CI&CEQ
Chemical Industry & Chemical Engineering Quarterly

Available online at
Association of the Chemical Engineers of Serbia AChE
www.ache.org.rs/CICEQ

Chem. Ind. Chem. Eng. Q. 28 (3) 191–201 (2022) CI&CEQ

BRUNA PRATTO [1]
MARTHA SUZANA RODRIGUES DOS SANTOS-ROCHA [2]
GUSTAVO BATISTA [1]
INTI DORACI CAVALCANTI-MONTAÑO [3]
CARLOS ALBERTO GALEANO SUAREZ [3]
ANTONIO JOSÉ GONÇALVES CRUZ [1]
RUY DE SOUSA JÚNIOR [1]

[1] Chemical Engineering, Federal University of São Carlos, São Carlos, SP, Brazil
[2] Federal Institute of Alagoas, Penedo, AL, Brazil
[3] Chemistry Institute, Federal University of Goias, Goiânia, GO, Brazil

SCIENTIFIC PAPER

UDC 66.094.941:60:66

RATIONAL FEEDING STRATEGIES OF SUBSTRATE AND ENZYMES TO ENZYMATIC HYDROLYSIS BIOREACTORS

Article Highlights

- Rational feeding strategies of substrate and enzyme using pretreated sugarcane straw
- Enzyme pulses keeping at least 70% of the initial reaction rate improved process economy
- Unproductivity adsorption of enzymes was the main reason for hydrolysis yield reduction
- Soybean protein boosted the glucose production to 190 g/L and the yield to 94%
- Substrate feeding profiles are useful in the application of automatic solid feeders

Abstract

Bioreactors operating in fed-batch mode improve the enzymatic hydrolysis productivity at high biomass loadings. The present work aimed to apply rational feeding strategies of substrates (pretreated sugarcane straw) and enzymes (CellicCtec2®) to achieve sugar titers at industrial levels. The instantaneous substrate concentration was kept constant at 5% (w/v) along the fed-batch. The enzyme dosage inside the bioreactor was adjusted so that the reaction rate was not less than a pre-defined value (a percentage of the initial reaction rate - r_{min}). When r reached values below r_{min}, enzyme pulses were applied to return the reaction rate to its initial value (r_0). The optimized feeding policy indicated a reaction rate maintained at a minimum of 70% of r_0, based on the trade-off between glucose productivity and enzyme saving. Initially, it was possible to process a 21% (w/v) solid load, achieving 160 g/L of glucose concentration and 80% of glucose yield. It was verified that non-productive enzyme adsorption was the main reason for some reduction of hydrolysis yield regarding the theoretical cellulose-to-glucose conversion. An increment of 30 g/L in the final glucose concentration was achieved when a lignin-blocking additive (soybean protein) was used in the enzymatic hydrolysis.

Keywords: enzymatic hydrolysis, fed-batch operation, rational feeding strategies, sugarcane straw, unproductive lignin-enzyme bonds.

Although the production of lignocellulosic-based ethanol has reached considerable technical maturity, it still faces bottlenecks regarding its feasibility, primarily due to the high enzyme costs and the low sugar levels obtained in the hydrolysis bioreactor [1,2]. In addition, the ethanol titer in the conventional sugarcane mill reaches approximately 80 g/L in the fermentation stage [3,4], which is hardly obtained in the isolated second-generation ethanol process. Thus, an efficient enzymatic hydrolysis process is imperative for implementing lignocellulosic biorefineries [5].

One way to obtain elevated sugar concentrations is to process high biomass loadings (> 15% w/v dry mass for most pretreated materials) in the bioreactor [6]. However, in this approach, soluble sugars and phenolic compounds (generated in the biomass pretreatment process) rapidly accumulate in the reaction medium, causing enzyme inhibition in the early stages of hydrolysis [7]. Besides, high solid contents lead to high

Correspondence: R. de Sousa Júnior, Chemical Engineering Department, Federal University of São Carlos, Rod. Washington Luís-Km 235, CEP: 13565-905, São Carlos, SP, Brazil.
E-mail: ruy@ufscar.br
Paper received: 2 December, 2020
Paper revised: 30 June, 2021
Paper accepted: 25 August, 2021

https://doi.org/10.2298/CICEQ201202030P

apparent viscosity of slurries, resulting in heat and mass transfer limitations and increased power consumption associated with the medium agitation [8-10]. These issues represent negatives impacts on hydrolysis yield, and the use of fed-batch operation offers an alternative approach to circumvent these problems.

Fed-batch hydrolysis has the advantage of processing a final high solid load at a controlled rate, maintaining low solids level inside the bioreactor at a time throughout the operation process. Thus, it is possible to get high sugar levels without compromising the hydrolysis yield and demanding less from the mixing system [8,10–12].

In addition to the benefits of substrate feeding, enzyme feed splitting can enhance enzymatic reaction performance by sustaining a high reaction rate throughout the process. On the other hand, one-batch enzyme addition subjects the enzymes to activity loss from the onset of the reaction [2].

Sugiharto *et al.* [13] evaluated different enzyme feeding strategies in the hydrolysis of empty fruit bunch. It was observed that proportional enzyme feeding (to substrate addition) increased the enzymatic digestibility and the glucose concentration, respectively, up to 26% and 12%, compared to the whole enzyme added at the beginning of the hydrolysis process. Corrêa *et al.* [8], employing steam-exploded sugarcane bagasse as substrate, also observed that enzyme addition along the hydrolysis time increased the glucose concentration by 10% and the energy efficiency (mass of glucose produced per energy consumed) by 25%, compared to all enzyme content added at the reaction onset.

Although the fed-batch mode presents better performance than simple batch, it is essential to define feeding profiles based on rational criteria. For instance, Modenbach and Nokes [6] raised some crucial questions regarding the proper fed-batch operation: at what point in the reaction time should subsequent substrate additions be applied to maintain a high conversion rate? Should enzymes be added in a single application, supplement the original application, or proportionally to the substrate?

Typically, the feeding strategies of substrate and enzyme are arbitrarily performed without considering reaction kinetics and parameters that affect it [11,12,14–16]. On the other hand, using rational feeding strategies of substrates and enzymes can improve product productivity, yield, and concentration.

