

Article

Mathematical Modeling of a Continuous Multistage Ethanol Production Bioprocess on an Industrial Scale

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Abstract

In this study, a mathematical model was proposed for a continuous, multistage, industrial-scale alcoholic fermentation process, comprising four vats in series with volumes equal to 600 m³, with separation, acid treatment, and cell recycling from the fourth to the first vat. The system was operated daily under variable volumetric flow rates and substrate concentrations in the feed stream, i.e., $F_0 = 93\text{--}127\text{ m}^3/\text{h}$ and $S_0 = 210\text{--}238\text{ g/L}$. The mathematical model consisted of mass balance equations for cells, substrate, and product in the vats, the separator, and the acid treatment unit. An unsegregated and unstructured approach was used to describe the microbial population, with the kinetics of cell growth, substrate consumption, and product formation represented by equations generally adopted for alcoholic fermentation. The model parameters were estimated by nonlinear regression, providing typical values for alcoholic fermentation. Model predictions agreed well with both the experimental data used in the parameter estimation step and those used in the model validation step.

Keywords: alcoholic fermentation; CSTR cascade; sugarcane; yeasts; kinetic models; parameter estimation



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1. Introduction

Ethanol is mainly produced in Brazil by fermentation using sugarcane as the raw material and yeast as the biological agent [1]. In this production, large amounts of residue are also generated, including biomass-based materials (bagasse and straw) and vinasse. Bagasse and straw represent rich potential sources of sugar, which have been extensively studied for the obtainment of cellulosic ethanol [2,3]. Vinasse has been commonly used for fertigation of the sugarcane crop itself and, recently, its potential for the production of biogas by anaerobic bacteria has been investigated [4].

Ethanol production in Brazil started with the traditional Melle-Boinot process, which is characterized by being a fed-batch fermentation, followed by cell separation by centrifugation, dilution, acid treatment, and recycling of the cells to the reactor to initiate a new bioprocessing cycle [1,5,6].

The development of cost-effective technologies for ethanol biotechnological production is a crucial objective to be achieved by the application of bioprocesses engineering principles, including kinetics and reactor design concepts [3,5,7]. In this context, it is highly desirable to intensify the fermentation process to increase the final ethanol concentration because, for the same volume of fermented broth, it allows for the reduction of both the water and

energy requirements for the preparation of the medium and for the distillation of ethanol, respectively [5,7,8].

Recently, Stremel et al. [5] presented several strategies for the intensification of chemical and biochemical processes, highlighting the cascading or compartmentalization of reaction zones integrated with the simultaneous removal of undesirable products, continuous operation mode, catalyst recycle, and immobilization.

The main technological innovation for ethanol production was the development of continuous fermentation processes, which were initially operated in a single stage [1]. Due to technical issues related to inhibition phenomena, continuous processes have evolved from single to multiple stages. It is well known that in alcoholic fermentation, inhibition by the substrate and by the product itself (ethanol) can occur [9,10]. Substrate inhibition is significant in very high fermentations (VHGs), which are characterized by using media with high sugar concentrations (above 250 g/L) to achieve high ethanol concentrations, causing strong stress for the cells due to high osmotic pressures at the beginning of the fermentation process [8]. When operating conditions are such that substrate inhibition can be neglected, a cascade of four to six continuous stirred tank reactors (CSTRs), connected in series, becomes the most recommended system to reduce product inhibition. Consequently, better process performance can be achieved in terms of substrate conversion and product formation, since the system will behave approximately like a plug flow reactor (PFR) [11]. Another advantage offered by this arrangement of bioreactors is the minimization of the residence time of the system, increasing process productivity [6]. In addition, the system can be improved by cell recycling using centrifuges to separate parts of the biomass and sending them to the first bioreactor of the cascade to intensify the fermentation with a higher cell concentration [1,11]. Since temperature is an important process variable that exerts a great influence on kinetic parameters, each reactor of the series is equipped with an external heat exchanger to remove the heat released by the fermentation, maintaining the temperature constant at the desired value [1,12].

Operation of the alcoholic fermentation processes in a continuous mode is desirable, since higher substrate conversions, yields, and productivities in ethanol can be attained [13]. However, the industrial implementation of a continuous process requires previous studies on its dynamic behavior, aiming at developing efficient control strategies [11,14]. When optimal operation is a target, a problem faced is the lack of process robustness in the presence of fluctuations in operational conditions, leading to changes in kinetic behavior, with impacts on process performance [15]. In ethanol plants, these changes are frequent and occur not only due to variations in the operating variables but also spontaneously, as a result of adaptive mechanisms of the cells themselves, generating oscillations in the main process variables. However, in industrial processes conducted in four to six bioreactors in series, such oscillations are attenuated until they disappear along the cascade of bioreactors, making the overall process performance mainly affected by fluctuations in the systems of fermentation, control, heat transfer, and separation [16].

The lack of process robustness in the face of fluctuations can be compensated by adjustments to both operational variables and control parameters, which must be based on a mathematical model sufficiently representative of the process [15].

Mathematical models for cell growth, substrate consumption, and product formation in alcoholic fermentations have been satisfactorily developed using an unsegregated and unstructured approach to the microbial population [9,17–21]. Given that such models are robust, they can be applied to describe the bioprocess under static and time-varying conditions, making them useful to predict the dynamic behavior of the system [22–24].

Although numerous studies have been published on the modeling and simulation of alcoholic fermentation bioprocesses, addressing different aspects such as bioprocess

kinetics, optimization, and control, most studies focused on the mathematical description of bioprocesses carried out in batch or fed-batch mode in a single fermenter at a bench scale [25]. Few studies have modeled continuous bioprocesses, let alone when these are conducted in bioreactors in series at an industrial scale, as in the present study, which generates significant academic and technological interest, as this operational configuration is used in some Brazilian plants [25–27]. Thus, the objective of this study was to develop a simple mathematical model, based on an unsegregated and unstructured approach, aiming at describing data from a continuous alcoholic fermentation process in a CSTR cascade with cell recycling. The originality of the study lies primarily in the industrial-scale dataset. Given that the bioprocess was carried out under fluctuating feed conditions, the developed mathematical model was indirectly tested for such conditions, without this being a previously desired objective.

2. Materials and Methods

2.1. Experimental Data

The experimental data used in this study are from the operation of a continuous multistage alcoholic fermentation process on an industrial scale, whose simplified flowchart for mathematical modeling purposes is presented in Figure 1.

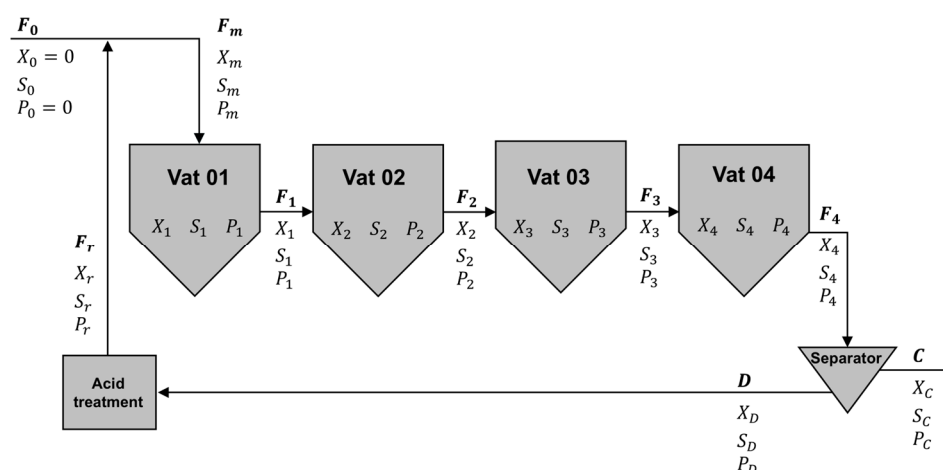


Figure 1. Simplified flowchart of the continuous multistage alcoholic fermentation process on an industrial scale.

