Growth kinetics of ***Bacillus subtilis*** (Bal3) for production of cellulolytic crudes used in the bioethanol production industry

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*Cinética del crecimiento de **Bacillus subtilis** (Bal3) para la producción de crudos celulolíticos empleados en la industria de producción de bioetanol*

*Cinètica del creixement del **Bacillus subtilis** (Bal3) per a la producció de crus cel·lulolítics emprats en la indústria de producció del bioetanol*

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ABSTRACT

Biotechnologically manageable microbiota that can produce enzyme complexes of interest is a thriving research field. After careful collection and laboratory culture, the microorganisms can develop hydrolysis and fermentation processes in bioreactors. Kinetic models for their growth are applied, being the most used those of Monod, Moser, Blackman and Powell, which combine mathematical models and experimental work.

The objective of this study was to determine the kinetic behavior of *Bacillus subtilis* (Bal3) to size the equipment needed to obtain enough amount of enzimes to produce 500 hl of ethanol at 70% per day. A medium composed of 60 g of sugarcane bagasse and 350 ml of whey in 1L of water with 0.02 g of inoculum was placed in a 2L Bioflo/CelliGen 115 bioreactor for 130 h at 20° C and 150 rpm. The pH ranged from 6.8 (start) to 5.8 (end). A biomass-substrate yield of 19.34% and a biomass productivity of 0.048 g/L per hour were obtained. With the kinetic models of Velhurs-Pearl, Pirt and Luedeking-Piret, the mass balance was performed and the equipment volume was calculated. Monod's model was considered to adequately represent the growth behavior of the bacteria.

Keywords: microbial kinetics, cellulases, *Bacillus subtilis*, kinetic models, mass balance

RESUMEN

Microbiota biotecnológicamente manejable productora de complejos enzimáticos de interés es un área de investigación en auge. Tras cuidadosa colección y cultivo en laboratorio, los microorganismos pueden efectuar hidrólisis y fermentación en biorreactores. Se aplican modelos cinéticos a su crecimiento, siendo los más usados los de Monod, Moser, Blackman y Powell, que combinan modelos matemáticos y trabajos experimentales.

El objetivo de este estudio fue determinar el comportamiento cinético de *Bacillus subtilis* (Bal3) para dimensionar los equipos necesarios para obtener suficiente cantidad de enzimas para producir 500 hl de bioetanol al 70% al día. Se colocó un medio compuesto de 60 g de bagazo de caña y 350 ml de suero de leche en 1L de agua con 0,02 g de inóculo en un biorreactor Bioflo/CelliGen 115 de 2L durante 130 h a 20° C y 150 rpm. El pH varió de 6,8 (inicio) hasta 5,8 (final). El rendimiento biomasa-sustrato fue 19,34 % y la productividad de biomasa 0,048 g/L por hora. Con los modelos cinéticos de Velhurs-Pearl, Pirt y Luedeking-Piret se realizó el balance de masa y el cálculo del tamaño de los equipos. Se consideró que el modelo de Monod representa adecuadamente el comportamiento del crecimiento de la bacteria.

Palabras clave: cinética microbiana, celulasas, *Bacillus subtilis*, modelos cinéticos, balance de masas

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RESUM:

Microbiota biotecnològicament manejable productora de complexos enzimàtics d'interès és una àrea de recerca en auge. Després d'una acurada col·lecció i cultiu en laboratori, els microorganismes poden efectuar hidròlisi i fermentació en bioreactors. S'apliquen models cinètics al seu creixement, sent els més utilitzats els de Monod, Moser, Blackman i Powell, que combinen models matemàtics i treballs experimentals. L'objectiu d'aquest estudi va ser determinar el comportament cinètic de *Bacillus subtilis* (Bal3) per dimensionar els equips necessaris per obtenir suficient quantitat d'enzims per produir 500 hl de bioetanol al 70% per dia. Es va col·locar un mitjà compost de 60 g de bagàs de canya i 350 ml de sèrum de llet en 1L d'aigua amb 0,02 g d'inòcul en un bioreactor Bioflo / CelliGen 115 de 2L durant 130 h a 20° C i 150 rpm. El pH va variar de 6,8 (inici) fins a 5,8 (final). El rendiment biomassa-substrat va ser de 19,34% i la productivitat de biomassa de 0,048 g / L per hora. Amb els models cinètics de Velhurs-Pearl, Pirt i Luedeking-Piret es va realitzar el balanç de massa i el càlcul de la mida dels equips. Es va considerar que el model de Monod representa adequadament el comportament del creixement del bacteri.

Paraules clau: cinètica microbiana, cel·lulases, Bacillus subtilis, models cinètics, balanç de masses

1. INTRODUCTION

Lignocellulosic biomass is a highly attractive and wide-ranging renewable source of energy and chemicals, available through agro-industrial waste, including sugarcane bagasse[1,2]. However, its rigid structure of cellulose, hemicellulose and lignin, very difficult to separate, hinders its usefulness for the industry[2,3]. Multiple studies centered on the enzymatic hydrolysis of bagasse for the production of bioethanol and by-products[4–8] recommend improving the technical and economic effectiveness of this technology through reducing the cost of enzymes[9].

Numerous microorganisms produce cellobiohydrolases, endoglucanases and other enzymes of interest that degrade lignocellulosic residues[6,10]. Fungi, especially, are increasingly being used in the industry[3], though bacteria are gaining protagonism in recent years[10,11]Bacteria have a higher growth rate, which warrants the constant search for new strains or species with greater cellulolytic efficiency[6,10].

Bacillus subtilis strains have proved to be excellent cellulase producers[12]. Often, they are genetically modified to improve their yield[13]. Otherwise, methods such as the preparation of mixtures of commercial enzyme cocktails with the enzyme extracts produced by microorganisms[14] are used.

Obtention of cellulolytic enzymatic cocktails promotes the study of kinetics and physiology of the microorganism. In recent stochastic and deterministic studies, techniques to maximize concentration, productivity and other optimization parameters in the fermentation process, such as mathematical models that allow describing relationships between biomass and substrate consumption[14], have been sought.

A complete enzymatic hydrolysis of cellulose needs combined action of many enzymes to finally obtain glucose[15], which can then be fermented into bioethanol and other products of interest. Bioethanol obtention has been researched in recent years due to its efficient production from hexoses and pentoses[16], aiming to develop cleaner energies[16–18].

Enzymatic hydrolysis of pretreated bagasse, combining use of commercial enzymes with enzymatic crude from native Ecuadorian bacteria and fungi, was recently studied through a Placket Bürman and Box-Hunter[19] design of experiment sequences, where the best performance was obtained with binary mixtures of commercial enzyme and enzymatic crude from the strain *Bacillus subtilis* (Bal3)[20]. However, kinetic behavior of this process, both for independent bacterial growth and for its combination with commercial enzymes, needs to be determined in order to adequately establish the technology to produce cellulolytic cocktails. This may allow reducing costs associated to bioethanol production from sugarcane bagasse in schemes using sugarcane industries as a second generation biorefinery.

In addition, almost all large-scale enzyme production facilities use the proven Submerged Fermentation (SmF) technology because it facilitates control and ease of handling of both the microorganism and the enzyme complex produced[21–23]. They use kinetic models to assess the conditions in which fermentation occurs[24].

The kinetics of growth in the substrate, be it bagasse or another raw material, is a basic tool in microbiology, with many environmental and industrial applications; it allows to understand more complex scenarios[26]. Therefore, the objective of the present research is to determine the kinetic behavior of the growth of *Bacillus subtilis* (Bal3) to generate cellulolytic crudes, intending to size the equipment necessary to obtain cellulases for a plant producing 500 hl/day of bioethanol at 70%.