Unrean *et al.* [17] developed a model of simultaneous saccharification and fermentation (SSF) process considering the metabolic network of *S. cerevisiae* and kinetics of both enzymatic hydrolysis and fermentation to define optimal feeding profiles of sugarcane bagasse and cells on ethanol production. Through the feeding profiles, ethanol production with a titer up to 65 g/L and a high yield of 85% of theoretical yield (related to the total sugar available in pretreated bagasse) were accomplished. Besides, the ethanol titer and productivity were increased by 47% and 41%, respectively, compared to the batch process.

Cavalcanti-Montaño *et al.* [18] simulated and validated substrate and enzyme feeding strategies in the hydrolysis of alkali-pretreated sugarcane bagasse. The first policy adopted a substrate feeding profile and enzyme pulses based on the reaction kinetics. The second one defined only a substrate feeding profile, using the classic theory of optimal control [19]. The implemented strategies provided a final glucose concentration of up to 160 g/L by processing 23% w/v of solids with 8.2 FPU/g cellulose (*Accellerase® 1500*).

In this light, the objective of this work was to study the enzymatic hydrolysis of sugarcane straw in fed-batch mode, using rational substrate and enzyme feeding strategies to improve the process efficiency by increasing the final product concentration or decreasing the enzyme consumption in the hydrolysis process. Moreover, the definition of optimal substrate feeding profiles for different lignocellulosic materials is beneficial for applying automatic biomass feeders since they need pre-defined profiles to track (open loop process operation approach).

MATERIALS AND METHODS

Simulations of feeding profiles of substrate and enzyme to the enzymatic reactor

Mathematical modeling of fed-batch enzymatic reactor

The following hypotheses and simplifications were considered for the modeling of the fed-batch bioreactor:

a) Bioreactor operates in semi-continuous mode: initially, the bioreactor is loaded with a certain amount of biomass, and substrate additions are performed until the fed-batch process is completed. At the end of the feeding phase, the bioreactor starts operating in batch mode until the hydrolysis reaction ceases;

b) Pseudo-homogeneous reaction system: the reaction is heterogeneous - insoluble substrate; soluble enzyme -however, as the insoluble substrate load is low ~5% w/v, it can be assumed a homogeneous reaction medium. This concentration was chosen because it is below the threshold level (< 10% w/v) to prevent a too high viscosity and severe mixing problem in the medium [17].

c) Substrates are uniformly accessible;

d) A single catalyst represents enzyme complex;

e) Enzyme is subjected to competitive inhibition by glucose;

f) Hemicellulose and lignin contents in the treated sugarcane straw are low and, therefore, disregarded since most were solubilized after hydrothermal + alkaline pretreatment (see Table 1).

A pseudo-homogeneous Michaelis-Menten (MM) kinetic model with inhibition by the product was considered. The kinetic parameters (K_m = 9.65 ± 1.51 g/L, K_i = 0.8 ± 0.08 g/L, and V_m = 0.214 ± 0.003 g/(L·min)) were obtained from previous work from hydrolyzed sugarcane straw assays in agitated flasks [20]. In the present study, the mathematical model will be used for experiments in bench-scale bioreactors and, therefore, a fine-tuning of the parameters was performed. After a manual fitting, the found values were: K_m = 9.65 g/L, K_i = 1.85 g/L, and V_m = 0.208 g/(L·min). Figure A.1 (Supplementary material) shows the very good fitting of the model to the experimental data in a way that the approach is validated.

Eqs. (1) to (4) describe the enzymatic reaction in fed-batch mode. Eq. (1) represents the mass balance for the substrate, Eq. (2) the mass balance for the product, Eq. (3) the total mass balance, and Eq. (4) the reaction rate of the enzymatic hydrolysis:

$$\frac{dC_s}{dt} = \frac{F_{S,fed} \cdot C_{S,fed}}{V} - r - \frac{F_{S,fed} \cdot C_S}{V} \quad (1)$$

$$\frac{dC_G}{dt} = r - \frac{F_{S,fed} \cdot C_G}{V} \quad (2)$$

$$\frac{dV}{dt} = F_{S,fed} \quad (3)$$

$$r = \frac{k \cdot C_E \cdot C_S}{K_m \cdot \left(1 + \frac{C_G}{K_i}\right) + C_S} \quad (4)$$

where $F_{s,fed}$ (L/min) indicates the fed substrate flow, $C_{s,fed}$ (g/L) is the concentration of substrate fed in terms of potential glucose, C_s (g/L) is the substrate concentration in the bioreactor in terms of potential glucose; C_G (g/L) is the concentration of glucose in the bioreactor, V (L) is the reaction volume at a given time, r (g/(L·min)) is the reaction rate, C_E (g/L) is the enzyme concentration required in the reaction medium, K_m (g/L) is the Michaelis-Menten constant, K_i (g/L) is the competitive inhibition constant by glucose, and k (1/min) is the specific reaction rate.

Definition of substrate and enzyme feeding profiles

The substrate and enzyme feeding strategies are described in Eqs. (5) to (7):

$$e_{fed} = \frac{C_E \cdot V \cdot 230}{1.19} - e_{accumulated} \quad (5)$$

$$C_E = \frac{r_0 \cdot \left[K_m \cdot \left(1 + \frac{C_G}{K_i}\right)\right] + C_{S,0}}{k \cdot C_{S,0}} \quad (6)$$

$$F_{S,fed} = \frac{r \cdot V}{C_{S,fed} - C_{S,0}} \quad (7)$$

where r_0 (g/(L.min)) corresponds to the initial reaction rate and e_{fed} (FPU) refers to the amount of the enzyme to be added to the medium to maintain the reaction rate (r) in a value not less than a pre-defined percentage of r_0 (r_{min}). When r reaches values below r_{min}, pulses of the enzyme are applied to return the reaction rate to its initial value (r_0). Enzyme pulses can enhance the performance of enzymatic reaction by sustaining high reaction rates throughout the process. Therefore, different percentages (from 50% to 100%) of r_0 were minimally kept (r_{min}) in the bioreactor and the fed-batch phase. The choice of r_{min} was based on the trade-off between using lower amounts of enzyme and not significantly impacting the decrease in the glucose productivity to the reference ideal condition (100% of r_0 - the enzyme fed continuously into the bioreactor). The $e_{accumulated}$ (FPU) is the enzyme concentration already fed at a given reaction time. $C_{s,0}$ refers to the substrate concentration at the start of the enzymatic hydrolysis. C_E must be multiplied by the reactor volume (V) and the enzymatic activity (230 FPU/mL) and divided by the enzyme density (1.19 g/mL) to obtain e_{fed} in FPU units.

