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Kinetic modeling of batch bioethanol production from CCN-51 Cocoa Mucilage

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ABSTRACT

Background: The aim of this study was the production of bioethanol generated during the fermentation of CCN-51 Cocoa mucilage with Saccharomyces cerevisiae yeast and the fitting of experimental data to the mathematical models: Logistic, modified Gompertz, and Andrews and Levenspiel. There are limited studies regarding the high energy potential of cocoa mucilage from Ecuador for bioethanol production as a gasoline additive. Currently, this by-product of the cocoa industry is considered as waste.
Methods: Discontinuous fermentation was performed in a batch bioreactor under different conditions of pH, temperature and yeast concentration. During the reaction, bioethanol concentration, yeast and consumed substrate were evaluated by means of microdiffusion, dry weight by lyophilization and UV spectrophotometry, respectively.
Significant Findings: The result of the final bioethanol concentration was 25.41 g/L at a temperature of 35 °C, pH of 4 and yeast concentration of 3 g/L. The models were fitted with determination coefficients greater than 0.9. From the results, the logistic model was used to describe yeast growth. Modified Gompertz model is considered appropriate for modeling bioethanol production. Both models fit the data adequately; however, Andrews and Levenspiel model, besides of the good adjustment, considered inhibition terms of the substrate and product.

Introduction

The use of primary fossil energy sources has been the driving force behind economic development and welfare of nations, and continues to be so, such that in 2018 they represented around 81% of the world's primary energy production, with a contribution of 31% of oil, 27% of carbon and 23% of natural gas [1]. However, the exploitation and intensive use of primary fossil energy sources has several disadvantages, among them, the certainty of its depletion in the medium-term and its important contribution to greenhouse gases emission which are causing significant environmental and climate imbalances in all areas [2]. In this sense, due to the changing world energy market and growing environmental concerns, alternative fuels such as biofuels represent perhaps the most attractive alternative, both due to their ability to substitute fossil fuels and their possibility for generating new markets for agricultural producers and for the valorization of biomass residues, of different origin, used in their production [3].

Most widely used liquid biofuels are biodiesel and bioethanol. Since these oxygenated additives are used for increasing the octane number of the fuel and reduce emissions (i.e., circular economy), they present an increase in power output [4,5]. In the case of liquid biofuels, bioethanol is the main vector because it represents 67% of the world production of biofuels, with an increase of 3.8% compared to 2016 [6], which is fully intended to the transport sector to use it alone or mixed with gasoline in different proportions. By these means, bioethanol has received much attention as a possible replacement for fossil fuels [7]. Bioethanol production processes vary significantly according to the type of residual biomass, its availability and its sugar content [8]. For these reasons, agricultural wealth provides renewable sources of high potential for energy use, such as African palm, banana, rice, and cocoa.

In the specific case of cocoa, Ecuador plays an important role in the world market in terms of volume and quality, since it is the largest fine cocoa producer, generating around 65% of the global supply [9], with one of the main residues being its mucilaginous pulp which contains sap cells rich in sugars capable of fermentation without modifications, transforming sugar into alcohol. The total volume of exudate is substantial, but to date, no suitable use has been found at an industrial level [10]. With a growing interest in industrial fermentation applications, mathematical modeling has been developed as a tool to help predicting and making decisions [11,12], reduce process expenses and avoid superfluous experimentation.

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In microbial growth, logistic model describes the changes in the number of organisms that appear from the initial to the maximum yeast concentration. However, this model does not consider substrate inhibition [13]. Modified Gompertz model is frequently used to describe bioethanol production during fermentation [7]. Andrews and Levenspiel model is a kinetic model proposed to represent the specific growth rate (μ) contemplating the mixed inhibition phenomenon. Andrews model was proposed in 1968 and it considers a substrate inhibition constant (K_{IS}) in a culture in an stirred tank reactor [14]; while Levenspiel model was proposed in 1980 and it considers the phenomenon of inhibition by product (α) [15]. However, during bioethanol fermentation, the leading inhibitory factors are high concentrations of sugar and bioethanol. Both agents affect yeast growth in a non-competitive inhibitory manner, so a model by Andrews and Levenspiel which includes terms for substrate and product inhibition, turns out to be a particularly suitable model.

