Process Biochemistry 129 (2023) 221–229

Contents lists available at ScienceDirect

Process Biochemistry

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

Monitoring biotechnological processes through quantitative image analysis: Application to 2-phenylethanol production by *Yarrowia lipolytica*

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ARTICLE INFO

Keywords:
2-phenylethanol monitoring
Quantitative image analysis (QIA)
Multilinear regression (MLR)
Yarrowia lipolytica
Yeast and cluster morphology

ABSTRACT

Quantitative image analysis (QIA) is a simple and automated tool for process monitoring that, when combined with chemometric techniques, enables the association of changes in microbiota morphology to various operational parameters. To that effect, principal component analysis, multilinear regression, and ordinary least squares methods were applied to the obtained dataset of the biotransformation conditions for *Y. lipolytica* through the monitor of yeast morphology, substrates (glycerol, L-phenylalanine - L-Phe) consumption and metabolites (2-phenylethanol – 2-PE) production was developed. Glycerol and L-Phe were successfully monitored by the proposed approach, though with a lower monitoring ability for 2-PE, and mostly related to yeast and cluster size and proportion, yeasts contents and cluster morphology. The chemometric approach also allowed to identify significant morphological modifications related with the change in the stirring speed in the experiments at 600 rpm, 600/400 rpm (600 rpm for 24 h, and 400 rpm until the end of the experiment) and in pH from 5.5 to 7.5. This work demonstrated, for the first time, that QIA combined with chemometric analysis can be considered a valuable tool to monitor biotechnological processes, namely the 2-PE production by *Y. lipolytica*, by analyzing yeast and cluster morphology.

1. Introduction

The establishment of the optimal conditions for attaining high yields and productivity is a frequent goal of biotechnological processes. This aim is typically translated into the challenge of determining the best control method for achieving the desired result. In every scenario, the goal is to offer the right environment conditions for the growth and/or maintenance of the microorganism and the simultaneous production of the desired metabolites [1]. An online control of growth and morphology is, therefore, an important requirement to achieve an optimal performance.

Microscopy inspection is commonly used for cell analysis since the microbial cell morphology can provide information about its overall and metabolic state. Moreover, the process productivity in a microbial culture is strongly related with the cells' physiology. Hence, the relationship between morphological characteristics and metabolic activity can be considered fundamental in biotechnological processes, helping for a better understanding of the system under study [2]. This relationship opens the door to employ the systematic survey of the microbial cell morphology as a means to monitor metabolic activity. In addition, monitoring the metabolites production in biotechnological processes is usually performed through laborious and time consuming off-line chemical analysis including high-performance liquid chromatography (HPLC) [3,4]. Thus, there is a clear need to develop alternative and green techniques to promptly monitor these processes without the use of chemicals and/or prior treatment of samples.

Quantitative image analysis (QIA) procedures have the potential to be a non-invasive and valuable tool for the morphological characterization of microbial cells, simplifying the evaluation of important biotechnological processes [5–8]. This technique has been successfully

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https://doi.org/10.1016/j.procbio.2023.03.019
Received 31 January 2023; Received in revised form 12 March 2023; Accepted 15 March 2023
Available online 20 March 2023

applied to the morphological characterization of *Yarrowia lipolytica* cells [9]. In addition, Braga et al. [10] showed that pneumatic agitation causes less impact in the cells' morphology than mechanical forces. Using QIA procedures, the dimorphic growth in different culture media, and the quantification and characterization of *Y. lipolytica* cells into different morphotypes, ranging from yeast (oval) single cells to (filamentous) hyphae cells has already been successfully accomplished [6]. Size, content, and morphological parameters may, therefore, be used to infer the state of the cell, its metabolic activity, and viability [11].

The large amount of data provided by QIA requires the use of chemometric techniques to evaluate and extract the most relevant information. Principal component analysis (PCA), multilinear regression (MLR), partial least squares (PLS), and ordinary least squares (OLS) methods have been recently applied in biotechnological processes to discriminate different operational phases and to predict a set of parameters and compounds [6–8,12–14]. However, up to date, the use of QIA coupled to chemometric techniques has never been applied to evaluate the biotransformation conditions and to the assessment of different metabolites during the production of 2-phenylethanol (2-PE) by *Y. lipolytica*.

Thus, this article describes the application of a QIA based methodology to the production of the rose-like aroma 2-PE by *Y. lipolytica*. This is a non-conventional yeast able to produce different metabolites widely used in the food and cosmetics industries, including flavor and fragrance compounds [15,16]. This microorganism presents a great potential for producing the rose-honey like aroma 2-PE due to several advantageous features, including its Generally Recognized as Safe (GRAS) status, outstanding performance in the use of various raw materials to grow and the fact that it has a Crabtree negative trait and does not produce ethanol, all of which are very important in the production of 2-PE [17–20].

The goal of this work is to demonstrate the feasibility of employing QIA, combined with chemometric analysis, to improve biotechnological processes monitoring, focusing on the assessment of essential metabolites, in a more cost-effective and eco-friendly procedure. This, in turn, will provide for a timely identification of deviations in the fermentation process and a faster response towards a more effective control strategy.

2. Materials and methods

2.1. Microorganism, media and culture conditions

The strain used in this work was *Y. lipolytica* CH1/5 (isolated from cheeses [21,22]. Bioconversion experiments were carried out in a 3.7 L bioreactor (RALF PLUS SOLO, Bioengineering) with a diameter of 17 cm and a height of 31 cm.

Cells were pre-cultivated as previously described in [17], and further used to inoculate 1.1 L of bioconversion medium (40 g L^{-1} of crude glycerol, 15 g L^{-1} of KH_2PO_4, 0.5 g L^{-1} of $MgSO_4{\cdot}7H_2O$, 0.02 g L^{-1} of Yeast Nitrogen Base (YNB) without amino acids, and 3 mg L^{-1} of thiamine, supplemented with 4 g L^{-1} of L-Phe), with an optical density at 600 nm of 0.5 [17].