The process was operated with different wort feed flow rates ($F_0 = 93\text{--}127\text{ m}^3/\text{h}$), different substrate concentrations (total reducing sugars) in the feed stream ($S_0 = 210\text{--}238\text{ g/L}$), and temperature (T) ranging from 30 to 35 °C, with the average value of 33 °C assumed as the constant temperature.

The fermented medium continuously removed from the fourth vat is directed to a separator, where the feed stream, after cell separation, is divided into two streams: a clarified stream (C), virtually free of cells, which is sent to the distillation unit for ethanol separation and recovery; and a dense stream (D), consisting mostly of biomass, which is sent to the acid treatment unit and subsequently recycled to the first vat. The ratio between the recycle flow rate (F_r) and the volumetric feed flow rate of the system (F_0) was 50%, resulting in a recycle ratio (RR) of 0.5, i.e., $RR = F_r/F_0 = 0.5$.

The experimentally measured response variables were the concentrations of substrate (S), and product (P) in the first and fourth vats, during 30 days of bioprocess operation, with the vat volumes being $V_1 = V_2 = V_3 = V_4 = 600\text{ m}^3$.

2.2. Analytical Methods

2.2.1. Determination of Cell Viability and Budding

The cell viability and budding of *S. cerevisiae* yeast were determined using the differential cell staining method with erythrosine solution, followed by counting viable (active) and nonviable cells, and viable buds in a Neubauer chamber, with the results expressed as percentages according to the following equations:

$$\text{Cell viability} = [(\text{Number of viable cells})/(\text{Total number of cells})] \times 100\% \quad (1)$$

$$\text{Budding} = [(\text{Number of viable buds})/(\text{Number of viable cells})] \times 100\% \quad (2)$$

2.2.2. Determination of Alcohol Content

To determine the alcohol content in fermentation vats 1 and 4, a common method used in plants with less advanced technology was used, which consists of collecting a 25 mL sample and placing it in a microdistiller, where the entire sample was distilled. The distilled product was then analyzed with a densimeter.

2.2.3. Determination of Total Reducing Sugars (TRSs)

TRSs represent all sugars in the sugarcane in the form of reducing sugars or inverted sugars. Lane-Eynon titrimetry, also known as the Fehling method, was used to determine the percentage of total reducing sugars in the sample at any given time instant.

2.2.4. Estimation of Cell Concentration

There is no available experimental data on cell concentration in the fermentation vats. The only available measurements of this state variable are the cell concentrations in the stream leaving the separator and going to acid treatment (after which the cells are recycled to the first vat), and the cell concentration in the clarified stream that goes to the distillation unit. Cell concentration is a fundamental variable in the kinetic analysis of bioprocesses, as it presents the equations that define specific rates of cell growth, substrate consumption, and product formation. This way, the measurement of the number of active cells described in item 2.2.1 was used to estimate the cell concentration in g/L, considering that the available measurement provides the number of yeast cells present in 1 mL and not the mass (in g) in this volume. A study conducted by the Brewers Friends Association concluded that a package of dry yeast contains approximately 8 to 20 billion cells per gram, a fairly wide range. Haddad and Lindegren [28] described an experimental method for determining the density and mass of an individual yeast cell and obtained mean values $\pm$ standard deviations of 1.087 ± 0.026 g/mL and $7.922 \times 10^{-11} \pm 3.188 \times 10^{-11}$ g, respectively. Assuming that 1 g of yeast contains approximately 10 billion cells, it was possible, based on the previous data, to establish appropriate relationships and estimate the cell concentration in the bioprocess, specifically in vats 1 and 4, obtaining values for this variable consistent with values reported in the literature for industrial-scale alcoholic fermentation processes similar to that investigated in the present study.

2.3. Equations of Mass Balance

For the mathematical modeling of the bioprocess, the original flowchart was adapted as shown in Figure 2.

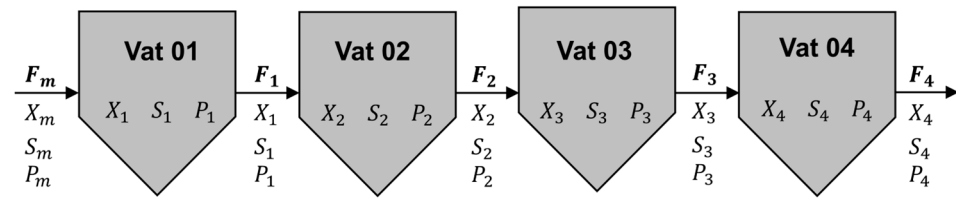


Figure 2. Adapted flowchart of the bioprocess for mathematical modeling purposes.

Due to the complete mixing promoted by both the evolution of CO₂ gas produced in the bioprocess and the agitation caused by the kinetic energy of outlet stream of heat exchanger when returning to the reactor, the hydrodynamic behavior of the fermentation vats is of a CSTR. However, the perfect-mixing assumption could be confirmed by data from hydrodynamic studies, which are not available. This way, based on the flowchart shown in Figure 2, the steady-state mass balance equations for each component in vats 2, 3, and 4 can be generalized in the form of the following equations, with $i = 2, 3, 4$:

$$\text{Cell : } 0 = F_{i-1} \cdot X_{i-1} - F_i \cdot X_i + (r_X)_i \cdot V_i; (r_X)_i = (\mu_X)_i \cdot X_i \cdot V_i \quad (3)$$

$$\text{Substrate : } 0 = F_{i-1} \cdot S_{i-1} - F_i \cdot S_i - (r_S)_i \cdot V_i; (r_S)_i = (\mu_S)_i \cdot X_i \cdot V_i \quad (4)$$

$$\text{Product : } 0 = F_{i-1} \cdot P_{i-1} - F_i \cdot P_i + (r_P)_i \cdot V_i; (r_P)_i = (\mu_P)_i \cdot X_i \cdot V_i \quad (5)$$

To elaborate the mass balances in the first vat, the scheme presented in Figure 3 was considered, obtaining Equations (6)–(8), in which the inlet conditions in this vat (F_m , S_m , X_m , and P_m) were determined from the properties resulting from the mixing of the system feed stream with the recycle stream.

$$\text{Cell : } 0 = F_m \cdot X_m - F_1 \cdot X_1 + (r_X)_1 \cdot V_1; (r_X)_1 = (\mu_X)_1 \cdot X_1 \cdot V_1 \quad (6)$$

$$\text{Substrate : } 0 = F_m \cdot S_m - F_1 \cdot S_1 + (r_S)_1 \cdot V_1; (r_S)_1 = (\mu_S)_1 \cdot X_1 \cdot V_1 \quad (7)$$

$$\text{Product : } 0 = F_m \cdot P_m - F_1 \cdot P_1 + (r_P)_1 \cdot V_1; (r_P)_1 = (\mu_P)_1 \cdot P_1 \cdot V_1 \quad (8)$$

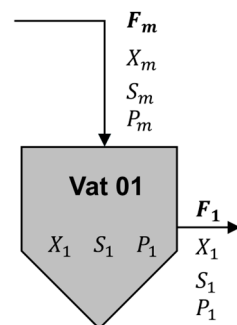


Figure 3. Scheme of the inlet and outlet streams in the first vat and respective concentrations of the components.

Figure 4 shows a scheme of the biomass separator, with the mass balances in this unit given by Equations (9)–(11):

$$\text{Cell : } 0 = F_4 \cdot X_4 - D \cdot X_D - C \cdot X_C \quad (9)$$

$$\text{Substrate : } 0 = F_4 \cdot S_4 - D \cdot S_D - C \cdot S_C \quad (10)$$

$$\text{Product : } 0 = F_4 \cdot P_4 - D \cdot P_D - C \cdot P_C \quad (11)$$

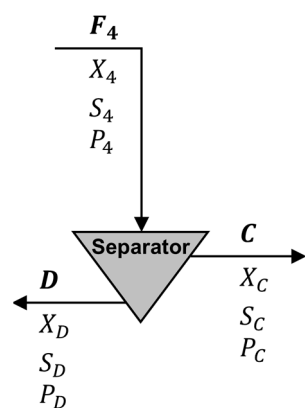


Figure 4. Scheme of the biomass separator with its inlet and outlet streams.

