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Alcoholic Fermentation From Sugarcane Molasses and Enzymatic Hydrolysates: Modeling and Sensitivity Analysis

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Ethanol competitivity can be enhanced when the total use of sugarcane portions is practicable, including bagasse and straw, through hydrolysis technology, in which the polysaccharides are processed to produce fermentable sugar and posteriorly ethanol. Among the problems of hydrolysate fermentation is the low sugar concentration in the medium (when hydrolysis is performed at low solids loading), which leads to low ethanol concentration, increasing the energy requirement in distillation. This can be solved through the concentration of hydrolysates with molasses. Kinetics of a mixture hydrolysates - molasses changes significantly from that of molasses, due to presence of other sugars and inhibitors. Thus, the kinetic models developed for molasses are not useful to predict fermentation data for hydrolysates. Considering this, in this work, a kinetic model for fermentation of a mixture of sugarcane bagasse hydrolysate and molasses was developed. For this purpose, data from batch fermentations at temperatures of 30, 32, 34, 36 e 38 °C were used. The model for hydrolysates was based on kinetic expressions previously developed for molasses fermentation, with addition of a term considering acetic acid inhibition on *Saccharomyces cerevisiae* growth. Also, to describe the data for fermentations with hydrolysate, a parameter re-estimation was necessary. Due to the large number of parameters in the model, a re-estimation methodology was proposed, in which the most sensitive parameters were adjusted and the less sensitive were kept fixed, making the re-estimation easier. A parametric sensitivity analysis through Plackett-Burman designs was performed, using the software Statistica, by varying the kinetic parameters and calculating their influence on the profiles of cell, substrate and ethanol concentrations. The model consisted of 13 parameters, of which 5 were considered as relevant on fermentation profiles (μ_{max}, P_{max}, Y_x, $Y_{p/x}$ and X_{max}) and chosen to be re-estimated. Through the use of this methodology, an accurate model for second generation bioethanol production was developed.

1. Introduction

One of the trends in the bioethanol sector is to use the whole portion of sugarcane (which includes sugarcane bagasse and straw) to produce ethanol through the hydrolysis of cellulosic material followed by hydrolysate fermentation. A common problem found in the hydrolysis step is that when low solid loadings are used, a diluted hydrolysate is obtained, leading to low ethanol concentration after the fermentation step, and consequently high energy costs in distillation (Hoyer, et al., 2010). On the contrary, when hydrolysis is performed at high solids loadings, low yield of fermentable sugars is obtained. A solution for this problem is to perform hydrolysis at low solids loadings adding sugarcane molasses to the hydrolysates to reach the sugar level required for fermentation. Performing fermentations at higher sugar concentration requires more compact bioreactors and reduces vinasse generation, and consequently the production cost.

The mixture of molasses and hydrolysates results in changes on fermentation kinetics (rates of cell growth, substrate concentration and ethanol formation) compared to the fermentation that uses pure molasses as raw material. This occurs due to the presence of inhibitors generated in hydrolysis of lignocellulosic

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material, such as weak acids (mainly acetic acid), furfural, hydroxymethylfurfural, and phenolic compounds (Palmqvist and Hahn-Hagerdal, 2000). These compounds, found in the hydrolysate broth, highly affect the performance and behavior of *Saccharomyces cerevisiae* strains in industrial media.
Considering the differences in fermentation kinetics when hydrolysate is added to molasses, the mathematical models developed and fitted with fermentation data for first generation ethanol (molasses or sugarcane juice) are not adequate to represent the second generation fermentation. Thus, a strategy to obtain an accurate model to represent fermentation of second generation ethanol is to perform a parameter estimation using data from fermentation of a mixture of hydrolysates and molasses. The updated model is important to be used in optimizations, simulations, studies of dynamic behavior and design of controllers for second generation processes (Morales-Rodriguez et al., 2011).
In this work, a kinetic model for fermentation of a mixture of sugarcane bagasse hydrolysate and molasses was developed. Data from fermentations, obtained from Andrade et al. (2013) at 30 - 38 °C were used. The model for hydrolysates was based on kinetic expressions previously developed for molasses fermentation, with addition of a term considering acetic acid inhibition on *Saccharomyces cerevisiae* growth. Also, to describe the data for fermentations with hydrolysate, a parameter re-estimation was also necessary. The re-estimation is considered a complex task due to the large number of kinetic parameters. Thus, in this work, a re-estimation methodology was proposed, in which the most sensitive parameters were adjusted and the less sensitive were kept fixed. A parametric sensitivity analysis (an extensively applied technique (Baraldi et al. (2012); Feng et al. (2013)) through Plackett-Burman designs (Plackett and Burman, 1946) was performed, using the software Statistica, by varying the kinetic parameters and calculating their influence on the profiles of cell, substrate and ethanol concentrations.