Biotransformation experiments were performed varying the pH (5.5, 6.5, 7.5 and without pH control), air flowrate (1 L min^{-1} and 3 L min^{-1}), and stirring speed (600 rpm, 900 rpm, and 600 rpm for 24 h, and 400 rpm until the end of the experiment (600/400 rpm)). The medium pH was automatically controlled by adding potassium hydroxide (2 mol L^{-1}) or hydrochloric acid (4 mol L^{-1}), using a Peripex peristaltic pump (Bioengineering AG, Wald, Switzerland). The crude glycerol was supplied by Prio Energy (Prio Biocombustíveis, S.A., Gafanha da Nazaré, Portugal) with the following composition (w/w), according to the supplier: 82% glycerol, 11.8% H_2O, 4.7% NaCl, less than 0.01% methanol, 0.5% organic matter (other than glycerol) and less that 1% of other impurities.

A total of 13 different bioconversion experiments were performed, according to the conditions presented in Table 2. Experiments 1, 2, and 3 were run without pH control. Experiment pairs 4 and 10, 5 and 8, 6 and 7, and 9 and 11 represent two runs performed for the corresponding conditions. L-Phe was further fed in experiments 12 and 13 at the run times identified in Table 2. Samples were taken at 0 h, 9 h, 24 h, 29 h, 33 h, 48 h, 53 h, 57 h, 72 h, 77 h, and 81 h for all experiments. In experiments 1 and 2, samples were also taken at 96 h and 99 h.

2.2. Analytical methods

Samples were collected throughout the bioconversion experiments to determine glycerol and L-Phe consumption as well as 2-PE synthesis. HPLC was used to determine the concentration of glycerol in a Jasco (Jasco Corporation, Tokyo, Japan) system associated with an RI-2031 detector [17]. The concentrations of 2-PE and L-Phe were determined in accordance with Braga et al. [17].

2.3. Image acquisition and QIA procedure

Samples were also collected throughout the bioconversion experiments for the QIA based *Y. lipolytica* morphological characterization in bright-field microscopy. The image visualization and acquisition procedure were performed by the use of an Olympus BX-51 microscope (Olympus, Shinjuku, Japan) and an Olympus DP 71 camera (Olympus, Shinjuku, Japan), is described in detail in Braga et al. [5 n]. The yeast cells, cell aggregates (clusters) and hyphae morphological descriptors were determined using a QIA procedure developed in Matlab R2022a (The Mathworks, Inc., Natick) language. The image pre-processing, segmentation, hyphae and yeast cells recognition, and image post processing steps are the main stages of the QIA program [5,24].

The image pre-processing step focus on background detection and removal. To reduce disparities in spatial illumination, the original image is first divided by a background image. Using a succession of closing and opening morphological operations, the background-corrected picture is further improved to increase the contrast of the cell borders. Next, yeast, hyphae and aggregates, are identified from the background, by segmentation using a predetermined 0.9 threshold value. Small holes inside the cells binary images are then filled. The hyphae morphotype is then established using a combined width and gyration radius technique [23] by a series of erosion and dilation morphological operations. The remaining objects, if above 3.5 μm in diameter, are classified as individual cells or cell aggregates. After elimination of debris cut off by the image boundaries, the resulting binary images are saved. These images were, finally, employed in the image analysis stage in order to describe the morphotypes' contents and the most pertinent size and morphological characteristics.

Size (in equivalent diameter, Deq) was used to identify individual ($<$ 6 μm) and aggregated ($>$ 6 μm) yeasts, whereas width ($<$ 9 μm) and gyration radius ($>$ 1) identified hyphae cells. The descriptors employed for the hyphae, yeast and cell aggregates morphotypes, are described in Table 1.

2.4. Multivariate statistical analysis

In biotechnological processes the use of multivariable statistical analysis is fundamental for enlightening interrelationships in the collected data, establishing dependencies and assess statistical significances, among others.

In the present case, an ordinary least squares (OLS) analysis was first performed for each collected QIA based parameter (presented in Table 3) regarding all studied compounds (glycerol, L-Phe, and 2-PE) concentrations over time, to identify the potentially meaningful parameters for monitoring purposes. OLS is a least squares technique used in linear regression models to predict a dependent (Y) variable from a set of independent (X) variables, by minimizing the sum of the residuals (differences between real and predicted Y values) squares [25]. In the present case, solely the QIA based parameters presenting a p-value

Table 1
Identification of the bioconversion experiments conditions.

Experiment	Stirring speed (rpm)		Air flowrate (L min^{-1})	pH	Run number
	(0–24 h)	(24 h - end)			
1	600	600	3	uncontrolled	–
2	900	900	3	uncontrolled	–
3	600	600	1	uncontrolled	–
4	600	600	1	6.5	1
5	600	600	1	7.5	1
6	600	600	1	5.5	1
7	600	600	1	5.5	2
8	600	600	1	7.5	2
9	600	400	1	5.5	1
10	600	600	1	6.5	2
11	600	400	1	5.5	2
12[a]	600	600	1	5.5	–
13 * *	600	600	1	5.5	–

[a] L-Phe addition at 24 h and 81 h; * * L-Phe addition at 24 h and 48 h and addition of 20 g L^{-1}Gly at 48 h

below 0.05 were considered to be statistically significant for monitoring purposes. Matlab R2022a (The Mathworks, Natick, USA) was employed for the OLS analysis.

A stepwise multilinear regression (MLR) analysis, encompassing interactions and up to quadratic terms, was also performed to predict the monitored compounds concentration values in the performed experiments. The overall dataset was divided into training (67% of the data points) and validation (33%) sets. The resulting models allowed to further determine the most influential QIA parameters for monitoring purposes. MLR analysis is a linear regression technique commonly used to predict dependent variables from a set of independent (predictor or influential) variables [26]. OLS was employed to determine the response variables coefficients in MLR. Matlab R2022a (The Mathworks, Natick, USA) was employed for the MLR analysis.