In Equations (3)–(11): F_k , D , C are volumetric flow rates (m^3/h); X_k , S_k , P_k are concentrations (g/L); r_k are volumetric reaction rates ($\text{g}/(\text{L}\cdot\text{h})$); and μ_k are specific reaction rates ($\text{g}/(\text{g}\cdot\text{h})$).

3. Results and Discussions

3.1. Experimental Data

3.1.1. Substrate Concentration in Feed

Figure 5 shows the daily values of the substrate concentration (sugars) in the feed stream of the fermentation system, which varied between 211 and 238 g/L of TRSs even during continuous operation, as it is common for variations in the composition of the juice to occur, since the composition of sugarcane can vary depending on the variety of sugarcane, the soil where it was planted, and the period and method (manual or mechanical) of harvesting.

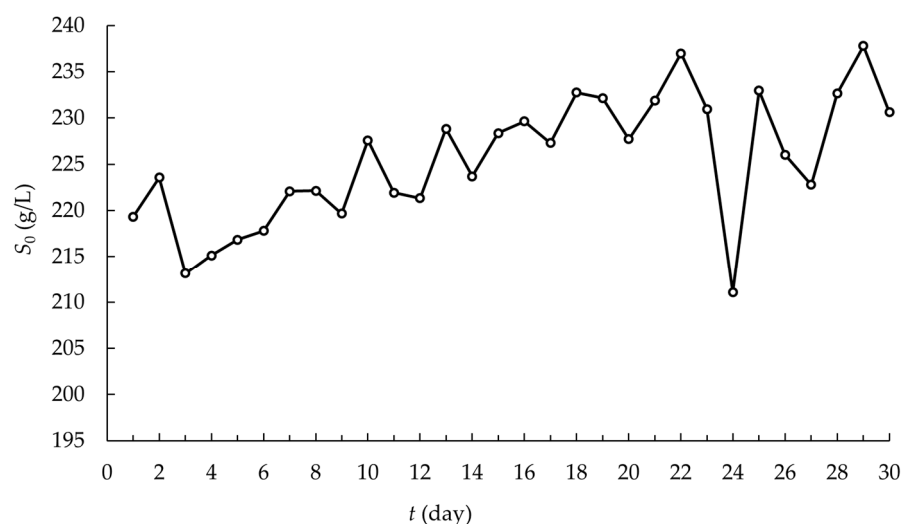


Figure 5. Daily substrate concentration in the fermentation system feed stream (S_0).

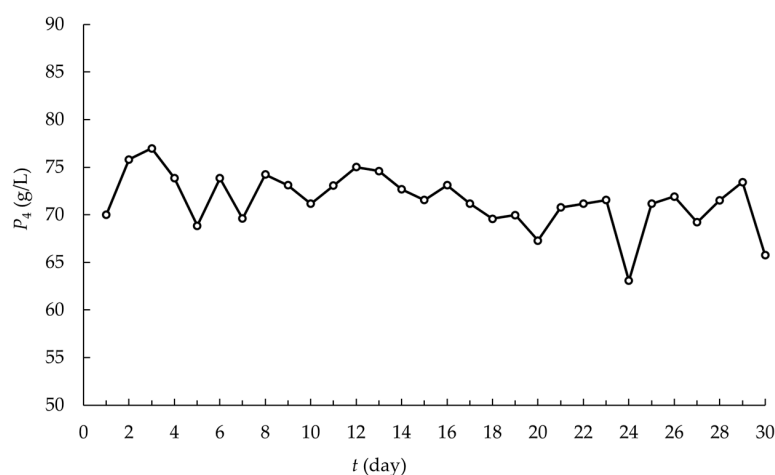
Regarding the amount of substrate consumed in the vats, Table 1 shows a significant substrate consumption in the first vat, followed by lower consumption in subsequent vats, resulting in nearly complete overall substrate consumption in the fermentation system. This is an expected performance for a good producer microorganism, i.e., high substrate conversion, as the cost of feedstock can represent more than 50% of the total production cost [29]. High levels of residual substrates are prohibitive, aiming at ensuring a favorable cost-effective relationship for the bioprocess.

Table 1. Daily values of volumetric flow rate and substrate concentration in some bioprocess streams and substrate concentration at the vat outlets.

t (day)	F_0 (m ³ /h)	F_m (m ³ /h)	S_0 (g/L)	S_m (g/L)	S_1 (g/L)	S_2 (g/L)	S_3 (g/L)	S_4 (g/L)
1	126	189	219.33	109.66	54.27	16.28	5.36	4.77
2	105	158	223.57	111.78	39.53	14.94	5.32	3.69
3	99	149	213.14	106.57	23.84	7.13	3.46	3.05
4	126	189	215.15	107.58	9.98	4.24	3.21	3.38
5	105	158	216.84	108.42	15.74	4.02	3.64	3.21
6	107	161	217.80	108.90	11.39	4.48	3.22	3.35
7	111	167	222.10	111.05	17.02	3.71	3.15	2.95
8	108	162	222.15	111.08	19.68	4.18	4.05	3.41
9	127	191	219.67	109.83	26.96	3.41	3.31	3.03
10	113	170	227.60	113.80	39.63	3.54	2.79	3.76
11	116	174	221.93	110.96	23.23	4.24	3.27	3.03
12	124	186	221.33	110.66	39.07	3.33	2.54	3.34
13	113	170	228.85	114.43	39.43	3.36	3.25	3.55
14	112	168	223.71	111.85	24.44	2.60	2.41	3.01
15	121	182	228.38	114.19	6.46	3.06	2.64	3.22
16	116	174	229.64	114.82	20.48	3.43	2.61	2.74
17	109	164	227.31	113.65	25.29	2.12	1.95	2.05
18	109	164	232.75	116.38	16.68	3.40	2.76	2.67
19	113	170	232.15	116.08	24.27	3.35	3.15	2.76
20	112	168	227.73	113.86	24.47	2.51	2.34	2.78
21	115	173	231.88	115.94	33.64	2.50	2.17	2.63
22	104	156	237.00	118.50	39.65	3.09	2.60	2.81
23	108	162	230.97	115.48	23.88	3.19	3.04	3.20
24	119	179	211.10	105.55	33.34	2.40	2.04	2.15
25	103	155	232.98	116.49	33.95	2.71	2.54	2.51
26	111	167	226.03	113.02	43.87	3.23	2.74	2.69
27	97	146	222.80	111.40	22.26	3.27	3.12	2.88
28	93	140	232.65	116.33	14.77	3.19	3.09	3.02
29	108	162	237.80	118.90	25.05	3.02	2.74	2.76
30	105	158	230.62	115.31	23.32	3.01	3.15	2.70

3.1.2. Product Concentration

Figure 6 shows the behavior of the daily ethanol concentration in the outlet stream of the fourth vat, which is equal to the concentration at the system outlet (P_4). Changes in this variable were most likely due to variations in S_0 , with direct effects on the concentration of ethanol produced.

**Figure 6.** Daily product (ethanol) concentration in the outlet stream of the fourth vat (P_4).

From the macrobalances around the fermentation system, the values of the product yield per substrate consumed ($Y_{P/S}$) and per biomass formed ($Y_{P/X}$), as well as the values of biomass yield formed per substrate consumed ($Y_{X/S}$) were calculated as follows: $Y_{P/S} = (P_4 - P_m)/(S_m - S_4)$, $Y_{P/X} = (P_4 - P_m)/(X_4 - X_m)$, and $Y_{X/S} = (X_4 - X_m)/(S_m - S_4)$, obtaining the data presented in Table 2.

