

Physiological state as transferable operating criterion to improve recombinant protein production in *Pichia pastoris* through oxygen limitation

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Short title: Physiological state control to improve protein specific productivity in *Pichia pastoris* with oxygen limitation.

Abstract

BACKGROUND: The yeast *Pichia pastoris* is widely used as a production platform for secreted recombinant protein. The application of oxygen-limiting conditions leads to an important increase on protein specific productivity driven by the *GAP* promoter.

RESULTS: The physiological and metabolic adaptation of the host to a wide range of oxygen availability has been systematically studied in glucose-limited chemostat cultivations producing an antibody fragment (Fab). A weighty increase of up to 3-fold of the specific Fab production rate (q_{Fab}) **and Fab yield (Y_{PX})** has been achieved for the optimal conditions. Besides the remarkable increase on both Fab yield and productivity, as a consequence of the metabolic shift from respiratory to respiro-fermentative pathways, a decrease on biomass yield and generation of several secreted by-products have been observed.

CONCLUSION: The accurate system characterization achieved throughout the bioprocess specific rates and the monitoring of the cell physiology allowed the determination of the optimal conditions to enhance bioprocess efficiency. This work also presents a versatile approach based on the physiological state of the yeast that can be used to implement the desired oxygen-limiting conditions to fermentations set-ups with different oxygen transfer capacities, alternative operating modes, and even for the production of others proteins of interest.

Introduction

In recent years the recombinant protein industry has been growing rapidly and bringing innovative products to market.^{1,2} In these production processes, genetic engineering, microbial physiology and bioprocess engineering, including up and downstream, must be combined with the objective of increasing the specific production rate of the desired recombinant proteins. Since there is often a lack of knowledge about the production pathway and its dynamic profile in the producing cells, detailed physiological studies are required for optimizing the overall bioprocess.³

Pichia pastoris is one of the most effective and versatile expression systems. This yeast is being widely and successfully used for the production of heterologous proteins.⁴⁻⁶ The combination of traits that makes *P. pastoris* a suitable expression system has been broadly reviewed in the literature.⁷⁻⁹ Although the use of the methanol inducible *AOXI* promoter (P_{AOXI}) is extensively used,¹⁰⁻¹² in the last decade, the glycolytic *GAP* promoter (P_{GAP}) has become an efficient alternative as a strategy to produce heterologous proteins on glucose or glycerol constitutively.¹³⁻¹⁵ The main advantages of P_{GAP} over P_{AOXI} such as lower oxygen requirements and heat production, can be found elsewhere.¹⁶ In the last years several works have been published assessing the performance of the *P. pastoris* P_{GAP} -based expression system for heterologous proteins in fermentation processes with different operational modes.¹⁶⁻¹⁹

The impact of oxygen supply on heterologous production has been studied for different recombinant production hosts as oxygen transfer rate is usually considered one of the most limiting bottlenecks for high cell density cultivations of microorganisms.²⁰ In *Escherichia coli* cultivations, oxygen limitation leads to a stress response and by-product formation including acetate, which inhibits both growth and recombinant protein production.^{21,22} The impact of oxygen limitation was also studied in *Saccharomyces cerevisiae* observing the production of ethanol and glycerol as by-products of the cultivation.²³

In *P. pastoris* cultures expressing a human antigen-binding fragment (Fab), an important increase of the specific production rate (q_p) at low oxygen supply was described in previous publications by our group.²⁴ In this work, three different oxygen-limiting conditions were studied in chemostat cultivations, observing a decrease in biomass production, generation of ethanol as a by-product and a significant increase in the specific production rate q_p . In addition, a primary strategy of fed-batch cultivation under hypoxic conditions was carried out, also showing a significant increase in the volumetric productivity, Q_p . Following inter-disciplinary systems biology studies, including transcriptomic, proteomic and metabolomics analyses, were performed with the same expressing strain under similar hypoxic conditions in order to extend the knowledge of the physiological and metabolic responses of the cells under oxygen-limiting conditions.²⁵⁻²⁷ However, no further

studies were carried out in order to generically identify the optimal culture conditions that lead to maximal productivities and yields for the protein of interest.

In the previously cited works, the different culture conditions, in terms of oxygen availability for the cells, have been indirectly related to the O₂ molar fraction in the inlet gas phase. This approach does not allow a proper comparison of the results among experimental set-up with different oxygen transfer capabilities, *k_{La}*, because this factor is intrinsic for each system and has a key impact in the oxygen transfer rate (OTR).²⁸ Thus, a systematic methodology that permits working with equivalent conditions of oxygen availability to the cells using different bioreactor configuration is needed in order to apply successfully the optimized cultivation strategies determined to different fermentation systems. In a previous work with *E. coli* growing under hypoxic conditions, an innovative indirect reporting parameter for oxygen availability was identified and presented. It was based on the determination of the minimal oxygen supply rate needed in each particular fermentation system for allowing the cell growth with a fully oxidative metabolism, and thus, in which no by-products are generated.²⁹ Hence, as novelty, this approach is based on the physiological behaviour of the culture rather than on cultivation settings itself.

A strain expressing the human 2F5 Fab, which is different than previously cited, 3H6,^{24,26} has been used as model protein. Fabs have a wide range of applicability as therapeutic agents,³⁰ and are complex proteins composed by different domains connected via disulphide bonds.¹⁷ Thus, it becomes a suitable model protein for studying the efficiency of recombinant protein production processes.

In the present work, a wide range of oxygen-limiting conditions has been assessed in *P. pastoris* chemostat cultivations searching for the best conditions to improve the recombinant protein yields and productivities. The determination of the key specific rates of the bioprocess, including a detailed characterization of the by-products generated, was carried out identifying new extracellular metabolites produced respect to the previously reported. In addition, cell viability and reactive oxygen species (ROS) analysis were also performed by flow cytometry in order to monitor the oxygen limitation effect on the physiological state of the cells. Therefore, a transferable methodology based on the control of physiological parameters such as the specific by-products generation rates or respiratory quotient is proposed. It will allow to work under equivalent oxygen-limiting conditions for different cultivation set-ups that differs in their oxygen transfer capabilities, *k_{La}*. Accordingly, this approach can also be used to achieve the desired oxygen-limiting conditions in fermentation processes under different operating modes, continuous or fed-batch, and even for other proteins of interest that could be positively affected by oxygen limiting conditions.

