Unstructured Kinetic Model for Tequila Batch Fermentation

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***Abstract*—** The desired product of the tequila fermentation process is ethanol. However, there are factors that may inhibit the alcohol production such as high substrate and ethanol concentrations present in the tequila must or culture medium. A model for predicting alcoholic fermentation behavior would be a valuable instrument for tequila research, due to the technical and economical implications. Therefore, an unstructured kinetic mathematical model taking into account substrate and product inhibition was proposed to predict tequila batch fermentation behavior. Several kinetic models were evaluated; the combination of the Moser and Luong kinetic model gave the best prediction. The nonlinear mathematical model performed satisfactory on biomass, substrate, and ethanol predictions.

***Keywords*—** Fermentation, kinetic model, substrate and product inhibition, tequila.

I. INTRODUCTION

THE fermentation stage is crucial in the production of alcoholic beverages because in this stage the ethanol is produced. Nevertheless, the fermentation is affected by environmental, physicochemical, and biological factors such as temperature, pH, dissolved oxygen, and growth inhibitors, among others [1]. In a fermentative process it is desired to maximize the production of a desired metabolite, however, without the accurate knowledge of the process this can not be attained. Modeling a fermentation process presents some advantages such as process knowledge improvement, decreasing the cost of expensive industrial experimentation [2] mathematical optimization [3] and process control [4].

Tequila is the most important distilled alcoholic beverage in México. It is produced from the fermented juice of cooked *Agave tequilana* Weber (blue variety). The tequila process involves multiple steps: fermentable sugars are obtained by steaming, milling, and pressing the agave head plants. During the steam cooking process, the polyfructans are hydrolyzed into a mixture of sugars which mainly consist of fructose. In some tequila distilleries, the fermentation occurs spontaneously while in others, the agave juice is inoculated using commercial or indigenous yeast cultures, often *Saccharomyces cerevisiae* species. After fermentation, the must is distilled twice and the final product is diluted to an alcohol content of 35 to 38% v/v. Maturation is carried out by storing the tequila in white oak barrels from two to 11 months to obtain “tequila reposado”, for more than 12 months is called “tequila añejo”, and beyond three years is known as “tequila extra-añejo” [5]. The tequila production is strictly regulated by the Mexican government by means of the Norma Oficial Mexicana del tequila [6].

In the tequila fermentation process ethanol and many volatile compounds that may influence the sensory characteristics of the final product are formed. Some compounds like ethanol are toxic to microorganisms and may cause product inhibition. Also, sluggish fermentation is a frequent problem in tequila distilleries. This problem is due to the lack of sugar consumption by yeast, mainly caused by stress conditions promoting an adverse environment. During wine alcoholic fermentation, the factors that may affect the microbial performance are: high sugar concentrations (osmosis stress), micro nutrient limitations (nitrogen and phosphate uptake), high ethanol concentration (produced during fermentation), low pH, low oxygen levels, poor mixing, extreme temperature, toxic substances (present on the must like sulphite, agro chemistry waste, killer toxins), and wild microorganisms not inoculated [7], [8]. Thatipamala reported that the most important cause of sluggish fermentations is the substrate and product concentration [9].

The effect of ethanol and sugar concentrations on the ethanol production is crucial in the tequila fermentation process. Therefore, the main aim of this work was to produce an unstructured mathematic model to predict the substrate and product inhibition on the tequila fermentation process. This model will allow better knowledge of the tequila fermentation process. A mathematical model which predicts alcoholic fermentation behavior will be a valuable instrument for tequila research due to the technical and economical implications.

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II. Fermentation Modeling Preliminaries

An unstructured model takes the cell as a uniform quantity without internal dynamics. The reaction rates depend only on the conditions presented inside of the bioreactor. Therefore these models only contain kinetics of growth, substrate uptake, and product formation [10]. Structured models are designed on the basis of biomass components such as concentrations of metabolites, enzymes, DNA, and/or RNA [11]. Nonsegregated models treat the culture as a collection of average cells, all with the same characteristics at any given time. Usually, unstructured models are also nonsegregated models. Segregated models treat each cell as independent, and a population as a collection of such distinct cells. They describe different morphological types of cells or cell aging and sometimes describe interactions between different cells [11]. There are other models called "black box" which use an artificial neural network to predict the kinetics of the fermentation from initial data [12]–[14]. At the industry level, most of the biochemical processes including alcoholic fermentation are carried out in batch cultures due to the simplicity of this process. In batch cultures the must or the culture medium is introduced into the bioreactor. The culture medium is inoculated with the selected microorganism and no additional must or culture medium is added during the fermentation. Furthermore the bioreactor volume remains constant. In the alcoholic fermentation, the microorganisms convert the organic compounds, mainly carbohydrates to biomass and simpler compounds, especially ethyl alcohols. The fermentation concludes when the substrates have been consumed by the microorganisms [15].