Table 2. Daily values of substrate, ethanol, and biomass concentrations at some key points of the fermentation system and yield factors.

t (day)	S_m (g/L)	S_4 (g/L)	P_m (g/L)	P_4 (g/L)	X_m (g/L)	X_4 (g/L)	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g)
1	109.66	4.77	20.59	70.01	48.40	49.34	0.47	52.77
2	111.78	3.69	20.22	75.81	27.45	30.93	0.51	15.97
3	106.57	3.05	19.48	76.98	47.68	56.61	0.56	6.44
4	107.58	3.38	20.13	73.87	63.55	70.22	0.52	8.05
5	108.42	3.21	19.12	68.84	52.25	56.65	0.47	11.31
6	108.90	3.35	20.13	73.84	49.95	55.11	0.51	10.41
7	111.05	2.95	18.08	69.61	65.81	76.13	0.48	5.00
8	111.08	3.41	19.38	74.24	74.41	85.45	0.51	4.97
9	109.83	3.03	15.88	73.10	58.75	80.97	0.54	2.57
10	113.80	3.76	17.73	71.16	75.95	91.38	0.49	3.46
11	110.96	3.03	17.54	73.07	70.14	87.81	0.51	3.14
12	110.66	3.34	19.76	75.01	75.05	85.77	0.51	5.15
13	114.43	3.55	18.46	74.61	43.36	52.63	0.51	6.06
14	111.85	3.01	16.44	72.67	64.16	84.92	0.52	2.71
15	114.19	3.22	16.17	71.55	55.23	73.53	0.50	3.03
16	114.82	2.74	17.00	73.10	56.31	72.38	0.50	3.49
17	113.65	2.05	17.54	71.18	44.85	54.38	0.48	5.63
18	116.38	2.67	17.99	69.59	60.66	70.14	0.45	5.44
19	116.08	2.76	16.25	69.98	43.61	56.47	0.47	4.18
20	113.86	2.78	15.61	67.30	48.37	62.73	0.47	3.60
21	115.94	2.63	16.35	70.78	48.06	62.27	0.48	3.83
22	118.50	2.81	17.63	71.16	65.53	79.25	0.46	3.90
23	115.48	3.20	16.53	71.55	56.65	73.84	0.49	3.20
24	105.55	2.15	16.63	63.07	63.38	72.25	0.45	5.24
25	116.49	2.51	18.55	71.16	46.95	53.90	0.46	7.57
26	113.02	2.69	20.31	71.93	92.70	98.13	0.47	9.50
27	111.40	2.88	20.32	69.21	70.99	72.25	0.45	38.92
28	116.33	3.02	16.99	71.53	56.57	71.33	0.48	3.70
29	118.90	2.76	19.29	73.44	42.35	48.30	0.47	9.10
30	115.31	2.70	17.92	65.76	55.11	60.81	0.42	8.39

The calculated values of $Y_{P/S}$ resulted in an average value of 0.47 g/g, corresponding to 92% of the stoichiometric value (0.511 g/g). The difference in relation to 100% can be explained by deviations in the use of substrate for other purposes such as cell growth, byproduct formation, and energy generation for maintenance. In calculating the average value of $Y_{P/S}$, data equal to or greater than 0.511 g/g were disregarded, since, as is known, it is not possible to achieve yields equal to or greater than the maximum due to metabolic deviations. *Saccharomyces cerevisiae* yeasts are typically used in alcoholic fermentations, often achieving ethanol yields of around 90% of the stoichiometric value [29]. This makes these yeasts the most commonly used microorganisms for this bioconversion, although several other microorganisms produce ethanol from glucose, but not with such high yields. This aspect is of great economic importance, given that the raw material, in this case, represents approximately 60% of the cost of ethanol [3]. Therefore, insufficient yields make the production of this product economically unfeasible, as its low added value

makes its profitability heavily dependent on high production and marketing volumes. It is always desirable for the microorganism to allow a high level of product accumulation in the medium without suffering more pronounced inhibition due to this higher product concentration. Greater product accumulation contributes to a reduction in recovery costs, which can also be quite significant.

Regarding $Y_{P/X}$, some values of this yield factor were well above those reported by other authors [22,30,31], which obtained values of $Y_{P/X}$ between 2.50 and 6.25 g/g. Discarding values outside the mentioned range, an average $Y_{P/X}$ value of 5.02 g/g was obtained, which is consistent with those reported in the literature.

The $Y_{X/S}$ average value was 0.1 g/g, similar to some values found in the literature [18,19], which is typical of alcoholic fermentation processes, and occurs in almost anaerobic conditions, with fermentative metabolism predominating over respiratory metabolism, reducing cell growth.

3.1.3. Cell Viability and Budding

In the unsegregated approach, the cell population is considered homogeneous, with all cells exhibiting the same behavior and no distinction is made between them in any aspect. However, it is known that in alcoholic fermentations, factors such as ethanol toxicity, nutrient limitations (especially sterols and unsaturated fatty acids), and osmotic stress, all of which compromise cell membrane integrity and metabolic activity can lead to the loss of cell viability in part of the microbial population, resulting in cell inactivation and consequently, decreased productivity [32].

Figure 7 shows the percentage of viable cells in the first vat of the fermentation system. It can be seen that high levels of cell viability were observed in this vat, resulting in high activity of the microbial population, which in turn provided a significant consumption of substrate in this vat.

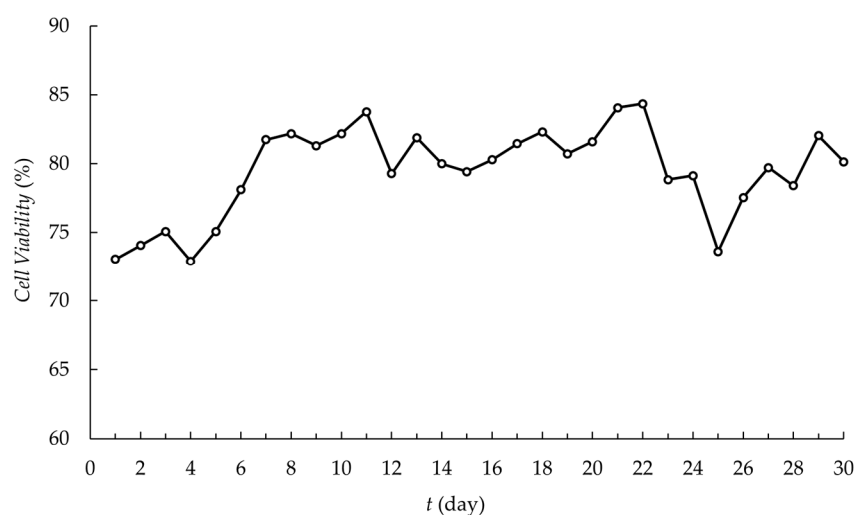


Figure 7. Percentage of viable cells in the first vat of the fermentation system.

Figure 8 shows the behavior of the cell budding percentage in the first vat of the fermentation system. This parameter is an indirect measure of biomass growth, and its changes are related to variations in cell concentration. It can be seen that, disregarding some outliers, the budding percentage fluctuated moderately, around 7%.

