



## Prediction of fermentative parameters from mathematical modeling using thermotolerant probiotic yeast



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### ABSTRACT

Probiotic yeasts are sources of food additives producing propionic acid (PA). Here, using metabolic data from p3 under a controlled aeration system was developed a mathematical model to estimate  $\alpha$  and  $\beta$  values by Luedeking-Piret equation. Maximum specific formation rate of PA, volumetric productivity of PA and maximum yield coefficient of product were demonstrated in microaerobic system. The formation rates of product (rp) were kinetically associated with the biomass formation rates (rx) and the concentrations of dry weight cell (DWC). The  $\alpha$  value was of 0.450639 ( $\text{mmol l}^{-1} \text{ PA g}^{-1} \text{ DWC}$ ) and 0.64479 ( $\text{mmol l}^{-1} \text{ PA g}^{-1} \text{ DWC}$ ), and  $\beta$  value of 0.000243 ( $\text{mmol l}^{-1} \text{ PA h}^{-1}$ ) and 0.00138 ( $\text{mmol l}^{-1} \text{ PA h}^{-1}$ ), in aerobic and microaerobic systems, respectively. Therefore, here, was possible to simulate the DWC and PA production and to describe that the PA production followed a semi-growth-associated model.

### 1. Introduction

Probiotic yeasts are administered in the feed of weaned piglets to improve adaptation to the growth phase, as well as to regulate food intake and weight gain. These yeasts have been associated with the production of antimicrobial compounds, competition for adhesion in the gut of animals, immunostimulatory and antibiotic activity, and for striking an ideal balance between beneficial and pathogenic microorganisms, as well as reduction of stress during the weaning period (Jin et al., 1998; Agarwal et al., 2000; Koutinas et al., 2009; Choi et al., 2011; Rai et al., 2016).

Probiotic yeasts also improve dry matter digestibility in ruminants and are important for balancing microbial activity and PA production. PA are produced in the gastrointestinal tract by microbial fermentation

from carbohydrates and endogenous substrates, together with acetate and butyrate in aerobic and microaerobic processes during synthesis of sterols (Bergman, 1990; Marbà-Ardébol et al., 2018; Noorae et al., 2010).

Importantly, the p3 culture employed has shown beneficial effects, including inhibition of swine pathogens (*Cryptococcus laurentii*), ability to grow under harsh conditions in the animal digestive tract, resistance to bile salts, and ability to survive rectal temperatures of 39 °C and pH 2; all these characteristics are desirable for probiotic yeasts (unpublished data).

The growth kinetics of p3 was evaluated under conditions of controlled pH, temperature, and aeration in a stirred tank reactor (STR) to better understanding your growth and metabolism as well as factors that are involved in PA production. The p3 was cultivated in three

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conditions of aeration to evaluate the interference of the PA production under level of aeration. A mathematical model was applied using the growth-associated constant  $\alpha$  and biomass-associated constant  $\beta$  of the Luedeking–Piret equation (Luedeking and Piret, 1959).

To the best of our knowledge, there are no mathematical modeling data or  $\alpha$  and  $\beta$  factors that explain PA production from probiotic yeast. The Luedeking–Piret kinetic model has been employed to describe inulinase production by *Kluyveromyces marxianus*, with  $\alpha$  and  $\beta$  of 0.75 and 0.033, respectively (Santharam et al., 2017).

However,  $\alpha$  and  $\beta$  correlate with the strains and end-products used, and therefore they vary and require analysis for each process (Nandasana and Kumar, 2008; Wu et al., 2015; Zhao et al., 2010). Furthermore, the biomass and the specific growth rate of p3 had partial influence on the increase in PA production, with  $\alpha$  and  $\beta$  factors directly involved in PA production, affected by the level of aeration employed.

Mathematical modeling is important for industrial application for  $\alpha$  and  $\beta$  factors for determining and optimization of PA production. For this reason, prediction of  $\alpha$  and  $\beta$  factors are indispensable for the optimization of PA production from cultures of p3 and its application as a food additive.

## 2. Materials and methods

### 2.1. Ethics in animal experimentation and maintenance of yeast p3

Probiotic yeast p3 was isolated from the enteric microbiota of newborn piglets on a pig farm at the Department of Animal Science at Federal University of Viçosa and were stored at the Microbial Physiology Laboratory, Biotechnology Applied to Agriculture Institute—Bioagro/FUV-Brazil.

Probiotic yeast p3 was isolated according to ethical guidelines for newborn piglets. Experiments were approved by the National Council for Control of Animal Experimentation, Federal University of Latin American Integration Animal Experimentation Committee (CEUA n° 001/2018). Animal care was provided according to standards of the Brazilian College of Animal Experimentation guidelines (COBEA).

Five piglets, three days old were anesthetized using ketamine (300 mg/kg) (Vetbrands, Brazil) and xylazine (22.5 mg/kg) (Syntec, Brazil). Glycerinated buffer was introduced with abdominal massage to stimulate intestinal evacuation.

The material was collected in sterile petri dishes and immediately transported to the laboratory. Samples of feces were diluted in 9 ml of peptone water. After vortexing for 60 s, successive dilutions were made up to 1:1000. Aliquots of 1 ml of original sample and each dilution were plated in duplicate in medium YPLC (1% yeast extract, 2% peptone, 2% lactose, chloramphenicol 0.02%). Plates were incubated at 30 °C for 72 h. Yeast colonies identified under a microscope were selected, isolated and stored at –80 °C in glycerol.

This yeast is resistant to environmental digestive tract conditions according to vitro testing (Bragança et al., 2015; Silveira et al., 2014). A stock of p3 cultures was stored at –80 °C in yeast-peptone-dextrose (YPD) medium with 20% glycerol. The medium was sterilized at 121 °C for 15 min and subsequently used in the experiments.

### 2.2. Batch fermentation in a shake flask

The p3 was pre-grown at 30 °C in YPD medium under agitation at 160 rpm. Yeast cultures were centrifuged at 5000 × g at 4 °C for 5 min and the pellets were suspended in YPD medium.