2. MATERIALS AND METHODS

Isolation and molecular identification of the microorganism were performed, followed by cultivation in suitable conditions, monitoring the behavior of operational parameters along time. Subsequently, through an analytical procedure developed for this purpose, the collected information was processed by adjusting the most probable kinetic models for this process, and mass balance and calculation of volumes and times were carried out.

2.1 Microorganism collection

The species *Bacillus subtilis* (Bal3) was collected from a bagasse reservoir stacked as fuel in Balsapamba, in the Ecuadorian Central Andes (SNI, 2015), coordinates

1°45'49.003"S 79°10'41.289"W. Whey was obtained from an artisan cheese factory near Quito, and it was frozen.

Both samples were received at the research laboratory of the Faculty of Chemical Engineering of Central University of Ecuador. The isolate was seeded in PCA medium (Plate Count Agar) at ± 37 ° C for 24 h.

2.2 Molecular identification

Bacterial identification allows having in Ecuador a database of industrially important strains, such as cellulolytic bacteria for bioethanol production. This in turn will hopefully strengthen the Ecuadorian industry through reduced dependency on imported strains.

Identification based on the 16S rRNA subunit was used. The DNA was extracted from approximately 2 x 10^9 CFU of Bacillus subtilis (Bal3), using a Genomic DNA Pure Link purification kit (Invitrogen). The final volume of 50 µl per reaction was amplified in a Multigene Gradient Thermal cycler (Labnet International, Inc.). 10 µl of DNA were obtained at a concentration of 5 ng/µl, 1 µM of each primer, 0.20 mM of dNTPs, 1.5 mM of $MgCl_{2,}$ and 1.25 U Taq Polymerase (Invitrogen). Primers used to sequence the 16S rRNA region were 8 Forward AGAGTTTGATCCTGGCTCAG and 1492 Reverse GGTTACCTTGTTACGACT. The amplified fragments were verified by electrophoresis in agarose gels at 1% w/v. PCR (Polymerase Chain Reaction) was purified with Pure Link PCR Purification Kit (Invitrogen). The sequences in base pairs (bp) Forward and Reverse were aligned using the alignment search tool BLAST from the National Center for Biotechnology Information, USA.

2.3 Culture and kinetic monitoring

To prepare the culture medium, 60 g of sugarcane bagasse composed of 44.9 % glucose, 28.24 % xylose, and 18.93 % lignin were employed as carbon source; 350 ml of whey as nitrogen source, and the volume was completed to 1L with water[26]. These substrates have been employed to study the growth of yeasts and other microorganisms in the Andes region[27].

An amount of prepared medium was homogenized and placed in a Bioflo/CelliGen 115 (New Brunswick) bioreactor of 2 L. It was inoculated with 0.02 g of *Bacillus subtilis* (Bal3) in dry weight, according to methodologies reported to the effect[19,28]. The bioreactor operated in batch culture during a total time of 130 hours at 20 °C and 150 rpm while it was being monitored. The strain entered the exponential growth phase after 80 to 90 hours of fermentation and reached a maximum value of 5.67 $g^2 h/L^2$ at 120 h and decreasing to 2.66 g^2h/L^2 at 130 h, at which time it was considered that the necessary data for analysis had been obtained. pH was measured every 4 hours throughout the entire process, being it 6.8 at the beginning and 5.8 at the end[26].

The generated biomass was measured from the calibration curve that related the absorbance as a function of Colony Forming Units (CFU), using an Agilent Cary 60 UV-Vis spectrophotometer, at an optical density of 540 nm.

The amount of consumed substrate was measured as lignocellulosic material, by the method of the NREL (2015)[29], USA, and as whey proteins, by the Biuret method, using fetal bovine serum concentration of 40 mg/l as standard[30].

The product, expressed in cellulolytic activity, was measured from the amount of glucose produced after breaking down crystalline cellulose (Avicel), carboxymethylcellulose (CMC) and filter paper, according to the methodology used at the Wallon Center for Industrial Biology (CWBI), University of Liège[31], Belgium. This last degradation promotes FPase activity, which was measured using the previously proposed method[32], for which the culture medium with the microorganism was filtered through a 0.45 µm membrane and the amount of glucose produced was measured on an Agilent 1600 (UV-Vis) spectrophotometer. To analyze the amount of glucose produced, a calibration curve was performed following the aforementioned procedure by the CWBI.

2.4 Selection of kinetic models for *Bacillus subtilis* (Bal3) growth in submerged fermentation.

Considering the amount of generated biomass of *Bacillus subtilis* (Bal3) as *X*, the glucose associated to metabolism as *P* and the consumption of an amount of substrate lignocellulosic as *S*, previously reported models for enzyme kinetics and fermentation can be applied to the kinetic monitoring of this process. Specific growth velocity (µ), based on biomass concentration, is effectively defined for the exponential phase with the Malthos model[33], as shown in equation 1:

$$\mu = \frac{1}{X}\frac{dX}{dt} \qquad (1)$$

Integrating in it, between a fermentative state (*i*) and its initial state (*i-1*), the expression to calculate specific speed results in equation 2:

$$\mu = \frac{\ln X_i - \ln X_{i-1}}{t_i - t_{i-1}} \qquad (2)$$

As the primary kinetic model, Monod model[33] was considered, defined in equation 3 as:

$$\mu = \mu_m \frac{S}{K_s + S} \qquad (3)$$

Where μ_m is the maximum specific growth velocity in units of $time^{-1}$ and K_S is the growth-limiting substrate concentration, in substrate concentration units, for which it is reached *($\mu = \mu_m/2$).*

This model assumes balanced conditions for cell growth and product formation, which may be inadequate to describe enzymatic hydrolysis of lignocellulosic materials and cellular response to growth-inhibiting phenomena. Therefore, fermentative kinetic models were selected, considering free inhibition per substrate and per product that adapt well to culture conditions in substrates of the sugar cane industry[33,34]. They are shown in Table 1. The models described below have been mainly studied in *Saccharomyces cerevisiae*[35]

Table 1. *Kinetic models considered for the growth of Bacillus subtilis (Bal3) in free inhibiting, per substrate and per product.*

Inhibiting phenomenon	*Autor*	*Kinetic model*	
Free	Teissier[36]	$\mu = \mu_m \left(1 - e^{-\frac{S}{K}}\right)$	(4)
	Moser[37]	$\mu = \mu_m \frac{S^n}{K_S + S^n}$	(5)
Per substrate	Andrews[38]	$\mu = \mu_m \frac{S}{K_S + S + \left[\frac{S^2}{K_{IS}}\right]}$	(6)
	Webb[39]	$\mu = \mu_m \frac{S\left[1 + \left(\frac{S}{K_{IS}}\right)\right]}{K_S + S + \left[\frac{S^2}{K_{IS}}\right]}$	(7)
Per product	Dagley and Hinshelwood[40]	$\mu = \mu_m \frac{S}{K_S + S}\left(1 - K_P^{'} P\right)$	(8)
	Aiba and Shoda[41]	$\mu = \mu_m \frac{S}{K_S + S} e^{-K_P^{'} P}$	(9)

In the Teissier model, K is the substrate concentration at which 63% of μ_m is reached[42]. In the Moser model, n is a correction coefficient of the Monod model that considers the inhibitory effect of high cell concentrations[37]. Kinetic models for inhibition per substrate and per product have been developed by modifying the expressions for the inhibition of enzymatic reactions and consider inhibition constants per substrate (K_{IS}) and inhibition per product (K_{IP} or K'_p)[43].