Concomitantly to the MLR analysis, a cross-correlation (CC) analysis between all the QIA based parameters was conducted, in order to assess possible interrelationships and further enlighten some results obtained in the MLR analysis. Cross-correlation analysis allows determining correlations between all pairs of variables in a given data set [22]. Cross-correlation values equal to, or above, 0.5 were set to select possible correlations. Microsoft Excel (Microsoft, Redmond, USA) was used for the cross-correlation analysis.

A Mann-Whitney (MW) test was performed for pairwise analysis concerning the differences on the studied compounds and QIA parameters due to pH (5.5, 6.5 and 7.5) and stirring speed (600, 600/400, 900 and 600 rpm-OT (OT referring to the conditions of experiments 12 and 13)). This test allows surveying for significant differences in the median values of two sets of samples. Samples were considered not significantly different from each other for a level of statistical significance (p-value) greater than 0.05, and significantly different otherwise. Matlab R2022a was employed for the MW analysis.

A principal components analysis (PCA) was also performed to analyze, in an ensemble manner, the data obtained during the different bioconversion experiments. PCA minimizes a set of highly connected, high-dimensional data by identifying the most important information about the new spaces created by the orthogonal vectors (principal components) describing a linear combination of the original variables (with each new PC possessing a lower explanatory ability than the preceding) [22]. Matlab R2022a was used for the PCA analysis.

3. Results and discussion

3.1. Ordinary least squares analysis

The OLS analysis was performed both in the ensemble of the results for each studied QIA parameter and compound (resulting in global p-values), as well as for each individual experiment. In the latter case, average p-values were obtained for each parameter and compound. The obtained results, for the QIA parameters found to be potentially most meaningful (lower p-values) for monitoring purposes, are presented in Table 3.

Considering the results, it could be found that both glycerol and L-Phe concentrations correlate negatively with the overall yeast contents, namely total area of aggregated and individual yeasts per volume (*TA/Vol*) and total number of both individual (*Nb/Vol(Y)*) and aggregated (cluster) (*Nb/Vol(Ag)*) yeasts per volume, as well as with the proportion of individual yeasts, namely area *(%Area(Y))* and number *(%Nb(Y))* percentage of individual yeasts. On the contrary, both glycerol and L-Phe concentrations correlate positively with the individual yeasts size, namely perimeter (*Per(Y)*), equivalent diameter (*Deq(Y)*) and length

Table 2
QIA descriptors obtained from samples. Detailed definition on each parameter can be found in Amaral [20].

Parameter	Descriptors	Formula
TL/Vol (mm/µL)	Total hyphae length per volume	$L = N_{Thn} + N_{Int} \times 1.1222 \times F_{Cal}$
TA/Vol (mm^2/µL)	Total area of aggregated and individual yeasts per volume	n.a.
TL/TA (mm/mm^2)	Total hyphae length per total area of yeast	$TL/TA = \frac{TL}{TA}$
D_{eq} (µm)	Yeast equivalent diameter	$D_{eq} = 2F_{Cal}\sqrt{\frac{A}{\pi}}$
Per (µm)	Yeast perimeter	$P = N_{Per} \times 1.1222 \times F_{Cal}$
Length (µm)	Yeast length	$Length = F_{\max} \times F_{Cal}$
Width (µm)	Yeast width	$Width = F_{\min} \times F_{Cal}$
FF	Yeast form factor	$FF = \frac{P^2}{4\pi A}$
Conv	Yeast convexity	$Conv = \frac{P_{Conv}}{P}$
Comp	Yeast compactness	$Comp = \frac{\sqrt{\frac{4}{\pi}A}}{F_{\max}}$
Round	Yeast roundness	$Round = \frac{4\pi A}{P_{Conv}{}^2}$
Sol	Yeast solidity	$Sol = \frac{A}{A_{Conv}}$
Ext	Yeast extent	$Ext = \frac{A}{W_{BB} \times L_{BB}}$
Ecc	Yeast eccentricity	$Ecc = \frac{(4\pi^2)(M_{2x} - M_{2Y})^2 + 4M_{2XY}{}^2}{A^2}$
Rob	Yeast robustness	$Rob = \frac{2er_{obj}}{\sqrt{A}}$
LrgC	Yeast largest concavity	$LrgC = \frac{2er_{comp}}{\sqrt{A}}$
RelArea	Yeast ratio between hole and object area	$RelArea = \frac{A_h}{A}$
Nb/Vol	Total number of yeasts per volume	n.a.
% Nb	Number percentage of individual / aggregated yeasts	$\%Nb = \frac{\sum_{i=1}^{Nclass} Nb_i}{T_{Nb}}$
% Area	Area percentage of individual / aggregated yeasts	$\%A = \frac{\sum_{i=1}^{Nclass} A_i}{TA}$

n.a.: not applicable; L is the hyphae length; NThn is the pixel sum of each thinned hyphae; NInt is the number of hyphae intersections; 1.1222 is used in order to homogenize the different angles of hyphae; FCal is the calibration factor (µm per pixel); NPer is the pixel sum of the yeast boundary; P is the yeast perimeter; Fmax is the yeast maximum Feret Diameter; Fmin is the yeast minimum Feret Diameter; Nobj is the yeast pixel sum; A is the yeast area; PConv is the yeast Convex Envelope perimeter; AConv is the yeast Convex Envelope area; WBB is the yeast bounding box width; LBB is the yeast bounding box length; M2X and M2Y are the yeast central second moments with respect to x-axis and y-axis respectively; M2XY is the yeast second horizontal and vertical order moment; erobj is the number of erosions needed to delete the yeast; ercomp is the number of erosions needed to delete the complement of the yeast in relation to its convex envelope; Ah is the yeast holes area; TNb is the total number of yeasts.

Table 3
Obtained p-values for potentially meaningful QIA parameters regarding the overall (global) and individual (average) analyses.