The organism was cultured at various temperatures and pHs using a batch system under agitation at 160 rpm (New Brunswick Scientific Co., USA), in 500-ml flasks containing 150 ml of YPD medium.

The initial cell concentration was adjusted to an OD600 of 0.05. The temperature and pH ranges were 28–45 °C and pH 3.8–5.5, respectively. The initial pH values were determined by considering the relationship of equidistance of the H<sup>+</sup> concentrations, and the pH was

adjusted using 1 N HCl and 3 N NaOH.

Samples were collected and cell concentrations were determined using OD600 spectrophotometry (Beckman DU 640) to determine the specific growth rate. According to the OD correlation, one unit of OD600 corresponded to 0.549 g l<sup>-1</sup> of dry weight of cells.

### 2.3. Batch fermentation in an STR bioreactor

The p3 was pre-grown in 250 ml YPD medium at 40 °C at 160 rpm and transferred with a cell concentration of OD600 0.5 into a BioFlo bioreactor (New Brunswick Scientific Co., Edison, NJ, USA). The bio-process was performed using a working volume of 2.5 l at 40 °C and a pH of 4.7 controlled with 1 N HCl and 3 N NaOH.

Submerged fermentation was studied in controlled aeration systems at three levels: aerobic, microaerobic and anaerobic. For the anaerobic system, the vessel with YPD without ergosterol supplementation was sterilized at 121 °C for 25 min to release dissolved oxygen.

Input of nitrogen gas was performed under agitation of 300 rpm for 20 min in Sparger tubes for residual dissolved oxygen release. Subsequently, nitrogen gas was maintained in the gas–liquid interface under agitation at 50 rpm. The microaerobic system was created without gas input and maintained agitation at 150 rpm. Air input at the liquid interface and constant agitation at 300 rpm was maintained in the aerobic system.

Samples were collected and cell concentrations, levels of ethanol, glycerol, and organic acids, and VFA production were analyzed using high-performance liquid chromatography (HPLC).

### 2.4. Fermentative parameters

The Arrhenius model (Eq. (1)) described the relationship between the activation energy ( $E_a$ ) and microbial growth ( $\mu$ ) at various temperatures ( $T$ ). The optimum temperature of p3 from can be obtained using a graph of the Arrhenius equation (Eq. (1)).

The specific growth rates at any time during the exponential phase were obtained using Eq. (2), where  $dX$  is the difference in OD600 at a particular time-point and a subsequent time-point,  $dt$  is the variation of time and  $X$  is the cell concentration at that particular time-point.  $R$  is the gas constant.

$$\ln(\mu) = \ln(A) - \frac{E_a}{R} \times \frac{1}{T} \quad (1)$$

$$\mu = \left( \frac{dX}{dt} \right) / X \quad (2)$$

The yield coefficients ( $Y$ ) of product formed per unit of substrate consumed can be obtained from Eqs. (3)–(7), where  $X$  represents the cell concentration and  $E$ ,  $G$  represent the levels of ethanol, glycerol, and  $P$  levels of PA measured by HPLC.  $S_i$  is the initial substrate and  $S_f$  is the residual concentration of substrate measured by HPLC. Eq. (8) defines the PA volumetric productivity ( $Q_p$ ) and ( $t$ ) the time.

$$Y_{X/S} = \frac{X_f - X_i}{S_i - S_f} (\text{g} \cdot \text{mmol}^{-1}) \quad (3)$$

$$Y_{E/S} = \frac{E_f - E_i}{S_i - S_f} (\text{mmol} \cdot \text{l}^{-1} (\text{mmol} \cdot \text{l}^{-1})^{-1} - 1) \quad (4)$$

$$Y_{G/S} = \frac{G_f - G_i}{S_i - S_f} (\text{mmol} \cdot \text{l}^{-1} (\text{mmol} \cdot \text{l}^{-1})^{-1} - 1) \quad (5)$$

$$Y_{P/S} = \frac{P_f - P_i}{S_i - S_f} (\text{mmol} \cdot \text{l}^{-1} (\text{mmol} \cdot \text{l}^{-1})^{-1} - 1) \quad (6)$$

$$Y_{P/X} = \frac{P_f - P_i}{X_f - X_i} (\text{mmol} \cdot \text{l}^{-1} \cdot \text{g}^{-1}) \quad (7)$$

$$Q_P = \frac{P_f - P_i}{t} \text{ (mmol}\cdot\text{l}^{-1}\cdot\text{h}^{-1}\text{)} \quad (8)$$

## 2.5. Mathematical modeling

A mathematical model was developed to describe the production of PA by submerged fermentation of p3 in controlled aeration systems. PA production can be determined by Luedeking-Piret equation as a semi-growth-associated process (Luedeking and Piret, 1959; Gonzalez-Garcia et al., 2017; Wu et al., 2015). In this section, the material balance for DWC and PA was described in Eq. (9).

$$r_p = \frac{dP}{dt} = \alpha r_x + \beta X \quad (9)$$

Eq. (9) describes the rate of PA production in terms of two factors: the DWC growth rate,  $r_x$  (with the proportionality constant  $\alpha$  and the DWC concentration,  $X$  (mediated by the constant  $\beta$ )). Eq. (9), known as the Luedeking-Piret equation, considers the partial coupling between cell growth and PA production. There are two curves in fermentation: one for DWC concentration and the other for product formation.

In the present study, cell growth was described by the following widely used logistic equation:

$$\frac{dX}{dt} = \mu_{max} \left( 1 - \frac{X}{X_{max}} \right) \cdot X \quad (10)$$

where  $X$  is the DWC concentration,  $X_{max}$  is the maximum DWC concentration,  $t$  is fermentation time,  $\frac{dX}{dt}$  is the cell growth rate,  $\mu$  is the specific growth rate, and  $\mu_{max}$  is the maximum specific growth rate. Integrating Eq. (10) with  $X_0$  as the initial biomass concentration gives  $X$  as a function of  $t$ , as follows:

$$X(t) = \frac{X_0 e^{\mu_{max} t}}{1 - \frac{X_0}{X_{max}} (1 - e^{\mu_{max} t})} \quad (11)$$

Eqs. (9)–(11) were employed in interactive software that simulates the experimental results.