2. Methods

2.1 Pretreatment of sugarcane bagasse with alkaline hydrogen peroxide

Sugarcane bagasse obtained from an industrial plant (Usina da Pedra, Serrana-Brazil) and dried in room temperature was pre-treated with a solution of alkaline hydrogen peroxide (7.355 % v/v, pH adjusted to 11.5 with NaOH) at 25 °C for 1 h, in an orbital shaker with agitation of 150 rpm. The pre-treated bagasse was extensively washed with water and dried at room temperature. In this step, the solid concentration used was 4 % WIS (water insoluble solid).

2.2 Hydrolysis

Enzymatic hydrolysis of dry pre-treated bagasse was performed at 50.0 °C, for 72 h, stirring of 100 rpm. The media was a sodium citrate buffer solution at 0.05 mol/L, pH 4.8. The enzymes used were celullase from *Trichoderma reesei* and β-glucosidase 188 from *Aspergillus niger* (both from Sigma-Aldrich). The enzyme loadings were 3.5 FPU celullase/g WIS; 25.0 CBU of β-glucosidase/g WIS (Rabelo *et al.* (2011)). The solid concentration was fixed at 3 % WIS. After 72 h, the broth was centrifuged at 8000 rpm, filtered in a membrane of 0.2 μm, and cooled to be posteriorly used in fermentations. The final hydrolysate composition was glucose (23.872 kg/m^3), xylose (2.659 kg/m^3), cellobiose (1.182 kg/m^3), arabinose (0.568 kg/m^3), furfural (0.026 kg/m^3), hydroxymethylfurfural (0.040 kg/m^3), and acetic acid (0.847 kg/m^3).

2.3 Fermentation

The microorganism used in fermentations was a *Saccharomyces cerevisiae* strain obtained from Usina Santa Adélia (Jaboticabal, SP, Brazil), preserved in the Bioprocess Engineering Laboratory, School of Food Engineering, Unicamp. This lineage is capable of metabolizing sucrose, glucose and fructose portions of the media. Details about the microorganism preservation and inoculum preparation are described in Andrade et al. (2013).
The production media used in fermentation was composed by 66.67 % (*v/v*) hydrolysate, 22.22 % of molasses solution and 11.11 % of inoculum. The fermentor was a Bioflo III (New Brunswick Scientific Co., Inc., Edison, NJ) stirred at 300 rpm by 2 flat blade turbines with 6 blades each. The temperatures used in the assays were fixed at 30, 32, 34, 36 and 38 °C. Previously to the fermentation, molasses was sterilized in an autoclave (121°C for 15 min) and the hydrolysate was subjected to cold sterilization using a 0.2 μm capsule filter (Minikap model, Spectrum Laboratories, Inc., FL, USA) to avoid evaporation of acetic acid, furfural and hydroxymethylfurfural.
The initial nominal composition of the medium was approximately: substrate (sum of sucrose, glucose and fructose - 150 (kg/m^3)); cells (2.8 kg/m^3) and acetic acid (0.7 kg/m^3).
Analysis of sugars, ethanol, and acetic acid of the samples was performed in a high performance liquid chromatography (HPLC). Cell concentration was gravimetrically determined according to Andrade et al. (2013).

