*SUSTAINABLE REUSE OF BANANA PSEUDOSTEMS: STUDY OF THE ENZYMATIC HYDROLYSIS PROCESS FOR BIOETHANOL PRODUCTION

Heitor Coelho Estevão, Iara Rebouças Pinheiro.

Universidade Federal do Espírito Santo/Center for Agricultural Sciences and Engineering, Alto Universitário, S/N - Guararema- 29500-000 - Alegre-ES, Brazil, heitorcoelho92@gmail.com, iararp.ufes@gmail.com.

Abstract
The increasing environmental impact of non-renewable energies stimulates the search for alternative sources enabling their replacement. Lignocellulosic biomass from agroindustrial residues, such as those from banana cultivation, emerges as a promising source for second-generation bioethanol production, possessing a high cellulose content that allows its conversion into bioethanol. The necessary steps for the conversion of banana cultivation biomass involve applying physicochemical pretreatments that remove components like hemicellulose and lignin, preparing the biomass for enzymatic hydrolysis to assess the optimal conditions for obtaining total reducing sugars for the fermentative process. Thus, the optimized process resulted in obtaining 159 g/L of total reducing sugars acquired via Rotational Central Composite Design (RCCD) and optimized enzymatic hydrolysis, with optimized conditions being: an enzyme concentration of 8.1 FPU/mL, a solids concentration of 4.6% (w/v), and a hydrolysis time of 12 hours.
Key words: Lignocellulosic biomass. Enzymatic hydrolysis. Central Composite Rotatable Design.
Knowledge Area: Engineering - Chemical Engineering

Introduction

The heavy reliance on non-renewable energy sources, driven by surging global energy demands, has resulted in significant socioeconomic and environmental impacts, including heightened greenhouse gas emissions, deforestation, and ecosystem degradation. To counteract these effects, there's a growing interest in researching sustainable energy alternatives derived from renewable sources (Balat, 2011; Nigam and Singh, 2011). Banana residues, with an annual production of approximately 140 Gt worldwide, represent an abundant and promising biomass for sustainable applications, especially as a source of cellulosic material for bioethanol production (Brodeur, 2011; Nigam and Singh, 2011; Kim et al., 2016; Vaz Junior, 2020).

Utilizing banana pseudostems as raw material for second-generation ethanol offers an environmentally sound and market-viable waste reuse option (Balat, 2011; Shimizu *et al.*, 2018). Regarding biomass composition, pseudostems contain cellulose (around 35%), hemicellulose (20%), lignin (18%), extractives (12%), proteins (5%), and starch (2%), varying by plant species. Extracting cellulose from such residues finds diverse applications, including biofuel production (Ogeda and Petri, 2010; Pelissari, Sobral and Menegalli, 2014; Pereira, Anjos and Magno, 2019).

Pre-treatments are necessary to enhance cellulose proportion by modifying structure and facilitating enzyme action (Brodeur, 2011; Sarkar *et al.*, 2012). Alkaline pretreatment with NaOH aims to remove hemicellulose and lignin under mild conditions, followed by bleaching with hydrogen peroxide to remove inhibitors (Kumar *et al.*, 2009; Sarkar *et al.*, 2012; Sun *et al.*, 2016). Second-generation ethanol production via enzymatic hydrolysis offers cleaner processes compared to acid hydrolysis (Kumar *et al.*, 2009; Carvalho, 2011; Pereira, Anjos and Magno, 2019).

Subsequent anaerobic fermentation converts sugars into ethanol and carbon dioxide, commonly utilizing Saccharomyces cerevisiae due to its superior performance (Nevoigt, 2008; Furlani, 2014; Pereira *et al.*, 2015; Rosa, 2021). This study aims to explore cellulose reuse from banana pseudostems in banana waste, focusing on investigating enzymatic hydrolysis for second-generation ethanol production.

Obtaining and pre-treatments

The biomass pretreatment methodology followed two approaches, both adapted from Shimizu *et al.* (2018). For the alkaline pretreatment, biomass was treated with a 1% (w/w) NaOH solution in a 1:10 (sample:solution) ratio, autoclaved at 121°C for 30 minutes, washed until neutral pH, dried at 70°C for 24 hours, and ground to obtain particles between 100 and 20 mesh (0.149 and 0.841 mm). For the hydrogen peroxide (H_2O_2) pretreatment, a 4% (w/w) solution was used, and the biomass was treated in a 1:20 (sample:solution) ratio in an SL 222 Shaker incubator at 25°C and 150 rpm for 4 hours. The material was then washed, dried under the same conditions as the alkaline pretreatment, and macerated to achieve the same particle size, ensuring homogeneity for both methodologies.