## 2.6. Analytical methods

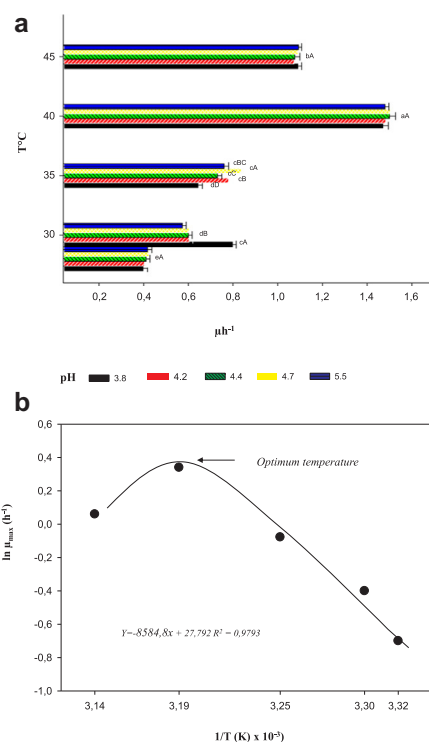
Concentrations of organic acids, sugars, ethanol, glycerol, and PA were determined using HPLC. Each sample was first diluted at 1:10 in 0.5 M  $\text{H}_2\text{SO}_4$  and filtered through 0.22- $\mu\text{m}$  membranes.

A Waters 410 differential refractometer was used with an ion exchange column (Aminex HPX87H). The column was eluted with 0.5 mM  $\text{H}_2\text{SO}_4$  at 50 °C and a flow rate of 0.6  $\text{ml}\cdot\text{min}^{-1}$ . The relevant concentrations were obtained from linear regression based on external standards.

## 2.7. Statistical analysis

The experiments to evaluate the specific growth rate under various temperature and pH conditions were conducted according to a completely randomized design. ANOVA was applied to the factorial design in three repetitions with a total of 75 observations.

In the analysis of the metabolism of p3, the fermentative parameters were determined using Student's  $t$ -test and a  $P$ -value  $< 0.05$  was considered significant. The mean values of three replications and standard deviations were calculated from the data obtained from the fermentation system using SigmaPlot version 10 statistical software. MATLAB version 2007 (MathWorks, Natick, MA, US) was used for the simulation and data analysis for kinetics models, using only the mean values from the experimental data.



**Fig. 1.** Microbial growth analysis of probiotic yeast as a function of temperature and pH in a shake flask system. (a) Specific growth rate versus temperature and pH. (b) The optimum temperature of p3 from a graph of the Arrhenius equation. Specific growth rate data were plotted for  $3.32 \times 10^{-3}$  K to  $3.19 \times 10^{-3}$  K to determine the ratio of the activation energy to the gas constant ( $Ea/R$ ). Each observation is the mean and standard deviation of three replicates ( $n = 75$ ). Coefficient of variation (CV) = 1.92%. Mean values followed by the same letter were not significantly different at the level of 5% probability (Tukey test using SigmaPlot version 10 statistical software).

## 3. Results

### 3.1. Kinetics of growth of p3 in extremes of pH and temperature in Erlenmeyer flasks

The growth kinetics of p3 were determined by examining the DWC and specific growth rate aiming to demonstrate its ability to resist extremes of pH and temperature (Fig. 1a).

An exponential increase of the specific growth rate within the linearity range was expected when data were plotted for  $3.32 \times 10^{-3}$  K (28 °C) to  $3.19 \times 10^{-3}$  K (40 °C), determining the ratio of the activation energy and the gas constant (Fig. 1b).

High specific growth rates and DWC were obtained at 40 °C without influence of medium pH values. Thus, was applied this temperature and pH 4.7 to evaluate the effects of cultivation of p3 in the controlled aeration system in a New Brunswick BioFlo Bioreactor of PA production (see subsequent sections).

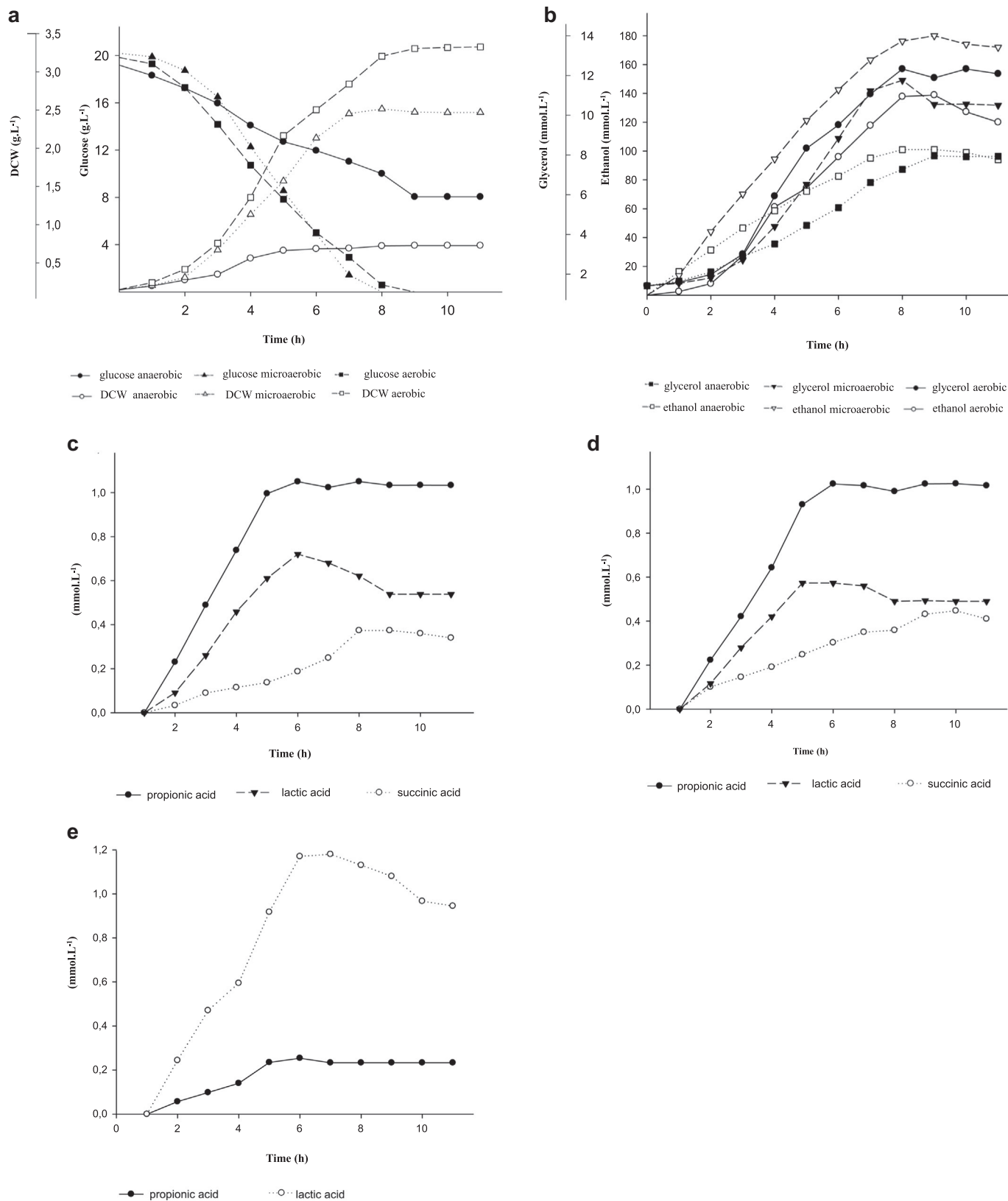
In this section, was demonstrated that the specific growth rate of p3 depended on temperature as described by the following equation:

$$\mu = \mu_0 \cdot e^{-Ea/RT} \quad (12)$$

$$\mu = 1.175 \times 10^{12} e^{-8584.8/T(\text{K})} \quad (13)$$

### 3.2. Performance of PA production and oxide-reductive metabolism from p3 under a controlled aeration system

To improve understanding of the growth and performance for the



**Fig. 2.** Batch fermentation and metabolic analysis of p3 in controlled aeration systems. (a) Growth and substrate consumption. (b) Ethanol and glycerol production. (c) Organic acid production in aerobic system. (d) Organic acid production in microaerobic system. (e) Organic acid production in anaerobic system.

PA production from probiotic yeast p3, were evaluated the metabolic data and fermentative parameters obtained under controlled aeration systems in an STR. The data obtained here were explored using mathematical models that explain the production of organic acids, aiming to

explain the production of PA from p3 under levels of aeration (Luedeking and Piret, 1959).

Oxidative-reductive metabolism analysis revealed lower DWC in the anaerobic system (0.58 g l<sup>-1</sup>), and 8.418 g l<sup>-1</sup> of residual substrate,

**Table 1**

Fermentative parameters, yields coefficients and carbon balance of p3 under controlled aeration system in STR. Fermentative parameters: Specific formation rate of PA,  $\mu P$  ( $\text{h}^{-1}$ ); Specific growth rate  $\mu X$  ( $\text{h}^{-1}$ ); Volumetric productivity,  $Q_P$  ( $\text{mmol}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$ ); Yields coefficients:  $Y_{E/S}$  ( $\text{mmol}\cdot\text{l}^{-1}$  ( $\text{mmol}\cdot\text{l}^{-1}$ ) $^{-1}$ );  $Y_{G/S}$  ( $\text{mmol}\cdot\text{l}^{-1}$  ( $\text{mmol}\cdot\text{l}^{-1}$ ) $^{-1}$ );  $Y_{P/S}$  ( $\text{mmol}\cdot\text{l}^{-1}$  ( $\text{mmol}\cdot\text{l}^{-1}$ ) $^{-1}$ );  $Y_{X/S}$  ( $\text{g}\cdot\text{mmol}\cdot\text{l}^{-1}$ );  $Y_{P/X}$  ( $\text{mmol}\cdot\text{l}^{-1}\cdot\text{g}^{-1}$ ); Products: E - ethanol ( $\text{mmol}\cdot\text{l}^{-1}$ ); G - glycerol ( $\text{mmol}\cdot\text{l}^{-1}$ ). Carbon balance (%): X - DWC concentration ( $(\text{g DWC})\cdot\text{l}^{-1}$ );  $\text{CO}_2$  ( $\text{mmol}\cdot\text{l}^{-1}$ ); Met - total metabolites ( $\text{mmol}\cdot\text{l}^{-1}$ ); S - residual substrates ( $\text{mmol}\cdot\text{l}^{-1}$ ). Mean values followed by the same letter were not significantly different at the level of 5% probability (Tukey test using SigmaPlot version 10 statistical software).

Controlled aeration systems	Fermentative parameters			Yields coefficients					Carbon balance					
	$\mu P$	$\mu X$	$Q_P$	$Y_{E/S}$	$Y_{G/S}$	$Y_{X/S}$	$Y_{P/S}$	$Y_{P/X}$	E	G	X	$\text{CO}_2$	Met	S
Anaerobic	0.370 <sup>b</sup>	0.52 <sup>c</sup>	0.050 <sup>c</sup>	1.7 <sup>a</sup>	0.11 <sup>a</sup>	0.027 <sup>c</sup>	0.0042 <sup>b</sup>	0.43 <sup>b</sup>	30.60	3.17	6.92	15.30	0.24	43.74
Microaerobic	0.497 <sup>a</sup>	0.85 <sup>b</sup>	0.210 <sup>a</sup>	1.6 <sup>a</sup>	0.09 <sup>a</sup>	0.020 <sup>b</sup>	0.0097 <sup>a</sup>	0.47 <sup>a</sup>	53.54	4.43	10.95	27.32	3.76	2.27
Aerobic	0.400 <sup>b</sup>	0.91 <sup>a</sup>	0.203 <sup>b</sup>	1.1 <sup>b</sup>	0.10 <sup>a</sup>	0.009 <sup>a</sup>	0.0093 <sup>a</sup>	0.34 <sup>c</sup>	42.08	5.08	30.83	21.11	0.90	0

Mean values followed by the same letter (a, b or c) were not significantly different at the level of 5% probability (Tukey test using SigmaPlot version 10 statistical software).

and in the microaerobic system, DWC of  $2.22\text{ g}\cdot\text{l}^{-1}$ ; and DWC production of  $3.02\text{ g}\cdot\text{l}^{-1}$  without residual substrate following fermentation, in aerobic system (Fig. 2a).