A meaningful way to be aware of the kinetic behavior of the microorganisms in the fermentation process is through the kinetic parameters. These parameters are obtained from the concentrations of biomass, products, and substrates consumed during the fermentation. These parameters provide information about the inhibition phenomenon as a result of high concentrations of substrates or products. Furthermore they give information about limitation phenomenon caused by low nutrient concentrations. The kinetic parameters more commonly used are the specific rate of: biomass, substrate, and product, as well as the biomass, and product yields. The information generated by the kinetic parameters may be used to construct a mathematical model of a fermentation that describes phenomenons such as substrate and product inhibition [15]. Numerous kinetic research models that have obtained satisfactory adjustments in the alcoholic fermentation in wine have been reported in enology [16]–[18].

III. Material and Methods

A. Microorganisms

In this work the indigenous AR5 *Saccharomyces cerevisiae* yeast isolated from the *Agave tequilana* Weber blue variety juice was used. The strain was stored at -70°C at the strain bank of the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C. (CIATEJ).

B. Fermentation Medium and Culture Conditions

The *Agave tequilana* Weber juice supplied by a distillery was filtered and frozen at -20°C. The inoculum was cultured for 12 h at 30°C and 250 r.p.m. in a diluted and sterilized at (121°C, 15 min.) agave juice containing 60 g/L of sugar and supplemented with 1 g/L of ammonium phosphate. The fermentations were performed in 500 mL Erlenmayer flasks, containing 300 mL of agave medium. Agave juice concentrations at 30, 60, 90, 150, and 200 g/L were adjusted and supplemented with 1 g/L of ammonium phosphate and sterilized at 121°C for 15 min. The fermentations were carried out in duplicate under anaerobic conditions at 33°C and 150 r.p.m. during 96 h. Sampling was performed every 2 h during the first 12 h. of fermentation, then every 4 h during the following 48 h, until the last sampling event at 72 h was accomplished.

C. Analytical Methods

Biomass concentration was obtained by dry weight measurement. A 5 ml sample of the fermented must was centrifuged for 15 min at 8000 r.p.m. and put in a plastic vessel. Prior to drying, the sample was weighted and dried for 24 h at 80°C. The plastic vessel was removed from the oven and placed in a desiccator until a constant weight was attained. The supernatant was used to determine the reducing sugar concentration by the DNS method [19] and ethanol concentrations were determinated by enzymatic measurement (YSI biochemical analyzer 2700, Yellow Spring Instruments).

D. Data Analysis

The response variables data (biomass, ethanol, and reducing sugar) of the two fermentations for each sugar concentration were compared using one-way variance analysis (ANOVA). The average or mean value of the duplicate experimental data for the biomass, ethanol and reducing sugar was used as the experimental data to validate the model. The mathematical model was simulated using the software MATLAB^{TM} 6.5. A linear regression between the experimental data and the predicted data were obtained for all the assays. The kinetic parameters were adjusted by trial and error method.

IV. Tequila Model Formulation

In order to quantify the alcoholic fermentation inhibition phenomenon anaerobic shake flask fermentations for 30, 60, 90, 150, and 200 g/L of initial substrate concentration under constant temperature (33°C) were carried out in duplicate. The indigenous AR5 *Saccharomyces cerevisiae* yeast isolated from agave juice was used in all the fermentation assays. The experimental data obtained was smoothed with an average filter described by:

$$\overline{x}_i = \frac{x_i + x_{i+1} + x_{i+2}}{3}, i = 1...n-1 \quad (1)$$

The following set of differential equations describes an unstructured batch fermentation process:

$$\frac{dX}{dt} = \mu X \tag{2}$$

$$\frac{dS}{dt} = -\left[Y_{x/s}^{-1} + Y_{p/s}^{-1} \right] \mu X - m_s X \tag{3}$$

$$\frac{dP}{dt} = \alpha Y_{p/s} \mu X + \beta X \tag{4}$$

where X is the biomass concentration, S is the substrate concentration , P is product concentration (ethanol), $Y_{x/s}$ and $Y_{p/s}$ are specific yield coefficients, m_s maintenance coefficient, α is a growth associated term, β is a non growth associated term, and μ is the specific growth rate, which gives the characteristic nonlinear behavior of fermentation processes. The microorganisms require an amount of time after inoculation to adapt to the new medium; this phenomenon is presented as a lag time of inactivity. To model this lag time the following equation was used:

$$Lag_t = 1 / 1 + e^{-(t - t_{lag})} \tag{5}$$

The following assumptions were made for the development of the tequila fermentation model:

H1. Although the substrate is a polyfructane formed by glucose and fructose, the glucose is a minority sugar and therefore fructose is assumed to be the unique substrate present in the medium.
H2. The only important fermentation product is the ethanol and other byproducts are neglected.
H3. Yield factors *Yxs* and *Yps* remain constant during the fermentation.

As commented in section II, there are several approaches to describe the kinetic microorganism behavior, for instance kinetic models that describe inhibition by high substrate concentration are shown in table I. In table II kinetic models that inhibit the specific growth rate by high product concentrations (ethanol) are shown.

TABLE I
KINETIC MODELS FOR SUBSTRATE INHIBITION

Reference	Equation	
[1]Haldane	$\mu(S) = \frac{\mu_m S}{Ks + S + S^2 K_i^{-1}}$	(6)
[2]Moser	$\mu(S) = \frac{\mu_m S^n}{Ks + S^n}, \quad n > 0$	(7)

[1]Haldane [20], [2]Moser [21]

Using each kinetic model given by (6-7) and (8-10) alone with the set of differential equations (2-4) can not describe satisfactorily the ethanol production in the tequila fermentation process as is shown in Fig. 1.

TABLE II
KINETIC MODELS FOR PRODUCT INHIBITION

Reference	Equation	
[1]Boulton	$\mu(P) = \frac{\mu_m Kp}{Kp + P}$	(8)
[2]Levenspiel	$\mu(P) = \mu_m \left(1 - \frac{P}{Kp}\right)^m$	(9)
[3]Luong	$\mu(P) = \mu_m \left(1 - \left(\frac{P}{Kp}\right)^m\right)$	(10)

[1]Boulton [22], [2]Levenspiel [23], [3]Luong [24]

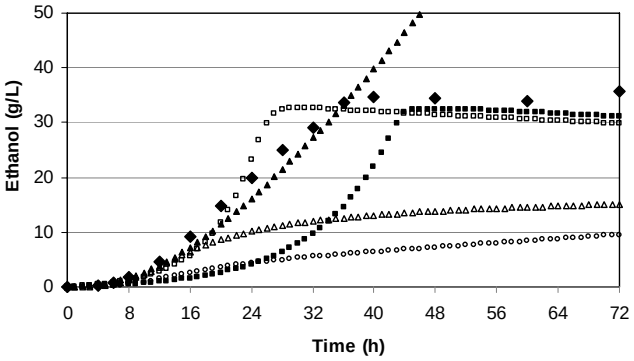

Fig. 1 ethanol prediction using the kinetic models from table I and table II. The symbol (♦) stands for experimental ethanol data, and the following prediction models, Haldane (□), Moser (■), Bulton (▲), Levenspiel (○), and Luong models (Δ).

However, the combinations of the substrate inhibition kinetic model with the product inhibition kinetic model may improve the model prediction. Table III contains different combinations of kinetic models with substrate and product inhibition (e.g. equation (11) uses the kinetic models of Haldane and Boulton, equation (12) uses the kinetic models of Haldane and Levenspiel, and so on). Fig. 2 shows the performance of the different kinetic models (11-16) on the prediction of ethanol tequila fermentation.

