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RESEARCH ARTICLE

Synergic kinetic and physiological control to improve the efficiency of *Komagataella phaffii* recombinant protein production bioprocesses

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Abstract

The yeast *Komagataella phaffii* (*Pichia pastoris*) is currently considered a versatile and highly efficient host for recombinant protein production (*RPP*). Interestingly, the regulated application of specific stress factors as part of bioprocess engineering strategies has proven potential for increasing the production of recombinant products. This study aims to evaluate the impact of controlled oxygen-limiting conditions on the performance of *K. phaffii* bioprocesses for *RPP* in combination with the specific growth rate (μ) in fed-batch cultivations. In this work, *Candida rugosa* lipase 1 (Crl1) production, regulated by the constitutive *GAP* promoter, growing at different nominal μ (0.030, 0.065, 0.100 and 0.120 h^{-1}) under both normoxic and hypoxic conditions in carbon-limiting fed-batch cultures is analysed. Hypoxic fermentations were controlled at a target respiratory quotient (*RQ*) of 1.4, with excellent performance, using an innovative automated control based on the stirring rate as the manipulated variable developed during this study. The results conclude that oxygen limitation positively affects bioprocess efficiency under all growing conditions compared. The shift from respiratory to respiro-fermentative metabolism increases bioprocess productivity by up to twofold for the specific growth rates evaluated. Moreover, the specific product generation rate (q_p) increases linearly with μ, regardless of oxygen availability. Furthermore, this hypoxic boosting effect was also observed in the production of *Candida antarctica* lipase B (CalB) and pro-*Rhizopus oryzae* lipase (proRol), thus proving the synergic effect of kinetic and physiological stress control. Finally, the Crl1 production scale-up was conducted successfully, confirming the strategy's scalability and the robustness of the results obtained at the bench-scale level.

INTRODUCTION

The yeast *Komagataella phaffii* (*Pichia pastoris*) is considered an efficient and versatile expression system for recombinant protein production (*RPP*). The wide range of well-known advantages presented by this cell factory include a large genetic toolbox, the ability to perform posttranslational modifications and grow at high densities in defined and inexpensive media, and the obtainment of high protein titres extracellularly while secreting very low levels of native proteins (García-Ortega et al., 2019; Looser et al., 2014). Altogether, they make this cell factory an extraordinary alternative for both research and industrial-scale *RPP*.

Despite the appealing features exhibited by *K. phaffii* as a cellular factory, considerable research endeavours have been undertaken in the field of bioprocess engineering to enhance *RPP* scalability and efficiency on a global scale. This is attributed to the exponential surge in demand for therapeutic proteins, food and feed proteins, as well as industrial enzymes of interest, which can be successfully produced using *K. phaffii* as an expression system (Barone et al., 2023; De Brabander et al., 2023).Bioprocess optimisation is intricately linked to the genetic construction of the production strain, wherein the selection and use of the promoter play a pivotal role in achieving the desired outcomes. Traditionally, in *K. phaffii*, most bioprocesses have relied on using the alcohol oxidase 1 promoter (P_{AOX1}). This choice derives from the robust and tightly regulated methanol-inducible promoter system, which enables the strong and controlled expression of target genes (Cregg et al., 2000; Ponte et al., 2016). However, the use of methanol as an inducer presents significant operational disadvantages, including the extraordinarily high need for heat removal and oxygen supply, as well as the negative impact of the potential for cell lysis and its subsequent proteolytic activity. Moreover, the transport and storage of large amounts of methanol is considered a relevant drawback for large-scale processes. Accordingly, new expression systems based on alternative methanol-free promoters have been developed, among which the glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}) is usually considered the classic benchmark (García-Ortega et al., 2019).

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In the literature, different articles have reported interesting bioprocess strategies to maximise P_{GAP}-based *RPP*. Specifically, previous studies have demonstrated that the application of various cell stress conditions, such as substrate starvation or oxygen limitation, has a significant positive impact on *RPP* under P_{GAP} regulation (Baumann et al., 2008; Garcia-Ortega et al., 2016).

P_{GAP}, the native promoter of the *TDH3* gene, is involved in a crucial glycolysis step. It is well-documented that metabolic fluxes within the glycolytic pathway, as well as ethanol production pathways, undergo significant enhancement during the transition from respiratory to fermentative metabolism. Previously published works also revealed notable alterations such as increasing transcription rates and, thus, protein levels of genes involved in this metabolic transition. Among them is the upregulation of the *TDH3* gene under the regulatory influence of the *GAP* promoter. This metabolic shift occurs when the organism encounters conditions of limited oxygen while utilising glucose as a substrate (Baumann et al., 2010; Gasset et al., 2022).

As described in previous works, there is a relationship between the degree of oxygen limitation applied to cultures and the respiratory quotient (*RQ*) determined by off-gas analysis. Other factors such as strain properties, carbon source and operating conditions can also significantly affect this parameter. Therefore, the *RQ* has been proposed as an advantageous metabolic reporter of hypoxic conditions as it can be calculated in real time via an external exhaust gas analysis and is independent of the fermenter employed and its oxygen transfer efficiencies (Alexeeva et al., 2002; Garcia-Ortega et al., 2017; Steinsiek et al., 2011). Hypoxic fermentations with a controlled *RQ* set-point value have been successfully implemented in chemostat cultures and even in dynamic systems, such as fed-batch cultures, resulting in an up to threefold higher specific production rate (q_p) compared with standard oxygen conditions (or normoxia) (Garcia-Ortega et al., 2017; Gasset et al., 2022).

Production kinetics often play a key role in optimising and scaling up bioprocesses, as well as ensuring efficient bioprocess management. Production kinetics is determined by the relationship between q_p and the specific growth rate (μ) (Looser et al., 2014; Nieto-Taype et al., 2020). Previous studies on P_{GAP} described that an increase in μ leads to significant enhancement of target gene transcriptional levels and, thus, the q_p of the secreted recombinant product. However, the magnitude of this increase varies depending on the specific protein, target gene dosage and operational mode employed (Garcia-Ortega et al., 2016; Rebnegger et al., 2014).

The implementation of carbon-limiting conditions, enabling the attainment of a pseudo-stationary state with a constant μ, has been demonstrated to notably enhance the robustness and reproducibility of bioprocesses in fed-batch cultures. This fact, coupled with the utilisation of a precise and reliable *RQ* control system for hypoxic conditions, contributes to a more uniform product quality, ultimately influencing essential target

attributes. These attributes include biological activity, half-life, immunogenicity, safety, protein glycosylation and aggregation — all of which can be effectively modified by controlling various cultivation parameters (Eon-Duval et al., 2012; Looser et al., 2014). The combination of C-limiting conditions and accurate *RQ* control represents a powerful approach to standardise and optimise bioprocesses, ensuring consistent and desirable product characteristics.