2.4 Mathematic model for alcoholic fermentation in a media with acetic acid

The mathematical model used to describe the fermentation was composed of a group of differential equations for microorganism growth, substrate consumption and ethanol production. Considering the mass balance in bath mode reactor, the resulting equations which relates cells (X), substrate (S), and ethanol (P) with rates of cellular growth (r_x), substrate uptake (r_S), ethanol production (r_P) and time (t) are given by Eqs (1)–(3), respectively:

$$\frac{dX}{dt} = r_X \tag{1}$$

$$\frac{dS}{dt} = -r_S \tag{2}$$

$$\frac{dP}{dt} = r_P \tag{3}$$

The rates of cell growth, r_x (kg/m^3.h), substrate uptake, r_S (kg/m^3.h) and product formation, r_P (kg/m^3.h) were expressed by a non-structured model, used by Andrade et al. (2013). The rate expression (Eq (4)), used by Andrade et al. (2013) considers the substrate *(S)* as limiting yeast growth; substrate, ethanol *(*P), cells (X) and acetic acid (C_{Ac}) concentrations as inhibitors:

$$r_x = \mu_{max} \frac{S}{K_s + S} \exp(-K_i S)\left(1 - \frac{X}{X_{max}}\right)^m \left(1 - \frac{P}{P_{max}}\right)^n \left(1 - \frac{C_{Ac}}{C_{Acmax}}\right)^{nn} X \tag{4}$$

The term of hyperbolic inhibition by acetic acid in Eq (4) was considered due to the presence of this component in sugarcane bagasse hydrolysates, which impacts the yeast growth. Furfural and hydroxymethylfurfural inhibitions were not considered in the model due to their low concentration in the hydrolysates (0.026 and 0.040 g/L, respectively).

In Eq (4), μ is the specific cell growth rate; $\mu_{máx}$ is the maximum specific growth rate; S is the substrate concentration (g/L); K_S is the constant of saturation by substrate (g/L); K_I is the constant of inhibition by substrate (g/L); P is the ethanol concentration (g/L), C_{Ac} the acetic acid concentration (g/L); $P_{máx}$ is the ethanol concentration were cell growth ceases (g/L), C_{Acmax}, the acetic acid concentration were cell growth ceases (g/L), n a parameter of ethanol inhibition, and nn, a parameter of acetic acid inhibition. For hydrolysates fermentation, it was found that Eq (4), which considers hyperbolic inhibition by acetic acid, represents satisfactorily the experimental data.

The expression (Eq (5)) describes the ethanol formation rate in relation to cell concentration (X) and rate of microbial growth (r_x), proposed by Luedeking-Piret (1959):

$$r_p = Y_{px} r_x + m_p X \tag{5}$$

The substrate consumption rate is represented by Eq (6):

$$r_s = (r_x / Y_x) + m_x X \tag{6}$$

In Eq (6), Y_X and m_X are the limit cellular yield (*g/g*) and the parameter of cell maintenance (g/g.h), respectively.

3. Results and discussion

3.1 Parameter estimation of fermentation considering a mixture of hydrolysates and molasses

The model for alcoholic fermentation in batch mode considering acetic acid presence in the media (Eqs (1)-(6)), is formed by 13 adjustable parameters. The parameters μ_{max}, X_{max}, P_{max}, Y_{Px}, Y_x vary with temperature, and the remaining ones are fixed at K_s= 4.1 g/L; K_I= 0.004 L/g; m_p = 0.1 g/(g.h); m_x= 0.2 g/(g.h); m=1.0; n=1.5; CAc_{max}=4.0 g/L, and nn=0.3, according to Andrade et al. (2013).

Experimental data obtained from 5 batch fermentations – found in (Andrade et al., 2013) for a mixture of hydrolysates and sugarcane molasses were used for parameter estimation. These data do not consider cell recycle and were obtained for temperatures of 30, 32, 34, 36 and 38 °C. A routine based on quasi-

Newton algorithm was developed in Fortran 90 to find the parameter values which minimizes the difference between experimental data and the model results, given by an objective function (Eq (7)).

$$E(\theta)=\sum_{n=1}^{np}\left[\frac{(X_n-Xe_n)^2}{Xe_{\max}^2}+\frac{(S_n-Se_n)^2}{Se_{\max}^2}+\frac{(P_n-Pe_n)^2}{Pe_{\max}^2}\right]=\sum_{n=1}^{np}\varepsilon_n(\theta) \quad (7)$$