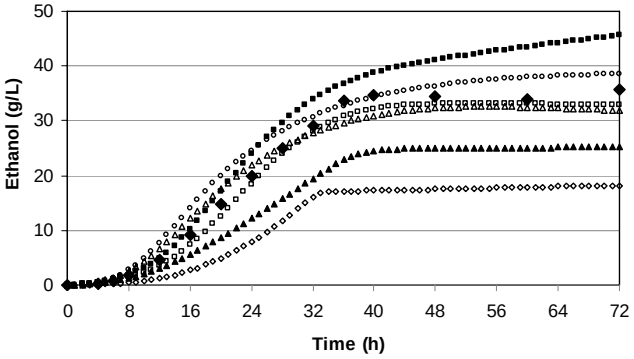

Fig 2 ethanol prediction using kinetic models from table I and II. The symbol (♦) stands for experimental ethanol data, Haldane and Bulton (◊), Haldane and Levenspiel (□), Haldane and Luong (■), Moser and Bulton (▲), Moser and Levenspiel (○), and Moser and Luong (Δ).

TABLE III

KINETIC MODELS FOR SUBSTRATE AND PRODUCT INHIBITION

Reference	Equation	
Haldane and Boulton	$\mu(S,P)=\frac{\mu_m S}{Ks+S+S^2K_i^{-1}}\frac{Kp}{Kp+P}$	(11)
Haldane and Levespiel	$\mu(S,P)=\frac{\mu_m S}{Ks+S+S^2K_i^{-1}}\left(1-\frac{P}{Kp}\right)^m$	(12)
Haldane and Luong	$\mu(S,P)=\frac{\mu_m S}{Ks+S+S^2K_i^{-1}}\left(1-\left(\frac{P}{Kp}\right)^m\right)$	(13)
Moser and Boulton	$\mu(S,P)=\frac{\mu_m S^n}{Ks+S^n}\frac{Kp}{Kp+P}$	(14)
Moser and Levespiel	$\mu(S,P)=\frac{\mu_m S^n}{Ks+S^n}\left(1-\frac{P}{Kp}\right)^m$	(15)
Moser and Luong	$\mu(S,P)=\frac{\mu_m S^n}{Ks+S^n}\left(1-\left(\frac{P}{Kp}\right)^m\right)$	(16)

Each model (11-16) was evaluated by means of a linear regression between the experimental data and the predicted data for the biomass, substrate, and product, with substrate concentrations of 30, 60, and 90 g/L. The correlation coefficient (R^2) of each set of fermentations was averaged and their values are shown in table IV.

TABLE IV
LINEAR CORRELATIONS FOR KINETIC MODELS

Kinetic Model	Mean Value among the correlation coeffient (R^2)
Haldane and Boulton	0.9374
Haldane and Levespiel	0.9832
Haldane and Luong	0.9885
Moser and Boulton	0.9796
Moser and Levespiel	0.9885
Moser and Luong	0.9886

Excellent correlation coefficients were obtained for models of Haldane and Levespiel (12), Haldane and Luong (13), Moser and Levespiel (15) and Moser and Luong (16). However, the model with the best correlation coefficients is the one with the combination of Moser and Luong. Therefore it was decided to use this model along with the equation 5.

V. TEQUILA MODELING VALIDATION

To validate the tequila mathematical model, experimental data were collected from a set of 5 batch fermentations with initial substrate concentrations of 30, 60 90, 150, and 200 g/L. The simulated data was obtained integrating the differential equations (2 – 4) along with the specific growth rate equation (16) and the parameter values shown in table V, using the Runge-Kutta method ode45 with MATLAB™. The biomass (X), substrate (S), and ethanol (P), profiles obtained with the unstructured kinetic model are shown on Fig. 3, 4, and 5. The hollow symbols correspond to experimental data and the solid symbols to simulated values. When the initial substrate corresponds to 60 and 90 g/L (Fig. 4 and 5) an excellent agreement among the predicted concentrations and the experimental values was observed for the biomass, substrate, and ethanol. In the fermentation for 30 g/L of initial substrate only the ethanol and substrate was predicted accurately. The biomass presented difficulty adjusting to the experimental data as can be seen on Fig. 3.