Numerous studies have reported successful expression and production of a large number of recombinant lipases using *K. phaffii* as the expression system. These industrial enzymes have demonstrated potential for diverse applications, including biodiesel production, food processing, pharmaceutical synthesis and detergent formulation. Among them, *Candida rugosa* lipase 1 (Crl1), *Candida antarctica* lipase B (CalB) and mature *Rhizopus oryzae* lipase (Rol) with 28 amino acids of the pro-sequence (proRol) have been established as relevant lipases for industrial applications and have generated considerable scientific interest (Eom et al., 2013; Ferrer et al., 2001; López-Fernández et al., 2020; Vanleeuw et al., 2019).

In this work, the isoenzyme Crl1 was selected as the model protein to evaluate the combined effect of two different but complementary bioprocess optimisation strategies. Specifically, a *K. phaffii* clone producing recombinant Crl1 under the regulation of the constitutive P_{GAP} was used to investigate the combined effect of hypoxia and growth rate. To accomplish this objective, hypoxic and normoxic fermentations were conducted at different μ under C-limiting fed-batch operating conditions. In hypoxic cultures, a novel and automatic controller of the *RQ* parameter was implemented to accurately and precisely regulate the degree of oxygen limitation that induces the desired *RPP* boosting effect. The outcomes of these combined strategies were analysed by determining the production kinetics and key physiological parameters of the bioprocess. Additionally, two other relevant lipases (CalB and proRol) were also tested to elucidate the protein dependence of the expression system with the proposed methodology. Finally, based on previous results, Crl1 production was scaled up to a 50 L bioprocess to assess the robustness and scalability of the methodology developed at the bench-scale level.

EXPERIMENTAL PROCEDURES

Strains

Three recombinant *K. phaffii* clones expressing different lipases were used in this study. The genes encoding the three lipases of interest are *Candida rugosa* lipase 1 (*Crl1*), pro-*Rhizopus oryzae* lipase (*proRol*) and *Candida antarctica* lipase B (*CalB*), all of which are regulated by the constitutive *GAP* promoter (P_{GAP}). The constructs contain the α-mating factor, from *Saccharomyces cerevisiae*, allowing the secretion of recombinant proteins to the extracellular media. Expression cassette gene dosage was determined using Droplet Digital PCR (ddPCR). Further details regarding the construction and copy number determination are described elsewhere (Garrigós-Martínez et al., 2021; López-Fernández et al., 2021; Nieto-Taype et al., 2020).

Cultivation methods

Inoculum preparation

The different recombinant producer clones were stored in cryostocks at −80°C and defrosted for each run. Shake flasks filled with 15% of their total volume of YPG medium and zeocin as pressure selection were inoculated with their corresponding cryostocks and grown for 18 h at 25°C with shaking at 120 rpm (*Multitron Shaker*, INFORS HT, Bottmingen, Switzerland). Biomass was then harvested by centrifugation and resuspended to inoculate the bioreactor.

Lab-scale fed-batch cultivation

The fermentations were performed in a 5 L *Biostat B* bioreactor (Sartorius Stedim, Göttingen, Germany) with an initial working volume of 2 L. The temperature was kept at 25°C, and pH was maintained at 6.0 using NH_4OH 15% (v/v). Fed-batch cultures were preceded by a batch phase with glycerol as the carbon source. Further details regarding batch media composition are described elsewhere (Garcia-Ortega et al., 2016). A pre-programmed exponential glucose-based feeding solution was implemented to maintain target-specific growth rates (μ) constant (between 0.030 and 0.120 h^{-1}) at the prefixed set-point under C-limiting conditions. Medium composition per litre of distilled water was glucose 400 g, $MgSO_4{\cdot}7H_2O$ 6.45 g, KCl 10 g, $CaCl_2{\cdot}H_2O$ 0.35 g, antifoam Glanapon 2000 kz 0.2 mL (Bussetti & Co. GmbH, Vienna, Austria), biotin 0.2 mg and PTM1 salt solution 1.6 mL. The PTM1 trace salts solution contained per litre: $CuSO_4{\cdot}5H_2O$ 6.0 g, NaI 0.08 g, $MnSO_4{\cdot}H_2O$ 3.36 g, $Na_2MoO_4{\cdot}2H_2O$ 0.2 g, H_3BO_3 0.02 g, $CoCl_2$ 0.82 g, $ZnCl_2$ 20.0 g, $FeSO_4{\cdot}7H_2O$ 65.0 g and 5.0 mL H_2SO_4 (98%).

This study aimed to compare hypoxic and normoxic fermentations while achieving similar biomass amounts and equivalent fed-batch durations for each specific growth rate. However, given the lower overall biomass-to-substrate yield ($Y_{X/S}$) under hypoxic conditions, normoxic fermentations were conducted with equivalent feeding profiles but different glucose concentrations to

achieve similar total biomass amounts while preserving similar fed-batch duration and volumes for each cultivation.

Dissolved oxygen (DO) was maintained above 30% of air saturation for normoxic conditions. In hypoxic conditions, the desired level of oxygen limitation was achieved by keeping the *RQ* at the targeted set-point value, which increases according to the hypoxic degree attained. To this end, an automated control of the stirring rate, which regulates the O_2 transfer rate (OTR), was designed and implemented. When the monitoring system detects *RQ* values over 1.4, the stirring rate is increased, thus also increasing O_2 transfer, which leads to a subsequent decrease in the *RQ*. The control loop operates in reverse mode when the system registers *RQ* values below 1.3.

In this work, *Eve* software (INFORS HT, Bottmingen, Switzerland) was used as a "Supervisory Control And Data Acquisition" or "SCADA" system (Brunner et al., 2021), acquiring and integrating data from the Sartorius fermenter and peripheral devices (gas analyser, methanol sensor, feeding system, etc.). This software allows the development of soft sensors, such as the one implemented for *RQ* control, which is based on CER and OUR online calculations.

Pilot-scale cultures

Two pilot-scale fermentations were carried out in a 50 L fermenter from ZETA GmbH (Lieboch, Austria) with an initial batch working volume of 30 L. The culture media and operating conditions were equivalent to lab-scale cultivation. However, for hypoxic fermentation, control of the *RQ* at a set-point of 1.4 was manually conducted, following the same heuristic rules described in the previous section. The *RQ* was calculated online via the "CER/OUR/*RQ*" Plugin from *BlueVis* software (BlueSens, Herten, Germany).