Eq. (7) shows the substrate feeding flow rate, $F_{s,fed}$ (g/L). It is calculated according to the substrate consumption rate, r (keeping dC_s/dt=0, i.e., the substrate is replaced as consumed). Processing a high biomass load is the way to obtain elevated sugar concentrations.

For computer simulations, Eqs. (1) to (7) were implemented in the software Matlab® R2017a.

Experimental validation

Substrate and enzyme

Sugarcane straw used in this work was provided by Ipiranga Agroindustrial S.A. mill (Descalvado, SP, Brazil). The biomass was dried at room temperature until 10% moisture content. Afterward, it was milled in a

Wiley-type mill (SP-30, Splabor, Presidente Prudente, SP, Brazil) to a particle size of 10 mesh (2 mm). A cellulase complex, *CellicCTec2®*, donated by Novozymes Latin America (Araucária, PR, Brazil), with 230 FPU/mL [21], was employed in the hydrolysis experiments.

Alkaline treatment of hydrothermally pretreated sugarcane straw

Samples of sugarcane straw pretreated with liquid hot water (195 °C/10 min), described elsewhere [22,23], were alkaline treated using a proportion of 1:20 of dry pretreated sugarcane straw per NaOH solution (4% w/v). The suspension was added to 2-L Erlenmeyer flasks and autoclaved for 30 minutes at 121 °C and 1 atm. After the operation, the remaining solid fraction was washed with tap water (room temperature) until neutral pH was reached. Compositional analysis of sugarcane straw before and after alkaline treatment was carried out to determine the cellulose, hemicellulose, and lignin content [24].

Enzymatic hydrolysis of sugarcane straw: application of substrate and enzyme feeding strategies

The validation experiment regarding the substrate and enzyme feeding strategies was conducted in 400-mL bench-scale reactors (in triplicate) with a 50-mL fed-batch initial volume. The reactors were operated at 50 °C, 250 rpm, and pH 5.0 (50 mM citrate buffer). The biomass and the enzyme were added into the bioreactor during the fed-batch process according to the feeding profiles obtained by the simulations described in the "*Definition of substrate and enzyme feeding profiles*" section.

The reactor was initially loaded with a solids content of 5% $w_{dry\ biomass}$/v (corresponding to 47.8 g/L of potential glucose concentration) and 38.5 FPU of *CellicCTec2®* (corresponding to an initial concentration of 18 FPU/$g_{cellulose}$) to avoid a high initial viscosity of the reaction medium. The substrate was manually fed every 0.5 h to keep the insoluble solids concentration constant at 5% w/v, processing a total (final) of 73 g biomass (final). Seven pulses of the enzyme were added over time, with 1450 FPU used. At the end of the reaction, the accumulated amounts of substrate and enzymes were equivalent to 21% w/v biomass and 22 FPU/$g_{cellulose}$, respectively.

Glucose quantification

The hydrolysis was monitored in terms of glucose released along the reaction process. Glucose concentration was determined by HPLC (LC-10AD, Shimadzu, Tokyo, Japan), equipped with a refractive index detector (RID-10A, Shimadzu) and an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) operated at 65 °C using 5 mM H_2SO_4 as a mobile phase (0.6 mL/min). Glucose yield (%) and productivity (g/(L·min)) were determined according to Eqs. (8) and (9), respectively. MG refers to the total mass of glucose released, and MC is the total mass of cellulose added into the bioreactor; 0.9 is the stoichiometric factor of glucose to equivalent cellulose.

$$\text{Glucose yield}(\%) = 0.9 \cdot \frac{M_G}{M_C} \cdot 100 \tag{8}$$

$$\text{Productivity}\left(g/(L \cdot \text{min})\right) = \frac{C_G}{t} \tag{9}$$

Measurement of crystallinity index

The crystallinity index of the biomass at the end of the reaction was analyzed by a Shimadzu XRD-6000 X-ray diffractometer (XRD-6000, Shimadzu, Tokyo, Japan), with Cu-Kα radiation (λ = 1.54 Å) generated at 30 kV and 30 mA. Scans were obtained in the 2θ range of 5–40° at a scan rate of 2°.min^{-1}. The crystallinity index (*CrI*) was calculated from the ratio between the area of the crystalline peak (I_{002} - I_{AM}) and the total area (I_{002}) of the crystalline peak 002, after subtraction of the background signal measured without cellulose. OriginPro 8 software was used for diffractogram peak fitting, assuming Gaussian functions for each peak [25]. *CrI* values were calculated according to Eq. (10):

$$CrI(\%) = \left(1 - \frac{I_{AM}}{I_{002}}\right) \cdot 100 \tag{10}$$

where I_{002} represents the crystalline peak intensity of the 002 crystal plane at 2θ = 22.8°; and I_{AM} is the minimum area between the 002 and the 101 crystalline peaks corresponding to the amorphous phase at 2θ = 18°.

Verification test of unproductive enzyme-lignin bonds

Isolated soy protein (90% protein, Bremil, Brazil) was added to the hydrolysis reaction to verify whether there were any enzymes bounded unproductively. The assay was carried out under the conditions already described, except that soybean protein (8% w/w) was added at the beginning of the reaction. This additive was chosen in the present study because it is one of the lowest-cost blocking agents of unproductive enzyme bonds [26].

RESULTS AND DISCUSSION

Hydrothermally pretreated sugarcane straw was submitted to alkaline treatment to remove the major content of lignin. Table 1 shows the chemical compositions of sugarcane straw before and after alka-

line treatment with NaOH.

It is worth noting that the alkaline treatment preserves a major part of the cellulose while removing significant lignin amounts from the sugarcane straw hydrothermally pretreated. The treated biomass was then used to validate the most suitable feeding strategy simulations.

Table 1. Chemical composition of sugarcane straw before and after alkaline delignification and the percentage of removal of the components

Component (%)	Before alkaline delignification* (Hydrothermally pretreated sugarcane straw)	After alkaline delignification* (Hydrothermally pretreated sugarcane straw, treated with 4% w/v NaOH)	Removal after alkaline delignification*
Cellulose	54.7 ± 0.3	86.2 ± 0.3	7.65 ± 0.01
Hemicellulose	8.21 ± 0.05	2.89 ± 0.01	79.37 ± 0.01
Lignin	26.7 ± 0.9	7.5 ± 0.9	83.5 ± 0.1
Ash	6.7 ± 0.1	2.1 ± 0.5	81.6 ± 0.2
Total	96.3 ± 0.9	98.6 ± 1.1	-
Solid recovery[a]	-	58.6 ± 0.2	-

[a] Solid recovery = $(m_{final}/m_{initial})x100$, where m_{final} (g) is the amount of dry biomass after treatment and $m_{initial}$ (g) is the amount of dry biomass before treatment;
* Values reported are average ± standard deviation of three replications

Simulations for different feeding strategies of substrate and enzymes to the enzymatic hydrolysis bioreactor

Figure 1 shows the results of computational simulations when different percentages (from 50 to 100 %) of the initial reaction rate (r_0) were minimally kept in the bioreactor and the fed-batch phase.