In Eq (7), θ is the vector of kinetic parameters; X_{en}, S_{en} and P_{en} are experimental data of cell, substrate and product concentrations at sampling time n. X_n, S_n and P_n are the concentrations computed by the model in each sampling time, and X_{emax}, S_{emax} and P_{emax} the maximum measured concentrations, n_p the number of samples. $e_n(\theta)$ is the minimized error. Eq (7) is subjected to the boundaries *lp* and *up* for each parameter. The obtained parameters are shown in Table 1.

Table 1. Estimated kinetic parameters for fermentations using a mixture of molasses and enzymatic hydrolysates as function of temperature

Parameters	*Temperature (°C)				
	30.0	32.0	34.0	36.0	38.0
μ_{max} (h^{-1})	0.150	0.18	0.19	0.179	0.145
X_{max} (Kg/m^3)	71.900	55.000	43.000	39.800	39.800
P_{max} (Kg/m^3)	96.000	81.340	75.000	72.400	72.020
Y_{px} (Kg/Kg)	12.000	13.300	14.330	15.200	16.000
Y_x (Kg/Kg)	0.039	0.038	0.034	0.031	0.025

Parameters C_{Acmax} and *nn* were estimated in 4.0 g/L and 0.3; respectively for all temperatures. The residual standard deviations between experimental data and the simulation of the model was 19.7 % at maximum for variable *P* at 32 °C. Most of values were next to 10 % (results not shown).

3.2 Sensitivity analysis of model parameters

Sensitivity analysis is a technique that when applied to mathematical models can indicate the relevance of parameters. This evaluation is useful because it assists the parameter re-estimation when necessary. The re-estimation procedure is required when changes in raw- material, or dominant microorganism lineage occur in fermentative processes. In this work, the mathematical model for fermentation of hydrolysate broth is constituted by 13 kinetic parameters that present interaction among them. Plackett-Burman methodology allows the sensitivity analysis varying all the parameters simultaneously, avoiding information losses, which makes this technique efficient.

The variables used in sensitivity analysis were substrate consumption (*dS*), product concentration (*P*) and cells (*X*) in each fermentation time (2, 5, 8, 10, 15, 20, 25, 30, 35 and 40 h). For simulations, values of parameters at a temperature of 34 °C were used (Table 1). These values were considered level (0). High (+) and low (-) levels were obtained by adding and deducting 10 % of the level (0). All the resulting levels for each parameter are shown in Table 2.

Table 2 – high (+) and low (-) level of kinetic parameters to build Plackett-Burman matrix.

Param.	$\mu_{máx}$	$X_{máx}$	$P_{máx}$	Y_x	$Y_{p/x}$	K_s	K_i	m_x	m	n	m_p	nn	Ac_{max}
Level (+)	0.209	47.3	82.5	0.0374	15.763	4.51	0.0044	0.22	1.1	1.65	0.11	0.33	4.4
Level(-1)	0.171	38.7	67.5	0.0306	12.897	3.69	0.0036	0.18	0.9	1.35	0.09	0.27	3.6

Data from Table 2 were used to build Plackett-Burman matrix with 20 lines and columns. The first 13 columns of the matrix are used to allocate kinetic parameters, and the remaining are dummy variables (not shown).

A number of 20 simulations of the mathematical model were performed with kinetic parameters varying according to a Plackett-Burman matrix. The initial conditions for *X*, *S* and *P* were fixed at 2.69; 150.628 and 2.436 *g/L*, and were obtained from an experiment at 34ºC, found in Andrade et al. (2013). The results of simulations of each variable (*dS*, *P* and *X*) for each time were used to calculate the effects of each parameter on the responses *dS*, *P* and *X*. The effects were calculated using the Software Statistica. The results for variables *X*, *dS* and *P* are shown in Figures 1, 2 and 3, respectively.