For the characterization of the pretreated material, the steps outlined and adapted by Pedrazzi *et al.* (2019), TAPPI standards, were followed to determine the moisture content, ash content, total extractives, lignin, holocellulose, alpha-cellulose, and hemicellulose contents.

Determination of the enzymatic complex activity and quantification of total reducing sugars

The method used to determine the activity of the enzymatic complex from the commercial crude extract Celluclast® 1.5 L was employed according to the methodology adapted from Ghose (1987) and Wood and Bhat (1988). Dilutions of 1:2000, 1:2750, 1:3500, 1:4250, and 1:5000 of the complex in 0.05 mol/L sodium citrate buffer at pH 4,8 were prepared. Aliquots of 1,5 mL of the dilutions and 50 milligrams of Whatman filter paper nº1 (1x6 cm) rolled to remain submerged were added to 6 test tubes, and additionally, 6 tubes were prepared for enzyme control, containing only 50 milligrams of filter paper, and 1 tube was prepared to calibrate the spectrophotometer, containing 1,5 mL of citrate buffer and 50 milligrams of filter paper. All tubes were capped and simultaneously placed in a water bath at 50°C for 1 hour, and after the end of the reaction time, 1,5 mL of the dilutions of the enzymatic complex were added to the enzyme control tubes

The quantification of total reducing sugars (TRS) was performed using the DNS method (Miller, 1959). A DNS standard curve was constructed. For the assays, 0.2 mL of the sample, 1 mL of standardized DNS, and 0.8 mL of distilled water were used, and the solutions were heated at 95°C for 5 minutes to observe the color change. The reaction was then stopped in an ice bath, and the samples were diluted to 25 mL. Readings were taken using a Kasuaki IL-227 spectrophotometer at 540 nm (Ghose, 1987). The TRS concentration in g/L was calculated using Equation (1), derived from the DNS standard curve, which showed an R^2 of 0.9975.

$$\square\square\square\square\square\square\square\square\square\square\square\ (\square\square\square) = 0.718 \times \square\square\square\ (\square/\square) - 0.0194 \qquad (1)$$

Enzymatic hydrolysis

The enzymatic hydrolysis process used the commercial enzyme complex Celluclast® 1.5 L (LNF Latin America). To optimize the experiments, a Rotational Central Composite Design (RCCD) was implemented, as described by Leal *et al.* (2022), with an alpha of 1.682. Hydrolysis was performed randomly in 250 mL Erlenmeyer flasks containing 100 mL of sodium citrate buffer (pH 4.8; 0.05 mol/L), along with substrate fractions and enzyme complex volumes defined by the RCCD and the cellulose percentage. The experiments were carried out in a shaking incubator at 170 rpm and 45°C, with varying reaction times (Nogueira, 2016; Rosa, 2021). Finally, the enzymes were denatured in a water bath at 100°C for 15 minutes, and the hydrolysate was filtered and refrigerated for TRS (Total Reducing Sugars) quantification. Analysis of Variance (ANOVA) was employed to assess the significance of the model and independent variables. Subsequently, the optimization of experimental conditions (solids concentration, enzyme concentration, and hydrolysis time) was performed by scanning the statistical subspace using the Python programming language within the ANACONDA program.

Results

According to Souza *et al.* (2021), the composition of banana fiber can vary due to climatic conditions, the geographic region of cultivation, and the type of banana variety, which results in different composition values found in the literature. Thus, the characterization data for the raw material used were: 20.2% extractives, 58.9% holocellulose, 28.2% cellulose, 30.7% hemicellulose, 15.6% lignin, and 5.3% ash. For the characterization of the pretreated material, the following was observed: 6.3% extractives, 79.2% holocellulose, 65.9% cellulose, 13.3% hemicellulose, 10.9% lignin, and 2.7% ash, demonstrating good applicability of the pretreatment methods adapted from the methodology of Shimizu *et al.* (2018), as there was a low discrepancy in values, around 1.5%. When comparing the raw and pretreated materials, a significant increase in cellulose content was observed, due to a substantial reduction in the recalcitrance of the material and a decrease in lignin and hemicellulose contents, enabling increased exposure area and enzyme access to monosaccharides. Regarding the hydrolysis process, the RCCD experimental design carried out yielded the results shown in Tables 1 and 2.