The information generated in the laboratory was validated through the use of previously reported predictive kinetic equations[35]but not limited to, substrate limitation, oxygen limitation, substrate inhibition, product inhibition, and cell death. The majority of these studies have used Saccharomyces cerevisiae and regular mathematical modelling in the form of unstructured unsegregated kinetic modelling. In this paper, the bioethanol fermentation kinetics of pentoses or hexoses or their combination are reviewed. The modes of culture (e.g., batch and continuous. For biomass, the Velhurs-Pearl equation[44]used since 1845 to describe the growth of populations, is interpreted from a functional point of view. More precisely, a simple problem of optimal growth is first formulated and then solved with the Euler-Lagrange equation. The analysis of the solution shows that the optimal growth is logistic if the functional to be minimized is defined over an infinite time horizon. The final result is that the logistic growth represents the best trade-off between deviation from optimal size and cost of growing of a population over its entire life. Such a functional interpretation of the Verhulst-Pearl equation is certainly much more simple and intuitive than those given by Volterra in 1939 and by Leitmann in 1972, which were not formulated ove an infinite time horizon was used, which assumes substrate saturation conditions during which the specific growth rate is not a function of S, where X_M is the value for which dx/dt = 0 for X > 0, as seen in equation 10:

$$X(t) = \frac{X_M}{1 - ((X_M - Xo)/e^{-\mu_m t}} \qquad (10)$$

For substrate consumption, Pirt expression[34] was used. It considers m as the maintenance coefficient in which S (t) vs X (t) produces a straight line with slope, $1 / Y_{(X/S)}$, as in equation 11:

$$S(t) = S_0 \frac{X - X_0}{Y(X/S)} - \frac{mX_M}{\mu M} ln\left[X_M - \frac{X_0}{X_M} - X\right] \qquad (11)$$

For the generated product, the Luedeking- Piret (1959) equation[45] was used, which considers the relationship between microbial growth and product formation, where is product yield based on biomass and k is the destruction factor, as established in equation 12:

$$P(t) = P_0 + Y_{\left(\frac{P}{X}\right)}(X - X_0) + \frac{kX_M}{\mu m} ln[X_M - X_0/X_M - X] \qquad (12)$$

2.5 Analytical procedure

The parameters of the kinetic models proposed for the case study were determined under the experimental conditions achieved using Velhurs-Pearl, Pirt and Luedeking- Piret models given in equations (5), (6) and (7). To validate the models, statistical analysis was run, using the software *Statgraphics Centurion XV* and formed by significance hypothesis tests of means and standard deviation, and by the Kolmogorov-Smirnov test to determine adjustment correction of probability

distributions of the values produced by equations (5), (6) and (7) and the experimental data of X, S and P obtained in the experimental procedure. As an experimental basis for the kinetic analysis, the expression rate of specific growth integrated in equation (1) was considered, with corrections in biomass calculation through equation (2). For the kinetic models, Monod's given in equation (3) was initially considered, adjusting by simulation of the experimental data in Excel version 2016 . Subsequently, the parameters of all the inhibitory models in Table 1 were determined by simulation (*in silico*). The values of X, S and P for the simulation in the kinetic modeling were adjusted according to the experimental values generated with equations (5), (6) and (7). For the simulation, the Hooke and Jeeves method was applied in the adjustment of the parameters by optimizing the results of integrating the sums of the differences between the values calculated by the models and the experimental data, in terms of the integral of the error defined through equation (13), as:

$$\text{INT E}_{model-exp} = \int_0^t (X_{Model} - X_{\mu\, experimental})^2 dt \qquad (13)$$

2.6 Technological scheme for the obtention of cellulolytic enzymatic crudes to supply a plant producing 500 hl/d of bioethanol at 70%.

The technological scheme was based on the methodology recommended by Vilbrandt (1963)[46] and by Peter and Timmerhaus (2003)[47] which comprises the following stages:

- considerations on the chemical process,
- specifications, and
- laboratory data.

For this study, it includes the microbial kinetics, a qualitative flow chart of the process, mass balance and equipment selection.

3. RESULTS AND DISCUSSION

3.1 Molecular identification

The bacterial strain isolated in Balsapamba corresponds to *Bacillus subtilis* (Bal3) with 99% similarity for the 16S region of rDNA[20]. The genetic sequencing pattern had 1415 bp.

3.2 Experimental and analytical procedure

In Table 2 and in Figure 1, the monitoring results of the growth parameters of *Bacillus subtilis* (Bal3) on the enzymatic hydrolysis of sugarcane bagasse are shown analytically and graphically with fermentation time. Results in Table 2 were determined experimentally and by applying the Velhurs-Pearl, Pirt and Luedeking-Piret equations [34]. In addition, results of the statistical processing that enables compare the experimental values with those calculated by using said equations (5), (6) and (7) are shown in Table 2.

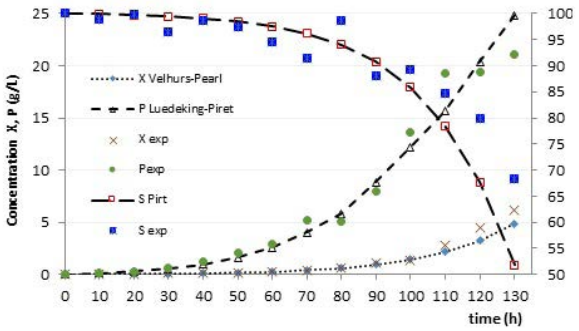

Figure 1. *Profile of experimental concentrations of X, S and P compared to calculated concentrations according to Velhurs-Pearl, Pirt and Luedeking-Piret models during growth of Bacillus Subtilis (Bal3) in enzymatic hydrolysis of sugarcane bagasse.*

In this process, generated biomass is enhanced, entering exponential growth phase after 80 to 90 hours of fermentation with a fairly long adaptation period, probably due to weak inoculation and the typical difficulties of lignocellulosic material degradation, assessment reinforced with analysis of substrate consumption and product generation, which undergo profound variations after 80 to 90 hours, and a severe increase in associated thermal effects, with an uptick of around 3° C in average temperature inside the bioreactor. Moreover, behavior of substrate consumption, product generation, experimental biomass-substrate performance (19.34%) and experimental biomass productivity (0.048 g/(L·h) are aligned with those obtained with other sugary substrates[9] such as rafinose, manose and lactulose despite no clear trend to reach the stationary phase, and suggest that in 130 hours of discontinuous fermentation, adequate results are achieved for this process. For this reason, longer fermentation times were not explored.

In addition, analysis of the previous results shows equations (5), (6) and (7) adequately describe the dynamic behavior of this process, reaching the highest dispersion in substrate consumption. However, for X, S and P, no statistically significant differences were found (at $\alpha = 0.05$) in means, standard deviation and dynamic distribution of the samples, given in the Kolmogorov-Smirnov test. On the other hand, for S, and after 100 hours of fermentation, its magnitude calculated by the Pirt equation drops faster than the experimental values do. This may be because Pirt's model considers that, in substrate-saturated microorganism cultures, specific growth rate is not a function of said concentration and is not applicable when substrate concentration is reduced due to growth[48]. Regardless, the product-substrate yield calculated from the values obtained from Pirt and Luedeking-Piret models (51.7%) adjusts better to the mentioned difficulties to metabolize lignocellulosic materials than that calculated according to experimental values (65.9%)[9,28,49].