Experimental

Strain and cultivation conditions

The *P. pastoris* strain X-33 $P_{GAP}Z\alpha A$ -Fab2F5 expressing both light and heavy chain genes of the human Fab 2F5 under control of the constitutive *GAP* promoter was used. Using the *S. cerevisiae* α -mating factor signal sequence the Fab is secreted to the medium. The strain construction was described in previous work.¹⁷

The preparation of the inoculum for bioreactor cultures were performed as described by Garcia-Ortega *et al.*¹⁶

Chemostat cultivations were carried out in a 2 L Biostat B Bioreactor (Braun Biotech, Melsungen, Germany) at a working volume of 1 L. Cells were grown under carbon-limiting conditions at a dilution rate (*D*) of 0.10 h⁻¹. Different oxygen molar fractions in the inlet gas of the bioreactor were used in order to apply different oxygen-limiting conditions. The cultivations were performed using the batch and chemostat medium composition detailed elsewhere.³¹ However, the slight differences detailed below were introduced in the used mediums. Glucose concentration was 50 g L⁻¹; Biotin 0.02% (1 mL), PTM1 (1.6 mL) trace salts stock solution (also described in ³¹) and antifoam Glanapon 2000kz (0.2 mL; Bussetti & Co GmbH, Vienna, Austria) were added per litre of chemostat medium.

Culture conditions were monitored and controlled at the following values: temperature, 25 °C; pH, 5.0 with addition of 15% (v/v) ammonium hydroxide; the pressure in the culture vessel was maintained at 1.2 bars using a pressure valve (GO Inc, Spartanburg, SC, USA); stirring rate, during the batch phase it was variable between 600 and 900 rpm in order to keep the pO₂ above 20% of saturation; on the contrary, during the continuous phase the stirring rate was always kept constant at 700 rpm for all the conditions, therefore being independent of the pO₂; the total gas flow was kept constant for all experiments at 0.8 vvm. In order to apply different controlled oxygen-limiting conditions and to keep constant the hydrodynamic behaviour of the system, air was partially replaced with nitrogen in the gas inlet to achieve the desired oxygen supply. Different hypoxic conditions were applied from high to low air ratio set points, using mixtures of the gases by means of thermal mass-flow controllers (TMFC; Bronkhorst Hi-Tech, Ruurlo, The Netherlands). An exhaust gas condenser with cooling water at 4 °C minimized mass losses by water evaporation and other volatile compounds. In all the experiments the continuous cultivation was performed for at least five residence times (τ) in order to assure reaching the steady state of the culture.

Analytical methods

Biomass determination by dry cell weight (DCW)

The *P. pastoris* biomass concentration of each steady state was determined as DCW by using the method described elsewhere.³² Determinations were performed by triplicate and the relative standard deviation (RSD) was about 4%.

Determination of biomass elemental compositions

Biomass samples for the determination of the elemental composition, as well as the ash content, were prepared and analysed as described by Carnicer et al.²⁶

Product quantification

The amount of 2F5 human Fab produced was quantified by ELISA as previously described.³³ Determinations were performed by triplicate and the RSD was about 4%.

Carbon source and by-products quantification

The concentration of the substrates and common by-products obtained such glucose, arabinol, glycerol and ethanol were determined by HPLC as previously described.³⁴ The estimated RSD was below 1% for all the analytes.

The concentration of the novel by-products identified observed at oxygen-limiting conditions, such α -ketoglutarate and succinate, was determined by means of LC-MS. The analysis of filtered supernatants were performed on a Shimadzu Prominence HPLC with a UV/VIS detector coupled to a Mass Spectrometry detector Shimadzu 2010A also coupled to an Electro Spray Ionization (ESI) interface operating at a wavelength of 210 nm. Metabolite compounds were separated on an ICsep ICE COREGEL 87H3 column (Transgenomic Inc., Omaha, NE, USA) using 15 mM formic acid in milliQ water. The analyses were performed at room temperature using a 20 μ l injection volume. The estimated RSD was about 3% for all the analytes.

Off-gas analysis

A quadrupole mass spectrometer (Balzers Quadstar 422, Pfeiffer-Vacuum, Asslar, Germany) was used for on-line exhaust gas analysis. Exhaust gas humidity was reduced by using a condenser (water at 4 °C) and two silica gel columns. The Faraday cup detector was used for its simplicity, stability, and reliability, determining responses of m/z corresponding to the major gas peaks (N₂: 28, O₂: 32, CO₂: 44, Ar: 40). Normalized mass spectrometer signals were used to reduce errors caused by variations of the operating conditions such pressure and temperature, as well as others that can generate some drift and noise on the signals. Multivariate calibration was performed by ordinary least squares (OLS) minimization with suitable standard calibration mixtures according to the components to be analysed and its concentration range.

The total humid off-gas flow rate was not measured directly; it was calculated by inert balance in the reactor. Inlet air composition was obtained from a 12 h measurement average before inoculating. Thus, through O₂ and CO₂ balances, accurate estimation of oxygen uptake rate (*OUR*), carbon dioxide production rate (*CPR*), and respiratory quotient (*RQ*) were carried out.³⁵

Flow cytometry measures and analyses

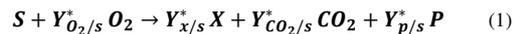
Cell counting, viability and measure of the stress caused by intracellular radical oxygen species (ROS) were determined by means of flow cytometry assays using the Guava EasyCyte™ Mini cytometer (Millipore, Hayward, CA, USA). All samples were always previously briefly sonicated in order to avoid the presence of cell clumps.