Analytical methods

Biomass analysis

Biomass concentrations, in terms of dry cell weight (DCW), were determined in four independent replicates following established protocols (Cos et al., 2005). The relative standard deviation (RSD) for these measurements was below 5%.

Carbon source and by-product quantification

Carbon sources (glycerol and glucose) and potential fermentation by-products (mainly ethanol, arabitol and succinate) were determined using HPLC. The specific column, software used and further details of these analyses are described elsewhere (Garcia-Ortega et al., 2017). The analysis RSD was below 2%.

Off-gas analyses

CO_2 and O_2 molar fractions from the fermenter exhaust gas were measured and recorded online using a *BlueInOne* FERM gas analyser (BlueSens, Herten, Germany). Recalibration was performed for every fed-batch culture, consisting of analysing different gas mixtures with known CO_2 and O_2 concentrations and establishing a linear correlation between CO_2/O_2 and the electronic signal provided by the analyser. The acquired data were used to calculate respirometric parameters: oxygen uptake rate (*OUR*), carbon dioxide evolution rate (*CER*) and respiratory quotient (*RQ*). Humidity reduction via a silica column in the inlet and off-gas lines improved the accuracy and precision of these respiration parameters. RSD was below 5% in all cases.

Lipolytic activity determination

The lipolytic activity of the lipases under study was assessed using a modified adaptation of a well-established enzymatic assay that relies on the hydrolysis of p-nitrophenyl butyrate (pNPB) as the substrate (Chang et al., 2006). The lipolytic activity measurement was based on the release of p-nitrophenol, with one activity unit (AU) defined as the quantity of enzyme required to release 1 μmol of p-nitrophenol per minute under the specified assay conditions. For all three lipases, the mixture comprising the sample under investigation and the reaction buffer was continuously monitored using a Specord 200 Plus instrument from Analytic Jena (Jena, Germany). The monitoring process lasted 120 seconds, measuring the absorbance at a wavelength of 348 nm, at 30°C. What differs between lipolytic assays is the reaction buffer; the reaction buffer used for the evaluation of Crl1 activity was 1 mM pNPB 50 mM phosphate buffer at pH 7.0 and 4% (v/v) acetone. A total volume of 980 μL of buffer was combined with 20 μL of the sample under investigation. For the measurement of proRol activity, the reaction buffer was also 50 mM phosphate with 1 mM of pNPB and 4% (v/v) acetone, adjusted to a slightly higher pH (7.25). A 500 μL aliquot of sample supernatant was diluted with 50 mM phosphate buffer (pH 7.25) and mixed with 800 μL of reaction buffer. The CalB enzyme assay reaction buffer consisted of 5.25 mM of p-NPB and 1% (v/v) acetone dissolved in 300 mM Tris-HCl at a pH of 7.00. For the assay, 100 μL of supernatant from the centrifuged samples was combined with 900 μL of reaction buffer. RSD was below 3% in all cases.

SDS-PAGE electrophoresis

Supernatant samples were also analysed by SDS-PAGE. Fifteen μL of supernatant was mixed with 5 μL of loading buffer (Laemmli buffer 4× + mercaptoethanol at a 10:1 proportion; Bio-Rad, Hercules, CA, USA) and incubated at 95°C for 5 min. Fifteen μL of the denatured sample was loaded onto a 4–15% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad, Hercules, CA, USA) and run at 120 V for about 80 min. Qualitative analysis and visualisation of the gel were performed using the software Image Lab (Bio-Rad, Hercules, CA, USA).

Process parameters

Mass balance and stoichiometric equations

The equations used to compute yields and rates in fed-batch cultures were previously described elsewhere (Ponte et al., 2016).

Data consistency and reconciliation

To ensure measurement consistency, standard statistical tests were conducted with carbon and redox balances as constraints. The combination of both online and offline measurements enabled the determination of seven key specific rates in the black-box process model, considering the lipase production negligible in carbon and redox balances. Thus, the specific rates determined were specific growth (μ), glucose uptake (q_S), oxygen uptake (q_{O2}), carbon dioxide production (q_{CO2}), and ethanol, arabitol and succinate production as by-products (q_{EtOH}, q_{Ara} and q_{Suc}, respectively).

A reconciliation data procedure was applied to all data sets for these specific rates, and the methodology was previously described in detail (Garcia-Ortega et al., 2017).

RESULTS AND DISCUSSION

Maintaining hypoxic conditions throughout the fed-batch phase

Previous studies were performed in chemostat operational mode to characterise the kinetics of P_{GAP}-based Crl1 production processes at different degrees of oxygen limitation and a specific $\mu = 0.100\,h^{-1}$ (Gasset et al., 2022). This work concluded that an *RQ* value between 1.4 and 1.6 corresponds to the most favourable oxygen-limiting conditions in terms of *RPP* increase, with no significant impact on cell growth, avoiding the inhibitory effect of ethanol and increasing the Crl1 q_P by up to twofold. For that reason, an *RQ* value of 1.4 was targeted in all the hypoxic cultivations of the present work.

To evaluate the combined effect of hypoxic conditions and μ in fed-batch operations, a set of eight cultures was performed. Four fermentations with targeted specific growth rates (0.030, 0.065, 0.100 and $0.120\,h^{-1}$) under normoxic conditions (dissolved oxygen (*DO*) > 30%) were conducted and compared with another four with the same μ set-points but under controlled hypoxic conditions (*RQ* = 1.4). The fed-batch operational strategy applied in these fermentations was based on C-limiting conditions with an exponential preprogrammed feeding profile, with glucose as the sole carbon source. This strategy maintains a constant μ during the feeding phase, reaching a pseudo-steady-state, enabling a detailed study of physiologic and production parameters (de Macedo Robert et al., 2019).