The global results of the simulation and adjustment of the considered kinetic models are shown in Table 3, where simulated parameters with the criterion of minimum adjustment error and the INT E value shown correspond to fermentation as a whole. According to

Table 2. *Results of growth parameters of Bacillus subtilis (Bal3) in enzymatic hydrolysis of sugar cane bagasse.*

$T(h)$	X_{exp} (g/L)	$X_{Velhurs-Pearl}$ (g/L)	S_{exp} (g/L)	S_{Pirt} (g/L)	P_{exp} (g/L)	$P_{Luedeking-Piret}$ (g/L)
0	0.020	0.02	100.000	100	0.000	0
10	0.039	0.031	98.891	99.89	0.119	0.113
20	0.038	0.047	99.820	99.72	0.246	0.289
30	0.086	0.073	96.486	99.47	0.641	0.557
40	0.100	0.111	98.684	99.08	1.211	0.969
50	0.198	0.171	97.495	98.48	2.077	1.598
60	0.301	0.262	94.643	97.57	2.866	2.559
70	0.339	0.403	91.362	96.17	5.148	4.022
80	0.567	0.616	98.732	94.03	5.036	5.856
90	1.102	0.942	88.057	90.78	7.953	8.837
100	1.360	1.432	89.305	85.87	13.682	12.216
110	2.820	2.169	84.791	78.51	19.272	15.668
120	4.430	3.257	79.795	67.623	19.352	20.37
130	6.193	4.838	68.389	51.81	21.097	24.82

Means significance tests Ho: ($\mu_{MODELS}=\mu_{exp}$: accepted for $\alpha=0.05$)

	$\mu X_{Velhurs\ Pearl}$ vs $\mu X_{exp.}$	μS_{Pirt} vs μS_{exp}	$\mu P_{Luedeking-Piret}$ vs $\mu P_{exp.}$
P_{value}	0.072 > 0.05 (t=0.3583)	0.674 > 0.05 (t=-0.4258)	0.984> 0.05 (t=-0.0199)

Standard deviation significance test Ho: ($\sigma_{MODELS}=\sigma_{exp}$: accepted for $\alpha=0.05$)

Significance test of the means Ho: ($\mu_{MODELS}=\mu_{exp}$: accepted for $\alpha=0.05$)

	$\sigma X_{Velhurs\ Pearl}$ vs $\sigma X_{exp.}$	σS_{Pirt} vs σS_{exp}	$\sigma P_{Luedeking-Piret}$ vs $\sigma P_{exp.}$
P_{value}	0.339 > 0.05 (F=1.7223)	0.1105 > 0.05 (F=2.503)	0.882 > 0.05 (F=1.092)

Kolmogorov-Smimov (K-S) test (without significant differences for $\alpha=0.05$ between parameters due to equations (3), (4) and (5) vs experimental values of X, S and P)

	$X_{Velhurs\ Pearl}$ vs $X_{exp.}$	S_{Pirt} vs S_{exp}	$P_{Luedeking-Piret}$ vs $P_{exp.}$
DN	0.0714	0.2142	0.0769
K-S statistic	0.1889	0.5669	0.1961
P_{value}	1.0 > 0.05	0.905 > 0.05	1.0 > 0.05

Table 3. *Results of simulation and adjustment of kinetic models considered in monitoring of Bacillus subtilis (Bal3) growth in enzymatic hydrolysis of sugarcane bagasse.*

Inhibiting mechanism	*Kinetic model*	*Parameters*	*INT $E_{model-exp}$ (g^2h/L^2) at 130 hours*
Free	Teissier	μ_m= 0.044 h^{-1} K= 20 g/L	21.27
	Moser	μ_m= 0.0438 h^{-1} Ks= 3.1 g/L n=1.13	6.45
Per substrate	Andrews	μ_m= 0.044 h^{-1} Ks= 2.8 g/L K_{IS}= 500 g/L	5.67
	Webb	μ_m= 0.0438 h^{-1} Ks= 3.9 g/L K_{IS}= 60 g/L	2.66
Per product	Dagley	μ_m= 0.045 h^{-1} Ks= 1.1 g/L K'_p= $1.5\cdot10^{-3}$ g/L	10.02
	Aiba	μ_m= 0.0438 h^{-1} Ks= 2 g/L K'_p= $1.55\cdot10^{-4}$ g/L	6.23

said INT E values, the best adjustment is reached with Monod model, followed by Webb, Andrews, Mosser and Aiba models. This suggests that inhibitory effects of free kind, per substrate and per product may be present. Nevertheless, for Andrews and Aiba models, although the achieved adjustment is adequate, their K_{IS} and K'_p parameters lack physical meaning. On the other hand, it is necessary to analyze the behavior of INT E criterion throughout fermentation time to verify the dynamic adjustment stage by stage.

For this, the results of simulation and adjustment to experimental values of Monod model are shown in Figure 2. Similar analysis is shown in Figure 3 for free inhibition models .

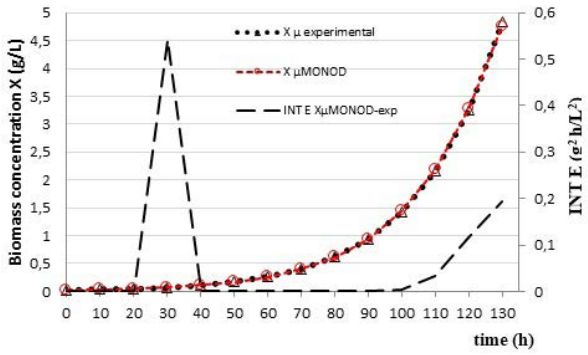

Figure 2. *Simulation and adjustment of Monod model to Bacillus subtilis (Bal3) growth in enzymatic hydrolysis of sugarcane bagasse.*

The coincidence of simulated and experimental values is reinforced with the values of INT E XµMONOD-exp criterion, well below one. This effect is generally not affected by the INT E XµMONOD-exp peak at 30 hours, which may be due to difficulties in the adaptation stage, due to low inoculation levels. This shows that this model adequately represents this process behavior. On the other hand, the Ks value suggests that with low substrate consumption requirements, it is possible to reach half of the maximum growth rate.

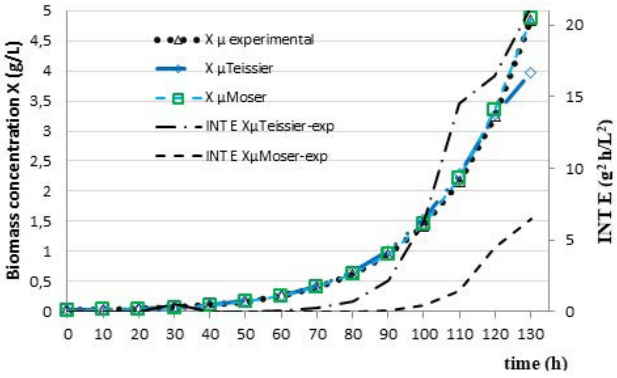

Figure 3. *Simulation and adjustment of free inhibition growth models for Bacillus subtilis (Bal 3) on enzymatic hydrolysis of sugarcane bagasse.*

Through simulation and adjustment of free inhibition models, when this mechanism responds to Moser model, it can also serve to adequately describe this process. Said model reaches very low INT E XµMoser-exp values in the first 100 h of fermentation that grow gradually up to 6.45 g^2 h/L^2 during development of the exponential phase, which took place between 90 and 130 h. In addition, as shown in Table 1, when *n* is close to the unit, this model includes Monod's, so, in this case, saving the slight increases in *n* and *Ks*, the kinetic mechanism can be considered as the one described by Monod with balanced growth and consumption. Also, through inspection and analysis of Figure 3, it is shown that Teissier model does not adjust with the same effectiveness to the case study, due to which it is not applicable.

Figures 4 and 5 show the results of the simulation and adjustment to experimental values of the inhibition models per substrate and per product, respectively.