The aim of this research was to study the possibility of using cocoa residues for bioethanol production by means of current technologies and the application of kinetic models to predict and control alcoholic fermentation, using Saccharomyces cerevisiae yeast. This research represents a step towards industrial-scale production, since it defines kinetic parameters [16] that will allow subsequent scaling to meet the energy demands of the countries in which cocoa-derived waste is generated.

Materials and Methods

Collection and Extraction of Cocoa Mucilage

CCN-51 cocoa mucilage, a residue of the growing cocoa industry, currently does not have a beneficial and cost-effective use. It belongs to geographical area number 6 of Ecuador, corresponding to Azuay, Cañar and Morona Santiago provinces. This variety was chosen due to its higher productivity. Cocoa was transferred from the cultivation land to University of Cuenca laboratories and underwent pretreatment processes such as those described by [17], the most important steps being harvesting, cutting, separation and softening to separate the mucilaginous pulp from the rest of its components. Subsequently, it was pasteurized at 88 °C for 5 minutes and stored at -18 °C [18].

Experimental design

The experimental design matrix used in this study corresponds to a two-level full factorial design 2^3, a maximum and a minimum. The variables considered were temperature, pH and Saccharomyces cerevisiae yeast concentration [19] (Table 1) due to their importance in fermentation reactions [20].

During the experimental design the software STATGRAPHICS® Centurion XV was used.

Substrate and microorganism

The cocoa mucilage is a liquid, viscous, whitish pulp, with a sweet and acid flavor that covers the beans of the fruit, it is mainly composed of cellulosic polysaccharides, gums and pectins. The fermentation volume is 80% of the total capacity of the fermenter under anaerobic conditions and the microorganism used is Saccharomyces cerevisiae, a commercial baker's yeast [21].

Table 1
Variables and levels for the experimental design.

Factor	Variable	Values	
		Minimum (-)	Maximum (+)
Temperature [°C]	X_1	25	35
pH [dimensionless]	X_2	4	5
Yeast concentration [g/L]	X_3	1	3

Alcoholic Fermentation

Alcoholic fermentation was carried out under the described operating conditions, in a homogenous 2-liter Biotron GX batch bioreactor, with a fermentation volume of 1.6 L and a stirring of 200 rpm to ensure homogeneity in the reaction mass and under anaerobic conditions. The experiment took between 30 to 96 hours, depending on the operating parameters.

Bioethanol Quantification

For the bioethanol quantification, the microdiffusion method was used; an alternative non-chromatographic method which uses a Conway chamber that allows determining substances susceptible to volatilization and the attachment to the appropriate medium to be quantified [22]. The chamber must be hermetically sealed and consists in two compartments. One of them contains the alcohol, which due to its high vapor pressure and the test temperature, volatilizes towards the second chamber. Ethanol oxidation to acetic acid occurs due to the presence of potassium dichromate dissolved in sulfuric acid; the excess of dichromate that remains unreacted is measured by the reaction with potassium iodide to form iodine, which is titrated with sodium thiosulfate in the presence of starch as an indicator.

Sugar Quantification

The phenol-sulfuric acid method allows quantifying several sugars such as polysaccharides, oligosaccharides, monosaccharides and their derivatives. Carbohydrates are generally sensitive to high temperatures and strong acids [7].

The yellow-orange color of the complexes formed by the reaction with phenol in concentrated sulfuric acid is very stable up to 24 hours. Color intensity is proportional to carbohydrate concentration and is measured by absorbance at wavelengths ranging from 488 nm to 492 nm. The samples were quantified in triplicate on a Ciba-Corning 2800 spectra scan UV and visible light spectrophotometer, at a wavelength of 490 nm, previously carrying out the calibration curves.

Yeast Quantification

Yeast content was determined by a direct counting method [23,24]. Dry cell weights were determined by lyophilization drying. The centrifuged samples were stored in liquid nitrogen at -190 °C to avoid degradation reactions. When the experimental process concluded, the samples were lyophilized in an Armfield FT 33 lyophilizer, remaining in it for 48 hours. Freezing was carried out in the first 24 hours and drying in the following 24 hours [25].

Mathematic Models

The experimental results obtained were adjusted to different mathematic models using a Levenberg-Marquardt nonlinear least squares method programmed in MATLAB® [7]. For the cases that required solving the differential models, a third-order Runge-Kutta method (ODE23) was used [24,26].