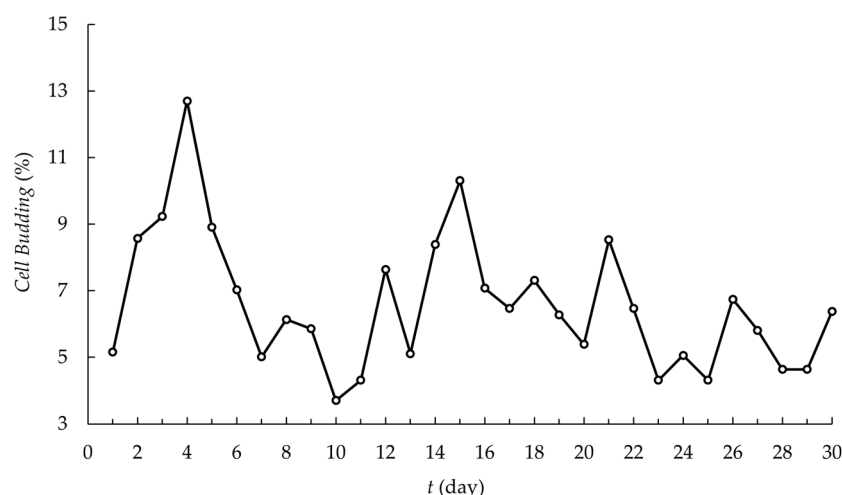


Figure 8. Percentage of cell budding in the first vat of the fermentation system.

3.2. Model Development

When developing a mathematical model for a given system, a preliminary step is model conception, in which several relevant aspects and characteristics of the system are identified for modeling purposes, simplifying assumptions are postulated, and complementary data are obtained from the literature.

A primary aspect to be discussed is whether or not to consider the loss of cell viability in the kinetic analysis of the bioprocess. Although this loss is reported by several authors, it is rarely considered in mathematical modeling. A theoretical study on the kinetics of continuous bioprocesses, considering loss of cell viability, established the concept of “minimum cell viability”, evaluating its consequences for the design and operation of continuous stirred tank reactors (CSTRs) with and without cell recycling in cultures whose growth kinetics were described by the Monod equation. According to this study, minimum cell viability is the minimum value of viability that the microbial population must present for a steady state other than the wash state to be reached and maintained [33]. Hojo et al. [34], studying ethanol production by flocculent yeasts in a CSTR with and without cell recycling, concluded that the consideration of cell viability is an important factor for establishing the kinetic model of the bioprocess with recycling and that cell death must be considered in long continuous fermentations with high hydraulic residence times. However, in the present study, due to the high levels of cell viability observed daily (70–85%) in the first vat, an unsegregated approach was used to describe the microbial population, as well as an unstructured approach to the cells, i.e., no intracellular components were considered in the description.

An event frequently observed in alcoholic fermentations is the inhibition by the ethanol, which begins at 8–10% *v/v*, approximately 64–80 g/L in the fermented medium [29]. Furthermore, the consideration of a possible inhibition by ethanol is due to this product being reported as a noncompetitive inhibitor of cell growth, with consequent inhibition of its own production, as it is a primary product of yeast metabolism, it is produced simultaneously with cell growth. This way, in the present study, μ_p was correlated with μ_x by using the classical equation of Luedeking-Piret, i.e., $\mu_p = \alpha\mu_x + \beta$, with $\alpha = Y_{P/X}$ and $\beta = 0$ for product formation coupled with cell growth [9].

In modeling ethanol fermentations, the kinetics of cell growth are generally given by $\mu_x = f(S) \cdot g(P) \cdot h(X)$, in which $f(S)$, $g(P)$, and $h(X)$ are functions that describe the effects of the concentrations of substrate, ethanol, and cell itself on μ_x , respectively. Since the concentration of residual sugars did not reach high levels in the fermentation vats, it was

not necessary to include an inhibition effect by the substrate on μ_x , allowing the $f(S)$ function to be represented by the Monod equation [9]:

$$f(S) = \frac{\mu_{max}S}{K_S + S} \quad (12)$$

To describe the inhibition effect of ethanol on μ_x , the following generalized nonlinear equation has been commonly used, where λ is called a “toxic power” and P_{max} is the ethanol concentration at which cell growth is completely inhibited [9]:

$$g(P) = \left(1 - \frac{P}{P_{max}}\right)^\lambda \quad (13)$$

Most models reported in the literature describe the growth rate (r_x) as a linear function of the cell concentration when this varies from 5 to 10 g/L in conventional fermenters [31]. However, it is well known that the use of yeast recycling leads to high cell concentrations in the fermenter, reaching 10 to 20 times higher than those in conventional fermenters [31]. This makes the linear relationship inadequate and in these cases, it is recommended to include in μ_x a generalized term $h(X) = (1 - X/X_{max})^\delta$, analogous to the product inhibition term $g(P)$, to describe the inhibitory effect of the biomass itself on cell growth, in which δ is a parameter that modulates the intensity of this inhibition and X_{max} is the maximum attainable cell concentration [31].

Based on the considerations previously made, the specific growth rate (μ_x) is given by Equation (14). This equation has been used by several authors to adjust data from various alcoholic fermentation processes, including data from alcohol plants for which it is necessary to incorporate into this kinetic expression a substrate inhibition factor that generally occurs in industrial vats, especially when molasses-based media are used [35].

$$\mu_x = \frac{\mu_{max}S}{K_S + S} \left(1 - \frac{P}{P_{max}}\right)^\lambda \left(1 - \frac{X}{X_{max}}\right)^\delta \quad (14)$$

Finally, to complete the kinetic modeling, the specific rate of substrate consumption (μ_s) was correlated with the specific rate of product formation (μ_p) through the product yield factor per substrate consumed ($Y_{P/S}$), i.e., $\mu_s = \mu_p / Y_{P/S}$.

3.3. Kinetic Parameter Estimation

Table 3 summarizes the experimental data available to estimate the kinetic parameters in Equation (14). Data for the three modeled state variables are available only in vats 1 and 4, while only substrate concentration data are available in intermediate vats 2 and 3. This required appropriate strategies for estimating the kinetic parameters, as well as for calculating the cell, ethanol, and substrate concentrations in the four vats.

These strategies initially consisted of using substrate, cell, and ethanol concentration data from vat 1 to estimate the kinetic parameters. Subsequently, it was assumed that the product yields $Y_{P/S}$ and $Y_{P/X}$ were equally valid in all vats, allowing the calculation of product, cell, and substrate concentrations in vats 2, 3, and 4 using Equations (15)–(20):

$$P_{2calc} = P_{1calc} + Y_{P/S}(S_{1calc} - S_{2calc}) \quad (15)$$

$$X_{2calc} = X_{1calc} + \frac{1}{Y_{P/X}}(P_{2calc} - P_{1calc}) \quad (16)$$

$$P_{3calc} = P_{2calc} + Y_{P/S}(S_{2calc} - S_{3calc}) \quad (17)$$

$$X_{3_{calc}} = X_{2_{calc}} + \frac{1}{Y_{P/X}}(P_{3_{calc}} - P_{2_{calc}}) \quad (18)$$

$$P_{4_{calc}} = P_{3_{calc}} + Y_{P/S}(S_{3_{calc}} - S_{4_{calc}}) \quad (19)$$

$$X_{4_{calc}} = X_{3_{calc}} + \frac{1}{Y_{P/X}}(P_{4_{calc}} - P_{3_{calc}}) \quad (20)$$

Table 3. Experimental data on substrate, cell, and ethanol concentrations available to estimate kinetic parameters.