The glucose concentration profiles are depicted in Figure 1a. At the end of enzymatic hydrolysis, the glucose concentration reached 202 g/L, representing an ideal cellulose-to-glucose conversion of 100%. The interruption of the substrate feeding can be seen through the discontinuity in the glucose formation curves (at approximately 150 g/L of glucose for the different simulation cases), that is, by a rapid increase in the glucose concentration due to the instantaneous reduction of the dilution effect associated with biomass addition. Figure 1b illustrates the volume inside of the bioreactor for different feeding strategies assessed.

Figure 1c shows the reaction rate over time. The sudden increases in the reaction rates refer to the enzyme pulses given to return the reaction rate to its maximum (initial) value (r_0). According to Figure 1d, continuous enzyme feeding is required during the fed-batch phase to keep the reaction rate at the maximum value (100% of r_0). For the other conditions, the enzyme addition was only carried out when the reaction rate dropped to less than a pre-defined percentage of r_0 (r_{min}), resulting in pulse additions of the enzyme to return the reaction rate to its initial value (r_0). The ideal portion of r_0 to be minimally kept in the bioreactor was based on a compromise solution by using the enzyme dosage as lower as possible without significantly reducing the glucose productivity.

Table 2 shows the values of glucose productivity and accumulated enzyme into the bioreactor, both respective to each minimum percentage of r_0 that should be maintained during the fed-batch phase. First, the glucose productivity was determined considering 90% of the theoretical yield. Then, the reduction in productivity and enzyme consumption was calculated concerning the reference condition (100% of r_0 - enzyme fed continuously in the bioreactor).

By sustaining the reaction rate above 80% of r_0 (condition 3), the enzyme consumption and the glucose productivity were reduced by 24% and 9.7%, respectively. On the contrary, for condition 4, where the reaction rate is kept above 70% of r_0, it could be experienced 65% of enzyme savings with only 15.3% of productivity reduction. Besides, the decrease in the amount of enzyme added into the bioreactor is almost 3-fold higher than under condition 3.

In condition 5 (r kept above 60% of r_0), the amount of enzyme added was higher than in condition 4. As enzyme additions were getting less frequent, adding a larger amount of enzyme was necessary to return the reaction rate to its initial value. For condition 6 (r kept above 50% of r_0), there is almost 90% of enzyme savings.

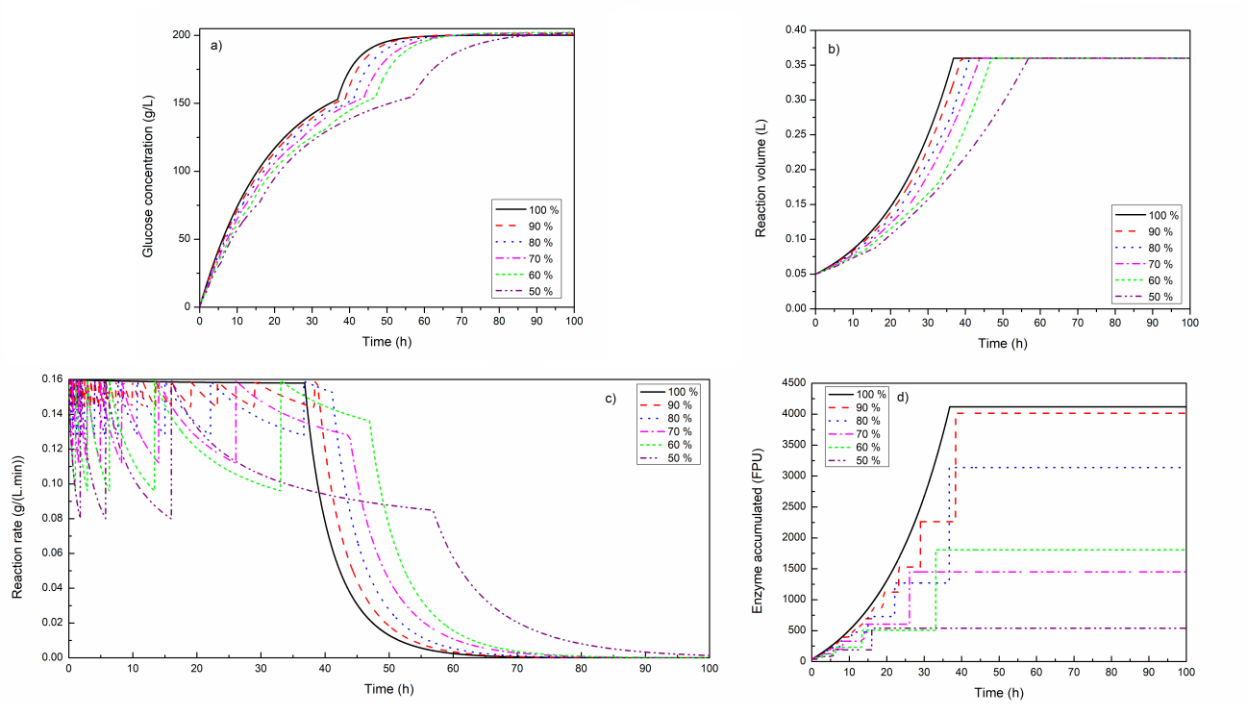

Figure 1. Simulations for different feeding strategies in the fed-batch bioreactor, keeping r at a minimum of pre-defined value (values from 50 to 100 % of r_0): (a) glucose concentration; (b) volume; (c) reaction rate; (d) enzyme accumulated in the bioreactor.