Specific growth rates ( $\mu X$ ) were  $0.91\text{ (h}^{-1}\text{)}$ ,  $0.85\text{ (h}^{-1}\text{)}$ , and  $0.52\text{ (h}^{-1}\text{)}$ , for aerobic, microaerobic, and anaerobic systems, respectively, with greater  $Y_{X/S}$  values in the aerobic systems (Table 1).

Carbon balance of all metabolites revealed high ethanol production in the microaerobic system and substantial glycerol production in the aerobic system (Fig. 2b and Table 1). However, the yield coefficient  $Y_{E/S}$  was significant in the anaerobic and microaerobic systems while  $Y_{G/S}$  was similar in the three systems (Table 1).

Metabolites produced during the submerged fermentation of p3 revealed the presence of lactic, succinic acid, and PA (Fig. 2c, d, e). PA production was greater in both aerobic and microaerobic systems (Fig. 2c, d). PA production in the anaerobic system was lower with lactic acid as the major metabolite produced (Fig. 2e). The specific formation rate of PA ( $\mu P$ ) was substantial in the microaerobic system ( $0.497\text{ h}^{-1}$ ), with greater PA volumetric productivity and  $Y_{P/X}$  values.  $Y_{P/S}$  values were substantial in the microaerobic and aerobic systems (Table 1).

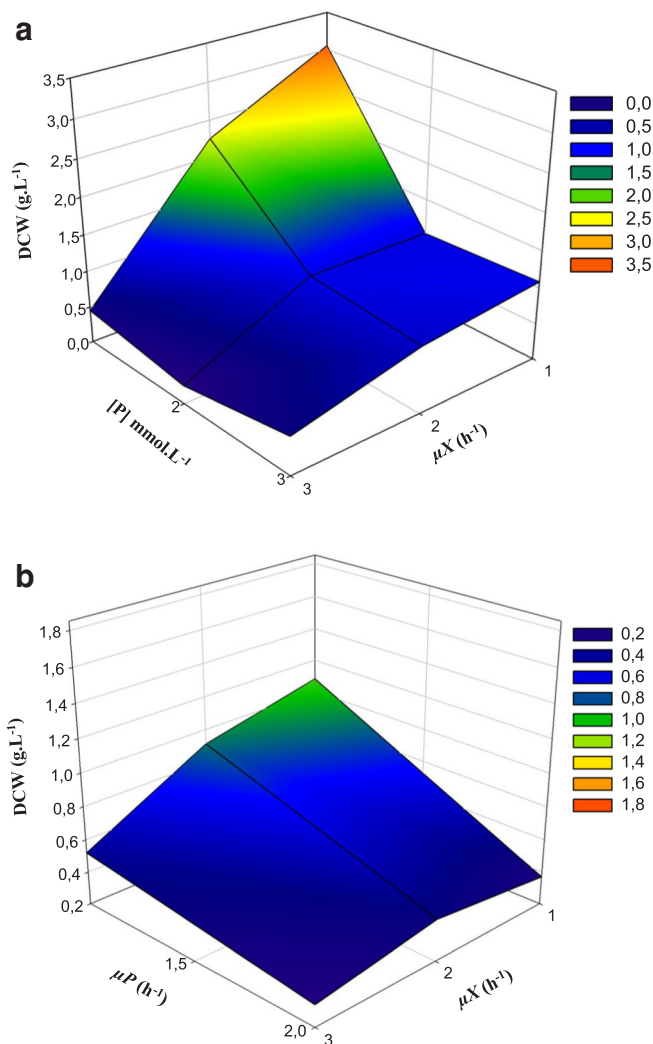
Approximately 96% of the substrate consumed was used as a source of metabolic energy in both microaerobic and aerobic systems and of 49% for the anaerobic system (Table 1). Residual substrates of 43.74% and 2.27% were obtained in the anaerobic and microaerobic systems, respectively. Redox balance closed at 0.98 for the microaerobic system and at 0.97 for the aerobic and anaerobic systems (Table 1).

### 3.3. PA production by p3 followed a semi-growth associated profile

The maximum PA production was obtained with 5 h of the process, with DWC continuing to increase, particularly in the microaerobic and aerobic systems. Response surface data showed that the increase of DWC and the specific growth rate had a partial influence on the increase in PA production and the specific formation rate of PA (Fig. 3a, b). Under aerobic and microaerobic systems, the effects were  $1.85\text{ g}\cdot\text{l}^{-1}$  and  $1.35\text{ g}\cdot\text{l}^{-1}$  of DWC, respectively. Thus, aiming to understand PA production, a mathematical model was built to describe the PA production by p3 under various aeration systems (Figs. 4, 5).

DWC and PA production predicted data, using by mathematical model built, were similar to these parameters observed from p3 cultivated in a stirred tank reactor (STR) under a controlled aeration system (CAS) (Figs. 4a, b and 5a, b). In addition, the parameter  $r_p$  is kinetically associated with the biomass production rates  $r_x$  and DWC. Therefore,  $r_x$  and  $r_p$  in the aerobic and microaerobic system have similar profiles for the both predicted versus observed data (Figs. 4c, 5c).

For the aerobic system, the slope of the straight line indicated that  $r_p = \beta X = 0.00074$ . Because the DWC ( $X$ ) is constant in this fermentation phase,  $\beta$  could be directly calculated as  $0.000243\text{ mmol}\cdot\text{l}^{-1}\text{ PA}\cdot\text{h}^{-1}$ . The  $\beta$  value was used to estimate the value of  $\alpha$  from the  $r_p$  profiles calculated by polynomial fitting. From this analysis, an average



**Fig. 3.** Mathematical modeling of PA production by p3 under controlled aeration systems. Response surface of the semi-growth-associated for PA production showing that the increase of DWC and the specific growth rate had a partial influence on the increase in PA production (a) and the specific formation rate of PA (b).

value of  $\alpha = 0.450639\text{ mmol}\cdot\text{l}^{-1}\text{ PA}\cdot\text{g}^{-1}\text{ DWC}$  was obtained. Therefore, PA production was partly growth-associated.