<i>t</i> (day)	<i>F_m</i> (m ³ /h)	<i>S_m</i> (g/L)	<i>X_m</i> (g/L)	<i>P_m</i> (g/L)	<i>S₁</i> (g/L)	<i>X₁</i> (g/L)	<i>P₁</i> (g/L)	<i>S₂</i> (g/L)	<i>S₃</i> (g/L)	<i>S₄</i> (g/L)	<i>X₄</i> (g/L)	<i>P₄</i> (g/L)
1	189	109.66	48.40	20.59	54.27	44.49	55.83	16.28	5.36	4.77	49.34	70.01
2	158	111.78	27.45	20.22	39.53	67.94	63.84	14.94	5.32	3.69	30.93	75.81
3	149	106.57	47.68	19.48	23.84	61.89	63.04	7.13	3.46	3.05	56.61	76.98
4	189	107.58	63.55	20.13	9.98	63.44	60.04	4.24	3.21	3.38	70.22	73.87
5	158	108.42	52.25	19.12	15.74	68.81	64.19	4.02	3.64	3.21	56.65	68.84
6	161	108.90	49.95	20.13	11.39	69.17	66.93	4.48	3.22	3.35	55.11	73.84
7	167	111.05	65.81	18.08	17.02	63.44	67.70	3.71	3.15	2.95	76.13	69.61
8	162	111.08	74.41	19.38	19.68	68.81	64.61	4.18	4.05	3.41	85.45	74.24
9	191	109.83	58.75	15.88	26.96	82.57	60.41	3.41	3.31	3.03	80.97	73.10
10	170	113.80	75.95	17.73	39.63	86.09	58.92	3.54	2.79	3.76	91.38	71.16
11	174	110.96	70.14	17.54	23.23	86.51	64.58	4.24	3.27	3.03	87.81	73.07
12	186	110.66	75.05	19.76	39.07	71.05	68.44	3.33	2.54	3.34	85.77	75.01
13	170	114.43	43.36	18.46	39.43	74.11	66.90	3.36	3.25	3.55	52.63	74.61
14	168	111.85	64.16	16.44	24.44	82.76	66.13	2.60	2.41	3.01	84.92	72.67
15	182	114.19	55.23	16.17	6.46	85.64	66.50	3.06	2.64	3.22	73.53	71.55
16	174	114.82	56.31	17.00	20.48	77.69	66.50	3.43	2.61	2.74	72.38	73.10
17	164	113.65	44.85	17.54	25.29	52.35	68.84	2.12	1.95	2.05	54.38	71.18
18	164	116.38	60.66	17.99	16.68	69.49	60.04	3.40	2.76	2.67	70.14	69.59
19	170	116.08	43.61	16.25	24.27	57.50	57.35	3.35	3.15	2.76	56.47	69.98
20	168	113.86	48.37	15.61	24.47	72.01	56.21	2.51	2.34	2.78	62.73	67.30
21	173	115.94	48.06	16.35	33.64	64.31	62.70	2.50	2.17	2.63	62.27	70.78
22	156	118.50	65.53	17.63	39.65	85.82	62.30	3.09	2.60	2.81	79.25	71.16
23	162	115.48	56.65	16.53	23.88	76.16	64.21	3.19	3.04	3.20	73.84	71.55
24	179	105.55	63.38	16.63	33.34	50.19	46.79	2.40	2.04	2.15	72.25	63.07
25	155	116.49	46.95	18.55	33.95	48.26	64.98	2.71	2.54	2.51	53.90	71.16
26	167	113.02	92.70	20.31	43.87	58.56	65.36	3.23	2.74	2.69	98.13	71.93
27	146	111.40	70.99	20.32	22.26	83.09	59.26	3.27	3.12	2.88	72.25	69.21
28	140	116.33	56.57	16.99	14.77	87.37	65.76	3.19	3.09	3.02	71.33	71.53
29	162	118.90	42.35	19.29	25.05	74.11	64.98	3.02	2.74	2.76	48.30	73.44
30	158	115.31	55.11	17.92	23.32	75.53	56.55	3.01	3.15	2.70	60.81	65.76

The estimation of the kinetic parameters by fitting the proposed model to the experimental data of concentration of substrate, cell, and product in the first vat provided the calculated values of these variables in this vat, i.e., $S_{1_{calc}}$, $X_{1_{calc}}$, and $P_{1_{calc}}$. Since the kinetic parameters were not estimated for the other vats, the respective calculated values of substrate concentration, i.e., $S_{2_{calc}}$, $S_{3_{calc}}$, and $S_{4_{calc}}$ could not be obtained, becoming degrees of freedom of the system, which were assumed to be their own experimental values.

By analyzing the data of S_1 , X_1 , and P_1 presented in Table 3, which were the data used in the parameter estimation, it is verified that the values of these concentrations vary in an interval whose order of magnitude is tens of g/L, not being necessary in the case of the normalization of residues, which is recommended when there are significant differences between the orders of magnitude of the values of the dependent variables (X , S , and P), avoiding advantages in the adjustment of those variables with greater absolute

values [36,37]. In this way, the estimation of the kinetic parameters was performed by nonlinear regression, using the Marquardt method to iteratively determine, from initial estimates extracted from the literature [9], the optimal values of the parameters that minimize the function ϕ , defined by Equation (21), in which i is the experiment number, corresponding to each day of operation (1st, 2nd, 3rd,, 30th day), n_e is the total number of experiments ($n_e = 30$) and the subscripts exp,i and $calc,i$ represent the experimental and calculated values of the concentration of residual sugars, ethanol, and cells in experiment i .

$$\phi = \sum_{i=1}^{n_e} (S_{exp,i} - S_{calc,i})^2 + \sum_{i=1}^{n_e} (P_{exp,i} - P_{calc,i})^2 + \sum_{i=1}^{n_e} (X_{exp,i} - X_{calc,i})^2 \quad (21)$$

The experimental data used in this study represent a historical series of the behavior of the main variables during a 30-day period of the continuous operation of the fermentation system, which extends for many more days until the sugarcane feedstock becomes unavailable. In the analyzed period, the feed conditions of the fermentation system varied slightly daily, leading to fluctuations in the daily behavior of the main bioprocess response variables. Excluding the occurrence of an abnormal event, such as bacterial contamination or a problem in some equipment, it is assumed that the changes in the behavior of process variables in other time periods are similar to that of the period under consideration. Thus, it is assumed that the process has a stationary average behavior, but fluctuations occur around this average behavior over time.

The values of the dependent variables for a particular set of kinetic parameter values and independent variables during the search were calculated by the “false transient” method, which has proven to be effective, not presenting the convergence difficulties of classical methods for solving systems of nonlinear algebraic equations due to inappropriate initial estimates of the roots [38]. It consisted, firstly, in formulating the mass balance equations in transient state, resulting in ordinary differential equations that, secondarily, were integrated numerically by the fourth order Runge-Kutta-Gill method with variable steps, until the steady state was reached for a given operational condition of the continuous system.

Table 4 presents the values and standard deviations of the estimated kinetic parameters. It can be observed that the estimates represent typical values of kinetic parameters in alcoholic fermentation [9]. Furthermore, except for K_S and λ , other parameters of the model were estimated with good precision, since the values of the standard deviations of their estimates are small when compared to the respective values of the parameters themselves.

Table 4. Values and standard deviations of the kinetic parameters of the proposed model.

Kinetic Parameter	Value $\pm$ Standard Deviation
μ_{max} (h ⁻¹)	0.48 $\pm$ 0.02
K_S (g/L)	0.83 $\pm$ 0.48
P_{max} (g/L)	59.52 $\pm$ 0.90
λ (—)	0.39 $\pm$ 0.22
X_{max} (g/L)	104.82 $\pm$ 7.33
δ (—)	0.30 $\pm$ 0.01

The large standard deviations observed for the K_S and λ parameters suggest that a parametric sensitivity analysis should be further performed to quantify the impact of parameter uncertainties on model predictions, aiming at accurately estimating the more influent parameters [10,39].

3.4. Model Verification and Validation

The verification of a mathematical model is a qualitative assessment of the model's adjustment to the experimental data, which precedes the quantitative one (validation) and aims at ensuring the proposed model adequately describes the tendency of behavior of the main variables of the system under study, detecting possible conceptual failures in the model conception process. Figure 9 shows the model's behavior compared to experimental data of the concentration of substrate, ethanol, and biomass in the first fermentation vat, which were used to estimate the kinetic parameters. It can be observed that the proposed model satisfactorily describes the behavior trend of the three modeled variables.