Table 2. Different feeding strategies on productivities and enzyme consumption to achieve 90% cellulose-to-glucose conversion

Condition	Minimal r (% r_0)	Productivity (g/(L min))	Decrease in productivity* (%)	$e_{accumulated}$ (FPU)	Decrease in $e_{accumulated}$* (%)
1	100	0.072	-	4118	-
2	90	0.069	4.2	4016	2.5
3	80	0.065	9.7	3137	23.8
4	70	0.061	15.3	1450	64.8
5	60	0.058	19.4	1807	43.9
6	50	0.046	36.1	540	86.9

[a] Decrease in relation to the reference condition (r maintained equal to r_0 throughout the fed-batch phase).

However, the productivity is reduced by 36%. The considerable drop in the use of enzyme was due to the reaction volume reaching the maximum capacity of the reactor before the next enzyme pulse (see Figures 1 (b) and (c)).

The values presented in Table 2 indicated that the strategy of maintaining the reaction rate at a minimum of 70% of r_0 is the most suitable since there was a critical enzyme saving without causing a significant drop in productivity to the reference ideal condition (100% of r_0 - enzyme fed continuously into the bioreactor). Figure 2 presents the profiles for the simulations considering 70% of r_0 to be minimally maintained over the fed-batch time.

The substrate concentration was kept constant until the maximum reactor capacity was reached (at 44 h, Figure 2a). After that, the substrate supply ceased, and the reactor started to operate in batch mode.

Figure 2b shows the enzyme additions and the enzyme accumulated along with the reaction. Seven pulses of enzymes were fed, totaling 1450 FPU of the enzyme accumulated inside the bioreactor. The enzyme addition was performed whenever the reaction rate reached 70% of r_0 (approx. 0.112 g/(L min)) (Figure 2c).

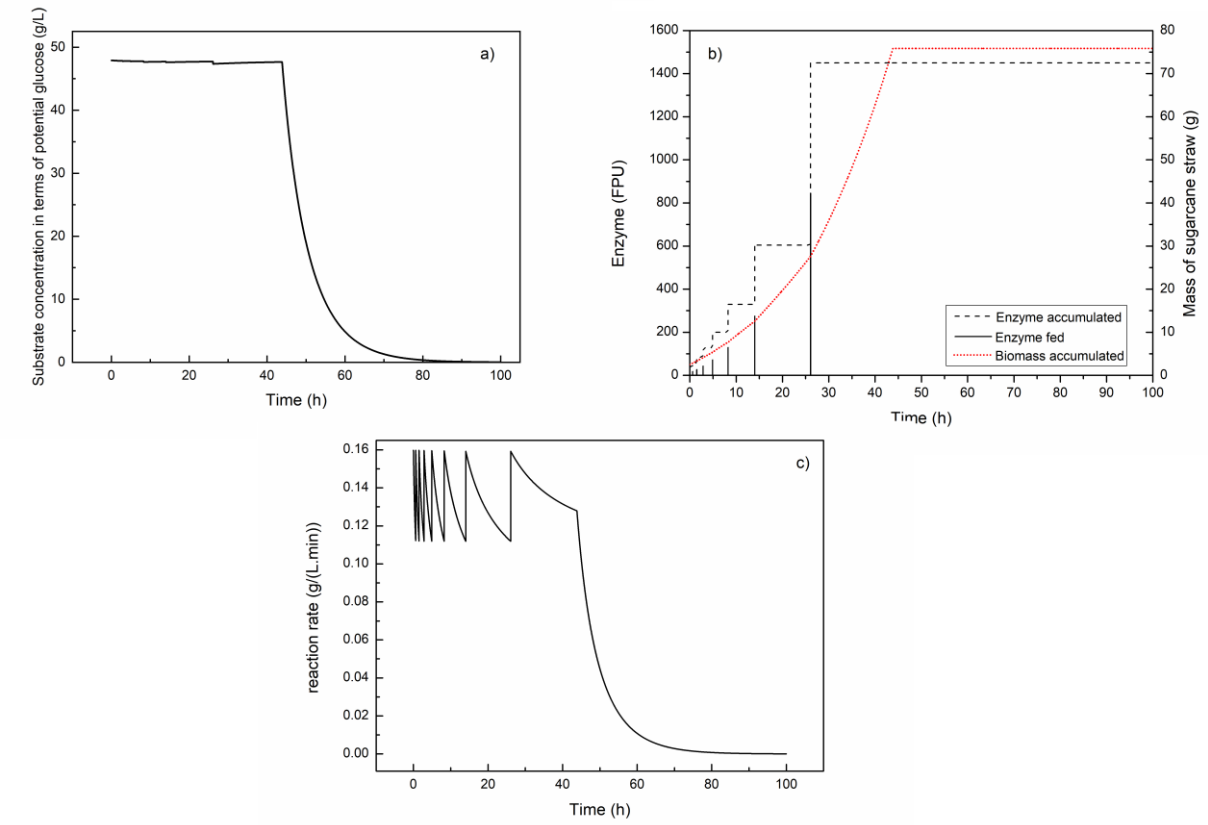

Figure 2. Simulations for fed-batch bioreactor with r maintained at a minimum of 70% of r_0: (a) substrate concentration; (b) enzyme fed, enzyme accumulated and biomass accumulated in the bioreactor; (c) reaction rate.

It can be observed in Figure 2b that the enzyme dosage was larger after each pulse. As glucose is being produced, the effects of enzyme inhibition become more significant [27,28]. Thus, higher enzyme amounts are needed for the reaction rate return to r_0. Figure 2b also shows the biomass accumulated during the reaction. It is observed that the mass of sugarcane straw remains constant after 44 h, indicating, once again, the end of the fed-batch phase.

Morales-Rodríguez *et al.* [29] optimized the fed-batch operation of a cellulose hydrolysis process. Three different feedback (PI) control strategies were developed and evaluated using the principles model of the hydrolysis process: the first one had insoluble solids as the control variable, handling the feed flow rate of the substrate; the second strategy additionally had the cellulose conversion as the control variable, adjusting the feed flowrate of enzyme for cellulose conversion; and the last one also considered the cellobiose conversion as the control variable, manipulating the enzyme feeding for cellobiose conversion. The third control strategy provided promising results regarding the hydrolysis performance with a substantial reduction in the amount of enzyme used in the process. Furthermore, as in the present study, Morales-Rodríguez *et al.* [29] also gave paramount importance to the economy in the enzyme consumption when applying a rational fed-batch strategy.

Hodge *et al.* [30] developed an optimization strategy for cellulose hydrolysis for solid percentages higher than 15%. In addition, a feeding substrate profile was developed (based on optimal control theory) to maintain the insoluble solids concentration at a manageable level. When this strategy was used, the 80% cellulose conversion and 140 g/L of glucose were achieved, close to the predicted values for the discussed strategies, i.e., the glucose concentration reaching 202 g/L, for the ideal cellulose-to-glucose conversion of 100%.