TABLE V
KINETIC PARAMETERS USED IN THE TEQUILA MODEL

Parameter	Value
μ_m = maximum specific growth rate, 1/h	0.37
Ks = Substrate affinity, g/L	20
Kp = Inhibition term, g/L	130
ms = maintenance coefficient, g/L	0.05
Yxs = yield coefficient, gX/gS	0.05
Yps = yield coefficient, gP/gS	0.44
τ = time delay constant, h	1
α = growth associated term	21
β = *non* growth associated term	0.01
n = exponential term for Moser model	1
m= exponential term for Luong model	9

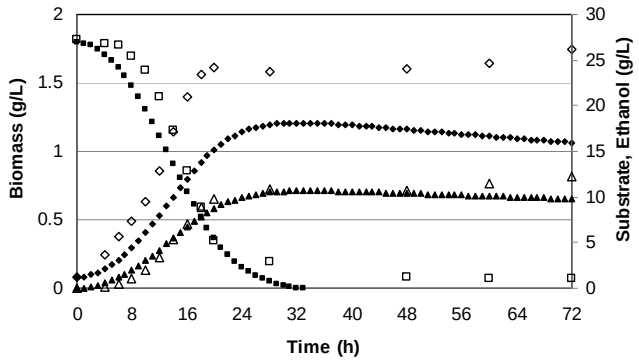

Fig. 3 comparison of the experimental and predicted kinetics of tequila fermentation for S_0 = 30 (g/L). Experimental biomass (◊), experimental substrate (□), experimental ethanol (Δ), predicted biomass (♦), predicted substrate (■), and predicted ethanol (▲).

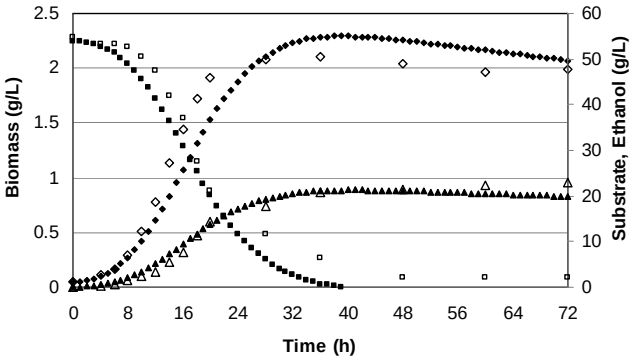

Fig. 4 comparison of the experimental and predicted kinetics of tequila fermentation for S_0 = 60 (g/L). Experimental biomass (◊), experimental substrate (□), experimental ethanol (Δ), predicted biomass (♦), predicted substrate (■), and predicted ethanol (▲).

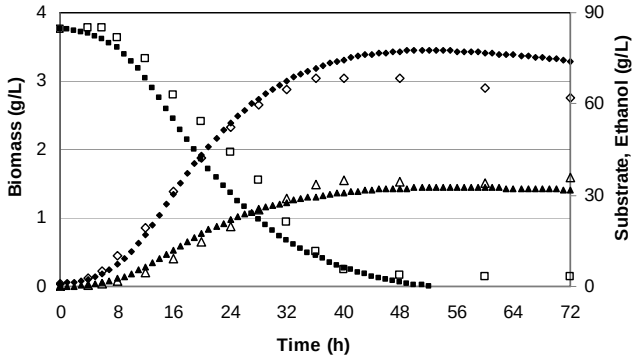

Fig. 5 comparison of the experimental and predicted kinetics of tequila fermentation for S_0 = 90 g/L. Experimental biomass (◊), experimental substrate (□), experimental ethanol (Δ), predicted biomass (♦), predicted substrate (■), and predicted ethanol (▲).

A linear regression for the predicted biomass, the substrate, and the ethanol with the experimental data was produced. The results for 90 g/L initial substrate concentrations are shown in figure 6. The quadratic correlation coefficients obtained were higher than 0.99, so the kinetic model then may be used to simulate the fermentation variables. The correlation coefficients for all the biomass, substrate, and ethanol concentrations for the kinetic model (16) are displayed in table VI.