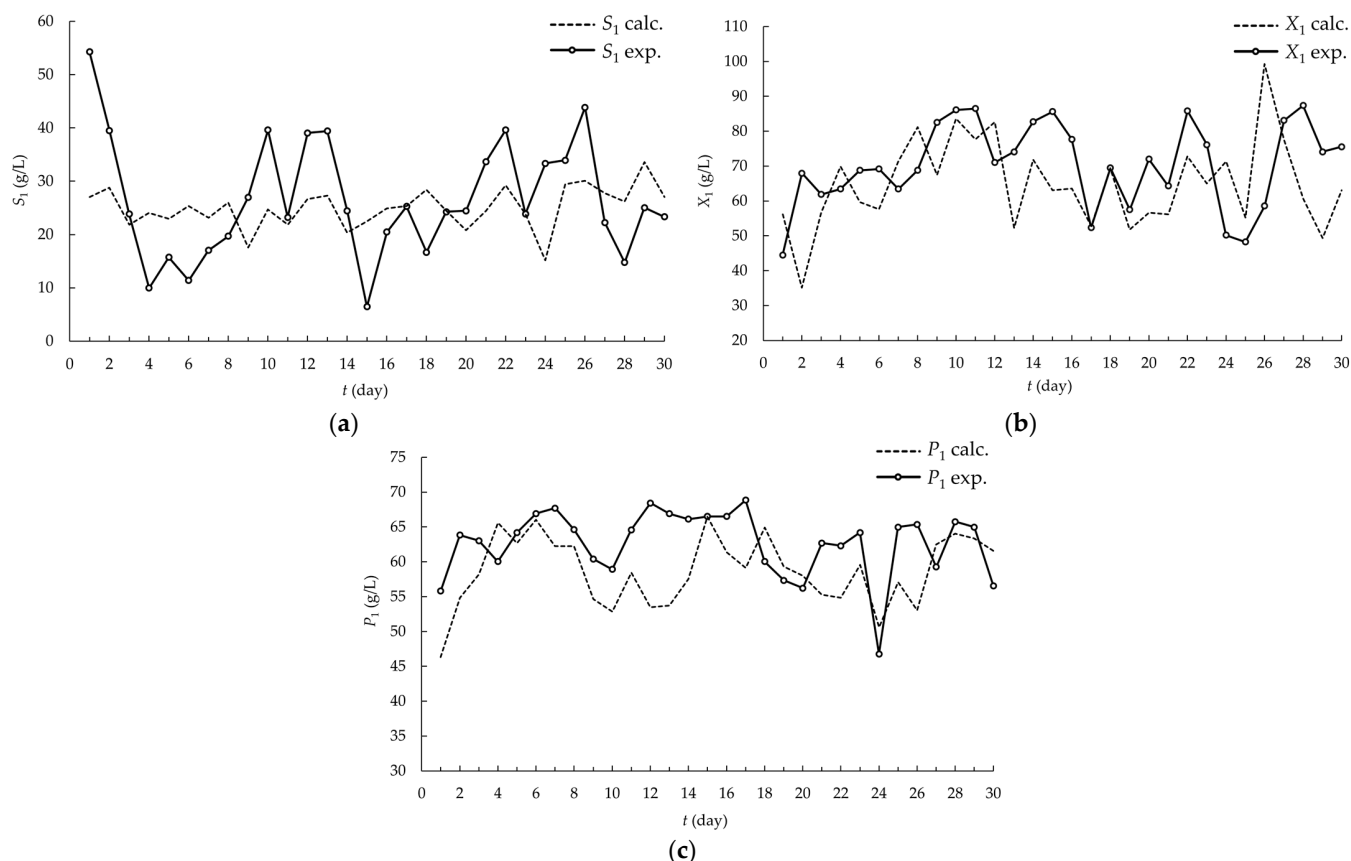


Figure 9. Model behavior compared to experimental data from vat 1: (a) substrate concentration; (b) biomass concentration; and (c) product concentration.

One way to better evaluate the mathematical model is to test it against experimental data that were not used in the parameter estimation step [22,40,41]. Figure 10 shows the model's behavior against experimental data for ethanol and biomass concentrations in the fourth vat, data not previously presented to the model. As can be seen in this figure, the model's adjustment to the experimental data was very good, especially for biomass concentration, whose behavior trend was accurately reproduced by the mathematical model. Quantitatively, the average experimental value for biomass concentration in the fourth vat was 68.20 g/L, while the average value predicted by the model was 67.88 g/L. For ethanol concentration, the average experimental value was 71.50 g/L, and the average value estimated by the model was 69.70 g/L. This significant agreement between experimental and predicted values qualifies the model for simulation, optimization, and control studies of the bioprocess.

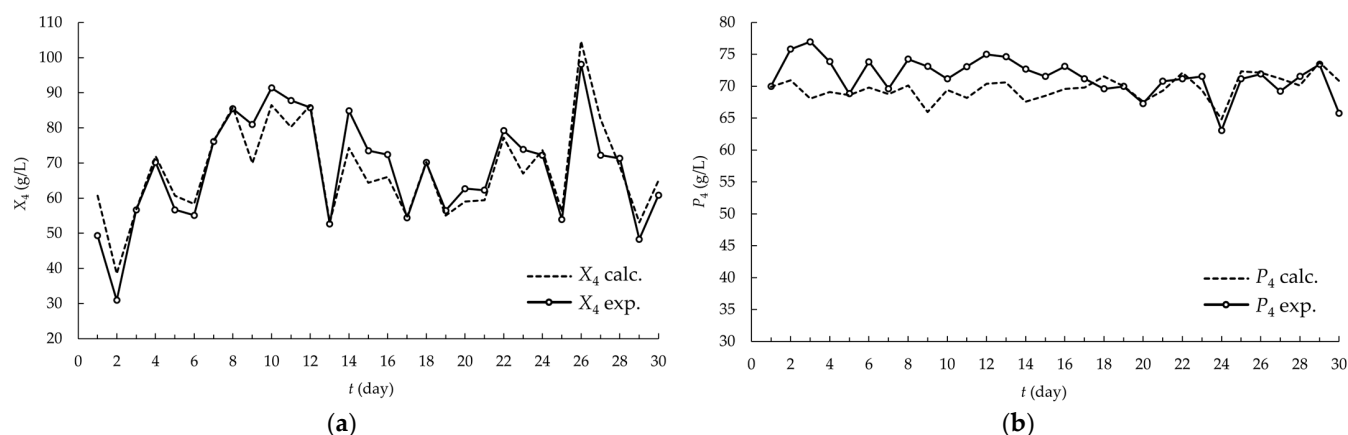


Figure 10. Adjustment of the model to experimental data (vat 4): (a) biomass concentration; (b) product concentration.

Another way of testing model validity is to plot predicted versus experimental values, generating a scatter plot [39]. This will allow for an assessment of the fit quality of the mathematical model, providing a 45° straight line for a perfect fit. Ideally, the points will cluster tightly around the 45° line, indicating a strong correlation and a well-fitting model. Deviations from this ideal line suggest bias or other forms of poor model performance, which can be quantified by the joint values of the coefficient of determination R^2 and the slope “ a ” of the straight $y_{calc.} = a \cdot y_{exp.}$ line obtained in the suggested linear regression, where y stands for variable X , P , or S . The closer these values are to 1, the better the model fits the experimental data. Table 5 presents the results of such procedure for the vats where predicted and experimental data were available. It can be observed that, according to the postulated criterion, the proposed mathematical model can be validated, since it presented statistical indicators that are favorable to its validation. The variable for which the model presented the lowest degree of predictability was the substrate concentration, although it was at an acceptable level. These results indicate high robustness of the model and high credibility in its predictions.

Table 5. Statistical indicators of the quality of the proposed model fit.

Variable/Vat	$a \pm \text{Standard Deviation}$	R^2
Substrate Concentration/Vat 1	0.82 ± 0.06	0.86
Cell Concentration/Vat 1	0.91 ± 0.04	0.95
Ethanol Concentration/Vat 1	0.93 ± 0.02	0.99
Cell Concentration/Vat 4	0.99 ± 0.02	0.99
Ethanol Concentration/Vat 4	0.97 ± 0.01	1.00

“ a ” and “standard deviation” they represent variables.