Achieving a constant degree of oxygen limitation in a dynamic system, such as fed-batch cultures, constitutes a challenge. Exponential biomass growth in the fermenter will require oxygen availability to increase at the same rate as the biomass. Therefore, control of the *RQ* parameter based on automated modification of the stirring rate was designed and implemented to adjust the oxygen transfer rate according to culture needs. As can be seen in Figure 1, the targeted *RQ*

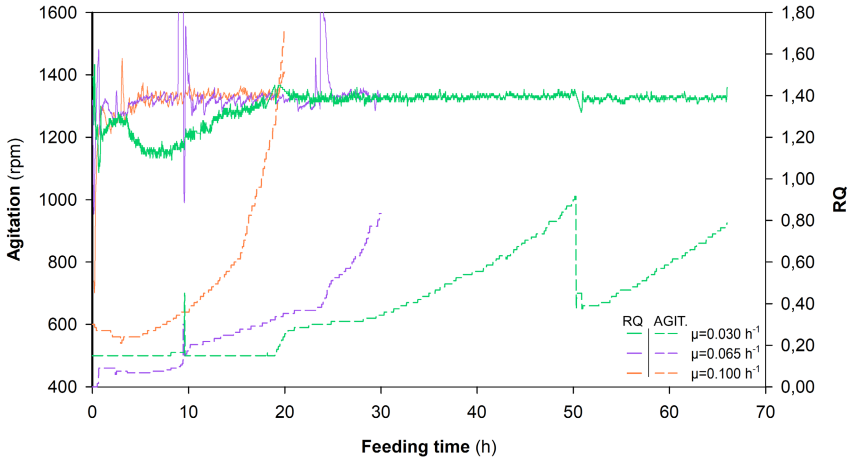

FIGURE 1 Time evolution of online controlled *RQ* at 1.4 and the stirring rate as a manipulated variable at three different specific growth rate set-points for hypoxic fermentations.

set-point was reached satisfactorily throughout the different fed-batch phases in the different μ-driven fermentations due to the accurate action of the controller. As expected, in a system where the biomass grows, oxygen transfer requirements will also increase correspondingly. Thus, the controller acted by increasing the stirring rate of the system, making small steps at the beginning of the phase and finalising with notably greater changes. Overall, only very small deviations from the *RQ* set-point were observed in all experiments at the different μ tested. The most relevant deviation was observed at the beginning of the culture targeted at a μ of 0.030 h^{-1}. In this case, changes in inlet gas composition had to be applied; the inlet air was mixed with pure nitrogen at a ratio of 3:1 (N_2/Air) during the beginning of the fed-batch phase and until 50 h, when the oxygen concentration of the inlet air was increased by reducing this ratio to 1:1. This change was necessary to achieve the desired oxygen-limiting condition without decreasing the stirring rate to below the 450 rpm lower limit, which could impact negatively on culture homogeneity. Interestingly, these air inlet changes did not negatively affect *RQ* control. On the other hand, for the $\mu = 0.100\ h^{-1}$ condition, pure oxygen supplementation of the inlet air was avoided by the use of the higher stirring rates ordered by the control system. Results corresponding to hypoxic conditions at $\mu = 0.120\ h^{-1}$ are not shown in Figure 1 because glucose accumulated in the culture broth. This fact indicates that this set-point exceeds the μ_{max} of the producer clone at the target oxygen-limiting conditions.

Accordingly, the innovative *RQ* controller effectively maintained the parameter around the desired set-point. In this study, for oxygen-limited fermentations, the values consistently fell within a narrow band around 1.4. On the other hand, in normoxic fermentations, the observed *RQ* values were around 1.1, which is consistent with those described in the literature for glucose-based normoxic cultures (Nieto-Taype et al., 2020). These results indicate that the control strategy implemented successfully maintained targeted *RQ* values, thus ensuring the desired oxygen conditions for the bioprocess.

Impact of oxygen-limiting conditions on the culture growth

The impact of oxygen limitation was assessed by comparing sets of two fed-batches at the same targeted μ during the feeding phase but with different oxygen availability. Due to the expected metabolic shift in hypoxic fermentations from oxidative to fermentative metabolism, a reduction in the overall biomass-to-substrate yield ($Y_{X/S}$) was also expected (Adelantado et al., 2017; Baumann et al., 2010). Accordingly, to compare processes with the same μ and volumetric feeding profiles achieving equivalent cell concentrations, a lower glucose concentration was added for the feeding media in normoxic fermentations. As seen in Figure 2A, a very similar biomass growth profile was observed for fermentations with the same μ set-point. The end-point criteria for all experiments were a biomass dry cell weight (DCW) concentration of about 85 $g_{DCW}\ L^{-1}$. Notably, the culture with a μ of 0.120 h^{-1} performed under hypoxic conditions could not maintain the targeted μ during the feeding phase. Glucose accumulation in the culture broth was detected after 10 h of fermentation (data not shown), showing that the μ_{max} of the producer strain under this hypoxic condition is lower than the targeted μ. This fact can be attributed to the negative impact on the maximum cell growth capacity under this oxygen-limiting condition and agrees with previously published results (Gasset et al., 2022; Nieto-Taype et al., 2020).

Effect of the oxygen-limiting conditions on the ethanol production

Ethanol production, as a fermentative by-product, was evaluated and is shown in Figure 2B; it was only detected in cultures under hypoxic conditions. Its presence confirmed the fermentative metabolism revealed by the *Crabtree* facultative yeast *K. phaffii*. Low concentrations of other fermentative by-products, such as succinic acid and arabitol, were also detected (data not shown) in all hypoxic fermentations, except at the lowest μ (0.030 h^{-1}), probably due to the more severe carbon-limiting conditions, which enabled cells to consume all carbon source available in the broth culture. Interestingly, comparable ethanol concentrations (≈11 $g\ L^{-1}$) were detected at the end of distinct cultures, confirming that an equivalent degree of oxygen limitation was applied.

Effect of μ on key bioprocess parameters

For both normoxic and hypoxic cultures, the glucose uptake rate (q_S), $Y_{X/S}$, ethanol production rate (q_{EtOH}) and *RQ* were determined as the main physiological parameters for all specific growth rates studied. Results are presented in Figure 3A,B.

In line with previous findings (Nieto-Taype et al., 2020), q_S shows a linear relationship with μ under normoxic conditions and, as expected, under hypoxic conditions too but with *slightly* higher values (Figure 3A). This fact could be attributed to the shift towards respiro-fermentative metabolism, where a greater amount of substrate (glucose) must be metabolised to support the same growth rate as cultures grown under normoxic conditions. Consequently, oxygen-limited fermentations also exhibit lower $Y_{X/S}$ values compared with oxygen-excess cultures. These findings are consistent