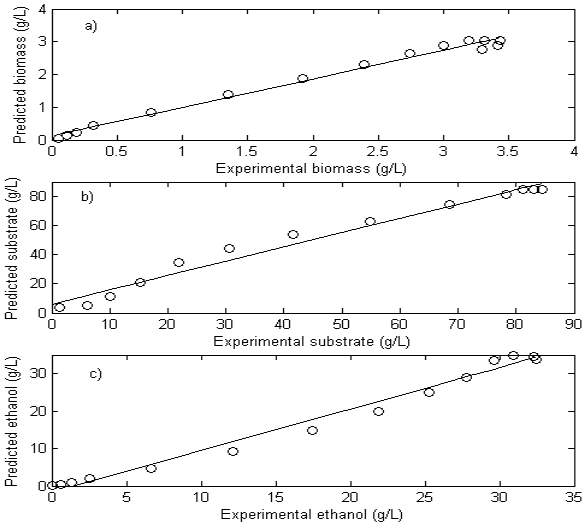

Fig. 6 linear regressions for experimental and predicted data in a fermentation with initial substrate concentrations of 90 g/L. a) experimental biomass (○), predicted biomass, R^2 = 0.9946, b) experimental substrate (○), predicted substrate, R^2 = 0.9909, c) experimental ethanol (○), predicted ethanol, R^2 = 0.9923.

The model proposed from the combination of Moser and Luong, and the lag time term (5) has the following form

$$\mu(S,P) = \frac{\mu_m S^n}{Ks + S^n}\left(1 - \left(\frac{P}{Kp}\right)^m\right)\frac{1}{1+e^{-(t-t_{lag})}} \qquad (17)$$

this model accurately predicts the fermentation in the 60-90 g/L substrate range; however for higher and lower substrate concentrations the model can not describe the biomass accurately. This situation can be justified due that the yield factor *Yxs* was assumed to be constant for all the assays; nevertheless, this parameter varies 0.06 to 0.02 gX/gS from 30 to 200 g/L of initial substrate concentrations respectively with a mean value and a stardar deviation of 0.0405 ± 0.02 gX/gS. By the other hand, the yield factor *Yps* remains almost constant for all the initial substrate concentrations (0.432 to 0.445 gP/gS), with a mean value and a standard deviation of 0.432 ± 0.006 gP/gS. This may explain the reason why the ethanol and the substrate achieve an excellent adjustment.

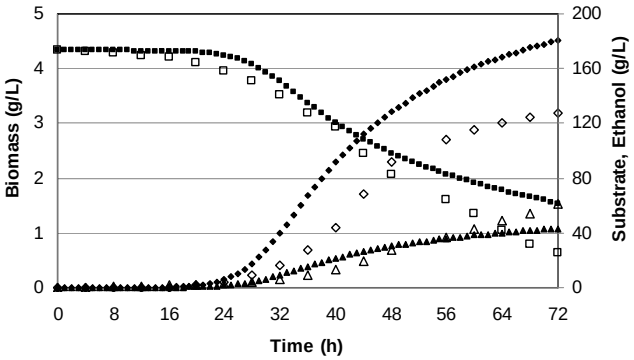

Fig. 7 comparison of the experimental and predicted kinetics of tequila fermentation for S_0 = 30 g/L. Experimental biomass (◊), experimental substrate (□), experimental ethanol (Δ), predicted biomass (♦), predicted substrate (■), and predicted ethanol (▲).

TABLE VI
LINEAR CORRELATIONS FOR KINETIC MODEL (17)

Concentration	*X*	*S*	*P*
30	0.9817	0.9920	0.9893
60	0.9697	0.9957	0.9910
90	0.9946	0.9909	0.9923
150	0.8930	0.9799	0.8515
200	0.9901	0.9948	0.9604

Although some works has been done on modeling alcoholic fermentation processes [25]-[28], currently there are no previous results reported on mathematical modeling of the tequila fermentation process.

VI. CONCLUSION

The proposed tequila unstructured mathematical model predicted satisfactorily the biomass, substrate, and the ethanol for the fermentations with initial substrates in the range 60–90 g/L. Although satisfactory substrate and ethanol prediction results were also obtained for higher substrate concentrations, the biomass could not be predicted accurately. The same situation was present on fermentations at 30 g/L. This situation can be justified due that the yield factor *Yxs* was assumed to be constant for all the assays; nevertheless, this

parameter varies in function of the initial substrate concentration. Nevertheless, the fermentations in the tequila factories used to start near a concentration of 90 g/L of substrate; therefore, this model could be a first approach on tequila fermentation modeling. Additional experimental work on bioreactor, a parametric sensititivity analysis, parameter estimation techniques, and variant yield parameters must be considered to improve the performance of the mathematical tequila model.

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