Viability assays were performed by means of the propidium iodide (PI) staining procedure as described elsewhere.^{36,37} The accumulation of ROS was also monitored since it has been described as critical factor that induces the mechanisms of apoptotic death of yeasts.³⁸ For ROS determination, intracellular superoxide anions were measured by using dihydroethidium (DHE) and dihydrorhodamine 123 (DHR), as previously described.³⁷

Process parameters determination, consistency check and data reconciliation

Mass balance and stoichiometric equations

The oxidative and oxidoreductive growth can be described on a C-molar basis by a single overall reaction, a so-called Black Box model, which is a simplification of all the biochemical reactions involved:



where *S* denotes one single limiting substrate as the carbon and energy source; O₂, oxygen; *X*, biomass; CO₂, carbon dioxide; *P*, products. *Y_{i/s}^{*}* are stoichiometric coefficients that can also be called overall “i” component-substrate yields.

Specific rates (*q_i*) and yields are parameters of capital importance to compare different culture conditions and allow the identification of changes in the physiological cell state that can impact into productivity and product quality.³⁹ Their calculation is based on the conversion rates (*r_i*) determined in the general mass balance of the cultivation. Specific rates are typically conversions rates related to the biomass concentration (equation 2). Yields are defined as ratios between rates (equation 3) and positive.

$$q_i = \frac{r_i}{X} \quad (2)$$

$$Y_{i/j} = \frac{r_i}{r_j} = \frac{q_i}{q_j} \quad (3)$$

For an ideal stirred tank-reactor, considering conversion rates of biomass formation, substrate uptake and product formation, the following mass balance equations for the continuous operation at steady state can be formulated according to equation 4.

$$\begin{bmatrix} \mu \\ q_S \\ q_P \\ q_{O_2} \\ q_{CO_2} \end{bmatrix} XV = \begin{bmatrix} F_{out} X \\ -F S_0 + F_{out} S \\ F_{out} P \\ OUR V \\ CPR V \end{bmatrix} \quad (4)$$

where μ is the specific growth rate (h^{-1}); q_S , specific substrate uptake rate ($g\ g^{-1}\ h^{-1}$); q_P , specific production rates ($\mu g\ Fab\ g^{-1}\ h^{-1}$ or $g\ g^{-1}\ h^{-1}$); q_{O_2} , specific oxygen uptake rate ($mol\ g^{-1}\ h^{-1}$); q_{CO_2} , specific carbon dioxide production rate ($mol\ g^{-1}\ h^{-1}$); F , substrate feeding rate ($L\ h^{-1}$); F_{out} , outlet flow rate ($L\ h^{-1}$); V , volume of broth in the reactor (L); X , biomass concentration ($g\ L^{-1}$); S , substrate concentration ($g\ L^{-1}$); S_0 , substrate feeding concentration ($g\ L^{-1}$); P , products concentrations ($\mu g\ Fab\ L^{-1}$ or $g\ L^{-1}$); OUR , oxygen uptake rate ($mol\ L^{-1}\ h^{-1}$); CPR , carbon dioxide production rate ($mol\ L^{-1}\ h^{-1}$). F_{out} , can be obtained by the total mass balance for an ideal stirred tank reactor in continuous operation at steady state (equation 5).

$$F_{out} = \frac{\rho_{Feed}F - \rho_{H_2O}F_{Evap} + \rho_{Base}F_{Base} - \rho_{Broth}F_O + M_{GAS}}{\rho_{Broth}} \quad (5)$$

where F_{Evap} is the water evaporation rate ($L\ h^{-1}$); F_{Base} , base feeding rate ($L\ h^{-1}$); F_O , withdrawal rate ($L\ h^{-1}$); M_{GAS} , net mass gas flow rate ($g\ h^{-1}$); ρ_{Feed} , substrate feed density ($g\ L^{-1}$); ρ_{H_2O} , water density ($g\ L^{-1}$); ρ_{Base} , base density ($g\ L^{-1}$); ρ_{Broth} , mean broth density ($g\ L^{-1}$). The net mass gas flow rate is calculated with the equation (6):

$$M_{GAS} = -(W_{O_2}OUR V + W_{CO_2}CPR V) \quad (6)$$

Where W_{O_2} is the oxygen molar mass ($g\ mol^{-1}$); W_{CO_2} , carbon dioxide molar mass ($g\ mol^{-1}$). In case of product stripping like ethanol or any other compound, an additional term is included in equations (4-5) in order to not underestimate its corresponding specific rate. Substrate and products concentrations were referred to the whole medium, including biomass volume.⁴⁰

Consistency check and data reconciliation

Specific rates and yields can be affected by random errors, drifts and even gross errors. Besides the propagation of random measurement errors, gross errors such as analyser miscalibration and drifts can alter their values. Mean values or moving average method is normally used to reduce random noise. Generally, applicable constraints such as elemental balances can remove measurement error by using very little prior knowledge.⁴¹

The consistency of the measurements was checked by standard statistical tests considering elemental balances as constraints.⁴² With the current experimental set-up it was possible the measurement of the key specific rates in the black-box process model: biomass generation (μ), glucose uptake (q_s), products formation (q_p), oxygen uptake (q_{O_2}), and carbon dioxide production (q_{CO_2}). In this work, the carbon balance and the redox balance were used as constraints. Protein production was considered negligible in these balances. **The uncertainties of the specific rates were estimated through error propagation from the uncertainties of the state variables. Thus, mean measurement errors associated to specific rates prior to the reconciliation procedure were: $\epsilon(\mu) = 1.0\%$, $\epsilon(q_s) = 3.8\%$, $\epsilon(q_p) = 5.7\%$, $\epsilon(q_{O_2}) = 6.8\%$, $\epsilon(q_{CO_2}) = 5.6\%$. The mean measurement errors associated to specific rates of by-products generation is $\epsilon(q_{BP}) = 3.9\%$.** Thus, the system is overdetermined and the degree of redundancy is the same as the number of constraints. This fact, respecting the covariance for each measurement,^{43,44} can be used to check the measurements for gross errors or pointing potential unidentified metabolites, and to improve the accuracy of the measured conversion rates by data reconciliation methods.⁴⁵ The h value given by the sum of the weighted squares of the residuals ϵ is the output of the statistical test for the presence of gross errors or neglected components.

$$h = \epsilon P^{-1} \epsilon \quad (7)$$

If h exceeds the threshold value, which depends on the significance level α (0.95 in this work) and degree of redundancy according to the χ^2 distribution, it is concluded that there are significant errors in the measurements and/or there is compound that has not been included in the black box process model. The variances of all specific rate measurements were considered uncorrelated and estimated by replicates and/or error propagation.