	Glycerol (g/L)			*2-PE (g/L)*			*L-Phe (g/L)*		
	Average	Global	Influence	Average	Global	Influence	Average	Global	Influence
TA/Vol (Y+Ag)	1.12E-02	7.19E-22	(–)	1.08E-01	9.22E-09	(+)	4.23E-02	1.57E-13	(–)
Deq (Y)	3.17E-01	7.39E-02	(+)	3.82E-01	2.35E-01	(–)	1.87E-01	2.63E-07	(+)
Per (Y)	1.21E-01	7.78E-11	(+)	1.59E-01	2.79E-06	(–)	9.42E-02	3.87E-11	(+)
Length (Y)	9.32E-02	6.82E-22	(+)	1.38E-01	1.84E-11	(–)	2.88E-01	1.83E-08	(+)
% Nb (Y)	1.42E-01	1.62E-06	(–)	2.05E-01	1.41E-05	(+)	5.66E-02	1.31E-17	(–)
% Area (Y)	1.56E-01	4.50E-06	(–)	2.27E-01	1.88E-05	(+)	5.94E-02	4.82E-16	(–)
Nb/Vol (Y)	1.23E-02	3.64E-22	(–)	9.68E-02	2.81E-09	(+)	3.72E-02	7.31E-14	(–)
Conv (Ag)	1.84E-01	1.52E-09	(+)	2.20E-01	9.52E-05	(–)	1.69E-01	8.27E-12	(+)
Rob (Ag)	2.10E-01	6.18E-10	(+)	2.49E-01	0.000139	(–)	1.97E-01	2.82E-14	(+)
Nb/Vol (Ag)	5.74E-02	1.45E-11	(–)	2.71E-01	0.000313	(+)	1.37E-01	4.84E-08	(–)

Y – Individual yeasts; Ag – Aggregated yeasts.

(*Length(Y)*), and with the cluster morphology, namely convexity (*Conv (Ag)*) and/or robustness (*Rob(Ag)*).

Regarding the 2-PE concentration, it was found to correlate positively with the overall yeast contents, namely total area of aggregated and individual yeasts per volume (*TA/Vol*) and total number of both individual (*Nb/Vol(Y)*) and aggregated (*Nb/Vol(Ag)*) yeasts per volume, as well as with the proportion of individual yeasts, namely area *(%Area (Y)*) and number *(%Nb(Y)*) percentage of individual yeasts. On the contrary, 2-PE concentration correlate negatively with the individual yeasts size, namely perimeter (*Per(Y)*) and length (*Length(Y)*), and with the cluster morphology, namely convexity (*Conv(Ag)*).

Thus, it seems licit to conclude that the decrease of both the glycerol and L-Phe, as well as the increase of 2-PE concentrations, within the experiments run time is closely correlated with an increase in the overall yeast contents in each experiment, as expected given that larger quantities of a given biomass, *ceteris paribus*, should lead to higher substrate consumption and product formation, as well as with a larger percentage of individual yeasts. On the other hand, it seems that larger yeast size

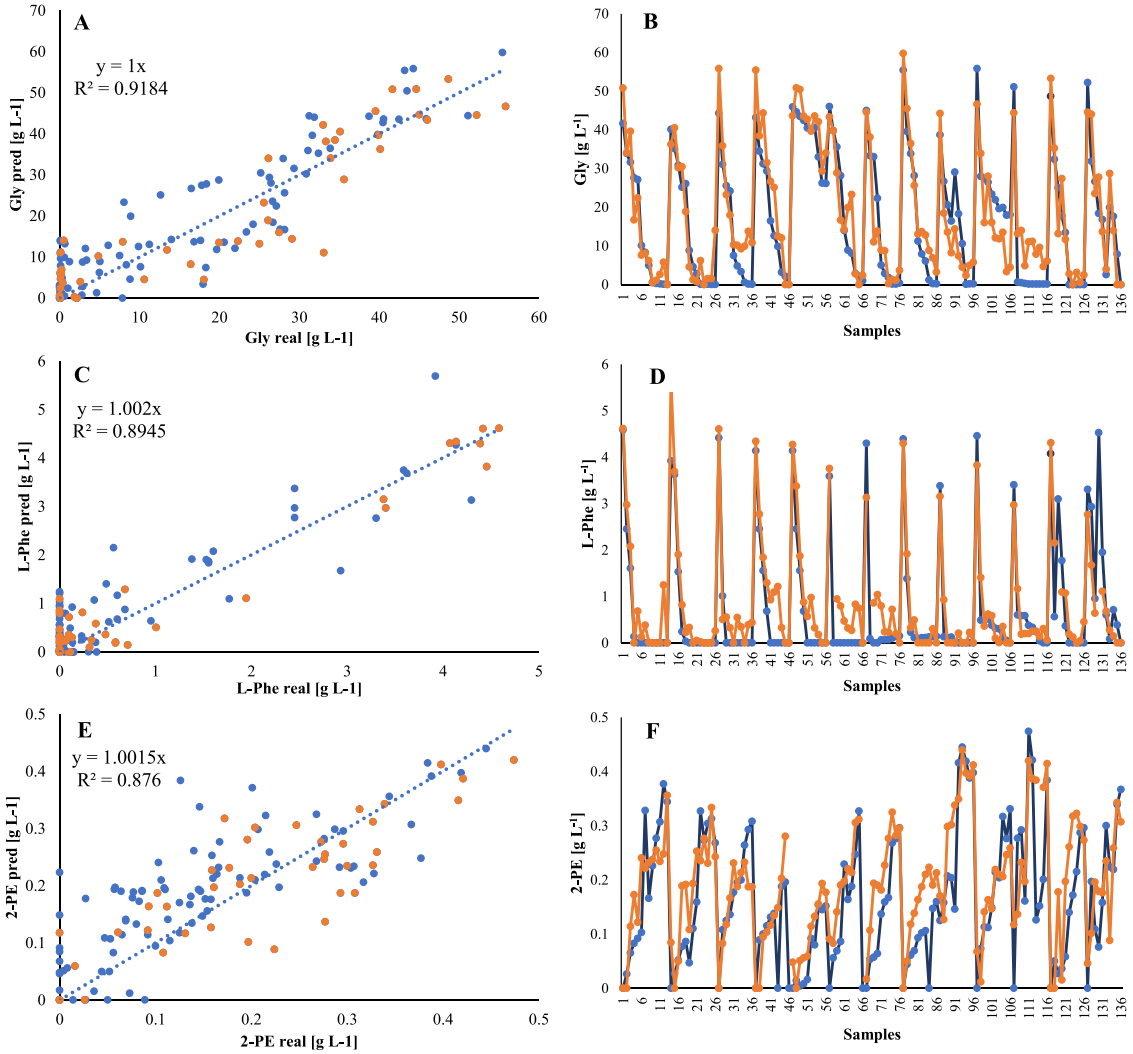

Fig. 1. Real (blue line) and predicted (orange line) concentrations values for glycerol (A, B), L-Phe (C, D) and 2-PE (E, F).