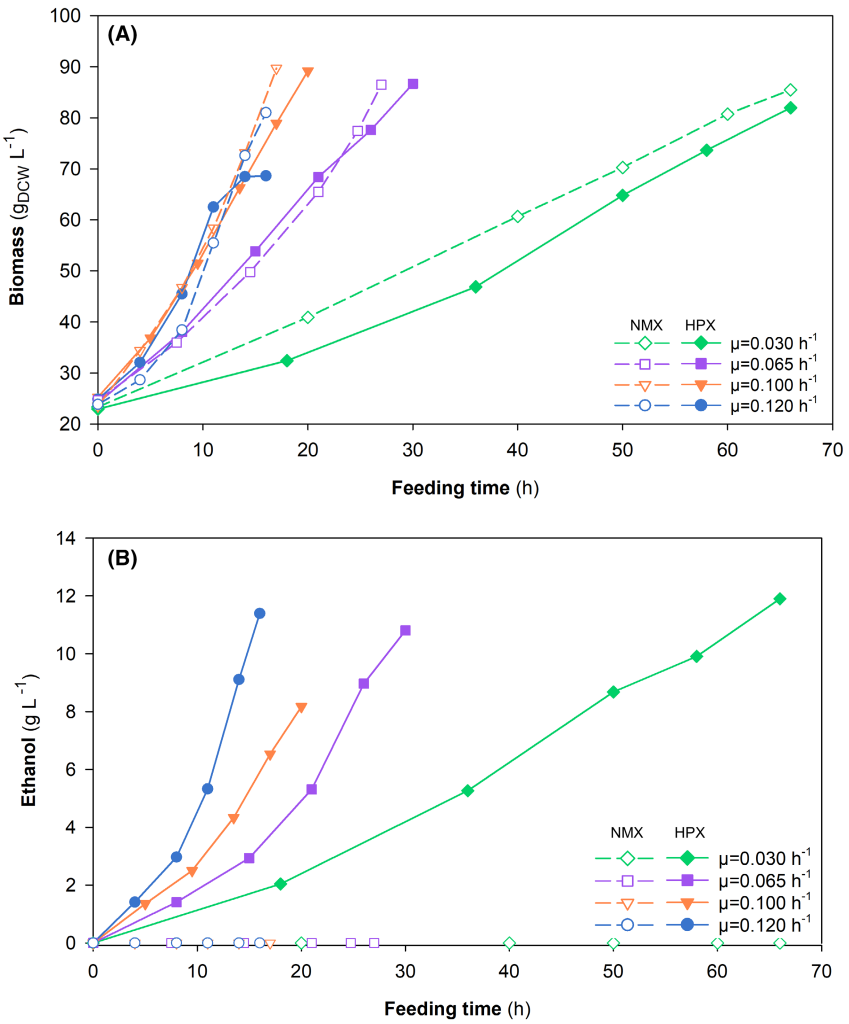

FIGURE 2 (A) Biomass and (B) ethanol concentration throughout the fed-batch phase at four different specific growth rate set-points under normoxic and hypoxic conditions.

with previous studies (Baumann et al., 2010), supporting that respiratory metabolism exhibits more complete substrate oxidation and produces fewer by-products compared with fermentative metabolism, where ethanol emerges as a prominent by-product; however, it is also accompanied by arabitol and succinic acid. In the case of normoxic experiments, the $Y_{X/S}$ values remain fairly constant with a value around 0.50 $g_{DCW}{\cdot}g_S^{-1}$; in the case of hypoxic fermentations, values are around 0.45 $g_{DCW}{\cdot}g_S^{-1}$. These results agree with previously published results (Garcia-Ortega et al., 2017; Gasset et al., 2022; Nieto-Taype et al., 2020). Both conditions of oxygen availability showed a slight decrease in $Y_{X/S}$ at the lowest ranges of μ, which can be explained due to the high energy requirements for maintenance (m_S) (Rebnegger et al., 2014; Zhu et al., 2019).

Another key parameter to evaluate the effect of μ in oxygen-limited fermentation is q_{EtOH}, which is linearly influenced by μ. Only a slight deviation from linear dependence can be observed under the $\mu = 0.10\,h^{-1}$ condition, according to the lower concentration detected at the end of this fermentation (Figure 2B). This could be mainly attributed to small deviations in gas analyser calibration, especially for O_2 determination, which can lead to small variations in actual RQ values, thus producing differences in the degree of oxygen limitation implemented to the system and, consequently, ethanol production. This is supported by the fact the *RQ* is slightly lower at this targeted μ (Figure 3B). Moreover, higher ethanol stripping due to the higher stirring rate is expected in this experiment (Figure 1).

Production parameter comparison among the culture strategies tested

Figure 4 shows the Crl1 titre and the specific Crl1 production rate, key production parameters to evaluate the efficiency of a bioprocess. Notably, two distinct levels of product titre can be clearly observed in Figure 4A. Under normoxic conditions, the values at the end of different feeding times consistently remain around 125 AU·mL^{-1} for every μ compared. In contrast, the values corresponding to hypoxic fermentations are significantly higher at the end of the bioprocess, in the range of 300 AU·mL^{-1}. The correlation between q_P and μ is presented in Figure 4B for both normoxic and hypoxic cultures. q_P is linearly influenced by μ under both

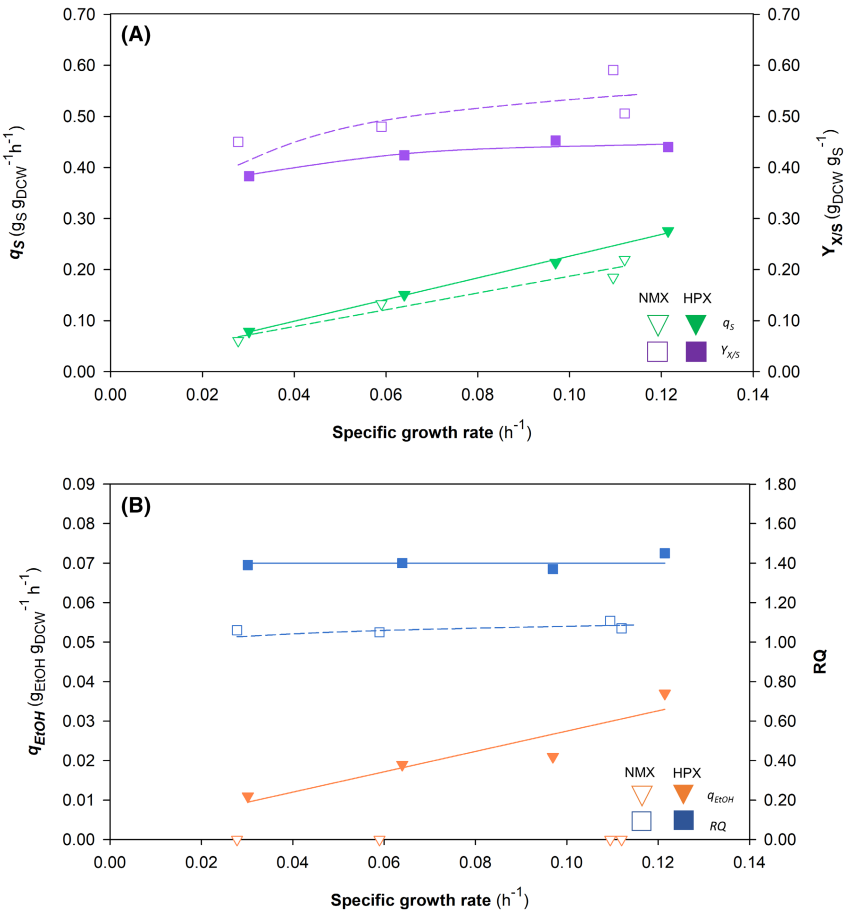

FIGURE 3 Main physiological parameters for fed-batch cultures under hypoxic and normoxic conditions at four different specific growth rates. (A) Specific glucose uptake rate and overall biomass-to-substrate yield. (B) Specific ethanol generation rate and respiratory quotient.