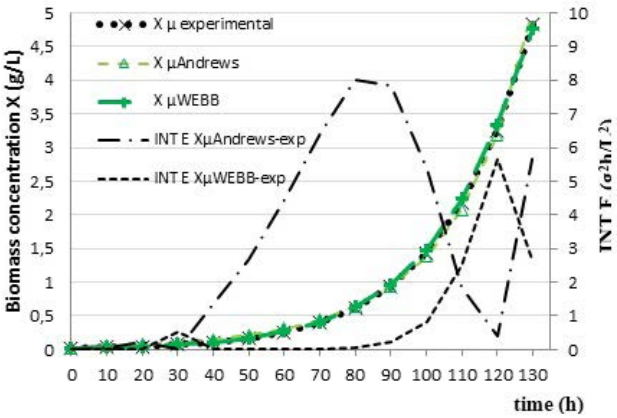

Figure 4 . *Simulation and adjustment of growth inhibition models per substrate for Bacillus subtilis (Bal3) in enzymatic hydrolysis of sugarcane bagasse.*

Analyzing Figure 4, inhibition per substrate described in the Webb model can be applied to the case study, taking into account the behavior of INT E X WEBB-exp., which grows progressively up to 120 h reaching a maximum value of 5.67 g^2 h/L^2 and decreasing to 2.66 g^2h/L^2 at 130 h. The peak formed for said variable is due to the fact that the new effects generated by inhibition per substrate, recorded in parameter K_{IS} = 60g/L, cause better adjustment in the fermentation final stage, where 11.4% of initial substrate is consumed (11.4 g/L) in 7.7% of total fermentation time (10 h).

In the case of Andrews model, no good adjustment to the experimental data is observed and the value of parameter K_{IS} lacks physical sense, due to which this model is not applicable to the case study. The magnitude of K_{IS}, the adjustment achieved by Monod model, the growth itself of INT E WEBB-exp. after 100 hours and, mostly, the impossibility of applying a traditional model with very good results on sugary substrates like that of Andrews[33] suggests the inhibition effects per substrate are evident only from the 100 hour mark until the end of the growth curve, that is, in the last 30 hours.

Results shown in Figure 5 make possible to state that the Aiba model adjusts best to the experimental data than Dagley's, with INT E Aiba-exp., which behaves almost zero and grows progressively after 120 h, reaching a maximum value of 6.23 g^2h/L^2 at 130 h of fermentation. However, the K'p parameter of both models lacks

physical meaning, due to which inhibition per product is not applicable to the case study. In view of this, the balanced behavior assumed by Monod's model adjusts better to the growth kinetics of *Bacillus subtilis* (Bal3) in sugarcane bagasse than those that consider inhibition per substrate and per product .

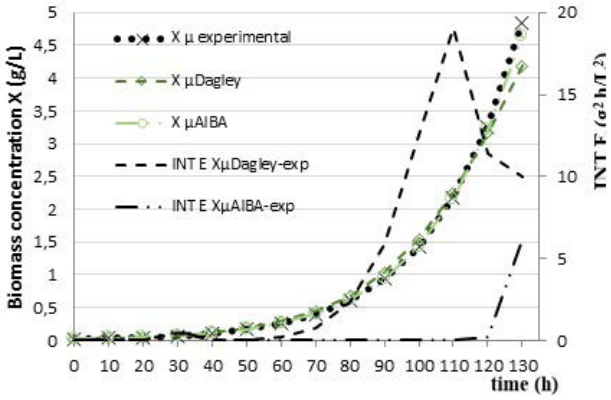

Figure 5. *Simulation and adjustment of growth inhibition models per product for Bacillus subtilis (Bal3) in enzymatic hydrolysis of sugarcane bagasse .*

Regarding the parameters of Monod model, growth rates have been found in sugary substrates such as sacarose with $\mu = 1.88\ h^{-1}$ for *Bacillus licheniformis*[50] and 1.5 h^{-1} for *Bacillus subtilis* with culture medium made of sunflower and corn kernels[51] . This allows to infer that the low µ max value of 0,043 h^{-1} may be due to possible low availability of nutriens such as vitamins and minerals in the substrate. It should be noted that sugarcane bagasse was used as the only source of nutrients with the aim to displace generalist species, as suggested by Mello et al. (2016)[52]it is possible to obtain a greater diversity of species in the laboratory setting when microorganisms are grown as mixed cultures. In order to mimic the environmental conditions, an appropriate growth medium must be applied. Here, we examined the hypothesis that a greater diversity of microorganisms can be sustained under nutrient-limited conditions. Using a 16 S rRNA amplicon metagenomic approach, we explored the structure of a compost-derived MC. During a five-week time course the MC grown in minimal medium with sugarcane bagasse (SCB.

3.3. Technological scheme for enzyme production: Considerations on the expected enzyme needs in the chemical process in a plant producing 500 hl/d of bioethanol at 70%.

The need to produce 2.8 m^3 of enzymatic crudes from *Bacillus subtilis* (Bal3) in order to obtain 500 hl/d of bioethanol at 70% from sugarcane bagasse was calculated from the following data: a proportion of 1.13 g of cellulolytic crude diluted in 25 mL of citrate buffer are used to degrade 1.6 g of bagasse.

Therefore, at industry level, 1,856,000 g of cellulases diluted in 41,061,946 mL of citrate buffer will be required to degrade 2,605,000 g of bagasse which correspond to 2320 L, considering that crude density as calculated in the laboratory was 0.8 g/ml. The amount of sugarcane bagasse was calculated by Mesa (2010)[4], who determined that 5.21 kg of sugarcane bagasse produce 1 L of bioethanol at 70% with this method.

The processes scale-up was based in the laboratory data described in the microbial kinetics. Afterwards, the equipment dimensions were calculated based on the experimental data. In this first application of the microbial kinetics, series of empirical relationships are presented, which must be confirmed with subsequent studies and through the application of more accurate mathematical models at higher scales. Nevertheless, it allows us to determine the worthiness of utilizing this strain to produce greater volumes of enzymatic crudes.

The process must be designed for large-scale production to be profitable. This criterion entails reducing imports and procuring local materials to the greatest extent possible, starting with the selected strain and continuing with the culture nutrients. For this reason, in the kinetic study, only sugarcane bagasse, whey, and water are used as substrate for bacteria inoculation.

3.3.1. Enzymatic characteristics of the crude produced from *Bacillus subtilis* (Bal3)

In sugarcane bagasse as a carbon source, enriched with whey, a maximum activity of endobetaglucanase of 0.30 Uml^{-1}, of exoglucanase of 0.91 Uml^{-1} and of 42 UPF was shown. These results are lower than those obtained by Ladeira et al.[53], in which 0.83 Uml^{-1} for avicelase activity and 0.29 Uml^{-1} for the CMCase activity were found. However, they hold relevance as they did not require imported raw material to produce both the microorganism and the enzymatic cocktail. To obtain these results, the calibration curve proposed in the methodology to determine enzymatic activity was that of glucose as a function of absorbance, presenting an $R^2 = 0.97$.

3.4 . Laboratory specifications and data-to-scale in a 2L fermenter

As explained in section 2.3, a homogenized medium was prepared and placed in a 2L Bioflo/CelliGen 115 (New Brunswick) bioreactor. Taking into account the integration equation of biomass and time, based on Monod's model, equation [14] was applied, wherein X and X_0 are the cell masses in time t and t_0. It can be expressed as:

$$lnX - lnXo = \mu.(t - to) \qquad (14)$$

This equation was used to identify one of the applications of the microbial kinetics in an industry that produces 500 hL/d of bioethanol. In this regard, the initial biomass of *Bacillus subtilis* (Bal3) that must be inoculated was calculated as $X_0 = 134$ kg/d to obtain the final biomass value of 376 kg/d.