The χ^2 -test performed for all the experimental data, obtained from chemostat cultivations, showed the measurements satisfied mostly the stoichiometric model and hence, both C-balance and e-balance.

Data reconciliation procedures are also based on the use of elemental balances according to a black box reaction scheme to improve the accuracy of the measured specific rates or yields and also to determine the unknown specific rates.⁴⁶ A measurement error vector δ is found by using a least squares approach to calculate the reconciled vector, which includes the best estimation of reaction rates to fit all the constraints imposed.

Results

Process variables

A set of chemostat cultivations with different oxygen supplies was carried out in order to describe the effect of oxygen-limiting conditions on the physiology of *P. pastoris*, as well as on the different productivity parameters of the bioprocess. The wide range of conditions studied was achieved by comparing the performance of different steady-state set points with different oxygen mole fraction in the inlet gas. This analysis led to a deep study of the hypoxic conditions effect on P_{GAP} -based recombinant protein production processes with *P. pastoris*. The working range was defined from 21% to 4% of oxygen mole fraction in the inlet air. The closure of carbon and redox balances determined for all the culture conditions compared in the present work were both always above 90%. Additionally, results were validated using the standard data consistency check and reconciliation procedures described in materials and methods section. These tests confirm the robustness and the reliability of the results obtained from the chemostat cultivations carried out.

From the main cultivation process variables plotted in Fig. 1A, in terms of oxygen effects on the growth, it can be proposed a distribution of the culture conditions that presents a similar correlated behaviour within differentiated areas of the graph. The first one is comprised of culture conditions in which the oxygen is not resulting in any limiting effect on the cultivation, thus it is only limited by the carbon source. The non-limitation by oxygen is evidenced by the positive values of pO_2 determined by the oxygen sensor. Both, the non-accumulation of glucose, as well as the non-production of ethanol as a by-product, indicates that cells are growing in fully aerobic glucose metabolism. In this area, the DCW is rather constant among the different set points, however a slight increase on Fab production can be observed, close to the transition area where oxygen also starts to limit the growth faintly.

A second pool of oxygen-limiting conditions can be grouped in an area in which glucose and oxygen are both limiting simultaneously the cultivation. For these culture conditions pO_2 is always 0%, which indicates that there is not an excess of dissolved oxygen in the culture broth. Thus, it should be assumed that within this area of set points, the total amount of oxygen that is being transferred to cultivation is being consumed by the biomass. However, although pO_2 is always 0% for all the conditions, actually different oxygen-limitation states can be achieved by

supplying different mixtures of air. Within this area, as the oxygen becomes a limiting growth factor, *DCW* clearly decreases and ethanol is produced up to concentrations of 9 g L^{-1} as a main by-product of the cultivation. This fact indicates a shift to a respiro-fermentative metabolism. The remaining glucose in the broth for all these conditions is always considered negligible, thus one can conclude that glucose-limitation is still being the main limiting factor of the culture. Among these points also a relevant peak of Fab titration can be observed. **The maximum product titer observed among this set of conditions is about $12 \mu\text{g}_{\text{Fab}} \text{ L}^{-1}$, which is significantly higher than the determined for moderate oxygen supplies $8 \mu\text{g}_{\text{Fab}} \text{ L}^{-1}$.**

Finally, a third area can be defined for the most severe oxygen-limiting cultivation condition. For this set point, since glucose is accumulated on the broth, it can be assumed that oxygen is the only factor that is truly limiting the growth. In comparison with the other areas, the *DCW* determined is the lowest, and ethanol production the highest. This behaviour follows the same trend that observed in the previous conditions. For the Fab titration, a drastic decrease is observed in comparison with less-restrictive oxygen conditions.

Since the biomass yield is not constant among all the conditions compared, in order to analyse the process parameters studied in this work, the *DCW* variations must be taken into account for determining the specific rates of each parameter. Thus, the parameters plotted in Fig. 1A are also shown in terms of specific rates in Fig. 1B. Although the main trends are similar, in the most restrictive oxygen conditions when the *DCW* amount is significantly lower, substantial differences between plots are observed. In terms of specific rates, the increase of ethanol production rate (q_{ethanol}) becomes rather linear and the peak of maximal Fab production is shifted to a stricter oxygen-limiting conditions. In this peak, q_{Fab} achieves a 3-fold increase upon non-limiting conditions. **For the Fab production yield (Y_{PX}) the increase observed under oxygen-limiting is equivalent.** In addition, since most of cultivations conditions are carbon-limited, the decrease of biomass yield under hypoxic conditions results in a notable rise of specific glucose uptake rate (q_{glucose}), which reaches up to 2-fold increase at low oxygen supplies. However, no significant differences were observed between the consumption rates of oxygen (q_{O_2}) for the different oxygen-limiting set points compared.

In Fig. 1C, the respiration parameters of the cultivations are presented. As it was commented in the previous paragraph, no significant differences were observed in q_{O_2} . However, a very high increase was detected in the specific production of CO_2 (q_{CO_2}) when oxygen-limiting conditions turn stricter. Consequently, a significant increase is also detected in the respiratory quotient (RQ). In the most severe conditions, both parameters can even double the values determined at normoxic conditions.

By-products observed at the oxygen-limiting conditions

One of the major impacts of the reduced oxygen supply on *P. pastoris* cultures growing on glucose is the generation of secreted fermentation by-products, which reflects the adaptation from a respiratory to a respiro-fermentative metabolism.