Table 1 - Design of factors used in the experimental planning of the research.

Factors	**Levels**				
	-α	-1	0	1	α
Time (h)	6	9.6	15.0	20.4	24
Solid Concentration (%w/v)	1	1.8	3.0	4.2	5
Enzyme Concentration (FPU/mL)	6	7.8	10.5	13.2	15

Source: Production by the authors.

Table 2 - Replications, axial, and central points used in the experimental design of the research.

Replications	**Experiments**	**Time (h)**	**Solid Concentration (%w/v)**	**Enzyme Concentration (FPU/mL)**	**TRS Concentration (g/L)**
1	1	9.6 (-1)	1.8 (-1)	7.8 (-1)	*18.569 ± 0.040*
	2	20.4 (1)	1.8 (-1)	7.8 (-1)	*43.291 ± 0.100*
	3	9.6 (-1)	4.2 (1)	7.8 (-1)	*69.680 ± 0.301*
	4	20.4 (1)	4.2 (1)	7.8 (-1)	*97.576 ± 0.100*
	5	9.6 (-1)	1.8 (-1)	13.2 (1)	*31.460 ± 0.100*
	6	20.4 (1)	1.8 (-1)	13.2 (1)	*115.903 ± 0.502*
	7	9.6 (-1)	4.2 (1)	13.2 (1)	*69.506 ± 0.174*
	8	20.4 (1)	4.2 (1)	13.2 (1)	*55.123 ± 0.100*
Axial	9	24 (α)	3 (0)	10.5 (0)	*72,696 ± 0.100*
	10	6 (-α)	3 (0)	10.5 (0)	*35,694 ± 0.100*
	11	15 (0)	5 (α)	10.5 (0)	*99,722 ± 0.742*
	12	15 (0)	1 (-α)	10.5 (0)	*23,514 ± 0.362*
	13	15 (0)	3 (0)	15 (α)	*74,493 ± 0.201*
	14	15 (0)	3 (0)	6 (-α)	*49,729 ± 0.100*
Central	15	15 (0)	3 (0)	10.5 (0)	*49.497 ± 0.174*
	16	15 (0)	3 (0)	10.5 (0)	*54.601 ± 0.100*
	17	15 (0)	3 (0)	10.5 (0)	*62.024 ± 0.174*

Source: Production by the authors.

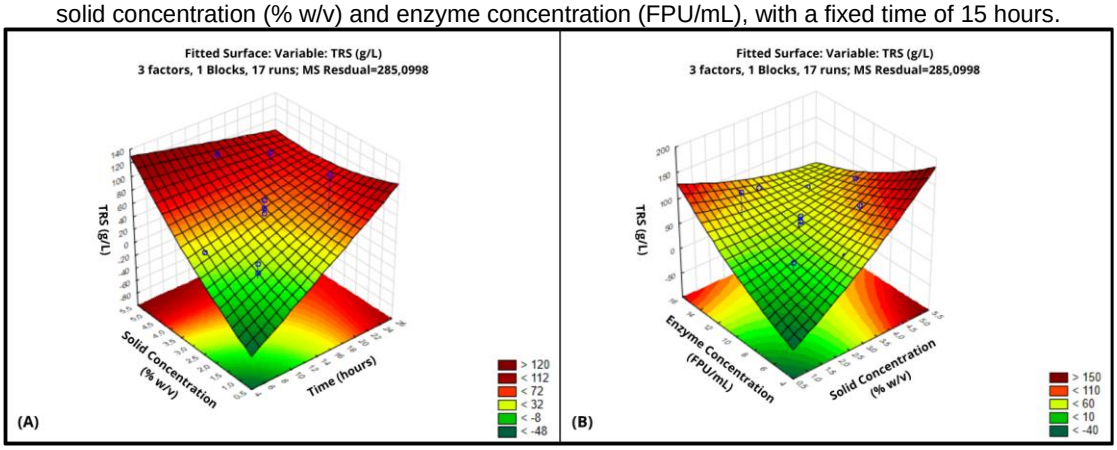
Regarding the hydrolysis process, a variation from 18.6 to 115.9 g/L of total reducing sugars (TRS) was observed, with reproducibility considered acceptable (standard deviation ≈ 6.3). The lowest values were associated with low solid concentrations, even when hydrolysis time and enzyme concentrations were moderate. On the other hand, the highest TRS levels were obtained either by increasing the solid concentration or by extending the hydrolysis time. Furthermore, statistical analysis showed that, at a 5%

significance level (p-value < 0.05), hydrolysis time, solid concentration, and the interaction between solids and enzymes significantly influenced TRS release.