Furthermore, from the calculated information, through the equations of biomass (X), substrate (S) and product (P), represented by equations [5], [6] and [7], it was found that 376 kg of *Bacillus subtilis* (Bal3) are required to be produced in order to obtain 2.35 m^3 of enzymatic cocktail.

3.5 Mass balance

From the experimental results obtained and from the information generated in the kinetic studies, it was found that $7E^{-6}$ g of cellulase crude diluted in water and whey were isolated in the laboratory to degrade 0.001 kg of sugarcane bagasse. Therefore, to degrade 260,500 kg of sugarcane bagasse, 1,856 kg of crude of native cellulase enzymes are required, which corresponds to 2.35 m^3 taking into account the crude density calculated in laboratory, which is 0.8 g/cm^3. Knowing the initial values of the products required to obtain the amount of enzymatic crude, and based on the enzymatic kinetics study, the values were calculated, extrapolating the data from the fermenter, as shown below in Figure 6, considering the criteria based on the principles of geometric, mechanic and cinematic, thermic, and chemical similarity[54].

3.6. Equipment selection

The chemical process will require laboratory-scale (a) and industrial-scale (b) equipment. The first part, (a), will have 25 0.05-m^3 Erlenmeyer flasks and 7 0.27-m^3 pre-fermenters. The second, (b), constitutes the production carried out in the industry, in which a 61.84-m^3 fermenter will be installed. This will be located on a filtering tank where there will be a bagasse conveyor mesh. The liquid mixture of serum, water, microorganisms and enzyme are collected in a storage tank of 2.35 m^3. The explained volumes are mentioned in Figure 6 and correspond to various mass balance studies carried out by Salvador (2018)[26].

3.7. Qualitative flow chart of enzymatic crude production process of Bacillus subtilis (Bal3)

Figure 7 shows the technological scheme represented in a block diagram of enzyme production from the mass balance of processes calculated by Salvador (2018)[26].

Equipment volumes were calculated from the amounts of substrate and bacteria required for a production of 500 hl/d of bioethanol at 70%, obtained through the aplication of the microbial kinetic equations.

The explained work scheme considered a discontinuous culture model, also known as batch fermentation, to produce cellulolytic enzymatic crudes from *Bacillus subtilis* (Bal3). Batch fermentation is widely applied in industrial biotechnology due to its simplicity, minimal control of temperature, humidity and pH, and monitoring based only on measuring cell growth. It also reduces the possibility of contamination[55,56]isolate PSK1

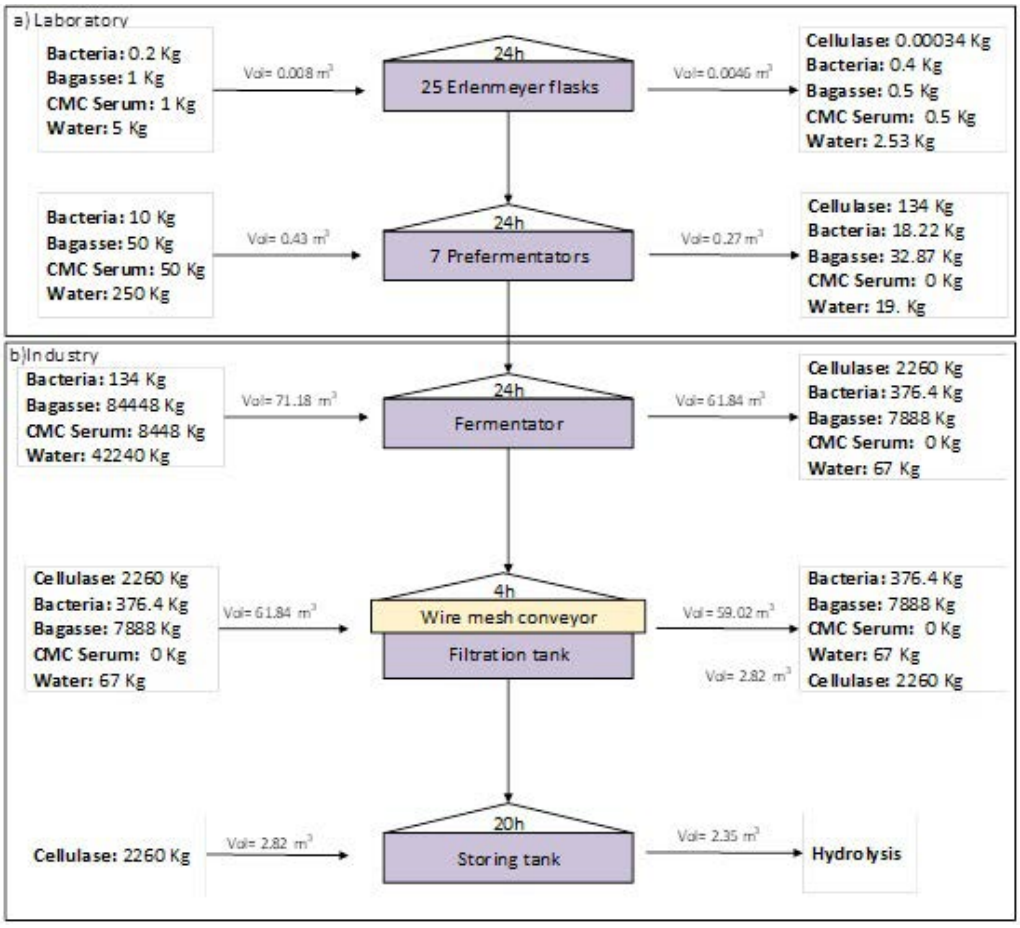

Figure 6. *Results of mass balance and calculation of the volumes and times for enzyme obtention. a) Laboratory, b) Industry*

was selected and identified by 16S rDNA sequencing as Bacillus aryabhattai. Growth optimization of PSK1 and physicochemical parameters affected bioflocculant production was carried out by Plackett-Burman design and resulted in increasing in the activity by 4.5%. Bioflocculant production by entrapped and adsorbed immobilized microbial cells was performed using different techniques and revealed enhancement in the activity in particular with pumice adsorption. HPLC analysis of sugars and amino acids composition, FTIR and the effect of different factors on the purified PSK1 biopolymer such as presence of cations, thermal stability, pH range and clay concentration was carried out. Scanning electron microscopy (SEM.

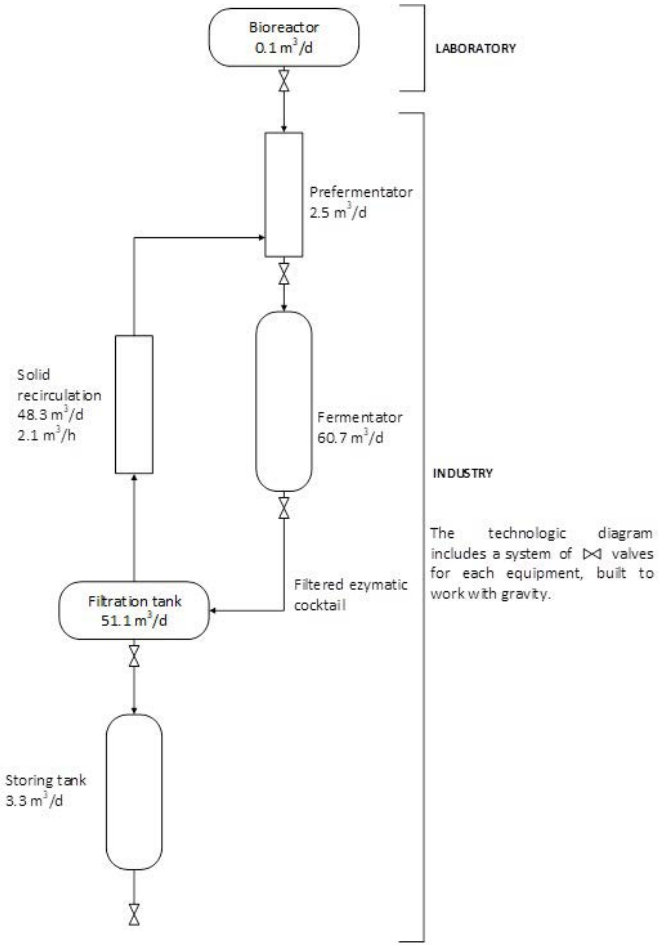

Figure 7. *Technological scheme of the production of enzymatic crude from the bacteria Bacillus subtilis (Bal3).*

4 . CONCLUSIONS

The application of microbial kinetics in this context allows to foresee application possibilities that working with the strain *Bacillus subtilis* (Bal3) may present.