In addition, was observed that  $\alpha$  was the dominant term, whereas the  $\beta$  term tended to zero, such that the product was formed during the growth phase, proportional to the growth rate ( $\alpha \neq 0$  and  $\beta \rightarrow 0$ ).

Under the microaerobic system, the slope of the straight line indicated that  $r_p = \beta X = 0.0031$ , with  $\beta$  equal to  $0.00138\text{ mmol}\cdot\text{l}^{-1}$

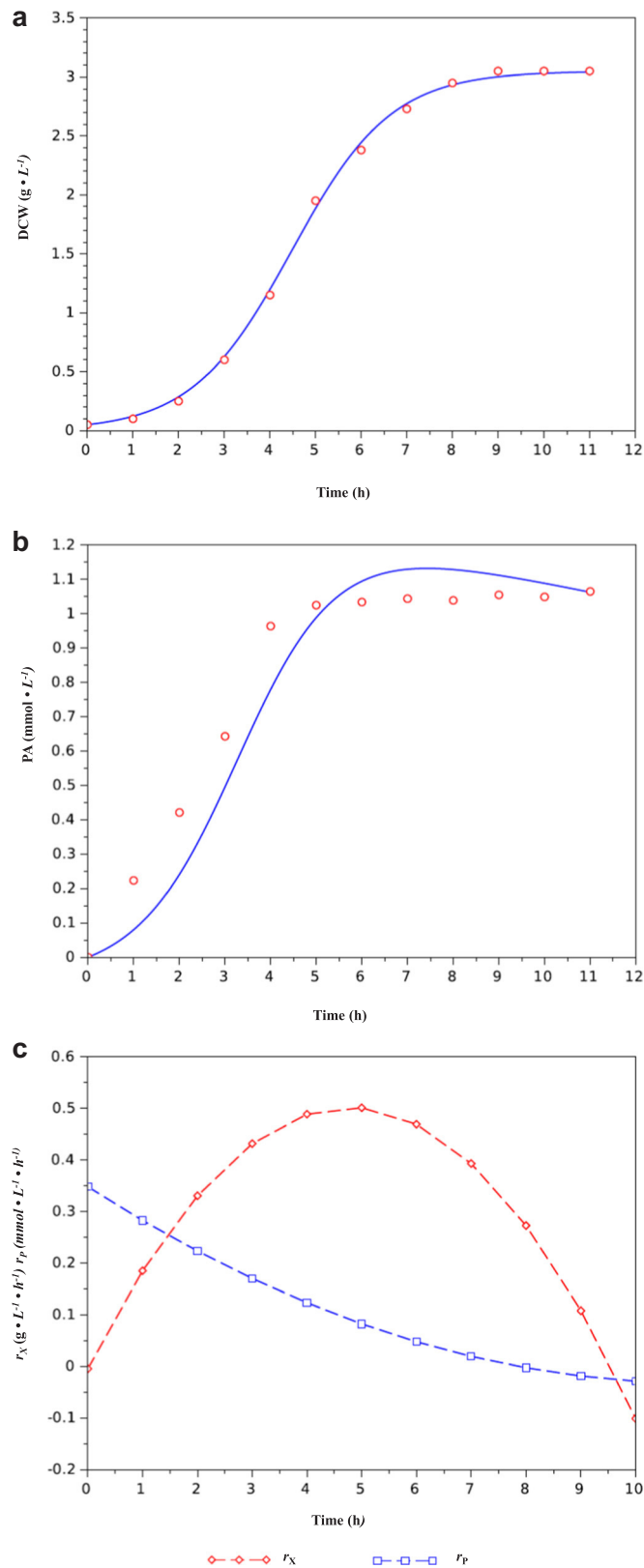


Fig. 4. Predicted data versus observed data from p3 cultivated in a stirred tank reactor (STR) under aerobic system for the DWC growth (a), PA production (b) and fermentative parameters ( $r_X$ ) and ( $r_P$ ) (c).