(especially length wise), and more regular clusters, act towards a slower decrease of both glycerol and L-Phe, and a slower increase of 2-PE, throughout the experiments. More regular clusters present a reduced surface area per volume, *i.e.* the specific area available for substrate uptake is smaller, which can lead to decreased substrate uptake, and hence product formation, for similar biomass contents. On the other hand, the morphological yeast changes towards a more hyphal-like cell (though still not considered as hyphae) seem to imply slower rates of substrate consumption and product formation. According to Soong et al. [27], under a nutrient-rich environment, *Y. lipolytica* cells grow in yeast-like form, whereas under stressful conditions (including pH and aeration changes) the cell morphology tend to be elongated (more hyphae-like). Hence, the observed morphological change, as a result of more stressful conditions, can be expected to be related to a decrease in cell metabolism.

glycerol consumption. One possible explanation for this relationship is that regular clusters may decrease the specific surface area available for substrate uptake, leading to a reduced glycerol consumption rate. On the other hand, the change on the yeast morphology towards a more elongated (and hyphae-like form) may imply a more stressful set of conditions for *Y. lypolitica*. In fact, it was already described that changes in the morphology of *Y. lipolytica* can affect nutrient uptake and metabolism [27].

Although the obtained results pointed towards increased glycerol consumption for higher initial stirring speed, and decreased glycerol consumption for higher final stirring speed, the performed statistical analysis (described in the next section) did not confirm this assumption. Yeasts perimeter and area percentage seem to appear as correction factors (given the opposing influence found in the OLS analysis).

$$\begin{aligned} Gly\left(g{\cdot}L^{-1}\right) = & -4.19\text{x}10^{2}\cdot + 8.89\text{x}10^{2}{\cdot}Length(Y) + 2.44\text{x}10^{3}{\cdot}Conv(Ag) - 2.64\text{x}10^{-3}{\cdot}Nb/Vol(Ag) + 1.11\text{x}10^{1}{\cdot}Per(Y)^{2} - 7.52\text{x}10^{1}{\cdot}Length(Y)^{2} - 1.48\text{x}10^{3}\cdot \\ & + 5.73\text{x}10^{-8}\cdot - 6.39\text{x}10^{-4}{\cdot}Str_{1}{}^{2} + 1.24\text{x}10^{-1}{\cdot}Str_{1}{\cdot}Per(Y) - 2.09\text{x}10^{-1}{\cdot}Str_{1}{\cdot}Length(Y) - 6.27\text{x}10^{-4}{\cdot}Str_{1}{\cdot}\%Area(Y) - 7.68\text{x}10^{-2}{\cdot}Str_{2}{\cdot}Per(Y) \\ & + 3.32\text{x}10^{-1}{\cdot}Str_{2}{\cdot}Length(Y) \end{aligned} \quad (1)$$

3.2. Multilinear regression analysis

A MLR analysis was also performed to infer the glycerol, 2-PE and L-Phe concentration values in the performed experiments and determine the most influential parameters for monitoring purposes. A stepwise procedure was adopted, fed with the parameters highlighted by the OLS analysis as well as the stirring speed (0 – 24 h and 24 h – end), air flowrate and pH, with a linear function encompassing interactions and up to quadratic terms. The glycerol, L-Phe and 2-PE prediction results obtained by the stepwise MLR analysis are presented in Fig. 1.

Glycerol concentration was able to be adequately monitored over time, by the proposed methodology, with a coefficient of determination (R^2) of 0.918 and a root mean squared error (RMSE) of 7.79 $g_{Gly}\ L^{-1}$ (13.95% of the studied glycerol range), including the training and validation sets, by Eq. 1. The validation set presented an RMSE of 7.60 $g_{Gly}\ L^{-1}$ (13.61% of the studied range). A total of 14 terms and 7 different parameters were found to be statistically significant (p-value $<$ 0.05).

Yeasts size (in terms of *Per.* and *Length*), alongside the stirring speed in the first 24 h, were found the most important parameters for glycerol assessment, trailed in importance by the stirring speed after 24 h, aggregates contents and morphology (in terms of *Nb/Vol(Ag)* and *Conv (Ag)*, respectively), and by the morphotype proportion (in terms of *% Area(Y)*). Higher aggregates contents seem to increase glycerol consumption, taking also into consideration the OLS results (confirming the influence found in the prediction model), whereas higher yeast length and aggregates convexity seem to decrease it. The dependence of glycerol consumption on overall *Y. lipolytica* contents was already expected, though the obtained results point towards a clear dependency on the cluster contents, which may represent a response towards stressful conditions, allowing for the yeast metabolism to be less affected. It is suggested that the dependency on yeast clusters may be a response to stressful conditions, as yeast clusters may provide a protective environment that allows the yeast metabolism to be less affected. It should be kept in mind, though, that the OLS analysis inferred a similar trend towards the yeast contents and increased dependence on the individual yeasts morphotype (rather than on the aggregated form), which was not confirmed by the MLR. Furthermore, it seems that more regular clusters (possibly decreasing the specific area available for substrate uptake, as mentioned before) and larger yeast size (especially length wise towards a more hyphal morphology, though still not hyphae) seem to decrease

L-Phe concentration was also able to be adequately monitored over time, by the proposed methodology, with a coefficient of determination (R^2) of 0.895 and a root mean squared error (RMSE) of 0.471 $g_{L\text{-}Phe}\ L^{-1}$ (10.27% of the studied L-Phe range), including the training and validation sets, by Eq. (2). The validation set presented an RMSE of 0.394 $g_{L\text{-}Phe}\ L^{-1}$ (8.59% of the studied range). A total of 15 terms and 10 different parameters were found to be statistically significant (p-value $<$ 0.05).