The integrated logistic equation (eq.1) was used to model the fermentation process and determine the maximum specific growth rate (μ_{max}), fitting the experimental data corresponding to the optimal operating conditions.

$$X = \frac{X_0 \cdot \exp(\mu_{\max} \cdot t)}{1 - \left[\left(\frac{X_0}{X_{\max}}\right) \cdot (1 - \exp(\mu_{\max} \cdot t))\right]} \quad (1)$$

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where:

X:Yeast concentration at time t [g/L]
X_0: Initial yeast concentration [g/L]
X_{max}: Maximum yeast concentration [g/L]
μ_{max}: Maximum specific growth rate [h^{-1}]
t: Fermentation time [h]

Modified Gompertz equation (eq.2) was used to model the fermentation process and determine the maximum bioethanol production rate (r_{pm}), fitting the experimental data corresponding to the optimal operating conditions.

$$P_E = P_{E\max} \cdot \exp\left\{-\exp\left[\frac{r_{pm} \cdot \exp(1)}{P_{E\max}} \cdot (t_l - t) + 1\right]\right\} \quad (2)$$

where: r_{pm}: Maximum bioethanol production rate [g/(L·h)]
P_E: Bioethanol concentration at time t [g/L]
P_{Emax}: Maximum bioethanol concentration [g/L]
t: Fermentation time [h]
t_l: Lag phase [h]

The Andrews and Levenspiel model (eq.3) was used to determine different kinetic parameters, adjusting the experimental data corresponding to the optimal operating conditions. The precision of the models was evaluated based on their determination coefficients (R^2).

During fermentation, the main inhibitory factors are high sugar and bioethanol concentrations. Both agents affect yeast growth in an inhibitory non-competitive way. A model that includes terms for substrate and product inhibition is the Andrews and Levenspiel model (eqn 3). Bioethanol is a primary metabolite of yeast growth under anaerobic conditions and its formation is associated with cell growth. Using the same philosophy based on the Andrews and Levenspiel equation, a product formation model is considered as evidence in (eq.4).

$$\mu = \left(\frac{\mu_{\max} \cdot S}{K_S + S + \frac{S^2}{K_{IS}}}\right) \cdot \left(1 - \frac{P_E}{P_{X\max}}\right)^{\alpha} \quad (3)$$

$$q_E = \left(\frac{q_{\max} \cdot S}{K_{SE} + S + \frac{S^2}{K_{IE}}}\right) \cdot \left(1 - \frac{P_E}{P_{E\max}}\right)^{\beta} \quad (4)$$

where:

μ: Specific growth rate at time t [h^{-1}]
μ_{max}: Maximum specific growth rate [h^{-1}]
S: Sugar concentration at time t [g/L]
K_S: Monod constant for growth [g/L]
K_{IS}: Substrate inhibition constant for growth [g/L]
P_{Xmax}: Maximum bioethanol concentration for yeast growth [g/L]
X: Yeast concentration at time t [g/L]
q_{max}: Maximum specific bioethanol production rate [g/(g·h)]
q_E: Specific bioethanol production rate [g/(g·h)]
K_{SE}: Bioethanol saturation constant [g/L]
K_{IS}: Substrate inhibition constant for growth [g/L]
K_{IE}: Substrate inhibition constant for bioethanol formation [g/L]
$Y_{X/S}$: Yeast yield [g_{yeast} /g_{sugar}]
$Y_{E/S}$: Bioethanol yield [$g_{ethanol}$ / g_{sugar}]
m: Cell maintenance coefficient [h^{-1}]
P_E: Bioethanol concentration [g/L]
P_{Emax}: Maximum bioethanol concentration for alcoholic fermentation [g/L]
α,β: Bioethanol inhibition constant [g/L]
t: Fermentation time [h]

Equation (eq.5) was used to describe yeast growth rate and (eq.6) to describe product formation rate.

$$\frac{dX}{dt} = X\left(\frac{\mu_{\max} \cdot S}{K_S + S + \frac{S^2}{K_{IS}}}\right)\left(1 - \frac{P_E}{P_{X\max}}\right)^{\alpha} \quad (5)$$

$$\frac{dP_E}{dt} = X\left(\frac{q_{\max} \cdot S}{K_{SE} + S + \frac{S^2}{K_{IE}}}\right)\left(1 - \frac{P_E}{P_{Emax}}\right)^{\beta} \quad (6)$$

In batch fermentation, substrate (sugars from cocoa mucilage) is used for cell growth and maintenance, as well as for bioethanol production. The substrate utilization rate was described by equation (eq.7).