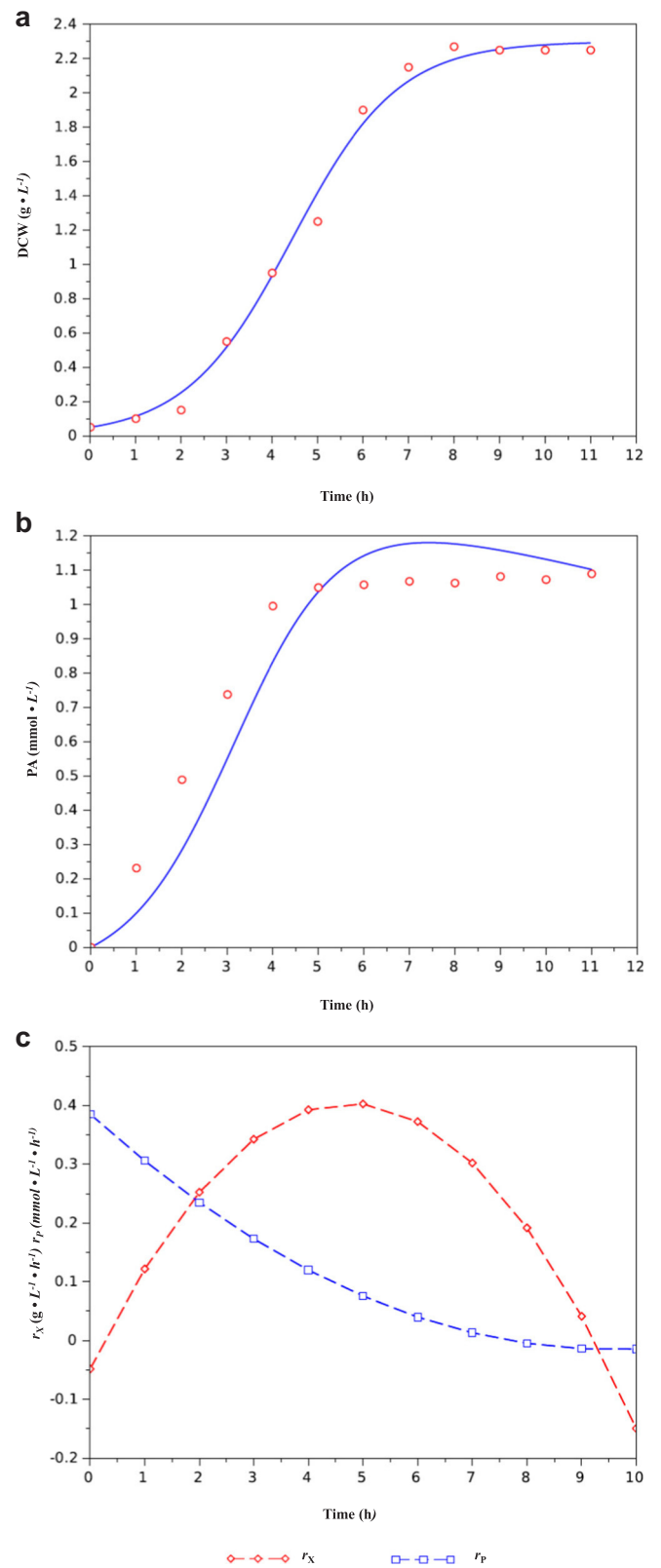


Fig. 5. Predicted data versus observed data from p3 cultivated in a stirred tank reactor (STR) under microaerobic system for the DWC growth (a), PA production (b) and fermentative parameters ( $r_X$ ) and ( $r_P$ ) (c).

**Table 2**

Simulation and data analysis for kinetics models, using only the mean values from the data experimental in MATLAB software version 2007 (MathWorks, Natick, MA). Kinetic parameters simulated of PA production using MATLAB. Recommended parameter values:  $\mu_0$  - Specific growth rate ( $\text{h}^{-1}$ );  $l$  - PA concentration ( $\text{mmol}\cdot\text{l}^{-1}$ );  $\alpha$  - Luedeking–Piret growth-associated parameter ( $\text{mmol}\cdot\text{l}^{-1}\text{ PA}\cdot\text{g}^{-1}\text{ DWC}$ );  $\beta$  - Luedeking–Piret non-growth associated parameter ( $\text{mmol}\cdot\text{l}^{-1}\text{ PA}\cdot\text{h}^{-1}$ );  $\mu_X$  - predicted specific growth rate ( $\text{h}^{-1}$ )  $\mu_P$  - predicted specific formation rate of PA ( $\text{h}^{-1}$ ).

Parameter	Recommended parameter values		
	Unit	Aerobic	Microaerobic
$\mu_0$	$\text{h}^{-1}$	2.271	2.687
$l$	$\text{mmol}\cdot\text{l}^{-1}\text{ PA}$	3.175	3.31
$\alpha$	$\text{mmol}\cdot\text{l}^{-1}\text{ PA}\cdot\text{g}^{-1}\text{ DWC}$	0.451	0.645
$\beta$	$\text{mmol}\cdot\text{l}^{-1}\text{ PA}\cdot\text{h}^{-1}$	0.00024	0.00138
$\mu_X$	$\text{h}^{-1}$	0.915	0.857
$\mu_P$	$\text{h}^{-1}$	0.413	0.554

$\text{PA}\cdot\text{h}^{-1}$ . In this system,  $\alpha = 0.64479\text{ mmol}\cdot\text{l}^{-1}\text{ PA}\cdot\text{g}^{-1}\text{ DWC}$ , again demonstrating a semi-growth-associated profile for PA production. Again,  $\alpha$  was the dominant term, whereas the  $\beta$  term tended to zero, with the product formed during growth phase proportional to the growth rate ( $\alpha \neq 0$  and  $\beta \rightarrow 0$ ).

Kinetic parameters and the mathematical model can be used for the simulation of DWC growth and PA production in both aerobic and microaerobic systems, respectively (Table 2), assuming that the PA production from p3 in these systems followed a semi-growth-associated model.

#### 4. Discussion

For the first time, aiming to better understanding the growth and metabolism of p3 to produce of food additives, was evaluated the kinetic of p3 growth under controlled pH, temperature, and aeration in a stirred tank reactor (STR). Previously, the effect of pH and temperature of p3 was evaluated and it was found that the maximum specific growth rate of thermotolerant yeast p3 occurred at 40 °C. Here, the activation energy was of 71.37 kJ·mol<sup>-1</sup> using the Arrhenius equation, similar to the activation energies of probiotic yeast that have been previously reported (Antoce et al., 1997; Rajoka et al., 2004; Urit et al., 2013). This condition of thermotolerant yeast is a very important parameter and suggests its use as a probiotic yeast. In industry, this could reduce operational costs (Choi et al., 2011; Maccaferri et al., 2012; Bengoa et al., 2018).

Maximum coefficient YP/X values determined under microaerobic systems suggest the synthesis of sterols from p3. Ergosterol synthesis by yeast occurs in the presence of oxygen and in oxygen-limiting conditions (Marbà-Ardébol et al., 2018). Another aspect of PA production by yeast is the ability of some yeast to convert organic acids to acetyl-CoA via the intermediary pathway of lipid biosynthesis using acetyl-CoA (Oberender et al., 2012; Kolouchová et al., 2015; Ratledge, 2004). Acetyl-CoA is an intermediary molecule that is also produced by acetic acid conversion by glyoxylate cycle, forming succinic acid via succinyl-CoA synthetase (Kolouchová et al., 2015; Marbà-Ardébol et al., 2018). Acetic acid was not detected. However, succinic acid was detected in trace amounts in the aerobic and microaerobic systems. It is possible that acetic acid conversion to acetyl-CoA may have occurred, forming organic acids and PA. These data, combined with the study of the maintenance of redox balance in metabolically active cells, demonstrated the p3 strategy for maintenance of redox balance and the re-oxidation of NADH (Fig. 2b). The aerated system can be used as a tool to optimize product yield and flux distribution by rebalancing NADH and NADPH utilization, as well as improving the efficiency of substrate conversion and increasing the yield of product (Dijken and Scheffers, 1986; dos Santos et al., 2013; Rodrussamee et al., 2011; Pentjuss et al.,

2017).