Yeasts size (in terms of *Per.* and *Length*), followed by the air flowrate, were found to be the most important parameters for L-Phe assessment, trailed in importance by the stirring speed in the first 24 h, pH, overall yeast and morphotype proportion (in terms of *TA/Vol, Nb/Vol(Y), Nb/Vol(Ag)* and *% Nb(Y)*, respectively), and by the stirring speed after 24 h and aggregates morphology (in terms of *Rob(Ag)*). Higher yeasts number percentage and aggregates contents seem to increase L-Phe consumption, taking also into consideration the OLS results (confirming the influence found in the prediction model), whereas higher air flowrate (within the studied range), yeast length and aggregates robustness seem to decrease it. Likewise the glycerol results, it seems that more regular clusters (mainly in terms of robustness) and larger yeast size (again length wise) seem to decrease L-Phe consumption. It should be also referred the high correlation (R^2 of 0.918) between the cluster robustness, evidenced in these results, and the cluster convexity of the glycerol analysis. Again, the obtained results point towards a clear dependency on the cluster contents of *Y. lipolytica*, though it is now clear also the increased dependency on the individual yeasts morphotype inferred by the OLS analysis. Furthermore, the two experiments with the larger employed air flowrates (3 L min^{-1}) seem to slow the rate of L-Phe consumption. In those experiments, the high air flowrates may increase the shear stress on the cells, which can also affect their metabolism and nutrient uptake [28,29].

Although the obtained results pointed towards increased L-Phe consumption for higher initial stirring speed, the performed statistical analysis (described in the next section) did not confirm this assumption. Overall and individual yeasts contents, and yeasts perimeter and area percentage seem to appear as correction factors (given the opposing influence found in the OLS analysis). No definite conclusions could be withdrawn for the pH, stirring speed after 24 h and aggregates robustness influence over L-Phe consumption.

$$L-Phe\left(g{\cdot}L^{-1}\right) = -2.36\text{x}10^{1}{\cdot}Per(Y)+5.27\text{x}10^{1}{\cdot}Length(Y)+1.29{\cdot}TA/Vol^{2}+6.76\text{x}10^{-3}{\cdot}Nb\Big/Vol(Y)+6.04\text{x}10^{-1}{\cdot}Per(Y)^{2}-4.46{\cdot}Length(Y)^{2}-8.06\text{x}10^{-3}{\cdot}$$
$$-5.61\text{x}10^{-5}{\cdot}Str_{1}{}^{2}+1.89{\cdot}Air_{F}+4.53\text{x}10^{-3}{\cdot}Str_{1}{\cdot}Per(Y)+8.27\text{x}10^{-3}{\cdot}Str_{2}{\cdot}Rob(Ag)-4.09\text{x}10^{-1}{\cdot}Air_{F}{\cdot}Per(Y)-5.58\text{x}10^{-5}{\cdot}Air_{F}{\cdot}Nb\Big/Vol(Ag)$$
$$-2.53\text{x}10^{-2}{\cdot}pH{\cdot}TA\Big/Vol-3.39\text{x}10^{-3}{\cdot}pH{\cdot}\%Nb(Y) \quad (2)$$

A somewhat poorer monitoring ability was obtained for 2-PE concentration over time, by the proposed methodology, with a coefficient of determination (R^2) of 0.876 and a root mean squared error (RMSE) of 0.086 $g_{2\text{-PE}}$ L^{-1} (18.05% of the studied 2-PE range), including the training and validation sets, by Eq. (3). The validation set presented an RMSE of 0.067 $g_{\text{L-Phe}}$ L^{-1} (13.99% of the studied range). A total of 12 terms and 8 different parameters were found to be statistically significant (p-value < 0.05).

Yeasts size (in terms of *Per.*, *Deq.* And *Length*) and stirring speed in the first 24 h, followed by the stirring speed after 24 h, were found to be the most important parameters for 2-phenylethanol assessment, trailed in importance by the overall and individual yeasts contents and morphotype proportion (in terms of *TA/Vol, Nb/Vol(Y),* and *% Nb(Y)*, respectively). Higher overall yeast contents seem to increase 2-PE production, taking also into consideration the OLS results (confirming the influence found in the prediction model), whereas yeasts diameter seems to decrease it.

The production of 2–PE was found to depend on the overall *Y. lipolytica* contents, as already expected. In this case, no increased dependency was found on neither the aggregated nor the individual form, though the OLS analysis inferred an increased dependence on the individual yeasts morphotype. Again, larger yeast size seems to not benefit the 2-PE production metabolism. However, unlike the glycerol and L-Phe consumption analysis, no definite influence of the yeasts' length could be confirmed, emerging instead the yeasts diameter as the driving force. This may be related to the surface area-to-volume ratio of the yeast cells. Yeast cells with larger diameters have a smaller surface area-to-volume ratio, which may limit the diffusion of nutrients and waste products into and out of the cell. This could affect the cell's metabolic activity and consequently, its ability to produce 2-PE [27]. Furthermore, a predominant yeast form was shown to be required for an efficient secretion of various heterologous proteins in *Y. lipolytica*, such as the case of fungal laccases and human interferons [30,31]. In addition, according to the cell morphotype, a different hydrocarbon degradation potential was observed in two *Y. lipolytica* strains: yeast cells of a marine isolate (NCIM 3589) metabolized hexadecane with higher efficiency than the mycelial form, whereas the mycelial form of a terrestrial strain (W29) metabolized the hydrocarbon more efficiently [32,33].