oxygen conditions, increasing its value as μ increases (Khasa et al., 2007). These results are consistent with previous studies focused on growth-coupled promoters and represent the linear kinetic function described by *Luedeking-Piret* (Barrigon et al., 2015; Garcia-Ortega et al., 2016). This production increase also correlates with the higher relative transcription levels observed at higher μ, when greater amounts of glycolytic enzymes are required (Nieto-Taype et al., 2020). In synergy with the μ effect, for hypoxic conditions, Gasset et al. (2022) described an additional boosting effect of oxygen-limiting conditions on the upregulation of P_{GAP}-driven transcription levels. Interestingly, this relative transcription level (RTL) increase can also be obtained with higher target gene dosages. However, the effect is not always synergistic and might eventually be limited when simultaneously applying different factors to boost transcription levels. Considering the results achieved, the optimal μ value for production purposes should be as close as possible to μ_{max}. Nevertheless, in this work we demonstrated that μ_{max} for cultures grown under hypoxic conditions is significantly lower than under normoxic fermentations; therefore, to identify this corner case scenario, it is essential to work below this value.

The main production and physiological parameters obtained in the fed-batch cultures performed under both hypoxic and normoxic conditions are summarised in Table 1. Productivities, yields and product titres are detailed as key performance indicators (KPI) along the different specific growth rates assessed.

As reported in previous figures, q_S increases linearly with μ under hypoxic conditions due to the metabolic shift towards fermentative metabolism. This linear increase was also observed in the main productive parameter, q_P. Fermentative metabolism carries higher glucose uptake rates, which translates to greater metabolic flux on the glycolysis pathway. Accordingly, the expression of glycolytic enzymes is significantly upregulated (Baumann et al., 2010). Since the recombinant product of interest is under the expression control of the *GAP* promoter — the native *TDH3* gene promoter involved in a key glycolysis step—the Crl1 production increment correlates with the q_S increase. In this study, for all the μ tested, was observed a twofold increase of q_p for Crl1 production when implementing oxygen-limiting conditions (hypoxia) in comparison with equivalent cultures that were carried out in non-limiting oxygen conditions (normoxia). This result is consistent with previous studies using the same model protein (Gasset et al., 2022). These results align with earlier studies involving the expression of different recombinant proteins such as an antibody fragment (Fab) (Garcia-Ortega et al., 2017). This enhancement of

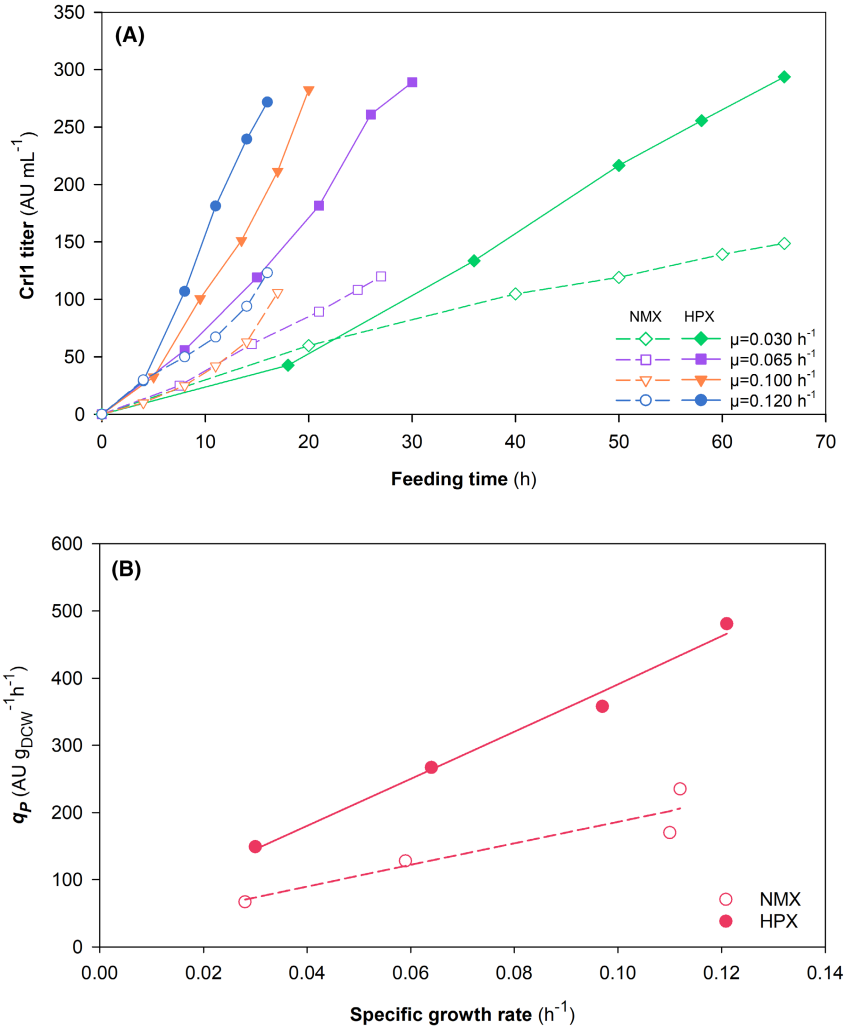

FIGURE 4 Crl1 production parameters for the fed-batch phase under hypoxic and normoxic conditions at four different specific growth rate set-points. (A) Crl1 titre production. (B) Specific production rate.

TABLE 1 Main bioprocess parameters obtained at different specific growth rates (μ) in fed-batch cultures under normoxic (NMX) and hypoxic (HPX) conditions. Lipolytic activity was determined using a colorimetric activity assay based on the use of p-nitrophenyl butyrate (PNPB) as substrate.