The kinetic values obtained from the validated models of Monod, Webb, Andrews, Mosser and Aiba support the feasability of using sugarcane bagasse and whey, substrates widely available in Ecuador, as a source of growth for *Bacillus subtilis* (Bal3) and potentially for other strains as well, depending on further studies..

Monod's model shows a μ max value of 0,043 h^{-1}, which adequately represents the growth behavior of the strain *Bacillus subtilis* (Bal3) used for this study in the described conditions of using only sugarcane bagasse and whey as nutrients.

In the experimental case, the growth decrease at 130 hours is due to the low level of inoculum applied and to difficulties of lignocellulosic material degradation. They influenced the strain entering the exponential growth phase after 80 to 90 hours of fermentation..

The identification of gene 16S of rDNA allowed to succesfully identify the local strain *Bacillus subtilis* (Bal3).

The saturation constant (Ks) value of 3.1 g/L suggests that, with low substrate consumption requirements, it is possible to achieve half the maximum growth rate.

The kinetic models and the application of equations from Velhurs-Pearl, Pirt and Luedeking-Piret contribute to perform the mass balance, the volume calculation for the equipment, and the technological scheme to obtain cellulolytic enzymatic crudes for the production of 500 hl/d of bioethanol at 70%. Said scheme consists of laboratory section and industry section.

REFERENCES

1. Albernas-Carvajal, Y., Gabriela, C., Layanis, M. G., Ronaldo, S. H. & Erenio, G. S. Estudio de la cinética de la hidrólisis enzimática del bagazo pretratado. *Afinidad* **72**, (2015).
2. Ji, Q. *et al.* Synergism of sweeping frequency ultrasound and deep eutectic solvents pretreatment for fractionation of sugarcane bagasse and enhancing enzymatic hydrolysis. *Ultrason. Sonochem.* **73**, 105470 (2021).
3. Bai, D.-P. *et al.* Metagenomics approach to identify lignocellulose-degrading enzymes in the gut microbiota of the Chinese bamboo rat cecum. *Electron. J. Biotechnol.* **50**, 29–36 (2021).
4. Mesa Garriga, L. Estrategia investigativa para la tecnología de obtención de etanol y coproductos del bagazo de la caña de azúcar. (Universidad Central 'Marta Abreu' de Las Villas, 2010).
5. Albernas-Carvajal, Y., J., P. G., G., C., L., R. R. & E., G. S. Primera aproximación a la cinética de la obtención de etanol mediante sacarificación y fermentación simultánea del bagazo. *Afinidad* **73**, (2016).
6. Buraimoh, O. M. *et al.* Sustainable generation of bioethanol from sugarcane wastes by Streptomyces coelicolor strain COB KF977550 isolated from a tropical estuary. *Sci. African* **11**, e00709 (2021).
7. Moodley, P. & Gueguim Kana, E. B. Bioethanol production from sugarcane leaf waste: Effect of various optimized pretreatments and fermentation conditions on process kinetics. *Biotechnol. Reports* **22**, e00329 (2019).
8. Wahono, S. K. *et al.* Optimization of Simultaneous Saccharification and Fermentation Incubation Time Using Cellulose Enzyme for Sugarcane Bagasse on the Second-generation Bioethanol Production Technology. *Energy Procedia* **65**, 331–336 (2015).

9. Salvador Pinos, C. A. *et al.* Obtención de celulasas en Ecuador para la disminución de gastos en enzimas en la hidrólisis de bagazo. *Rev. Cent. Azúcar* **46**, (2019).
10. Nelson, K., Muge, E. & Wamalwa, B. Cellulolytic Bacillus species isolated from the gut of the desert locust Schistocerca gregaria. *Sci. African* **11**, e00665 (2021).
11. Su, T., Zhao, D., Khodadadi, M. & Len, C. Lignocellulosic biomass for bioethanol: Recent advances, technology trends, and barriers to industrial development. *Curr. Opin. Green Sustain. Chem.* **24**, 56–60 (2020).
12. Irfan, M., Mushtaq, Q., Tabssum, F., Shakir, H. A. & Qazi, J. I. Carboxymethyl cellulase production optimization from newly isolated thermophilic Bacillus subtilis K-18 for saccharification using response surface methodology. *AMB Express* **7**, 29 (2017).
13. Imran, M., Anwar, Z., Irshad, M., Asad, M. & Ashfaq, H. Cellulase Production from Species of Fungi and Bacteria from Agricultural Wastes and Its Utilization in Industry: A Review. *Adv. Enzym. Res.* **04**, 44–55 (2016).
14. Sant'Ana da Silva, A. *et al.* High-solids content enzymatic hydrolysis of hydrothermally pretreated sugarcane bagasse using a laboratory-made enzyme blend and commercial preparations. *Process Biochem.* **51**, 1561–1567 (2016).
15. Morales Zamora, M. Estrategia para la reconversión de una industria integrada de azúcar y derivados para la producción de etanol y coproductos a partir del bagazo. (Universidad Central 'Marta Abreu' de Las Villas, 2012).
16. Zhou, M. & Lü, X. Chapter 9 - Strategies on simultaneous fermentation of pentose and hexose to bioethanol. in *Woodhead Publishing Series in Energy* (ed. Lü, X. B. T.-A. in 2nd G. of B. P.) 161–211 (Woodhead Publishing, 2021). doi:https://doi.org/10.1016/B978-0-12-818862-0.00010-8.
17. Sanusi, I. A., Suinyuy, T. N. & Kana, G. E. B. Impact of nanoparticle inclusion on bioethanol production process kinetic and inhibitor profile. *Biotechnol. Reports* **29**, e00585 (2021).
18. Kuhad, R. C., Gupta, R., Khasa, Y. P., Singh, A. & Zhang, Y.-H. P. Bioethanol production from pentose sugars: Current status and future prospects. *Renew. Sustain. Energy Rev.* **15**, 4950–4962 (2011).
19. Salvador, C. A., Albernas, Y., Mesa, L. & García, A. Obtaining the kinetic parameters of the enzymatic hydrolysis of sugarcane bagasse using a new enzyme mixture from commercial Aspergillus niger and a local strain of Bacillus subtilis (Bal3). *Afinidad* **78**, (2021).
20. Batallas Merino, F., Salvador Pinos, C. A., Villavicencio Montoya, J., González Gavilánez, H. & González Suárez, E. Hydrolysis of sugarcane bagasse to obtain ethanol using native and commercial enzymes. *Cent. Azúcar* **45**, 90–100 (2018).
21. Rani Singhania, R. Chapter 8 - Production of Celluloytic Enzymes for the Hydrolysis of Lignocellulosic Biomass. in (eds. Pandey, A., Larroche, C., Ricke, S. C., Dussap, C.-G. & Gnansounou, E. B. T.-B.) 177–201 (Academic Press, 2011). doi:https://doi.org/10.1016/B978-0-12-385099-7.00008-5.
22. Soccol, C. R. *et al.* Recent developments and innovations in solid state fermentation. *Biotechnol. Res. Innov.* **1**, 52–71 (2017).
23. Osemwegie, O. O. *et al.* Exopolysaccharides from bacteria and fungi: current status and perspectives in Africa. *Heliyon* **6**, e04205 (2020).
24. Torres, N. V, Voit, E. O., Glez-Alcón, C. & Rodríguez, F. An indirect optimization method for biochemical systems: Description of method and application to the maximization of the rate of ethanol, glycerol, and carbohydrate production in Saccharomyces cerevisiae. *Biotechnol. Bioeng.* **55**, 758–772 (1997).
25. Kovárová-Kovar, K. & Egli, T. Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. *Microbiol. Mol. Biol. Rev.* **62**, 646–666 (1998).
26. Salvador Pinos, C. A. Obtención Y Uso De Enzimas Celulolíticas Nativas De Ecuador Para La Producción De Etanol De Segunda Generación. (Universidad Central 'Marta Abreu' de Las Villas, 2018).
27. Aguilar, J. *et al.* Evaluación de la cinética de crecimiento de saccharomyces cerevisiae utilizando un medio de cultivo a base de melaza de caña y suero lácteo. *Agroindustrial Sci.* **5**, 37–47 (2015).
28. Villavicencio Montoya, J. *et al.* Use of native cellulolytic enzymes from Ecuador, in the enzymatic hydrolysis for the ethanol production. *Centro Azúcar* vol. 45 91–100 (2018).
29. Resch, M. G., Baker, J. O. & Decker, S. R. *Low Solids Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP), Issue Date: February 4, 2015*. www.nrel.gov/publications. (2015).
30. Villar Illescas, A. B. Validación de un método analítico para determinación del contenido total de proteínas en leche. (Universidad de Jaén, 2018).
31. Salvador Pinos, C. A. Recherche des activités enzymatiques chez Eisenia foetida. (Université de Lie`ge-Gembloux).
32. Adney, B. & Baker, J. *Chemical Analysis and Testing Task. Laboratory Analytical Procedure. Measurement of cellulase activities*. https://www.nrel.gov/docs/gen/fy08/42628.pdf (2008).
33. Trejos Montoya, V., Fontalvo, J. & GARCIA, M. Descripción matemática y análisis de estabilidad de procesos fermentativos. *Dyna* **76**, (2009).
34. Pirt, S. J. *Principles of microbe and cell cultivation.* (Blackwell Scientific Publications., 1975).
35. Nosrati-Ghods, N., Harrison, S. T. L., Isafiade, A. J. & Leng Tai, S. Mathematical Modelling of Bioethanol Fermentation From Glucose, Xylose or Their Combination – A Review. *ChemBioEng Rev.* **7**, 68–88 (2020).
36. Tessier, G. Croissance des populations bactériennes et quantité d'aliment disponible. *Rev. Sci. Paris* **80**, 209 (1942).
37. Moser, H. Structure and dynamics of bacterial populations maintained in the chemostat. in *Cold*