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}} \cdot \left(\frac{dX}{dt}\right) + \frac{1}{Y_{E/S}} \cdot \left(\frac{dP_E}{dt}\right) + m \cdot X \quad (7)$$

Finally, productivity of each experiment is calculated with the maximum value of bioethanol concentration and the time to reach that concentration. It is described by equation (eq.8).

$$Productivity = \frac{Maximum\ Bioethanol\ Concentration}{Time\ to\ achieve\ Maximum\ Bioethanol\ Concentration} = \frac{\left[\frac{g}{L}\right]}{[h]} \quad (8)$$

Results and Discussion

The 2^k experimental design allowed to establish the best bioreactor operating conditions considering temperature, pH and yeast concentration to obtain the highest bioethanol concentration. These variables were selected due to their relevance in fermentative processes, both for yeast development and for control during fermentations. Moreover, for the higher productivity experiment, substrate and yeast concentration was evaluated throughout the fermentation.

Table 2
Experimental results of concentration, time and productivity of bioethanol in CCN-51 cocoa mucilage fermentative processes for three operating parameters.

Experiment	Operating Parameters			P_E** [g/L]	Time to maximum bioethanol concentration [h]	Process productivity [g/(L · h)]
	Temperature [°C]	pH	X_0 [g/L]			
1	25	4	1	22.84	70	0.32
2	35	4	1	23.40	50	0.46
3	25	5	1	18.72	30	0.62
4	35	5	1	22.84	47	0.48
5	25	4	3	24.51	80	0.30
6	35	4	3	25.41	36	0.70
7	25	5	3	18.94	96	0.19
8	35	5	3	21.17	44	0.48
9-1*	30	4.5	2	22.28	57	0.39
9-2*	30	4.5	2	23.95	46	0.52

*A replica of experiment 9 was performed.
** Concentrations were estimated in triplicate throughout the reaction. The average is shown.

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Table 3
Yeast, bioethanol and sugars concentration for the experiment with the highest production and productivity (experiment 6 of Table 2).

Time [h]	X [g/L]	P_E [g/L]	S [g/L]
0	0.41 ± 0.07	0.00 ± 0.00	231.48 ± 2.04
6	3.09 ± 0.11	7.19 ± 0.67	185.58 ± 10.26
12	5.18 ± 0.17	9.11 ± 2.04	110.18 ± 9.42
18	5.39 ± 0.28	16.51 ± 0.13	81.71 ± 3.86
24	5.85 ± 0.76	21.10 ± 0.68	33.01 ± 1.89
30	6.20 ± 0.13	24.45 ± 0.68	28.02 ± 2.46
36*	6.20 ± 0.25	25.41 ± 0.67	31.99 ± 0.87
42	6.20 ± 0.09	25.39 ± 0.19	30.68 ± 1.42
48	6.20 ± 0.17	25.40 ± 0.19	30.11 ± 0.69

*Time at which the maximum concentration of biomass and bioethanol is considered.

Table 4
Kinetic parameters of CCN-51 cocoa mucilage batch obtained by fitting to Andrews and Levenspiel model.

Parameter	Value	Standard deviation	Units
μ_{max}	0.34	±0.05	[h^{-1}]
q_{max}	2.48	±0.33	[h^{-1}]
K_S	1.32	±0.37	[g/L]
K_{SE}	39.82	±0.22	[g/L]
K_{IS}	938.99	±48.63	[g/L]
K_{IE}	99.72	±2.69	[g/L]
$Y_{X/S}$	0.15	±0.03	[g/g]
$Y_{E/S}$	0.1	±0.004	[g/g]
m	5.71E-06	±5.35E-06	[h^{-1}]
$P_{X.max}$	23.14	±1.07	[g/L]
α	1.49	±0.004	[g/L]
β	1.39	±0.09	[g/L]

The key limitation of this study was that obtained kinetic parameters correspond only to the fermentation of CCN-51 cocoa mucilage and they are not suitable to any other type of yeast strain but Saccharomyces cerevisiae. Table 2 shows the main results obtained. Experiment labeled 6 (shaded area of the table), is the one in which the maximum bioethanol concentration was generated after 36 hours. Other experiments such as 2 and 5 reached close concentrations, nonetheless, reaction times were increased. Also, experiments that present lower values of temperature, pH and yeast concentration generate a lower alcohol concentration, low productivities and greater reaction time.

Table 3 indicates yeast growth; bioethanol production and substrate consume results for experiment 6. They were observed during fermentation up to 48 hours. Concentrations stabilize between 30 to 36 hours.