	0.030			0.065			0.100			0.120		
μ set-point	NMX	HPX	x-fold	NMX	HPX	x-fold	NMX	HPX	x-fold	NMX	HPX	x-fold
Experimental μ (h^{-1})	0.028	0.030		0.059	0.064		0.107	0.097		0.115	0.121	
q_S ($g_S\ g_{DCW}^{-1}\ h^{-1}$)	0.06	0.08	1.3	0.13	0.15	1.1	0.18	0.21	1.2	0.22	0.28	1.3
q_P (AU $g_{DCW}^{-1}\ h^{-1}$)	67	144	2.1	128	267	2.1	164	358	2.2	235	481	2.0
q_{EtOH} ($g_{EtOH}\ g_{DCW}^{-1}\ h^{-1}$)	–	0.011		–	0.019		–	0.021		–	0.037	
RQ	1.06	1.39		1.05	1.40		1.11	1.37		1.07	1.46	1.4
$Y_{P/X}$ (kAU g_x^{-1})	2.39	4.80	2.0	2.17	4.17	1.9	1.53	3.69	2.4	2.04	3.97	1.9
$Y_{X/S}$ ($g_{DCW}\ g_S^{-1}$)	0.45	0.38	0.8	0.48	0.42	0.9	0.59	0.45	0.8	0.51	0.44	0.8
Q_V (kAU $L^{-1}\ h^{-1}$)	2.5	4.8	2.0	5.1	10.7	2.1	7.7	15.3	2.0	9.0	17.6	1.97
Final Crl1 titre (AU mL^{-1})	187	352	1.9	173	358	2.1	179	331	1.8	168	297	1.8

q_p is also maintained in terms of volumetric productivity (Q_V). This outcome is realistic considering that the volumes and biomass concentrations at the end of each experiment are similar. The metabolic shift from respiratory to respiro-fermentative metabolism has other implications regarding physiological parameters; while *RQ* is increased under oxygen-limiting conditions, $Y_{X/S}$ presents a decrease in comparison with oxygen-excess conditions. The higher specific production rates for CO_2 (q_{CO2}) and fermentative

by-products (mostly ethanol but also succinic acid and arabitol) support these findings and are reliable based on previous research (Baumann et al., 2010). Remarkably, final product titre and overall product-to-biomass yield ($Y_{P/X}$) are two time-independent parameters focused on production, and it can be established that they have similar values in all the experiments grown under hypoxic as well as normoxic conditions. Comparable to q_P and Q_V, in the case of oxygen-limiting conditions, both $Y_{P/X}$ and the final Crl1 titre are increased by up to twofold when compared with normoxic conditions.

To contextualise the productivity outcomes of this work, a comparative analysis of the specific production rates (q_P) achieved in the present study using the constitutive *GAP* promoter has been compared with previously published works of our research group based on the use of the strongly inducible *AOX1* promoter, traditionally the most utilised in *Pichia* bioprocesses (Garrigós-Martínez et al., 2019).

To assure a fair comparison, numerous details have been taken into consideration. The results are obtained using the same operational mode (fed-batch), based on the same platform strain (X33), and producing the same recombinant protein of interest using the same DNA coding sequence, and same gene dosage (single copy integration) but with the different gene expression regulation since the promoter used is different. Accordingly, the results presented by Garrigós-Martínez et al. (2019) offer a valuable comparative framework for assessing production parameters.

The study's analysis reveals that q_P values, obtained at different specific growth rates in fed-batch cultures utilising P_{AOX1}, exhibit q_P values in the same range that the obtained in the hypoxic fermentations presented in the current study (up to 460 AU $g_X^{-1} h^{-1}$ for P_{AOX1}-driven culture at the optimal μ tested vs 481 AU $g_X^{-1} h^{-1}$ for P_{GAP} hypoxic fermentations at the optimal μ tested). In terms of volumetric productivity (Q_P), the values are also notably higher for the P_{GAP}-based system (17.6 AU $L^{-1} h^{-1}$ vs. 10.9 AU $L^{-1} h^{-1}$).

Accordingly, the enhanced production levels achieved through oxygen limitation conditions have been shown to yield specific production rates comparable to the reference methanol-based expression system, regulated by the inducible P_{AOX1}. These findings are significant for the *Pichia* community since this operational methanol-free strategy becomes an efficient alternative to traditional methanol-based processes, which often come with higher operational costs and numerous drawbacks for large-scale production.

Additional model enzymes and Crl1 production change of scale

In addition to the production boost observed for Crl1 described in the previous sections, the positive impact of hypoxic culture conditions was also successfully evaluated by expressing two additional recombinant lipases of industrial interest: proRol and the widely used CalB. The producer clones' performance was tested by implementing the same strategy as detailed in the previous section, with μ set at 0.065 h^{-1} and compared under both normoxic and hypoxic conditions. The results of the key process parameters obtained in these fermentations are shown in Table 2. Similar growth parameters and biomass yields were obtained among the compared strains, under both normoxic and hypoxic conditions, suggesting that equivalent physiological stress levels were achieved in all cases compared. However, the impact of oxygen limitation on *RPP* varied between reporter proteins.

The proRol-producing strain exhibited the least significant effect on the specific product formation rate (2.0-fold higher) compared with the 2.2- and 2.6-fold increases observed for Crl1 and CalB, respectively, suggesting that the expression increment achieved is protein-specific. Previous studies reported that overexpression of recombinant Rol negatively affects cell growth and fitness (Amara et al., 2016; Resina et al., 2007). Variations in the final concentrations of

TABLE 2 Main bioprocess parameters obtained with *K. phaffii* clones expressing different recombinant lipases (Crl1, CalB and proRol) in fed-batch at a specific growth rate (μ) of 0.065 h^{-1}, under normoxic (NMX) and hypoxic (HPX) conditions. Lipolytic activity was determined using a colorimetric activity assay based on the use of p-nitrophenyl butyrate (PNPB) as substrate.