While carbon limitation is the only acting on the system, no by-products can be detected. However, for oxygen-limiting conditions, different by-products were determined. Ethanol is the main extracellular metabolite, reaching concentrations up to 10 g L⁻¹ in the most restrictive conditions (Fig. 1A). Arabitol, a C5 sugar alcohol present in the pentose phosphate pathway, was also detected at concentrations significantly lower than ethanol. Both metabolites were previously described as by-products of *P. pastoris* during fermentation at low oxygen supply.^{25,26} However, other significant peaks that could be related to other unknown metabolites were observed in the HPLC analysis performed. This fact, coupled with the significant carbon balance mismatches determined in hypoxic conditions, lead to think about other missing compounds are being generated as a fermentation by-products. Carrying out LC-MS coupled with HPLC analysis, two major peaks were identified from the molecular weights detected in the unknown chromatogram peaks. The new compounds identified were succinate (MW=118) and α -ketoglutarate (MW=146), both related with the Tricarboxylic Acids (TCA) cycle.

In Fig. 2 the specific production rates of the extracellular metabolites detected in oxygen-limiting fermentation samples are presented. The generation of ethanol is notably higher than the others by-products, and has a rather linear increase accordingly to the reduction on oxygen supply. This fact makes the specific production of ethanol an interesting indirect reporting parameter of the oxygen availability for the cells, which is required for the implementation of oxygen-limiting conditions to different cultivations set-ups and operational modes. The rest of by-products have a similar behaviour between them. Low specific production rates while glucose and oxygen limitations are both acting on the cultivation. However, a notable increase in the specific production rates is triggered for the most restrictive oxygen-limiting condition.

Physiology study based on flow cytometry analyses

Flow cytometry is a powerful tool that enables to determine the physiological state of the cells growing in a culture with high accuracy and reproducibility. Since in this work the effect of a critical limiting factor such oxygen availability in the *P. pastoris* growth has been studied, these analyses provide additional valuable information about how the physiology of the yeast can be affected. These results are shown in Fig. 3, in which also are indicated the same areas defined by the limitations that are acting the cultivation.

For the viability determination, it is considered that propidium iodide (PI) stained cells are dead, thus will not further participate in cell growth and product formation. No critical differences were observed in the ratio of cell viability when the O₂ supply was conducted with a higher concentration than 6% mole fraction, viability results were always rather constant above 95%. Only the most severe hypoxic conditions a significant drop of cell viability to a ratio under 90% was observed.

In the flow cytometry procedures carried out to determine the presence of ROS, DHR and DHE were used to monitor the stress effects on the cells caused by the oxygen-limiting conditions. In the different set points of normoxic conditions, no stained cells were detected by using neither DHE nor DHR protocols, thus was considered that ROS stress was not affecting the cells in these growing conditions. In the phase where oxygen and glucose were both limiting the culture, a significant rise in the level of ROS was observed. It was increasing progressively as the oxygen supply was being reduced. DHR protocol detected fractions of stressed cells by ROS between 15 and 20%; DHE protocol determined that the fractions of stressed cells were between 25% and 35%. In contrast, as other parameters commented previously, an abrupt change was observed for the most severe oxygen-limiting conditions. In this set point, the determined fraction of stressed cells by ROS was up to 30% and 50% by applying the DHR and DHE procedures respectively.

Discussion

In this work, a thorough study on the global adaptive response of recombinant *P. pastoris* to a wide range of oxygen availability has been carried out. As previously described, a very strong positive effect of oxygen-limiting conditions on specific productivity of recombinant proteins driven by P_{GAP} was observed.²⁴⁻²⁶ Nevertheless, in the mentioned works only two limiting conditions for specific set-up cultivations were characterized. In contrast, in this study, a high number of different degrees of oxygen availability have been compared in order to deeply characterize the system describing accurately the effect of the oxygen limitation on the physiology and the metabolism at macromolecular level of the yeast. Hence, it allowed also determining the specific conditions that lead to the maximum productivity of the process. In addition, alternative strategies to implement equivalent oxygen-limiting conditions to different cultivation set-ups, operating modes and other recombinant proteins of interest have been proposed.

The main causes that lead to the prominent increase of specific recombinant protein production under oxygen-limiting conditions were extensively discussed in a previous work of our group, in which transcriptomic, proteomic and metabolic fluxes analyses were integrated to understand the adaptation of cellular mechanisms to low oxygen availability in a recombinant *P. pastoris* strain.²⁵ This study hypothesized that the significant increment of the recombinant protein specific

productivity may be due to the overall increase of transcriptional levels of genes involved in the glycolytic pathway, hence genes under the control of glycolytic promoter such as P_{GAP} . In addition, this work also described other effects due to hypoxia conditions such changes in membrane fluidity and increased transcription of genes related with the unfolded protein responses (UPR), e.g. *PDH1*, *Ero1* and *Hac1*, which may also contribute to enhance specific productivity of secreted recombinant proteins.⁴⁷

Besides the mentioned specific productivity increase of P_{GAP} -regulated recombinant protein expression, most of the adaptation effects to low oxygen supply on *P. pastoris* cultivations are caused by the metabolic shift from a respiratory to a respiro-fermentative pathways, which leads to a decrease in the biomass yields, generation of secreted by-products (ethanol, arabitol, α -ketoglutarate and succinate), increment of the specific uptake rate of the carbon source ($q_{glucose}$), as well as of the q_{CO_2} and RQ . These metabolic effects increased progressively as the oxygen availability decreased. In contrast, q_{O_2} is rather constant among the different conditions of oxygen limitation. Thus, while in normoxic conditions all the carbon provided by glucose are directed to biomass and CO_2 formation, in oxygen-limiting conditions a notable fraction of carbon goes to ethanol, arabitol, α -ketoglutarate and succinate that are secreted into the fermentation broth. The different C-distribution in function of the oxygen supply is shown in Fig. 4.

