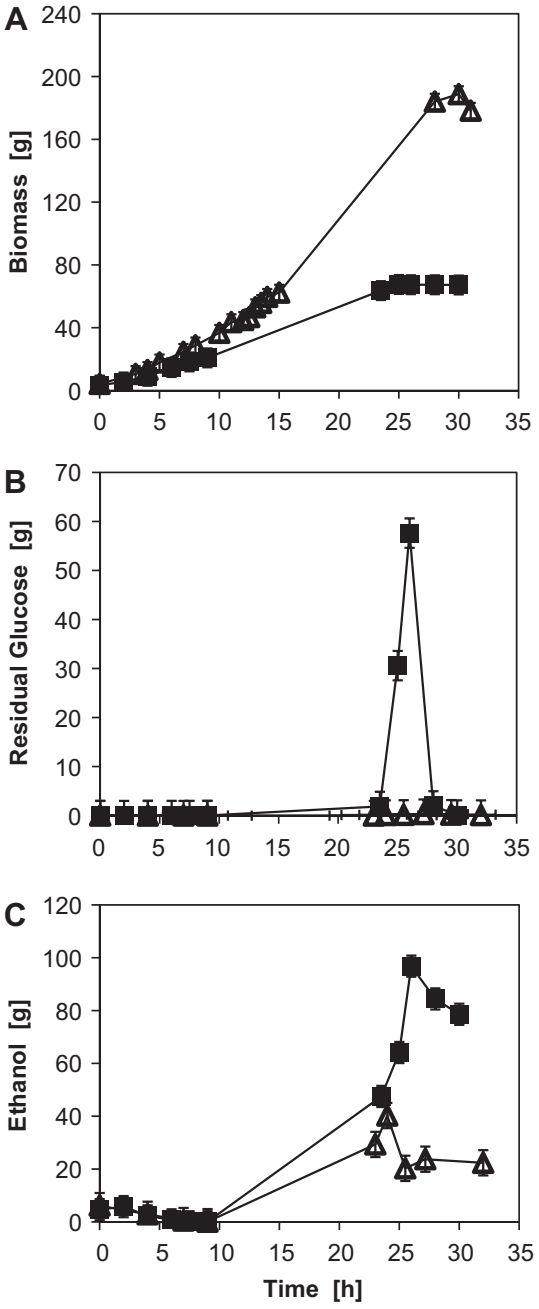


**Fig. 1.** Growth in aerated fed-batch reactor of one-auxotrophy bearing *S. cerevisiae* CEN.PK strains. Auxotroph for uracil (empty trangles) auxotroph for leucine (full square); (A) biomass profile over the entire time-course of exponentially increasing feed; (B) time-course of residual glucose; and (C) time-course of ethanol consumed (first 7 h of feeding) and that produced during the run.

In terms of total biomass obtained, the yeast strain performance decreased with the number of auxotrophies (Fig. 2A), even though the difference among the three strains considered was not as great as that observed between CEN.PK *ura*$^-$ and *leu*$^-$ (Fig. 1A). Glucose supplied to the reactor was completely consumed during almost the whole runs except for CEN.PK *ura*$^-$*leu*$^-$*his*$^-$*trp*$^-$ strain (Fig. 2B) which accumulated ethanol in the medium at the end of the feeding phase.

Towards the end of the run, all the strains produced ethanol in a significant amount (Fig. 2C). Ethanol was produced notwithstand-