The increase of yeast concentration throughout the fermentation suggests that yeasts are constantly producing new cells by mitosis, thus generating a higher concentration of them throughout the experiment until reaching a steady state point.

Substrate consumption is because yeasts use it as food to grow and reproduce, metabolizing glucose to produce alcohol and carbon dioxide under anaerobic conditions of the environment. Since the yeasts are going to process the glucose available in the substrate, it decreases throughout the fermentation process.

When applying the integrated logistic model (eq.1) and Modified Gompertz (eq.2) a good fit was obtained to the experimental data, with R^2 values of 0.97 and 0.98 respectively. A maximum specific rate (μ_{max}) of 0.39 ± 0.03 h^{-1}, lag phase (t_l) of 2 hours and a maximum bioethanol production rate (r_{pm}) of 1.11 ± 0.06 g/(L·h) were achieved. These models do not include substrate consumption and product inhibition terms, as Andrews and Levenspiel (eq.3) does. For this method, good results were obtained for yeast production (eq.4) (R^2 = 0.90), bioethanol (eq.5) (R^2 = 0.95), and substrate consumption (sugars) (eq.6) (R^2 =0.98).

Table 4 shows kinetic parameters obtained by adjustment of Andrews and Levenspiel model. It was carried out using Levenberg-Marquardt algorithm developed in MATLAB®, this model is suitable to several experimental systems among which is CCN-51 cocoa mucilage fermentation with Saccharomyces cerevisiae.

As an example of the results of the parametric fitting to the empirical results, Figure 1 indicates the comparison between the temporal evolution of bioethanol, yeast and sugars concentrations for

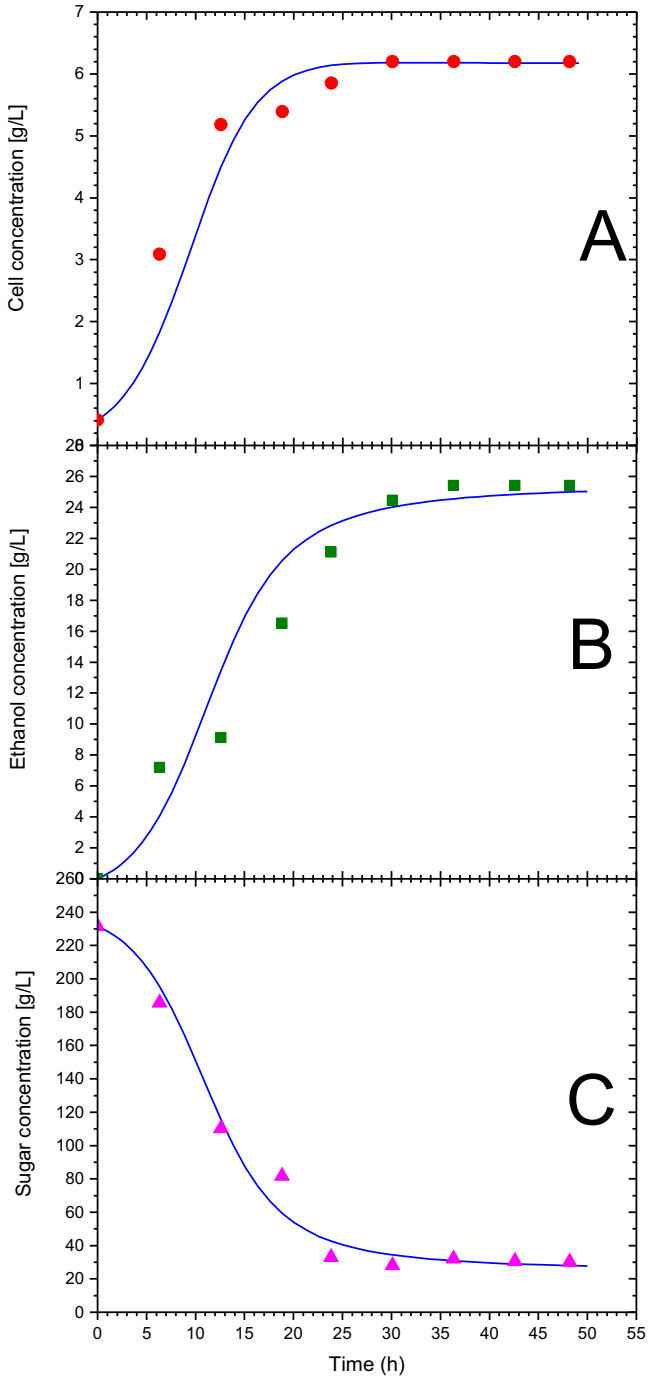

Figure 1. Yeast concentration (A), bioethanol concentration (B), sugars concentration (C), in CCN-51 cocoa mucilage discontinuous fermentation, adjusted with Andrews and Levenspiel model (solid lines).