Having proven the good predictive capacity of the proposed model, it was used to simulate the daily behavior of ethanol and biomass concentration in intermediate vats 2 and 3, with the results of these simulations being presented in Figure 11. Regarding the predicted behavior for these variables, it can be seen that it is very similar in both vats, with average values of biomass and ethanol concentration, respectively, equal to 67.77 g/L and 69.14 g/L in vat 2 and 67.88 and 69.69 g/L in vat 3. Considering that the average predicted and experimental value in vat 1 was 65.68 g/L and 70.31 g/L for X_1 and 58.66 g/L and 62.66 g/L for P_1 , it can be inferred that practically no cell growth occurs in vats 2 and 3, with a predominance of cellular maintenance metabolism in these vats, with low values of ethanol production associated with this metabolism. That is, the majority of sugar consumption for significant ethanol production occurs in the first vat, with the

remaining vats functioning as hydraulic detention tanks in which residual sugars are almost completely converted, preventing damage caused by the potential caramelization of these sugars in the distillery equipment.

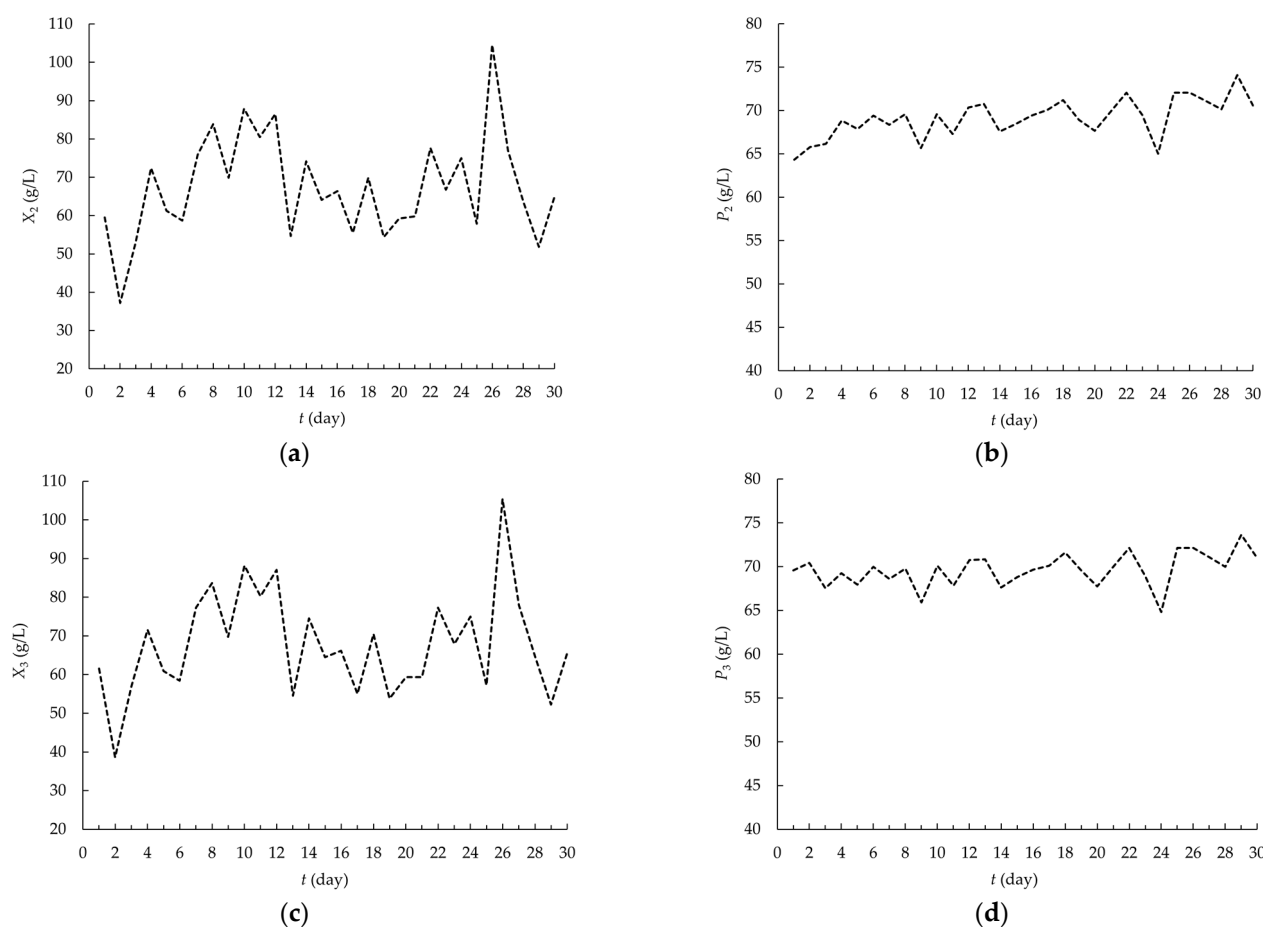


Figure 11. Simulation of the daily behavior: (a) biomass concentration (vat 2); (b) ethanol concentration (vat 2); (c) biomass concentration (vat 3); and (d) ethanol concentration (vat 3).

3.5. Model Limitations

In this subsection, some of the main limitations of the proposed mathematical model are presented and discussed, as follows.

The model does not incorporate the kinetics of heat and mass transfer processes or phase equilibrium conditions. Investigating and specifying the hydrodynamic behavior of the bioreactor is also essential to define an appropriate equation for its design.

Calculations based solely on mass balance equations cannot provide the basis for the design of hydromechanical equipment (separators) and mass transfer equipment (reactors) for industrial production. Likewise, designing heat exchangers is impossible without making the corresponding energy balances.

The choice of an unsegregated and unstructured model is justifiable by the high viability observed. Nevertheless, such simplifications neglect the loss of viability, intracellular metabolic dynamics, and stress-induced effects, all of which are highly relevant in prolonged industrial operations. This may result in the overestimation of productivity, underestimation of byproduct formation, and limited generalizability to alternative feedstocks (e.g., molasses, lignocellulosic hydrolysates).

The parameters K_S , P_m , and X_m are mass flow rate-dependent. Therefore, they can only be considered as apparent instead of intrinsic kinetic parameters. The kinetic parameters of biochemical processes are indicators that characterize the rate and mechanisms of

synthesizing chemical substances from organic raw materials under the influence and direct participation of microorganisms and the enzymes they produce. Kinetic parameters are primarily constants (coefficients) of the rate of reaction, diffusion between phases, equilibrium constants, and other parameters that describe the dependence of the rate of a chemical reaction on environmental conditions (temperature, concentration of reagents, and presence of enzymes).

Regarding model calibration, the dependence on limited measurements of substrate and product concentrations with very sparse data raises concerns about identifiability and robustness. Cell concentration is indirectly estimated from viability and the literature-derived cell mass, introducing considerable uncertainty. Furthermore, byproducts such as glycerol, organic acids, or biomass degradation products were not monitored, even though they are known to influence ethanol yields. The absence of replicate runs further reduces confidence in the reproducibility of the results and affects parameter estimation and predictive reliability.

4. Conclusions

In this study, a mathematical model was initially conceived for a continuous, multistage alcoholic fermentation process on an industrial scale, aiming at using it in future optimization and control studies to increase bioprocess productivity.

The next step consisted of translating the model conception into corresponding mathematical equations, followed by estimating its various parameters aiming at comparing the model predictions with experimental data and, thus, validate or revise the proposed model, depending on the quality of the fit to the experimental data. The estimated kinetic parameters were consistent with values commonly reported in the literature for alcoholic fermentation processes, constituting typical parameters of this bioprocess.

Despite the various simplifications made during the conception of the mathematical model, it managed to incorporate the essential behavior of the bioprocess, accurately describing the behavior trend of the main modeled variables.

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