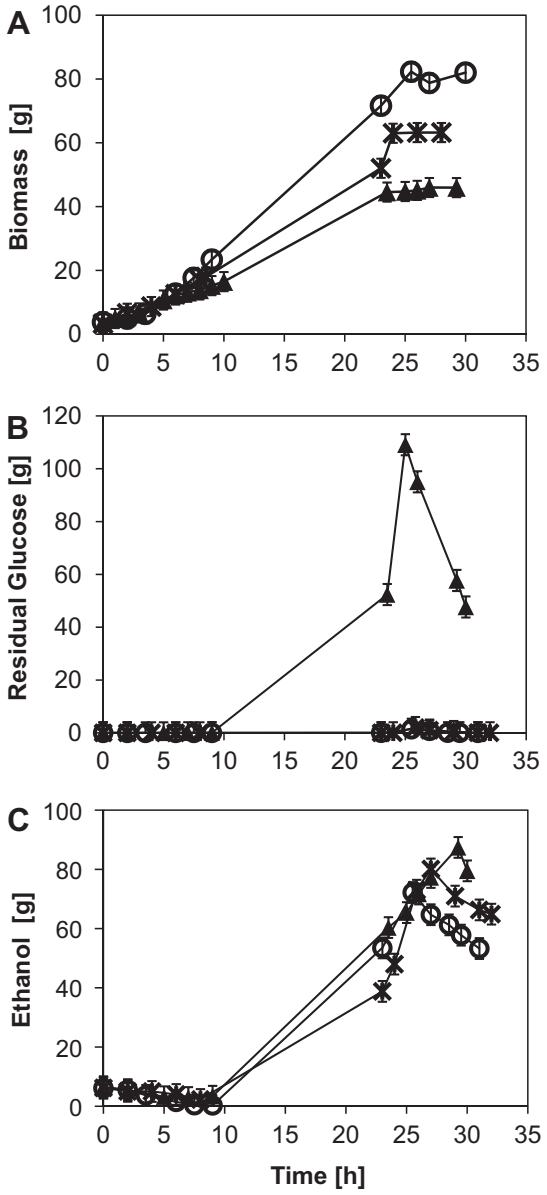


**Fig. 2.** Growth in aerated fed-batch reactor of *S. cerevisiae* CEN.PK strains carrying more than one auxotrophy. CEN.PK *ura*$^-$*leu*$^-$ strain (empty cyrcles), CEN.PK *ura*$^-$*leu*$^-$*his*$^-$ strain (stars), and CEN.PK *ura*$^-$*leu*$^-$*his*$^-$*trp*$^-$ strain (full triangles). (A) Biomass profiles over the entire time-course of exponentially increasing feed; (B) time-course of residual glucose; and (C) time-course of ethanol consumed (during first 7 h of feeding) and that produced in the run.

ing the oxygen demand by yeast cells was high, as evidenced by the continuous opening of the valve for oxygen enrichment of air, necessary to maintain the set point of 30% air saturation (data not shown).

The unsatisfactory performance exhibited by the yeast strains bearing the auxotrophy for leucine or more than two auxotrophies might be related to their incapability to cope with the stressful environmental conditions determined by the prolonged and vigorous aeration of the fed-batch reactor. Therefore, a test was performed to highlight the occurrence of oxidative stress. To this end, cells from two of the strains examined, namely CEN.PK *ura*$^-$ and CEN.PK *ura*$^-$*leu*$^-$*his*$^-$*trp*$^-$, were collected at different time intervals during the run, and analyzed to point out the presence of intracellular reactive oxygen species (ROS). ROS were more evident in the cells of the strain carrying four auxotrophies than in the *ura*$^-$cells and increased during the run (Fig. 3A). Since ROS accumulation might be accompanied by loss in cell viability, this latter was estimated from the specific death rate of yeast cells during fed-batch operation. The viability test was performed with all the yeast strains including the prototrophic one. From Fig. 3B, it was possible to infer that the prototrophic strain and the strain carrying the single auxotrophy for uracil, were viable over the entire time-course of the fed-batch run ($K_d = 0$), whereas the strain carrying the single auxotrophy for leucine exhibited a significantly high specific death rate ($K_d = 0.018\ h^{-1}$). The viability of the other auxotrophic strains diminished as well if compared to the prototrophic and *ura*$^-$strain. The strain carrying four auxotrophies exhibited the highest $K_d$ value (Fig. 3B).