Experimental validation

Figure 3 shows the values of glucose concentration obtained along the experimental hydrolysis reaction time and in the simulation, for *r* maintained at a minimum of 70% of r_0.

From 144 h of reaction, no glucose release is observed. At this point, the hydrolysis reaction achieved 80% of yield and 160 g/L of glucose concentration. The titer obtained is adequate for large-scale processes since up to 81.8 g/L of ethanol could be generated in the fermentation stage. It is worth

mentioning that the increment of glucose concentration due to the hydrolysis reaction was not expressive after 84 h, indicating that the enzymatic hydrolysis could be ended at this time but still obtain a high glucose level (~ 140 g/L). Some further increase in concentration is mainly related to liquid fraction evaporation.

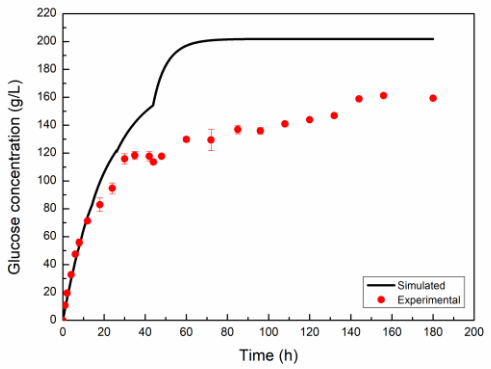

Figure 3. Experimental (•) and simulated (—) data of released glucose in the enzymatic hydrolysis of sugarcane straw for r maintained at a minimum of 70% of r_0 over reaction time.

The glucose yield obtained with the fed-batch enzymatic hydrolysis of sugarcane straw was higher than that of other authors for sugarcane bagasse. For example, Godoy *et al.* [12] attained 127 g/L of glucose concentration and 66.2% of glucose yield after 144 h of the fed-batch hydrolysis using the alkali-pretreated sugarcane bagasse (initial substrate content of 10% w/v followed by three additions of 5% (w/v) each). By processing the alkali-pretreated sugarcane bagasse in the fed-batch mode (initial load of 12% (w/v) and three more additions of 7% (w/v)), Gao *et al.* [15] obtained 129.50 g/L of glucose concentration with 60% of yield after 120 h of reaction.

The present results indicated that the rational substrate and enzyme feeding strategies enhanced the process efficiency compared to the arbitrarily feeding substrate (as adopted in the works mentioned above). Besides, in this work, lower solids content (5% w/v) was kept during the reaction process, implying a lower power requirement to agitate the system. Corrêa *et al.* [8] and Santos-Rocha *et al.* [10] verified that smoother feeding is more suitable for the formed glucose content and the required energy consumption.

The detachment of the experimental data to the simulation model (100% glucose yield) can be linked to the unpredicted phenomena by the kinetic model considered, such as an unproductive binding between enzymes and lignin, the remaining (crystalline) cellulose resistance to hydrolysis, and the internal diffusive effects. Regardless of the physical and chemical phenomena that caused the hydrolysis yield reduction, the reaction reached equilibrium at 80% of conversion. Decreasing yield at high solids concentration is generally called solids-effect [31].

Factors affecting enzymatic hydrolysis of lignocelluloses

The already mentioned phenomena were investigated to understand the factors associated with the incomplete cellulose-to-glucose conversion, such as:

1. Substrate depolymerization: cellulose recalcitrance is increased during the reaction. Fractions of the amorphous cellulose are depolymerized in the early stages of the reaction, leaving more crystalline parts in the final stages [32];

2. Deactivation of the enzymes: cellulases can become inactive due to unproductive adsorption onto lignin. Cellulases have a high affinity not only for cellulose but also for lignin. In the case of cellulose, they are usually released into the liquid fraction once the cellulose chains are hydrolyzed. In contrast, cellulases adsorbed onto lignin do not naturally desorb from it, a mechanism usually designated as non-productive binding [33].

One way to verify the effect of substrate depolymerization was to analyze the residual solid after the enzymatic hydrolysis reaction by XRD. Figure 4 shows the XRD diffractogram for the treated sugarcane straw after 156 h of fed-batch enzymatic hydrolysis. It shows the typical peaks of cellulose with a *CrI* of approximately 75%. This value is similar to the value found by de Aguiar *et al.* [34] for the alkali-pretreated sugarcane straw submitted to the enzymatic hydrolysis for 96 h.

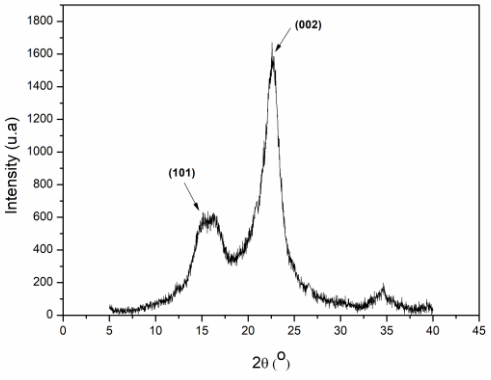

Figure 4. X-ray diffractograms of residual sugarcane straw after 156 h of fed-batch enzymatic hydrolysis of sugarcane straw maintaining *r* at a minimum of 70% of r_0.

Li *et al.* [35] verified a strong negative correlation

between reducing sugar yield and crystalline cellulose when enzyme loading was not a limiting factor (> 140 FPU/$g_{cellulose}$). However, at low enzyme loads (< 28 FPU/$g_{cellulose}$), there was no direct correlation between *CrI* and cellulose digestibility. In the present study, an enzyme loading of 22 FPU/$g_{cellulose}$ was used, which limits, according to the criteria of Li *et al.* [35], the evaluation of crystallinity effect on hydrolysis efficiency. It is consensus that the decreased *CrI* values could facilitate the cellulose-to-sugars conversion. However, some studies [36,37] show that crystallinity is not a dominant factor for reducing cellulose digestibility.

Kristensen *et al.* [31] evaluated the effect of high-solids loading on the enzymatic hydrolysis of filter paper. There was a strong correlation between decreasing the adsorption of cellulases and conversion, indicating that the inhibition of the adsorption of the enzyme onto substrate was the main cause of the hydrolysis yield reduction.