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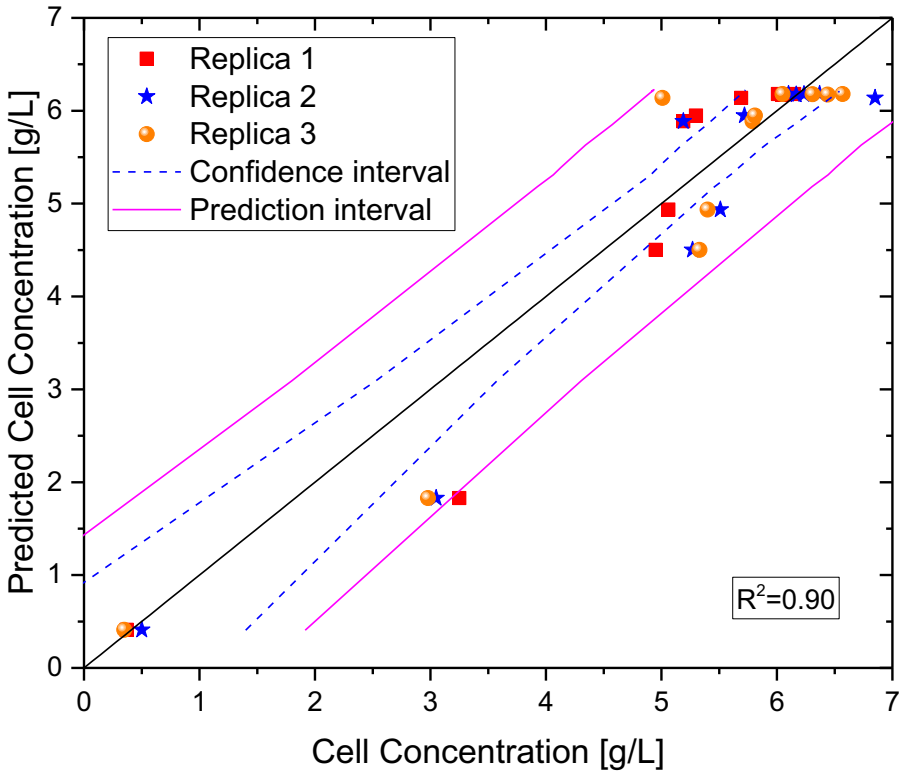

Figure 2. Correlation between yeast concentration calculated by Andrews and Levenspiel model (eq.5) and the experimental data. Solid line (black) represents a linear correlation coefficient of 1. Dashed lines (blue) represent a 95% confidence interval. Solid line (magenta) represents the prediction interval.

experiment 6 of Table 1 (pH=4, temperature= 35 °C y X_0= 3 g/L) and the results obtained for the parameters shown in Table 4.

Figures 2, 3 and 4 show the correlation between yeast, bioethanol and sugars concentration calculated by Andrews and Levenspiel model and experimental data. They have a high R^2 valued and data points are in the 95% prediction band, this suggested that Andrews and Levenspiel model can be used to predict and control fermentation systems with CCN-51 cocoa mucilage, as well as it could have