The average biomass yield on glucose ($Y_{X/G}$), and the volumetric productivity ($P_X$) together with the final total biomass ($X_{tot}$), are reported in Table 3. These parameters were evaluated over the entire feeding phase (25 h). The highest value of each parameter was obtained with the prototrophic strain, and, among the auxotrophic strains, with the strain carrying the single auxotrophy for uracil.

### *3.3. Mathematical model reveals the relation between number of auxotrophies and maintenance*

The mathematical model ensured a good fitting between the experimental data and the simulation curves (data not shown) and was capable to highlight many aspects of fed-batch runs carried out with the CEN.PK strains considered such as:

[i] strain ability to grow (in the first 7 h of the run) in accordance with the glucose feeding profile (Table 1)
[ii] ethanol consumption according to the Monod kinetics (Table 1);
[iii] deviation from the ideal behavior occurring after the first 6–7 h of the run (Tables 1 and 2, see $\Gamma$ parameters).

The deviation from the ideal behavior for all the auxotrophic CEN.PK strains examined, occurred after about 7 h of the feeding phase and consisted in a sudden decrease in the specific growth rate, which remained constant from that time on. The decrease in the $\mu$ value was greater the higher the number of auxotrophies, as evidenced by the different values assumed by the parameter $\Gamma_2$ (Table 2) and depended on the diversion of the specific glucose consumption towards the production of energy for maintenance ($q_m$) (Fig. 4A–D). The decrease of $q_G$ (the specific glucose consumption to produce biomass) in the first hours of feeding for the prototroph (Fig. 4A), the *ura*$^-$ (Fig. 4B) and *leu*$^-$ (Fig. 4C) strains, was ascribable to a larger biomass than expected. Indeed, in addition to glucose, also ethanol produced during batch phase, was used (data not shown). In the case of the strain carrying four auxotrophies, the $q_G$ decrease did not occur because the strain was unable to completely metabolize ethanol (Fig. 4D).

The mathematical model highlighted that the diversion of carbon flux increased dramatically in the strain carrying the auxotrophy for leucine (Fig. 4C) and even more in the strain carrying four auxotrophies (Fig. 4D). In both cases, the auxotrophic strains performance lowered because an increasing amount of glucose was utilized for maintenance instead of being channeled into biosynthetic pathways to be converted into new biomass.

## 4. Discussion

The *S. cerevisiae* strains used in this work belong to the CEN.PK family making part of an interdisciplinary German research project aiming at meeting the needs of physiologists, geneticists, and engineers [10]. CEN.PK strains are considered excellent hosts for