Lignin-blocking agents can be added to the reaction medium to block the exposed lignin surface and verify the presence of the enzyme-lignin bonds [38]. These agents, in general, also disintegrate the hemicellulose-lignin bond, making the cellulose more accessible to the enzymatic attack [39]. Several additives, such as non-ionic surfactants (Tween 20 and 80), polyethylene glycol (PEG), and non-enzymatic proteins (bovine serum albumin, soybean protein, peptone), have been evaluated to avoid the unproductive enzymes bonds [26,39]. Here, the isolated soybean protein (ISP) was chosen due to the justification mentioned in the "*Verification test of unproductive enzyme-lignin bonds*" section.

Figure 5 shows the effect of soybean protein supplementing on the glucose concentration released

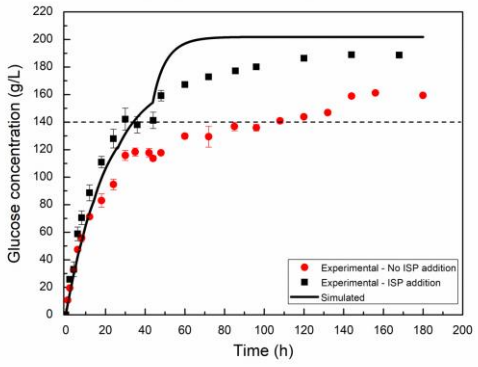

Figure 5. Effect of the soybean protein addition (•) on the enzymatic hydrolysis of sugarcane straw in the same conditions of enzymatic hydrolysis without ISP addition (●), compared to simulated data (—).

along the reaction course, compared with the assay with no addition of the blocking agent (data from Figure 3).

The ISP additive increased glucose concentration from approximately 160 to 190 g/L, resulting in a gain of around 19% in cellulose-to-glucose conversion. The dashed line reveals that, in 48 h of reaction, the glucose amount released by the ISP-added assay was virtually the same as that obtained in 140 h of hydrolysis in the assay with no ISP addition. This behavior supports that enzymes were inactive (due to non-productive bonds), resulting in a slower reaction rate. A similar improvement was found by Brondi *et al.* [26] using 8% w/v of soybean protein in the enzymatic hydrolysis of steam-exploded sugarcane bagasse: the glucose amount released was increased by 25% after 96 h of reaction. Despite the great improvement obtained with the use of the additive, some detachment between experimental and simulated data persists, mainly between 40 and 60 h. It is justified because, experimentally, the effect of reducing the dilution at the end of the substrate feed (i.e., at the end of the fed-batch phase) was not so expressive.

Based on the observations above-mentioned, the main reason for the decrease of hydrolysis yield is the enzyme deactivation caused by the unproductive bonds. It is worth highlighting that the objective of using soybean protein, at this moment, was only for investigation purposes (verification of the presence of lignin-enzyme bonds) rather than process improvement. However, an optimized additive concentration can be obtained to establish the economic feasibility of its use on an industrial scale.

CONCLUSION

Rational feeding strategies of substrate and enzymes were investigated to improve the enzymatic hydrolysis of pretreated sugarcane straw. With the feeding strategy adopted (reaction rate maintained at a minimum of 70% of r_0), it was possible to process a total of 21% w/v biomass load (always keeping approximately 5% w/v of insoluble solids in the reaction medium at a time), achieving 80% of glucose yield and 160 g/L of glucose concentration. The hydrolysis yield decrease was mainly associated with unproductive enzyme adsorption. On the other hand, the lignin-blocking agent boosted the glucose production to 190 g/L, making it an alternative process enhancer for biorefinery applications. Moreover, the feeding profiles obtained here are beneficial from bioreactors engineering, such as, for example, in the application of an automatic substrate feeder.

Acknowledgment

The authors would like to thank CNPq (National Council for Scientific and Technological Development, Brazil, Processes #312903/2018-9, and #140761/2017-9), and FAPESP (São Paulo State Research Funding Agency, Brazil, Process #2016/10636-8) for the financial support.

This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

REFERENCES

[1] H. Shokrkar, S. Ebrahimi, M. Zamani, Cellulose. 25 (2018) 6279-6304.

[2] A.S. da Silva, R.P. Espinheira, R.S.S. Teixeira, M.F. de Souza, V. Ferreira-Leitão, E.P.S. Bon, Biotechnol. Biofuels. 13 (2020) 58.

[3] K.C.S. Rodrigues, J.L.S. Sonego, A. Bernardo, M.P.A. Ribeiro, A.J.G. Cruz, A.C. Badino, Ind. Eng. Chem. Res. 57 (2018) 10823-10831.

[4] R.D. Pereira, A.C. Badino, A.J.G. Cruz, Energy & Fuels. 34 (2020) 4670-4677.

[5] B. Pratto, M.S.R. dos Santos-Rocha, A.A. Longati, R. de Sousa Júnior, A.J.G. Cruz, Bioresour. Technol. 297 (2020) 122494.

[6] A.A. Modenbach, S.E. Nokes, Biomass and Bioenergy. 56 (2013) 526-544.

[7] D.B. Hodge, M.N. Karim, D.J. Schell, J.D. McMillan, Bioresour. Technol. 99 (2008) 8940-8948.

[8] L.J. Corrêa, A.C. Badino, A.J.G. Cruz, Bioprocess Biosyst. Eng. 39 (2016) 825-833.

[9] D.H. Fockink, M.B. Urio, J.H. Sánchez, L.P. Ramos, Energy & Fuels. 31 (2017) 6211-6220.

[10] M.S.R. Santos-Rocha, B. Pratto, L. Jacob, A. Colli, R. Maria, R. Garcia, A. José, G. Cruz, Ind. Crop. Prod. 125 (2018) 293-302.

[11] Y.H. Jung, H.M. Park, D.H. Kim, J. Yang, K.H. Kim, Appl. Biochem. Biotechnol. 182 (2017) 1108-1120.

[12] C.M. de Godoy, D.L. Machado, A.C. da Costa, Fuel. 253 (2019) 392-399.

[13] Y.E.C. Sugiharto, A. Harimawan, M.T.A.P. Kresnowati, R. Purwadi, R. Mariyana, Andry, H.N. Fitriana, H.F. Hosen, Bioresour. Technol. 207 (2016) 175-179.

[14] M.R. Mukasekuru, P. Kaneza, H. Sun, F.F. Sun, J. He, P. Zheng, Ind. Crops Prod. 146 (2020) 112156.