As mentioned in the results section, it is particularly interesting to highlight that the specific consumption rate of oxygen (q_{O_2}) is rather constant among the different oxygen-limiting conditions compared as well as the non-limiting. Consequently, since $OTR=OUR=q_{O_2} \cdot X$, the lower OTR results into a lower biomass yield, which is in accordance with the formation of the different by-products described. It is directly related with the different C-distribution mentioned above.

As an adaptive response of the yeast to the environmental stress condition, the reduced oxygen availability leads to a strong transcriptional induction of glycolysis and fermentative pathways as well as the downregulation of the pentose phosphate pathways (PPP) and the tricarboxylic acid (TCA) cycle,²⁵ the central carbon metabolism. The generation of ethanol, the main by-product in the culture, was clearly defined as a metabolic shift in the pyruvate breaching point from the pyruvate dehydrogenase pathway, the respiratory flux through the TCA cycle, to the pyruvate decarboxylase pathway, which leads to the ethanol production.

The formation of the other by-products should be related to the adaptation towards a fermentative metabolism in which cells have to remove the excess redox equivalents that are accumulated in the biomass synthesis and the secretion of oxidized metabolites.⁴⁸ **Actually, the previously cited**

work,²⁵ also relates directly the presence of by-products with alterations in the transport phenomena between the cytosol and mitochondria, specifically the partially oxidized metabolites derived from the low concentration of oxygen in the cytoplasm. Previous works described the generation of arabitol as a mechanism to maintain the redox balance during the fermentative growth and as a kind of protection to osmotic stress.^{26,49} The generation of succinate during growth under oxygen-limiting conditions in yeasts has been widely described, especially those related with wine production.^{50,51} This formation was also related with the need to maintain the redox balance in hypoxic conditions.^{52,53} The production and secretion of α -ketoglutarate, also another intermediate in the TCA cycle as succinate, was discussed by Otto et al. as a fermentation by-product of bacteria and yeasts cultures including *Pichia* species.⁵⁴ This generation may be related with the decrease of carbon flux through the TCA cycle due to the limitation of oxygen availability, as well as the growth in presence of significant concentrations of ethanol.^{55,56}

In previous works, small fractions of all the mentioned metabolites were also detected in glucose limited chemostat cultivations of *P. pastoris* and *S. cerevisiae*.^{57,58} Other extracellular central metabolites described in these studies might also be present in the cultivation broth of the oxygen limited cultivations of the present work. However their concentration levels would be under detection limit of the analytical techniques used in the presented work. Interestingly, different from other authors that described the formation of acetate under non-limited glucose conditions,^{59,60} in the present study no detectable amounts of this metabolite could be quantified by means of any analytical technique detailed in the materials and methods sections, neither using enzymatic kits nor gas chromatography analysis. Therefore, as described in our previous work, the production of acetate in this system should be related with cultures grown on excess of glucose and low-moderate oxygen availability.¹⁶

The application of flow cytometry analysis enabled a more thorough understanding of the oxygen availability effect on the physiology of *P. pastoris* producing recombinant proteins. Therefore, by comparing the viability and the accumulation of ROS among samples of several steady-state chemostat cultures, it was possible to determine the stress effects on cells caused by oxygen-limiting conditions. From the results, it was shown that the percentage of viable cell that are growing in glucose-limited chemostat is close to 100%, which is in accordance with other results published.¹⁸ Only when very low oxygen fractions were supplied to the cultivations, a significant decrease on the viability up to around 88% could be detected.

On the other hand, from the very beginning of the application of non-severe oxygen-limiting conditions, significant levels of cell stress that caused a relevant accumulation of ROS were detected. This accumulation was progressively increasing as the oxygen availability was being

reduced. However, for the most restrictive condition, the accumulation was triggered to levels significantly higher, thus indicating principal changes on the physiology in which cells were exposed to a prominent oxidative stress. Although the significant quantitative differences observed between both reporting indicators use for each method, DHE and DHR, the similar behaviour observed between them leads conclude that both are valid for the qualitative detection of ROS accumulation. Nevertheless, in order to improve the accuracy for a reliable quantitative determination, the procedures should be revised and improved.

In the literature, higher cell viability has been described in continuous cultures respect to batch and fed-batch processes.⁶¹⁻⁶³ It was attributed to the absence of accumulated substances that, unlike non-continuous cultivations, are continuously washed out. Other important factor is the aging phenomenon of fed-batch processes, what makes the cells more sensitive to stress.³⁹ Thus, the relevant effects observed in flux cytometry analysis even though that the cells were grown in a chemostat set-up, leads to conclude that oxygen-limiting conditions causes a relevant stress on the physiological state of *P. pastoris*.

By the rational analysis of the results obtained from the different hypoxic conditions carried out in this study, it was able to determine the optimal conditions that maximize the productivity of recombinant protein regulated by P_{GAP} . As was described above, the conditions that maximize q_{Fab} are the most severe oxygen-limiting while glucose limitation is still the major limiting factor of the culture. Thus, equivalent physiological states should be achieved in order to reach the maximum levels of protein expression in similar protein production processes. When oxygen is the major limiting factor, besides a significant decrease of q_{Fab} , it has also been observed a considerable rise of oxidative stress that leads to an increase of cell mortality and accumulation of ROS. Also for the most severe limiting conditions, a weighty metabolic shift that triggers the generation of big amounts of culture by-products was observed, which could be caused by the collapse of the respiratory pathways due to the very low levels of oxygen availability.

As it is stated in the introduction section, developing a methodology that allows applying equivalent oxygen-limiting conditions to experimental set-up with different oxygen transfer capabilities is necessary to exploit the relevant increment of protein production using this strategy in different equipment. Otherwise, the full study correlating the O_2 molar fraction in the input gas with the real oxygen availability for the culture and its effects should be carried out for every fermentation system and operating mode used for the implementation of this cultivation strategy. In this sense, some of the parameters studied in this work could be selected as a reference or reporting indicators of the degree of oxygen limitation applied to *P. pastoris* cultivations.