Spring Harbor symposia on quantitative biology vol. 22 121–137 (Cold Spring Harbor Laboratory Press, 1957).
38. Andrews, J. F. A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates. *Biotechnol. Bioeng.* **10**, 707–723 (1968).
39. Webb, J. L. Enzyme and metabolic inhibitors. (1963).
40. Dagley, S. & Hinshelwood, C. N. 363. Physicochemical aspects of bacterial growth. Part I. Dependence of growth of Bact. lactis aerogenes on concentration of medium. *J. Chem. Soc.* 1930–1936 (1938).
41. Aiba, S., Shoda, M. & Nagatani, M. Kinetics of product inhibition in alcohol fermentation. *Biotechnol. Bioeng.* **10**, 845–864 (1968).
42. Sönmezişik, M., Tanyola, D., Seker, S. & Tanyola, A. The double-substrate growth kinetics of sulfolobus solfataricus, a thermophilic sulfur-removing archeabacterium. *Biochem. Eng. J.* **1**, 243–248 (1998).
43. Albernas Carvajal, Y., Pedraza Gárciga, J. & Corsano, G. *Contribución De La Ingeniería De Las Reacciones Químicas Al Desarrollo De Nuevos Procesos Industriales*. (Ingeniería Química, Desarrollo industrial, Docencia de postgrado, 2019).
44. Gatto, M., Muratori, S. & Rinaldi, S. A functional interpretation of the logistic equation. *Ecol. Modell.* **42**, 155–159 (1988).
45. Luedeking, R. & Piret, E. L. A kinetic study of the lactic acid fermentation. Batch process at controlled pH. *Biotechnol. Bioeng.* **1**, 393–412 (1959).
46. Vilbrandt, F. C., Dryden, C. E. & Barnes, J. G. *Ingeniería química del diseño de plantas industriales*. (Grijalbo, 1963).
47. Peters, M. S., Timmerhaus, K. D. & West, R. E. *Plant design and economics for chemical engineers*. vol. 4 (McGraw-Hill New York, 2003).
48. Rincón Santamaría, A., Cuellar Gil, J. A., Valencia Gil, L. F. & Sánchez Toro, O. J. Cinética de crecimiento de gluconacetobacter diazotrophicus usando melaza de caña y sacarosa: evaluación de modelos cinéticos. *Acta Biológica Colombiana* vol. 24 38–57 (2019).
49. Mesa, L. *et al.* The effect of organosolv pretreatment variables on enzymatic hydrolysis of sugarcane bagasse. *Chem. Eng. J.* **168**, 1157–1162 (2011).
50. Rizo-Porro, M., González, N. & Ramos-Sánchez, L. Macroscopic growth model, in a sucrose-based medium, for a probiotic bacterium using on rearing of shrimp postlarvae. *Congreso Internacional sobre azúcar y derivados. Diversificación. Memorias.* (2017).
51. Caraman, S., Barbu, M. & Munteanu, C. Expert System Based on Fuzzy Rules for Alpha-Amylase Production with Bacillus subtilis. *IFAC Proc. Vol.* **37**, 487–492 (2004).
52. Mello, B. L., Alessi, A. M., McQueen-Mason, S., Bruce, N. C. & Polikarpov, I. Nutrient availability shapes the microbial community structure in sugarcane bagasse compost-derived consortia. *Sci. Rep.* **6**, 38781 (2016).
53. Ladeira, S. A., Cruz, E., Delatorre, A. B., Barbosa, J. B. & Martins, M. L. L. Cellulase production by thermophilic Bacillus sp. SMIA-2 and its detergent compatibility. *Electron. J. Biotechnol.* **18**, 110–115 (2015).
54. Dueñas-Rivadeneira, A., Alcívar-Cedeño, U., Sacon-Vera, E., Casdelo-Gutiérrez, N. & Villanueva-Ramos, G. Escalado y dimensionamiento de una planta piloto para la producción de un extracto acuoso de Chuquiraga jussieui JF Gmel. *Tecnol. Química* **36**, 255–266 (2016).
55. Abd El-Salam, A. E. *et al.* Isolation, characterization, optimization, immobilization and batch fermentation of bioflocculant produced by Bacillus aryabhattai strain PSK1. *J. Genet. Eng. Biotechnol.* **15**, 335–344 (2017).
56. Liu, S. Chapter 14 - Cell cultivation. in (ed. Liu, S. B. T.-B. E. (Third E.) 593–658 (Elsevier, 2020). doi:https://doi.org/10.1016/B978-0-12-821012-3.00014-2.