	Crl1			CalB			proRol			Crl1 scale-up		
rLipase	**NMX**	**HPX**	**x-fold**	**NMX**	**HPX**	**x-fold**	**NMX**	**HPX**	**x-fold**	**NMX**	**HPX**	**x-fold**
Experimental μ (h^{-1})	0.059	0.064		0.065	0.070		0.065	0.068		0.066	0.062	
q_S ($g_S\ g_{DCW}^{-1} h^{-1}$)	0.13	0.15	1.2	0.12	0.16	1.3	0.12	0.16	1.2	0.11	0.14	1.3
q_P (AU $g_{DCW}^{-1} h^{-1}$)	128	267	2.1	4.15	10.8	2.6	8.81	17.5	2.0	84	198	2.3
q_{EtOH} ($g_{EtOH}\ g_{DCW}^{-1} h^{-1}$)	–	0.019		–	0.020		–	0.021		–	0.027	
RQ	1.05	1.40		1.01	1.35		1.03	1.37		1.10	1.45	
$Y_{X/S}$ ($g_{DCW}\ g_S^{-1}$)	0.48	0.42	0.9	0.54	0.46	0.8	0.52	0.42	0.8	0.58	0.43	0.7

each lipase were observed at the end of the fermentations, indicating that bioprocess efficiency is indeed protein-specific. For a qualitative comparison of these results, please refer to the SDS-PAGE gel presented in the Figure S1.

The protein-specific variability is also corroborated by the total protein results analysed using the Bradford assay for total protein determination. Variations in the total protein concentrations were observed along the different fermentations expressing the three distinct recombinant lipases.

As the most relevant results, the processes performed at $\mu = 0.065\,h^{-1}$ producing Crl1 and CalB yielded total protein concentrations of 0.17 and 0.35 $g{\cdot}L^{-1}$, respectively, under non-limiting oxygen conditions. In contrast, under hypoxic conditions, the same recombinant proteins resulted in total protein concentrations of 0.33 $g{\cdot}L^{-1}$ for Crl1 and 0.43 $g{\cdot}L^{-1}$ for CalB. These results are coherent with the increase of enzymatic activity determined by the specific analysis included in the previous section, as well as with the SDS-PAGE presented as Figure S1.

Furthermore, based on the SDS-PAGE results, it could be observed that the increase in total protein is mostly due to the overexpression of the target protein since the native host cell proteins do not present relevant differences between normoxic and hypoxic conditions.

Additionally, as an alternative approach to validate the boost achieved in Crl1 production by implementing the hypoxic-based strategy described, we also scaled up to a 50 L pilot-scale system of the normoxic and hypoxic processes at the target $\mu = 0.065\,h^{-1}$. *RQ* was also selected as the transferable operating parameter in this new system to scale up the process in a system with different oxygen-transfer capacities (Garcia-Ortega et al., 2017). The key results of the aforementioned fermentations are also included in Table 2. After comparison with the lab-scale results obtained for Crl1 production, similar values for all key parameters, for example, μ, q_S, q_P, $Y_{X/S}$ *and* q_{EtOH}, were obtained, indicating that equivalent culture conditions could be achieved in a system at a different scale. Thus, this could be considered a successful physiology-based scale-up, which was one of the project's aims. According to lab-scale results, a 2.2-fold increase in q_P was observed when applying hypoxic conditions, and a 20% reduction in $Y_{X/S}$ under hypoxic conditions was also confirmed. Furthermore, ethanol (17 $g{\cdot}L^{-1}$) and low concentrations of around 1 $g{\cdot}L^{-1}$ of arabitol and succinate were detected in the culture broth. Interestingly, in the lab-scale results, of all the metabolites only ethanol accumulated in the $\mu = 0.065\,h^{-1}$ hypoxic fermentation, whereas arabitol and succinate were only detected at higher μ ($\mu = 0.100\,h^{-1}$ and $\mu = 0.120\,h^{-1}$). This may suggest a lower accuracy of the non-automated *RQ* control with repeated manual adjustments of the stirring rate since *RQ* fluctuations could also be related to the formation of greater amounts of by-products (Gasset et al., 2022).

The scale-up methodology carried out in this work relies on a non-invasive exhaust gas analysis that is less affected by eventual substrate heterogeneities or oxygen gradients within the fermenter, which is often described in large-scale fermenters. This feature makes it suitable for large-scale bioprocesses and easier to implement in existing production biosystems. Providing an innovative physiology-based scale-up criterion contributes to resolving the lack of universally defined criteria for microbial fermentation scaling up in the industrial field (De Brabander et al., 2023; García-Ortega et al., 2019; Takors, 2012). Future research should involve testing this strategy with different strains and proteins to validate its efficiency. Additionally, exploring alternative glycolytic promoters may yield similar improvements when applied under hypoxic conditions.

CONCLUSIONS

From the results obtained, it can be concluded that synergic hypoxic conditions and specific growth rates have a relevant impact on both the physiological and production parameters of P_{GAP}-based *K. phaffii RPP* processes. The results provide insights into optimising conditions for improved productivity and understanding the metabolic shifts associated with oxygen availability and its impact on glycolytic gene expression upregulation.

Furthermore, an automated control system to maintain the desired oxygen-limiting conditions was successfully implemented. The system effectively kept the targeted *RQ* within a narrow band throughout the bioprocesses without significant deviations. This represents an innovative control strategy with high accuracy and precision based on the variation of the stirring rate and its effect on oxygen transfer, particularly when applied in dynamic systems such as fed-batch cultures.

To conclude, this strategy can be widely applied to produce recombinant proteins with reproducible results, making the *K. phaffii* P_{GAP}-based system more efficient and competitive. These findings highlight the potential of the control strategy implemented for improving bioprocess efficiency and productivity when applying hypoxic conditions, especially considering the results obtained with different clones expressing different recombinant lipases (Crl1, CalB and proRol) under normoxic and hypoxic conditions. For all the clones evaluated, the improvement in bioprocess efficiency and productivity was around twofold. This increment was also successfully achieved in a scale-up demonstration test in a 50 L Crl1 production bioprocess.

Finally, it has been demonstrated that controlled cellular stress, in this case hypoxia which relies on a non-invasive measurement methodology, can be applied in an *RPP* process to enhance its efficiency. The potential of this new generation control strategy focused on physiological parameters has been proven and could be used for future transferable and scale-up criteria.

AUTHOR CONTRIBUTIONS

Albert Sales-Vallverdú: Data curation (equal); formal analysis (equal); investigation (lead); methodology (lead); software (equal); visualization (equal); writing – original draft (lead); writing – review and editing (equal). **Arnau Gasset:** Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); software (equal); writing – original draft (equal); writing – review and editing (equal). **Guillermo Requena-Moreno:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal). **Francisco Valero:** Conceptualization (equal); funding acquisition (lead); project administration (lead); resources (lead); supervision (equal); validation (equal); visualization (equal); writing – review and editing (equal). **José Luis Montesinos-Seguí:** Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); software (equal); supervision (equal); validation (equal); visualization (equal); writing – review and editing (equal). **Xavier Garcia-Ortega:** Conceptualization (equal); funding acquisition (equal); investigation (equal); project administration (equal); resources (equal); supervision (lead); validation (lead); visualization (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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