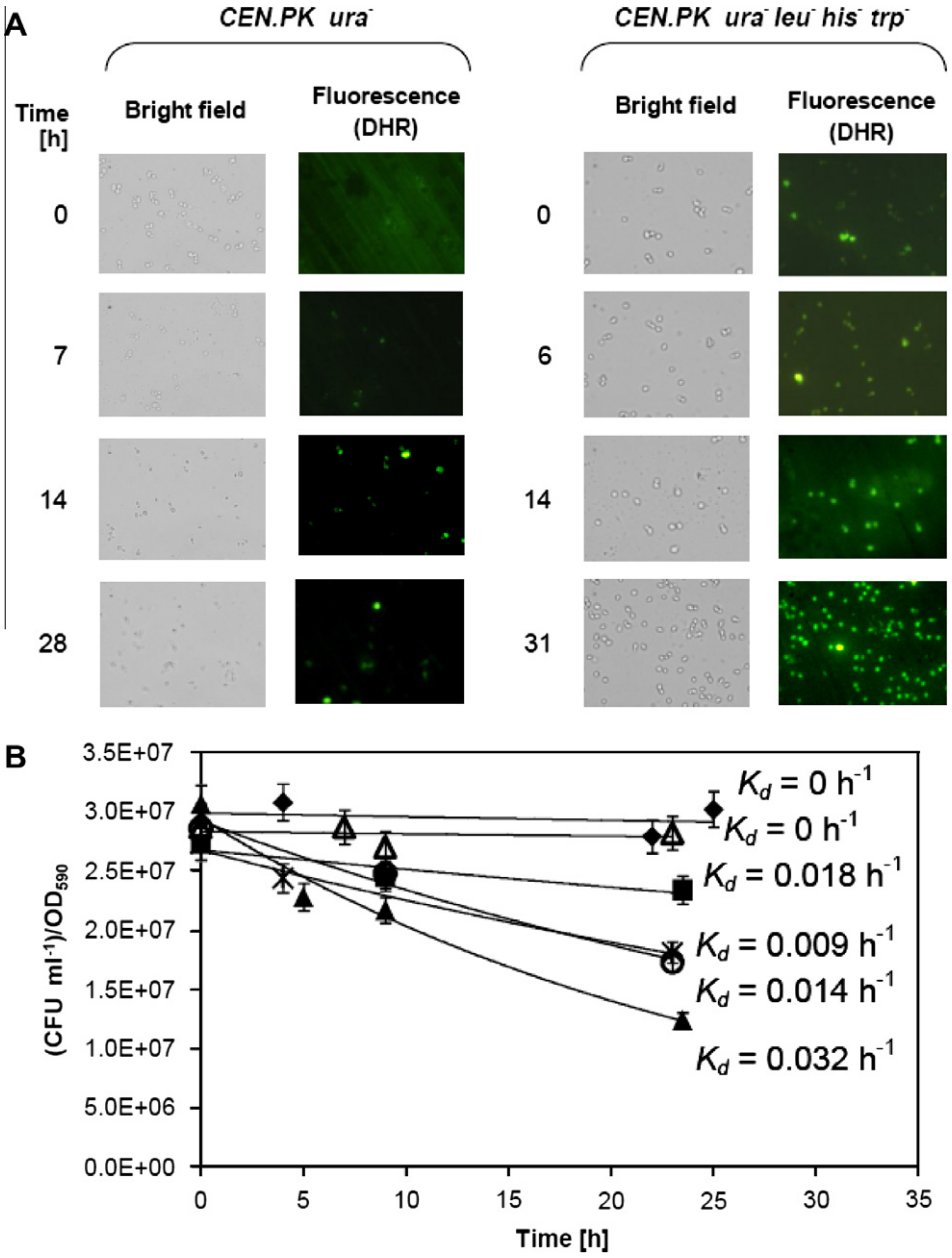


**Fig. 3.** ROS detection by diihydrorodamine fluorescence test and viability loss during aerated fed-batch runs. (A) Optical microscopic observation of cells samples of *S. cerevisiae* CEN.PK *ura*$^-$ strain (on the left), and *S. cerevisiae* CEN.PK *ura*$^-$*leu*$^-$*his*$^-$*trp*$^-$ strain (on the right), collected at different times during fed-batch runs; (B) viability loss of *S. cerevisiae* CEN.PK strains in aerated fed-batch reactor. $K_d$ values were determined from the slope of the curves (CFU ml$^{-1}$/OD$_{590}$) vs. time. CEN.PK prototrophic strain (full rhombus), $R^2$ = 0.085; CEN.PK *ura*$^-$ (empty triangles), $R^2$ = 0.076; CEN.PK *leu*$^-$ (full squares), $R^2$ = 0.983; CEN.PK *ura*$^-$ *leu*$^-$ (empty circles) $R^2$ = 0.993; CEN.PK *ura*$^-$*leu*$^-$*his*$^-$ (stars), $R^2$ = 0.949; CEN.PK *ura*$^-$*leu*$^-$*his*$^-$*trp*$^-$ (full triangles) $R^2$ = 0.969. The standard deviation never exceeded 5%.