$$\square = 55.124 + 13.600\,\square_1 + 0.273\square_1^2 + 15.468\,\square_2 + 2.946\square_2^2 + 6.208\square_3 + 3.123\square_3^2 - 11.956\,\square_1\square_2 + 2.180\square_1\square_3 - 16.016\square_2\square_3 \quad (2)$$

When analyzing the statistical model, equation (2) (where $\square_1$ represents time in hours; $\square_2$ the solid concentration in % w/v; and $\square_3$ the enzyme concentration in FPU/mL), the model showed a good fit between predicted and observed values, as confirmed by the scatter plot. According to the Pareto chart, solid concentration and hydrolysis time contributed positively to TRS production, while the interaction between solids and enzymes had a reductive effect. Additionally, analysis of the surface response curves revealed that the production of total reducing sugars (TRS) increases with higher solid content and enzyme concentration, as illustrated in item "A" of Figure 1. This behavior is attributed to greater efficiency in the cleavage of polysaccharides and depolymerization of cellulose, favored by increased enzyme availability, as highlighted by Rosa *et al.* (2021). Furthermore, in item "B" of Figure 1, a positive interactive effect between solid concentration and enzyme concentration can be observed. Even with lower amounts of enzymes, high solid concentrations can lead to high TRS yields and, consequently, greater ethanol production, according to Kiefer (2018).

Figure 1 – (A) Response surface of TRS production as a function of time (hours) and solid concentration (% w/v), with an enzyme concentration of 10 FPU/mL; (B) Response surface of TRS production as a function of solid concentration (% w/v) and enzyme concentration (FPU/mL), with a fixed time of 15 hours.



Source: Production by the authors.

Discussions

From the optimization of equation (1), using coded variables, the optimal conditions were determined as follows: 6 hours of enzymatic hydrolysis, 4.6% (w/v) solids, and 8.1 FPU/mL of enzymes, resulting in an estimated yield of 104.81 g/L of TRS. In validation experiments, these conditions produced 87.137 ± 0.100 g/L (6 h) and 158.962 ± 0.201 g/L (12 h). Compared to the literature, Shimizu *et al.* (2018) obtained a maximum of 80 g/L of TRS using a 4% H_2O_2 pretreatment and 30 g/L of TRS using 5% NaOH, both after 48 hours of hydrolysis and with 15 FPU/mL of enzymes.

Furthermore, the values obtained can also be justified by the increase in particle size range, as pointed out by Alves (2018), since smaller particles allow for greater accessibility, surface area, and hydrolysis efficiency, thereby accelerating the reaction. Rosa *et al.* (2021) studied the enzymatic hydrolysis of orange bagasse using cellulase and pectinase enzymes, based on a cellulose content of 19.52%, and achieved a maximum conversion of 21.06 g/L of TRS with 5% (w/v) solids, 12.5 FPU/mL of cellulase, and 13.2 U/mL of pectinase, in 32 hours. Comparing the results highlights the significant

influence of cellulose content in the medium and, as reaffirmed by Rosa *et al.* (2021), the critical role of time in the generation of monosaccharides.

Conclusions

This study, grounded in robust quantitative data, demonstrates that combined and adapted pretreatments (1% (w/v) NaOH and 4% (w/v) □□□□) significantly increased cellulose content by reducing lignin, hemicellulose, and extractives. This indicates a promising, low-cost approach for large-scale application. Enzymatic hydrolysis, using Celluclast® 1.5 L, yielded relevant quantities of total reducing sugars (TRS), notably influenced by time, solids concentration, and the interactive effect of solids and enzyme concentrations. The developed empirical model explained 82.9% of the variability, and ANOVA confirmed its statistical relevance, with predicted results aligning well with observed ones. The calculated optimal conditions (160 g/L of TRS in 12 hours of hydrolysis, with 8.1 FPU/mL enzyme and 4.6% (w/v) solids) provide a solid foundation for future research on bioethanol production from banana pseudostem, emphasizing the effectiveness of pretreatments and the influence of variables like time and solids concentration.

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