Although the obtained results pointed towards decreased 2-PE production for higher initial stirring speed, the performed statistical analysis (described in the next section) did not confirm this assumption. On the other hand, an increased 2-PE production, for higher final stirring speed, could be confirmed. Changes in the stirring speed result in differences in the stress yield which may affect yeasts size and cluster formation/disaggregation dynamics. This fact is evidenced by both the yeast size and the individual/aggregated yeasts proportion results (as described in the next section) and was further translated into 2-PE production (as above mentioned). The impact of shear stress in *Y. lipolytica* morphology was already reported by Braga et al. [10]. Yeasts perimeter, length, individual contents and number percentage seem to appear as correction factors (given the opposing influence found in the OLS analysis).

$$2-PE\left(g{\cdot}L^{-1}\right) = 3.56\text{x}10^{-1}{\cdot}Per(Y)-1.055\text{x}10^{-1}{\cdot}Nb\Big/Vol(Y)+1.91\text{x}10^{-3}{\cdot}TA/Vol^{2}-4.40\text{x}10^{-4}{\cdot}Per(Y)^{2}+1.44\text{x}10^{-1}{\cdot}Length(Y)^{2}-2.10\text{x}10^{-5}{\cdot}Str_{1}{}^{2}$$
$$+6.19\text{x}10^{-3}{\cdot}Str_{1}{\cdot}Diam(Y)-7.79\text{x}10^{-4}{\cdot}Str_{1}{\cdot}Per(Y)+1.85\text{x}10^{-4}{\cdot}Str_{1}{\cdot}\%Nb(Y)-6.30\text{x}10^{-3}{\cdot}Str_{2}{\cdot}Diam(Y)+2.81\text{x}10^{-3}{\cdot}Str_{2}{\cdot}Per(Y)$$
$$-3.29\text{x}10^{-3}{\cdot}Str_{2}{\cdot}Length(Y) \quad (3)$$

3.3. Stirring speed and pH effect

Mann-Whitney tests were performed for pairwise analysis concerning the operational conditions (pH and stirring speed). The selected pairs were 600 *vs.* 600/400 rpm, 600 *vs.* 900 rpm and 600 *vs.* 600 rpm-OT (regarding the stirring speed) and 5.5 *vs.* 6.5, 5.5 *vs.* 7.5 and 6.5 *vs.* 7.5 (with respect to pH). The obtained results for glycerol, L-Phe and 2-PE concentrations, as well as for the most important QIA parameters found by the OLS analysis, are presented in Table 4.

Table 4
Statistical significance analysis for stirring speed and pH change regarding glycerol, 2-PE, L-Phe and selected QIA parameters.

	Gly	*2-PE*	*L-Phe*	*TA/Vol (Y+Ag)*	*Deq (Y)*	*Per (Y)*	*Length (Y)*	*% Nb (Y)*	*% Area (Y)*	*Nb/Vol (Y)*	*Conv (Ag)*	*Rob (Ag)*	*Nb/Vol (Ag)*
Stirring speed													
600 *vs* 600/400		X		X	X	X		X	X	X	X	X	
600 *vs* 900					X	X		X	X				X
600 *vs* 600-OT			X								X		
pH													
5.5 *vs* 6.5					X	X		X	X		X		
5.5 *vs* 7.5			X	X		X	X			X	X		X
6.5 *vs* 7.5					X								X

X – Statistically different (p-value<0.05).

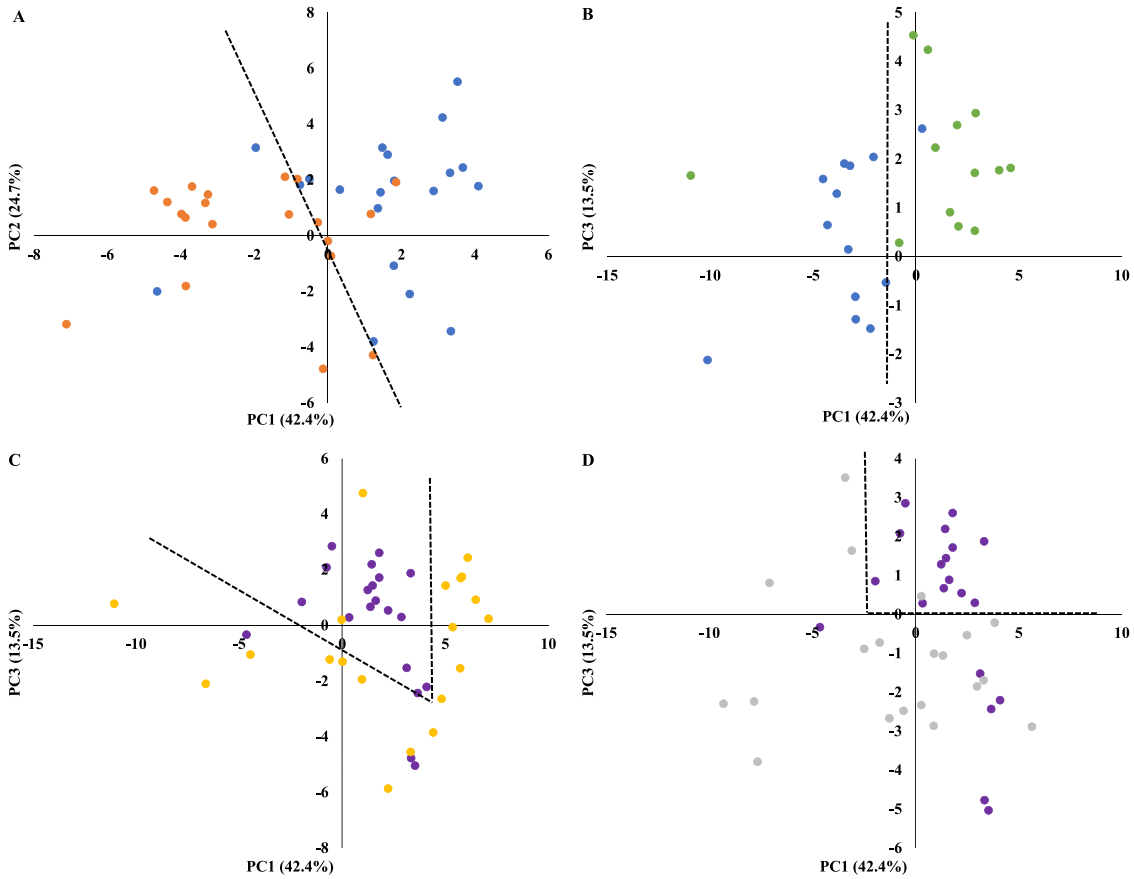

Fig. 2. PCA analysis regarding the 600 (●) *vs.* 600/400 rpm (●) (A) stirring speeds, 600 (●) *vs.* 900 rpm (●) (B) stirring speeds, pH 5.5 (●) *vs.* pH 7.5 (●) (C) and pH 5.5 (●) *vs.* pH 7.5 (●) (D).