**Table 3**
Performance of *S. cerevisiae* CEN.PK strains cultivated in aerated fed-batch reactor.

| | Total biomass $X_{tot}$ (g) | Biomass yield $Y_{X/G}$ | Volumetric productivity $P_X$ (g l$^{-1}$ h$^{-1}$] |
|---|---|---|---|
| CEN.PK prototroph | 245 | 0.601 | 4.60 |
| CEN.PK *ura*$^-$ | 189 | 0.410 | 3.24 |
| CEN.PK *leu*$^-$ | 67.2 | 0.140 | 1.35 |
| CEN.PK *ura*$^-$*leu*$^-$ | 70.0 | 0.146 | 1.39 |
| CEN.PK *ura*$^-$*leu*$^-$*his*$^-$ | 63.2 | 0.131 | 1.56 |
| CEN.PK *ura*$^-$*leu*$^-$*his*$^-$*trp*$^-$ | 46.6 | 0.093 | 0.95 |

heterologous protein production [10]. The CEN.PK strains used in this work present an increasing (up to four) number of auxotrophies. Two of them differ only for the type of auxotrophy (uracil or leucine).

The aerated fed-batch reactor was chosen to cultivate the CEN.PK strains because it better resembled the semi-continuous cultivation systems employed in the industry. This kind of reactor is capable to exert, by means of sugar limitation, the metabolic

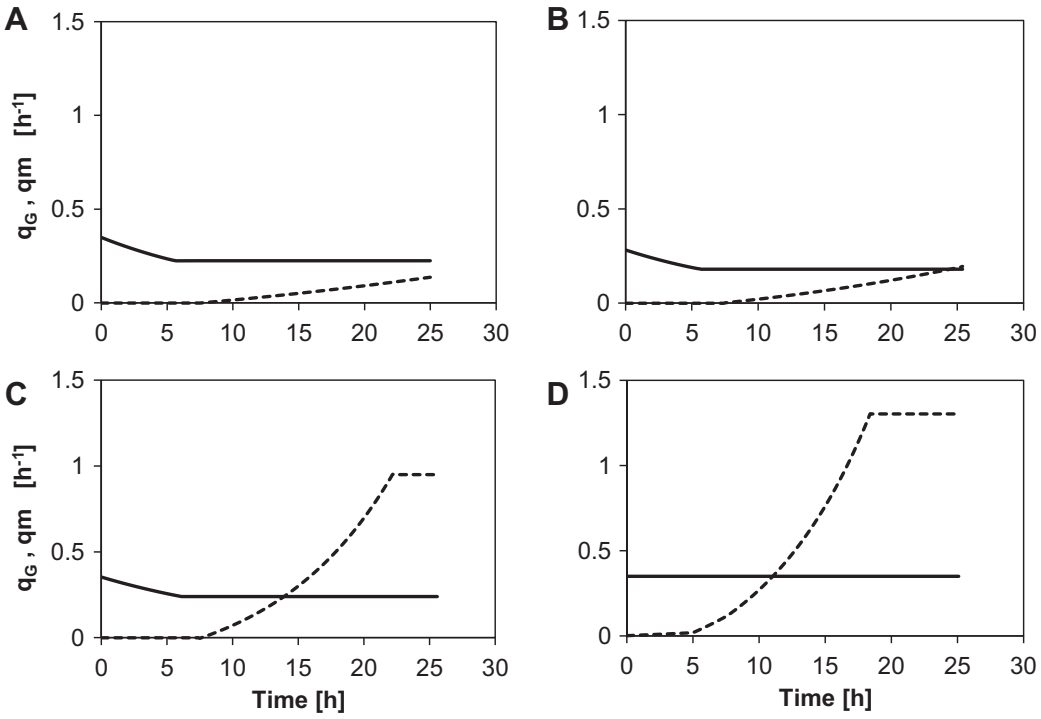


**Fig. 4.** Modeling of the distribution of specific glucose consumption rates. Time-course of specific glucose consumption rate for growth, $q_G$ (continuous line), and for maintenance, $q_m$ (dashed line) in the case of (A) *S. cerevisiae* CEN.PK prototrophic strain, (B) CEN.PK *ura*$^-$, (C) CEN.PK *leu*$^-$ and (D) CEN.PK *ura*$^-$*leu*$^-$*his*$^-$*trp*$^-$.