[15] Y. Gao, J. Xu, Z. Yuan, Y. Zhang, Y. Liu, C. Liang, Bioresour. Technol. 167 (2014) 41-45.

[16] C. Xu, J. Zhang, Y. Zhang, Y. Guo, H. Xu, J. Xu, Z. Wang, Bioresour. Technol. 292 (2019) 121993.

[17] P. Unrean, S. Khajeeram, K. Laoteng, Appl. Microbiol. Biotechnol. 100 (2016) 2459-2470.

[18] I.D. Cavalcanti-Montaño, C.A.G. Suarez, U.F. Rodríguez-Zúñiga, R. de Lima Camargo Giordano, R. de Campos Giordano, R. de Sousa Júnior, Bioenergy Res. 6 (2013) 776-785.

[19] W.F. Ramirez, Process control and identification, Academic Press, Boston, 1994.

[20] B. Pratto, R.B.A. de Souza, R. Sousa, A.J.G. da Cruz, Appl. Biochem. Biotechnol. 178 (2016) 1430-1444.

[21] T. Ghose, Pure Appl. Chem. 59 (1987) 257-268.

[22] G. Batista, R.B.A. de Souza, B. Pratto, M.S.R. Santos-Rocha, A.J.G. da Cruz, Bioresour. Technol. 275 (2019) 321-327.

[23] M.S.R. Santos-Rocha, B. Pratto, R. de Sousa, R.M.R.G. Almeida, A.J.G. Cruz, Bioresour. Technol. 228 (2017) 176-185.

[24] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, Determination of structural carbohydrates and lignin in biomass. Technical Report NREL/TP-510-42618., (2008).

[25] S. Park, J.O. Baker, M.E. Himmel, P.A. Parilla, D.K. Johnson, Biotechnol. Biofuels. 3 (2010) 10.

[26] M.G. Brondi, V.M. Vasconcellos, R.C. Giordano, C.S. Farinas, Appl. Biochem. Biotechnol. (2018) 1-13.

[27] F. Xu, H. Ding, Appl. Catal. A Gen. 317 (2007) 70-81.

[28] R. Sousa, M.L. Carvalho, R.L.C. Giordano, R.C. Giordano, Brazilian J. Chem. Eng. 28 (2011) 545-564.

[29] R. Morales-Rodriguez, A.S. Meyer, K. V. Gernaey, G. Sin, Bioresour. Technol. 102 (2011) 1174-1184.

[30] D.B. Hodge, M.N. Karim, D.J. Schell, J.D. McMillan, Appl. Biochem. Biotechnol. 152 (2009) 88-107.

[31] J.B. Kristensen, C. Felby, H. Jørgensen, Biotechnol. Biofuels. 2 (2009) 11.

[32] X. Zhao, L. Zhang, D. Liu, Biofuels Bioprod. Biorefining. 6 (2012) 465-482.

[33] D. Gomes, J. Cunha, E. Zanuso, J. Teixeira, L. Domingues, Polysaccharides. 2 (2021) 287-310.

[34] J. de Aguiar, T.J. Bondancia, P.I.C. Claro, L.H.C. Mattoso, C.S. Farinas, J.M. Marconcini, ACS Sustain. Chem. Eng. 8 (2020) 2287-2299.

[35] L. Li, W. Zhou, H. Wu, Y. Yu, F. Liu, D. Zhu, BioResources. 9 (2014) 3993-4005.

[36] S.C. Pereira, L. Maehara, C.M.M. Machado, C.S. Farinas, Renew. Energy. 87 (2016) 607-617.

[37] C. Rezende, M. de Lima, P. Maziero, E. DeAzevedo, W. Garcia, I. Polikarpov, Biotechnol. Biofuels. 4 (2011) 54.

[38] J.K. Ko, E. Ximenes, Y. Kim, M.R. Ladisch, Biotechnol. Bioeng. 112 (2015) 447-456.

[39] C. Florencio, A.C. Badino, C.S. Farinas, Bioresour. Technol. 221 (2016) 172-180.

BRUNA PRATTO [1]
MARTHA SUZANA RODRIGUES DOS SANTOS-ROCHA [2]
GUSTAVO BATISTA [1]
INTI DORACI CAVALCANTI-MONTAÑO [3]
CARLOS ALBERTO GALEANO SUAREZ [3]
ANTONIO JOSÉ GONÇALVES CRUZ [1]
RUY DE SOUSA JÚNIOR [1]

[1] Chemical Engineering, Federal University of São Carlos, São Carlos, SP, Brazil

[2] Federal Institute of Alagoas, Penedo, AL, Brazil

[3] Chemistry Institute, Federal University of Goias, Goiânia, GO, Brazil

NAUČNI RAD

RACIONALNE STRATEGIJE DOLIVANJA SUPSTRATA I ENZIMA U BIOREAKTORE ZA ENZIMSKU HIDROLIZU

Bioreaktori sa dolivanjem poboljšavaju produktivnost enzimske hidrolize pri visokim koncentracijama biomase. Ovaj rad je imao za cilj da primeni racionalne strategije dolivanja supstrata (prethodno obrađene slame šećerne trske) i enzima (CellicCtec2®) za postizanje prinosa šećera na industrijskim nivoima. Trenutna koncentracija supstrata je održavana konstantnom na 5% (m/v) tokom dolivanja. Doziranje enzima u bioreaktor je podešeno tako da brzina reakcije nije manja od unapred definisane vrednosti (procenat početne brzine reakcije - r_{min}). Kada r dostigne vrednosti ispod r_{min}, dodavan je enzim da bi se brzina reakcije vratila na početnu vrednost (r_0). Optimizovana politika dolivanja je pokazala da se brzina reakcije održava na minimum 70% od r_0, na osnovu kompromisa između produktivnosti glukoze i uštede enzima. U početku je bilo moguće obraditi 21% (m/v) čvrstog supstrata, postižući koncentraciju glukoze od 160 g/l i prinos glukoze od 80%. Verifikovano je da je neproduktivna adsorpcija enzima glavni razlog za izvesno smanjenje prinosa hidrolize u odnosu na teorijski stepen konverzije celuloze u glukozu. Povećanje konačne koncentracije glukoze od 30 g/l postignuto je kada je u enzimskoj hidrolizi korišćen aditiv koji blokira lignin (protein soje).

Ključne reči: enzimska hidroliza, operacija dolivanja, racionalne strategije dolivanja, slama od šećerne trske, neproduktivne veze lignin-enzim.