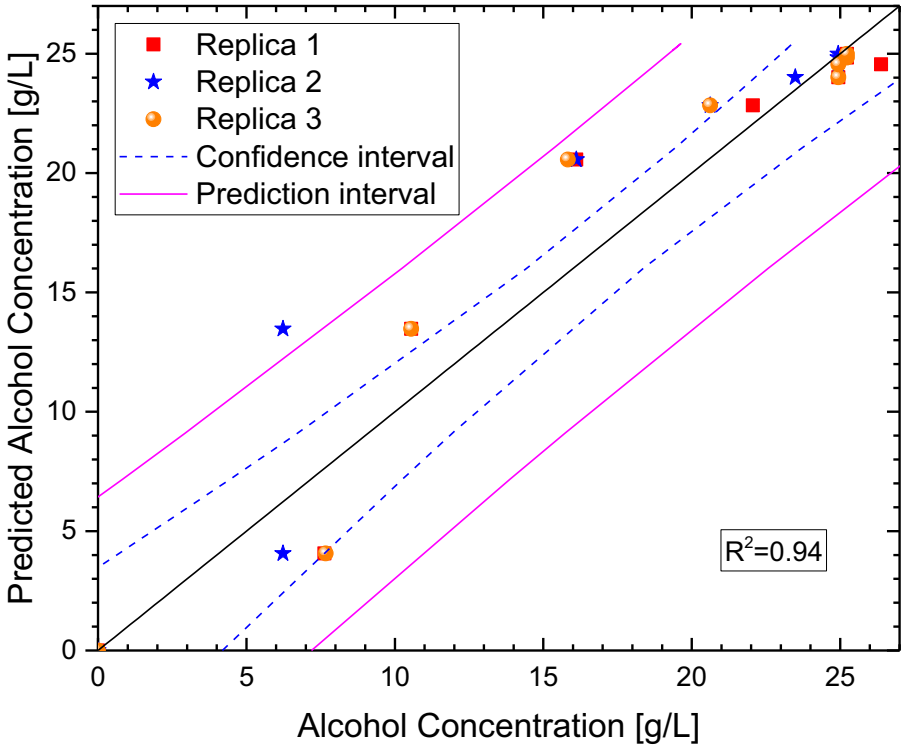

Figure 3. Correlation between bioethanol concentration calculated by Andrews and Levenspiel model (eq.6) and the experimental data. Solid line (black) represents a linear correlation coefficient of 1. Dashed lines (blue) represent a 95% confidence interval. Solid line (magenta) represents the prediction interval.

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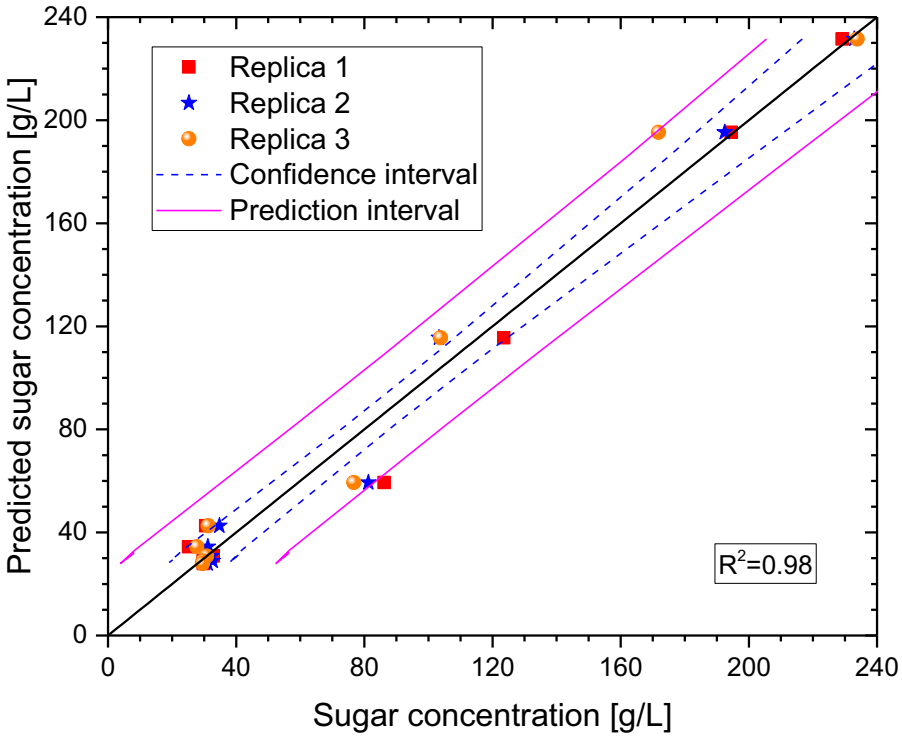

Figure 4. Correlation between sugars concentration calculated by Andrews and Levenspiel model (eq.7) and the experimental data. Solid line (black) represents a linear correlation coefficient of 1. Dashed lines (blue) represent a 95% confidence interval. Solid line (magenta) represents the prediction interval.

wide feasibility to systems operating under other conditions and with several carbon sources, so it could possibly be used to predict and control those systems as well.