control on *S. cerevisiae* strains, as generally reported in the case of glucose-sensitive yeasts [19,20]. Sugar limitation avoids over-flow metabolism and favors oxidative metabolism with high biomass yield and recombinant product [7,19].

The results of this work demonstrate that in the aerated fed-batch reactor, the performance of the strains, in terms of final biomass, biomass yield on glucose, volumetric productivity and the capacity of maintaining the $\mu$ value imposed during the run, declines with the increase in the number of auxotrophies.

Also the origin of auxotrophy has a profound effect on the performance of the auxotrophic strains as shown by the strong deleterious effect of the auxotrophy for leucine, in accordance with other observations regarding leucine auxotrophic strains [21,22]. This implies that CEN.PK *ura*$^-$ strain should be preferably selected as auxotrophic host for the expression of a heterologous protein, in the perspective to set-up the production in an aerated fed-batch reactor.

The intracellular accumulation of reactive oxygen species (ROS) in the cells of CEN.PK *ura*$^-$*leu*$^-$*his*$^-$*trp*$^-$ during the run is indicative of an oxidative stress response induced by exposure of the cells to the peculiar conditions of the aerated fed-batch cultivation. Indeed, fed-batch operations are generally carried out under conditions of vigorous and continuous aeration, so that oxygen stress is almost unavoidable. It is known that ROS accumulation is responsible for serious damages to biomolecules and cellular structures [23] and, therefore can be evoked for the loss in cell viability (high $K_d$ value) encountered during the process. Apparently, CENPK *ura*$^-$*leu*$^-$*his*$^-$*trp*$^-$ and the other auxotrophic strains, are unable to face the vigorous aeration applied during the fermentation run to maintain the oxygen concentration at saturating levels.

The specification and quantification of the maintenance coefficient ($q_m$) for glucose permitted to point out that $q_m$ increases over time and its maximum values ($q_{mmax}$) depends on the number and type of auxotrophies, being the lowest value that of the prototrophic strain. Apparently, the auxotrophic strains use most of the available carbon source to satisfy an increasing energy demand for maintenance during the fermentation run, in order to face the environmental stress conditions of the aerated fed-batch cultivation. In this view, the values of $q_{mmax}$ can be considered as a measure of their sensitivity to those conditions.

On the whole, the results obtained in this work show that the behavior of an auxotrophic strain is the result of the interaction between biological (genotype, metabolic properties, etc.) and environmental determinants. Therefore, before setting up heterologous protein production by yeast cells, the auxotrophic host has to be tested in a cultivation system as close as possible to that of production, since the expressed phenotype may vary from system to system.

In conclusion, the experimental and theoretical approach, proposed in this work, has allowed us to elucidate the behavior of a complex environment such as that arising in an aerated fed-batch reactor, and highlight an emergent property of the system itself [24], i.e. the increase of maintenance coefficient in the auxotrophic strains which can be considered as indicative of the strain sensitivity towards the environmental adverse conditions of cultivation. In the next future, the dynamics of metabolic interactions which characterize the system would deserve a deeper insight, through the study of the networks that originate from the interconnections among genes, proteins and metabolites [24].

## Acknowledgment

This work was supported by the University of Salerno funds (ex 60%, 2009) to Palma Parascandola in the framework of the research project *"Proteine eterologhe da lievito: lo stress ambientale e la fisiologia dell'ospite durante la produzione in sistemi fed-batch aerati"*.

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