The stirring speed change from 600 to 600/400 rpm resulted in statistical differences for the 2-PE concentration, hyphae, yeast and cluster contents, most of the QIA parameters related to yeasts and clusters and all the parameters identified in the OLS analysis except for length (*Length(Y)*) and total number of aggregated yeasts per volume (*Nb/Vol(Ag)*). The stirring speed change from 600 to 900 rpm resulted in statistical differences for hyphae and cluster contents, most of the QIA parameters related to yeasts and clusters and the equivalent diameter (*Deq(Y)*), perimeter (*Per(Y)*), number *(%Nb(Y))* and area *(%Area(Y))* percentage of individual yeasts and total number of aggregated yeasts per volume (*Nb/Vol(Ag)*), identified in the OLS analysis. The stirring speed change from 600 to 600 rpm-OT resulted in statistical differences solely for the L-Phe concentration and the convexity (*Conv(Ag)*) identified in the OLS analysis. Overall, the most significant changes resulting from the stirring speed variation were felt in the yeast size and individual/aggregated yeasts proportion.

The pH change from 5.5 to 6.5 resulted in statistical differences for the yeast size, individual/aggregated yeasts proportion and cluster morphology, and the equivalent diameter (*Deq(Y)*), perimeter (*Per(Y)*), number *(%Nb(Y))* and area *(%Area(Y))* percentage of individual yeasts and convexity (*Conv(Ag)*), identified in the OLS analysis. The pH change from 5.5 to 7.5 resulted in statistical differences for the L-Phe concentration, hyphae, yeast and cluster contents, yeast size, and the total area of aggregated and Individual yeasts per volume (*TA/Vol*), perimeter (*Per (Y)*), length (*Length(Y)*), convexity (*Conv(Ag)*) and total number of individual (*Nb/Vol(Y)*) and aggregated (*Nb/Vol(Ag)*) yeasts per volume, identified in the OLS analysis. The pH change from 6.5 to 7.5 resulted in statistical differences for the hyphae and cluster contents, yeast size and morphology and the equivalent diameter (*Deq(Y)*) and total number of aggregated yeasts per volume (*Nb/Vol(Ag)*), identified in the OLS analysis. Nonetheless, no clear separation between the studied pH pairs was able to be obtained by the PCA analysis. Overall, the most significant changes resulting from the pH variation were felt in the hyphae and cluster contents and yeast size.

Glycerol concentration was not affected by any of the studied pairwise comparisons regarding the stirrer speed and pH changes, whereas solely the change from 600 to 600/400 rpm significantly altered the 2-PE concentration and both the change from 600 to 600 rpm-OT and from pH 5.5–7.5 altered the L-Phe concentration. The stirring speed change from 600 rpm to 600/400 rpm resulted in the most significant modification in the QIA parameters highlighted by the OLS analysis (8 parameters and also significant in 2-PE concentration), followed by the pH change from pH 5.5 and 7.5 (6 parameters and also significant in L-Phe concentration), the change from 600 to 900 rpm (5 parameters) and the change from pH 5.5–6.5 (4 parameters).

Principal components analysis (PCA), presented in Fig. 2, was run for the overall dataset allowing to separate the 600 rpm stirring speed data from both the 600/400 rpm and the 900 rpm, and pH 5.5 from both pH 6.5 and pH 7.5. No clear separation between the 600 rpm and the 600 rpm-OT data, and from pH 6.5 from pH 7.5, was able to be obtained by the PCA analysis.

4. Conclusions

The proposed methodology allowed to obtain promising results with respect to glycerol and L-phenylalanine consumption, and even 2-phenylethanol production monitoring over time, with RMSE values of 13.95%, 10.27% and 18.05% of the studied glycerol, L-phenylalanine and 2-phenylethanol ranges, respectively. Higher overall (and mainly clusters) contents and yeasts proportion were found to increase glycerol

and L-phenylalanine consumption, whereas larger yeast size (especially length wise, configuring an hyphal approximation trend) and more regular clusters were found to decrease it. On the other hand, higher final stirring speed and overall yeast contents were found to increase 2-PE production and larger yeast size to decrease it.

The most significant changes resulting from stirring speed variation (600, 600/400, 900 and 600 rpm-OT) were felt in the yeast size and individual/aggregated yeasts proportion. On the other hand, the most significant changes resulting from pH variation (5.5, 6.5, 7.5) were felt in the hyphae and cluster contents and yeast size. The stirring speed change from 600 rpm to 600/400 rpm resulted in the most significant modification in the QIA parameters highlighted by the OLS analysis, followed by the pH change from pH 5.5 and 7.5, the change from 600 to 900 rpm and the change from pH 5.5–6.5. Except for the L-Phe (change from 600 to 600 rpm-OT and from pH 5.5–7.5), and 2-PE (change from 600 to 600/400 rpm), no significant alterations were observed for the studied compounds concentration with the change in operational conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors thank the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UIDB/04469/2020 unit, and by LABBELS – Associate Laboratory in Biotechnology, Bioengineering and Microelectromechnaical Systems, LA/P/0029/2020. The authors also acknowledge Dr. D.Q. Nguyen and Dr. E. Nagy from Szent István University, Budapest, Hungary, for kindly providing *Y. lipolytica* CH 1/5 strain. The authors wish to express their gratitude to Prio Energy – Prio Biocombustíveis, SA for providing the glycerol used in this experimental work.

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