To define parameters of mathematical modeling and to explain the PA production, it is important to obtain data regarding growth, ethanol, and glycerol production, as well organic acids from p3 culture, under controlled aeration systems in a STR. It is also important to determine whether DWC formation took place concomitantly with PA production (Fig. 3a, b). Using the growth-associated constant  $\alpha$  and biomass-associated constant  $\beta$ , it was determined that the biomass and the specific growth rate of p3 had partial influence on the increase in PA production, with  $\alpha$  and  $\beta$  factors being directly involved in PA production, according to the level of aeration employed (Luedeking and Piret, 1959; Sansonetti et al., 2013; Zhang and Vadlani, 2013).

In both bioprocesses,  $\alpha$  was the dominant term, whereas the  $\beta$  term tended toward zero, with the product formed during growth phase proportional to the growth rate ( $\alpha \neq 0$  and  $\beta \rightarrow 0$ ). The growth-associated constant  $\alpha$  and biomass-associated constant  $\beta$  are commonly obtained using the Luedeking–Piret equation, in that both  $\alpha$  and  $\beta$  correlate with the strains and end-products used, and therefore vary, requiring analysis of each process (Nandasana and Kumar, 2008; Wu et al., 2015; Zhao et al., 2010). Here, there were higher yields of DWC and specific growth rate of p3 in STR;  $\alpha$  was 0.450639 and 0.64479  $\text{mmol}\cdot\text{l}^{-1}\text{ PA}\cdot\text{g}^{-1}\text{ DWC}$ , while those of  $\beta$  were 0.000243 and 0.00138  $\text{mmol}\cdot\text{l}^{-1}\text{ PA}\cdot\text{h}^{-1}$  in the aerobic and microaerobic systems, respectively. Other investigators described  $\alpha$  values of 0.26 g/g for lactic acid production from molasses using *Enterococcus faecalis* RKY1 (Anjana and Surendra, 2008). The  $\alpha$  and  $\beta$  factors can be modified for the same product (lactic acid), with  $\alpha$  of 10.48 g/g and  $\beta$  of 1.36 g/(g·h) (*Sporolactobacillus* spp.) or  $\alpha$  of 3.2631 g/g and  $\beta$  of 0.5980 g/(g·h) using *Rhizopus oryzae* (Zhao et al., 2010; Wu et al., 2015), and with a different product (inulinase), with  $\alpha$  and  $\beta$  of 0.75 and 0.033, respectively, using *K. marxianus* (Santharam et al., 2017). Our findings suggest that it is indispensable to predict  $\alpha$  and  $\beta$ , the constants of proportionality of Luedeking–Piret factors, so as to optimize PA production. PA production followed a semi-growth-associated with biomass and that for the specific growth rate of p3,  $\alpha$  and  $\beta$  were affected by aeration levels employed. This study revealed the importance of these metabolic analyses for the prediction of factors from a mathematical model, aiming toward the optimization of PA production from cultures of p3 in aerated systems.

#### 5. Conclusion

The thermotolerant yeast p3 showed better growth at 40 °C. And under CAS in STR, better specific formation rates of PA and yield coefficients of product in microaerobic system. The rp of p3 was kinetically associated with the biomass formation rates ( $r_x$ ), and the DWC in both aerobic and microaerobic system. The DWC and the specific growth rate caused a partial effect on increases in PA production, varying  $\alpha$  and  $\beta$  of the Luedeking–Piret equation. From this work, using the predicted values of  $\alpha$  and  $\beta$  from yeast p3 under CAS, it was possible to predict the PA production optimization.

#### Abbreviations

CEUA	Federal University of Latin American Integration Animal Experimentation Committee
COBEA	Brazilian College of Animal Experimentation
STR	stirred tank reactors
GRAS	Generally Regarded as Safe
FDA	Food and Drug Administration
DWC	dry weight of cells
Acetyl-CoA	acetyl coenzyme A
HPLC	high performance liquid chromatography
X	biomass
$\mu$	specific growth rate, ( $\text{h}^{-1}$ )
$\mu_P$	Specific formation rate of PA, ( $\text{h}^{-1}$ )

$Q_P$	volumetric productivity of PA ( $\text{mmol}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$ )
$\text{Ln}(\text{OD})$	natural logarithm of optical density, ( $600_{\text{nm}}$ )
$E_a$	activation energy, ( $\text{KJ}\cdot\text{g}\cdot\text{mol}^{-1}$ )
$R$	gas constant, ( $\text{J}\cdot\text{K}^{-1}\cdot\text{g}\cdot\text{mol}^{-1}$ )
$\alpha$ and $\beta$	constant of proportionality, Luedeking–Piret
$r_x$	instantaneous specific growth rate.
$r_p$	instantaneous rate of product formation

### Compliance with ethical standards

The authors declare that they followed all the ethical standards for publication. This article does not contain any studies with human participants. All experiments of isolation of microorganisms were performed in accordance with the ethical guidelines for experiments in newborn piglets, and the protocols were approved by the National Council for Control of Animal Experimentation, Federal University of Latin American Integration Animal Experimentation Committee (CEUA n° 001/2018). The guidelines for animal use and care were based on the standards established by the Brazilian College of Animal Experimentation (COBEA).

### CRediT authorship contribution statement

ASRC performed the experiments using p3 culture in STR bioreactor. ASRC, FMLP, FJVP, GSSP, CAM, and WBS analyzed data, isolated microorganisms and wrote the manuscript. PAC, RWSA, GRS, IVB participated in the analysis and in the development mathematical modeling. EMS, KVF, FSC and PAN revised the manuscript for English language and technical aspects of the work.

### Declaration of competing interest

There is no conflict of interest to declare.

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