The rather linear increase of $q_{ethanol}$ as the oxygen supply decreases becomes this specific rate into a feasible indirect reporting parameter of the oxygen availability for the cells. In contrast, other specific rates of metabolites generated as by-products are not as suitable to be used as a reference due to their lower production and non-linear dependence respect to oxygen limitation. Thus, in Fig. 5 the behaviour of the main parameters studied in this work are presented in function of $q_{ethanol}$. In this plot, there are only shown the areas in which oxygen limitation is affecting the cell growth, otherwise the ethanol would not be generated. Interestingly, the plot also shows a linear correlation between the RQ and $q_{ethanol}$. Consequently, RQ could be also a useful reporting parameter of the oxygen availability for the cells with its own pros and cons. One of the major advantages is that RQ gathers information associated to q_{O_2} and q_{CO_2} into one single parameter not dependent on biomass concentration. This fact is relevant in the reporting parameter selection since the determination of biomass cannot be considered straightforward. Both cell physiology and central carbon metabolism effects, caused by hypoxic conditions as well as the metabolic burden due to recombinant protein expression, affect significantly the biomass generation.

The application of determined oxygen-limiting conditions into different set-ups working on continuous mode, which advantages have been recently reviewed for industrial bioprocesses,^{2,64} can be carried out by using as a reporter indicator either the $q_{ethanol}$ or RQ . Since this cultivation mode works ideally in steady-state conditions, the process variables are rather constant and the implementation should be straightforward. Ethanol concentration can be determined off-line, by using HPLC or equivalent analytical methods,⁶⁰ or on-line, by NIRS,⁶⁵ MS⁶⁶ or sensors able to monitor either methanol or ethanol, which are commonly used in *P. pastoris* processes that use methanol.^{10,67} RQ can be easily determined from off-gas data analysis. Therefore, according to the reporting parameters selected, the manipulated variable would be the molar fraction of O_2 in the inlet gas, air flow rate or stirring rate required for applying the desired oxygen-limiting conditions in the system. Due to the specific characteristics of the chemostat cultures, working at steady-state conditions, this operating condition would be kept constant in time, in order to obtain a maximal Fab production. For large-scale production to attain the desired O_2 availability it would be recommended to manipulate either air flow and/or stirring rate and not by N_2 supply, thus reducing operating costs.

The implementation of this cultivation strategy to other operational modes different from continuous mode broadens the versatility of the proposed approach. In this sense, fed-batch cultivation, in which the process parameters are time dependent, should be considered more challenging. Opposite to the continuous mode, along fed-batch cultivations the molar fraction of O_2 , air flow rate or stirring rate should be continuously modified by means of reliable monitoring and control strategies. It would allow maintaining the key oxygen availability to the cells

throughout the process in which the amount of biomass and its requirements are continuously changing.

In the new proposed approach in this work it is intended to mimic continuous conditions to give it high versatility, and not only to be transferable from other fermentation systems but also between different operating modes and scales. The proposed fermentation strategy aims to achieve pseudo-steady-state conditions for cell growth (μ) and substrate uptake (q_s) as reached in continuous mode. Hence, a pre-programmed exponential feeding rate profile for substrate addition derived from mass balance equations to maintain a constant specific growth rate (μ) would be implemented.¹⁶ In the simplest scenario purposed in a previous works, if the concentration of ethanol is taken as indirect physiological indicator its control does not guarantee to keep constant neither $q_{ethanol}$ nor μ .^{24,68,69} Only in chemostat, when the concentration of a component for a given dilution rate at steady-state is unvarying, their specific rate and productivity are constant.

As it was described for continuous mode, again either $q_{ethanol}$ or RQ could be selected as reporting parameters of the degree of oxygen limitation. The control of $q_{ethanol}$ would require the estimation of both ethanol production rate and biomass concentration. From the measurements of ethanol concentration and application of mass balances, the production rate of ethanol could be calculated. However, in order to estimate the $q_{ethanol}$, the biomass determination is also required, and on the contrary, there is not currently available a reliable standard method for the on-line determination of biomass. Each available technique has its own advantages and disadvantages.⁶⁶ Alternatively, real-time determination of biomass can be conducted by means of different estimation algorithms and techniques,⁷⁰⁻⁷² but always incorporating some complexity and even instability in the system. In contrast, control of RQ is *a priori* not so complex because its on-line determination is commonly carried out from off-gas analysis and mass balancing of CO_2 and O_2 . Thus, the simplicity, portability and robustness makes the RQ determination the best alternative to be considered for a real-time application.

Conclusions

As summary, since the bioprocess efficiency is strongly affected by changes in the cellular state, it should be monitored, and properly manipulated. In this study, a generic methodology to work systematically with different oxygen-limiting conditions has been presented. It allows the control of the physiological and metabolic state of the cells by means of monitoring either the specific generation rate of ethanol or the respiratory quotient in *P. pastoris* cultures. The versatility of the proposed approach has been discussed for three scenarios. First, in a more general way, it can be

applied to work under equivalent oxygen-limiting conditions for different cultivation set-ups although may differ in their oxygen transfer capabilities. Second, the understanding of the physiological state of the cell gained from continuous mode could be migrated to fed-batch operation, which is intrinsically time variant. Third, the whole approach could be applied for the production of other recombinant proteins of interest regulated by P_{GAP} in order to exploit the positive effects of oxygen-limiting conditions in the protein expression.

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Figure 1: **A:** Main cultivation parameters at different oxygen supply levels: dry cell weight (*DCW*, ●); 2F5 Fab titration (○); glucose concentration (▲); ethanol concentration (▽); and dissolved oxygen (*DO* or *pO₂*, ■). **B:** Biomass yield and main specific rates of the cultivation at different molar fraction of oxygen in the inlet gas: biomass yield (*Y_{XS}*, ●); specific 2F5 Fab production rate (*q_{Fab}*, ○); specific glucose uptake rate (*q_{glucose}*, ▲); specific ethanol production rate (*q_{ethanol}*, ▽); and specific oxygen uptake rate (*q_{O₂}*, ■). **C:** Specific oxygen uptake rate (*q_{O₂}*, ■); specific carbon dioxide production rate (*q_{CO₂}*, □); and respiratory quotient (*RQ*, ◆) at different molar fraction of oxygen in the inlet gas.