The primary objective of this study was to determine the best conditions to operate a bioreactor to ferment CCN-51 cocoa mucilaginous residue. The results show that the optimum operating conditions correspond to a temperature of 35 °C, pH of 4 and an initial yeast concentration of 3 g/L. These findings are similar to other experimental systems such as those reported by [20] and [27], where the temperature varies between 30 and 35 °C, pH between 4 and 6; the main difference being the culture medium.

The present study found that lag phase (t_l) is around 2 hours with an initial concentration of sugars of 230 [g/L], and biomass concentration stabilizes after 30 [h]. Comparable results were reported by [7], who studied the kinetic models for batch bioethanol production from sweet sorghum juice and indicate that lag phase is generated during the first 2 hours of the fermentation and the stationary phase was observed after 24 hours of reaction.

When applying the integrated logistic model, a maximum specific growth (μ_{max}) rate of 0.39 ± 0.03 h^{-1} was reached, while when applying Andrews and Levenspiel model, μ_{max} was 0.34 ± 0.05 h^{-1}, being lower because the logistic model did not include the substrate consumption and the product inhibition terms. However, both values are higher than the reported by [13] whose value of μ_{max} is 0.19 h^{-1} for a bioethanol fermentation from sugar beet juice containing 136 g/L of total sugar without nutrients supplementation, this variation may be due to elements like yeast strain type, substrate type, substrate concentration and the bioreactor operating conditions.

The highest bioethanol concentrations were reached after 36 hours with a value of 25.41 g/L and a productivity of 0.70 g/(L·h). By applying the Modified Gompertz model, a maximum bioethanol production rate (r_{pm}) of 1.11± 0.06 [g/(L·h)] was determined. All these values were lower than the reported by [7], where maximum bioethanol concentration may improve if the initial amount of sugars is increased as reported by [28] and [29].

P_{Xmax} was 24 g/L. This finding indicates the alcohol concentration at which yeast stops growing. On the other hand, P_{Emax} for the fermentation was 25.41 g/L. This value suggests the bioethanol concentration from which yeasts inhibit their growth.

If compared with other results found in different culture media and other yeast strains, different values of P_{Xmax} and P_{Emax} were obtained, being 112 g/L and 115 g/L for [30] respectively, and 83.35 g/L and 107.79 g/L, respectively, in [24] study. Results shown are superior to those obtained in this study. This difference may be due to the type of strain used and its immobilization [24], in addition to the existence of a risk of inhibition when using thick juice and molasses [21]. Substrate inhibition constant for bioethanol formation (K_{IE}) was 99.72 ± 2.69 g/L, and substrate inhibition constant for growth (K_{IS}) was 938.99 ± 48.63 g/L. Both substrate inhibition constants were relatively lower compared to substrate concentration squared S^2 (231.48 g/L)^2, indicating that the substrate at higher concentrations could inhibit products formation, especially yeast growth [8].

Biomass yield ($Y_{X/S}$) was 0.15 ± 0.03 g_{yeast}/g_{sugars}, and ethanol yield ($Y_{X/S}$) was 0.1 ± 0.004 $g_{ethanol}/g_{sugars}$, like the experimental 0.12 $g_{ethanol}/g_{sugars}$. However, both values are lower than the theoretical value of 0.51 $g_{ethanol}/g_{sugars}$ [31], since reaching this value is difficult because yeast uses glucose to produce other metabolites [26].

Conclusions

In this research, the objective was to evaluate CCN-51 cocoa mucilage, a residue from the cocoa industry, as a raw material with great potential to obtain bioethanol, since it contains fermentable sugars and is plentifully found in Ecuador.

The study has identified that the application of a 2^3 complete experimental design, makes it possible to establish the optimal operating conditions for a discontinuous bioreactor. The results show that the highest bioethanol production is generated at an operating temperature of 35 °C, pH 4 and yeast concentration of 3 g/L.

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This work contributes to the existing knowledge, since kinetic models for fermentation with yeast of the type of Saccharomyces cerevisiae were successfully applied, allowing to predict and control CCN-51 cocoa mucilage fermentative systems. Although all the evaluated models had a reasonable adjustment ($R^2 > 0.90$), the Andrews and Levenspiel method has the additional advantage of considering the substrate and product inhibition terms.

Future research should be directed towards the application of these models with immobilized cells to predict and compare results of bioethanol production and subsequently its industrial scaling.

Declaration of Competing Interest

None

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