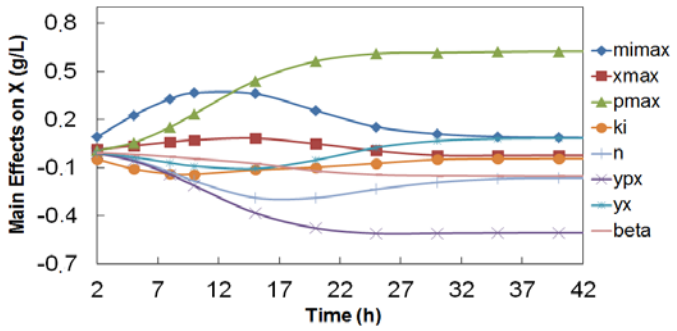

Figure 1: Effect of kinetic parameters on cell concentration, X

Figure 1 shows that the influence of the relevant kinetic parameters on cell concentration varies with fermentation time for batch cultivation. The most relevant parameters (from 0 to 40 h), for variable *X*, were $Y_{p/x}$ and P_{max}. It is important to mention that although the minor relevance of $\mu_{máx}$ in the final fermentation time, this parameter showed to be the most relevant for the first 12 h. the remaining parameters such as X_{max}, K_i, Y_x e m_p showed to have minor influence on *X* profile for all the times, except *n*.

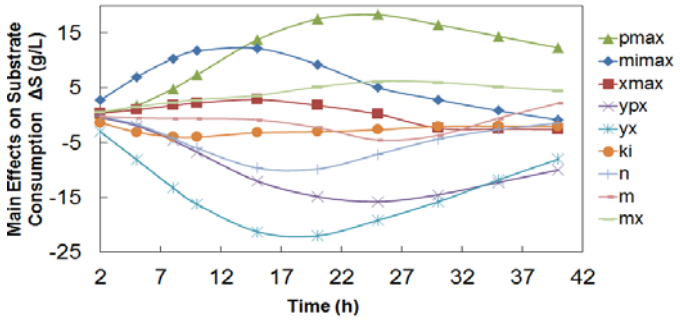

Figure 2: Effect of kinetic parameters on substrate consumption, ΔS

The impact of parameters on substrate consumption was represented by *S* variation for each time. The most relevant parameters from 0 to 30 h were Y_x, followed by P_{max} and $Y_{p/x}$. Similarly to Figure 1, in Figure 2, μ_{max} presents low relevance for final fermentation times (40 h), but for first 12 h it was one of the most important, as well as the parameter *n*.

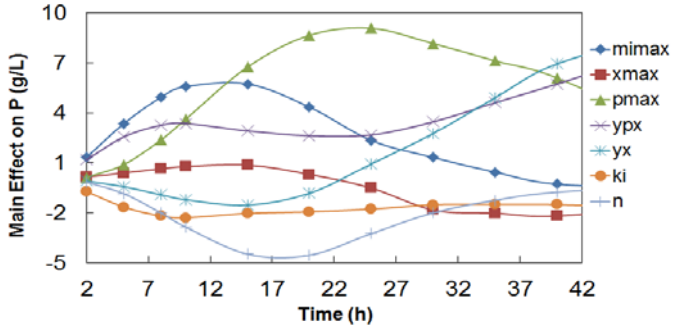

Figure 3: Effect of kinetic parameters on ethanol concentration, P

For ethanol concentration, P_{max} was the most relevant parameter (22 h), followed by Y_x and $Y_{p/x}$, in 40 h. The parameter μ_{max} presents higher relevance up to 12 h, according to Figure 3.
Thus, for *X*, *S* and *P* profiles, the most relevant parameters for all fermentation times were Y_x, $Y_{p/x}$, P_{max}, μ_{max} and *n*. Although the high influence of *n*, this parameter can be maintained fixed in re-estimation procedures because predicting new values of *n* is difficult (graphic methods related to its prediction are not accurate).

4. Conclusions

The results from sensitivity analysis suggest that in case of changes in proportions of hydrolysates - molasses, the most relevant parameters (Y_x, $Y_{p/x}$, P_{max}, μ_{max} and n) must be re-estimated. The remaining parameters considered as non-relevant can be fixed without significant losses in model accuracy. The reduction of number of parameters to be re-estimated makes this procedure easier.

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