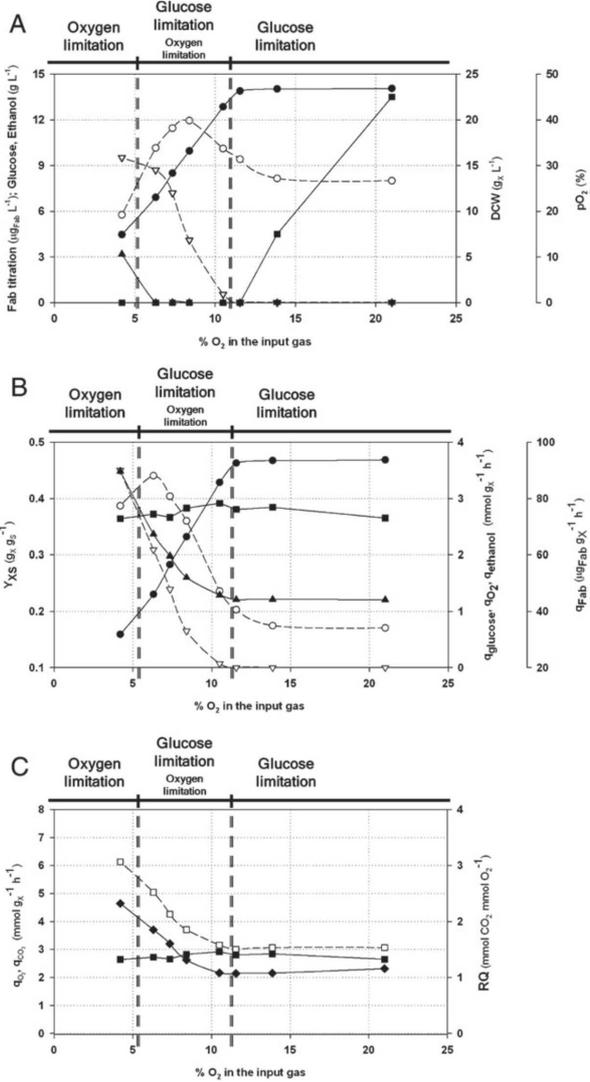


Figure 2: Comparison of the specific production rates of the 2F5 Fab and the by-products monitored at different molar fraction of oxygen in the inlet gas: specific 2F5 Fab production rate (q_{Fab} , \circ); specific ethanol production rate ($q_{ethanol}$, ∇); specific arabitol production rate ($q_{arabitol}$, Δ); specific α -ketoglutarate production rate ($q_{\alpha-KG}$, \blacktriangledown); and specific succinate production rate ($q_{succinate}$, \blacksquare).

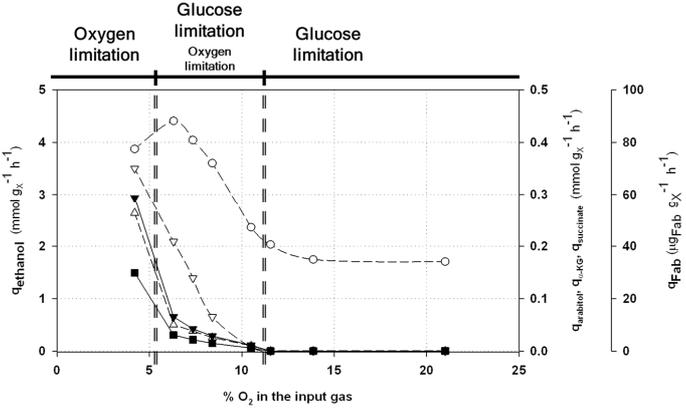


Figure 3: Cell viability monitored with PI (●), ROS monitored with DHE (▲) and ROS monitored with DHR (○) at different molar fraction of oxygen in the inlet gas.

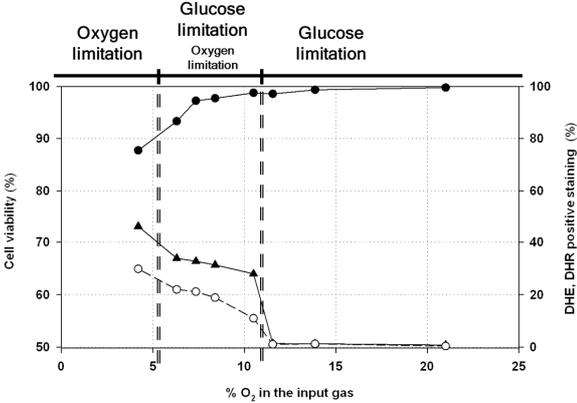


Figure 4: Consumed C-mol distribution at different molar fraction of oxygen in the inlet gas: biomass (●); CO₂ (□); ethanol (▽); arabitol (△); α-ketoglutarate (▼) and succinate (■).

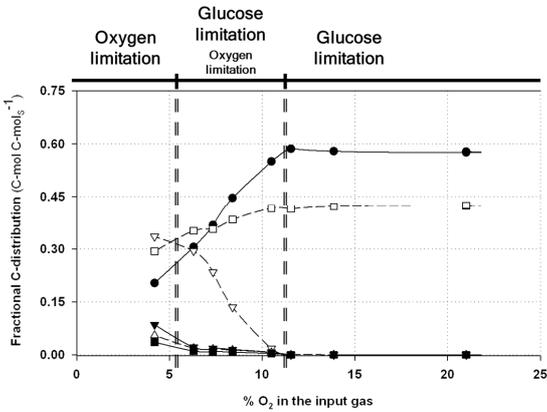


Figure 5: Biomass yield (Y_{XS} , ●); specific 2F5 Fab production rate (q_{Fab} , ○); respiratory quotient (RQ , ◆); and specific arabinol production rate ($q_{arabitol}$, △) respect to specific ethanol production rate ($q_{ethanol}$, ▽).

