



OPTIMIZATION OF THE ENZYMATIC HYDROLYSIS OF BREWER'S SPENT GRAIN AIMING AT THE PRODUCTION OF SECOND-GENERATION ETHANOL

OTIMIZAÇÃO DA HIDRÓLISE ENZIMÁTICA DE BAGAÇO DE MALTE VISANDO A PRODUÇÃO DE ETANOL DE SEGUNDA GERAÇÃO

DOI: 10.5281/zenodo.22949592



Roberto Guimarães da Cunha Filho¹

Daniel de Freitas Rodrigues²

Renata Carolina Zanetti Lofrano³

Boutros Sarrouh⁴

ABSTRACT

Brewer's spent grain has high potential for application in second-generation bioprocesses due to its lignocellulosic composition. This study investigated optimization strategies for sequential acid-alkaline pretreatment and enzymatic hydrolysis, aiming at the release of fermentable sugars. Pretreatment was evaluated through a central composite rotatable design (CCRD), considering solid content, NaOH concentration, and reaction time. Solid content was identified as the most significant factor for lignin solubilization, reaching up to 5.6 g/L of total phenolics under mild conditions (12.99% solids, 1.95% NaOH, 19.8 min) with minimal sugar loss. Subsequently, another CCRD was conducted for enzymatic hydrolysis as a function of solids, enzyme loading, and time, resulting in a well-fitted model ($R^2 = 93.4\%$; $\text{adj-}R^2 = 84.8\%$). The optimal condition was predicted at 9.96% solids, 61.3 FPU/g, and 71.7 h, estimating up to 25 g/L of reducing sugars. Experimental validation confirmed the model trend, achieving 19.3 ± 0.8 g/L. These findings demonstrate that integrating sequential pretreatment with

¹ Master student. Post-Graduate Program in Chemical Engineering at the Federal University of São João del-Rei, Ouro Branco, Minas Gerais, Brazil. E-mail: betolga.cunha@hotmail.com.

² Master student. Post-Graduate Program in Chemical Engineering at the Federal University of São João del-Rei, Ouro Branco, Minas Gerais, Brazil. E-mail: daninhofr@gmail.com.

³ PhD in science. Department of Chemical Engineering, Federal University of São João del-Rei. Ouro Branco, Minas Gerais, Brazil. E-mail: renataczlofrano@gmail.com.

⁴ PhD in science Department of Chemistry, Biotechnology and Bioprocess Engineering. Federal University of São João del-Rei. Ouro Branco, Minas Gerais, Brazil. E-mail: bsarrouh@ufsj.edu.br.





enzymatic hydrolysis enhances the conversion of brewer's spent grain, reinforcing its applicability as a feedstock for bioethanol and other value-added bioproducts.

Keywords: Enzymatic Hydrolysis; Brewer's Spent Grain (BSG); Lignocellulosic Biomass; Process Optimization; Acid-Alkaline Pretreatment.

RESUMO

O bagaço de malte apresenta alto potencial para aplicação em bioprocessos de segunda geração devido à sua composição lignocelulósica. Este estudo investigou estratégias de otimização para O pré-tratamento ácido-alcálico sequencial e hidrólise enzimática, visando à liberação de açúcares fermentáveis. O pré-tratamento foi avaliado por meio de um planejamento composto central rotacional (CCRD), considerando o teor de sólidos, a concentração de NaOH e o tempo de reação. O teor de sólidos foi identificado como o fator mais significativo para a solubilização da lignina, atingindo até 5,6 g/L de fenólicos totais em condições brandas (12,99% de sólidos, 1,95% de NaOH, 19,8 min) com perda mínima de açúcares. Posteriormente, outro CCRD foi conduzido para a hidrólise enzimática em função dos sólidos, da concentração de enzima e do tempo, resultando em um modelo bem ajustado ($R^2 = 93,4\%$; R^2 ajustado = 84,8%). A condição ideal foi prevista em 9,96% de sólidos, 61,3 FPU/g e 71,7 h, estimando até 25 g/L de açúcares redutores. A validação experimental confirmou a tendência do modelo, atingindo $19,3 \pm 0,8$ g/L. Esses resultados demonstram que a integração do pré-tratamento sequencial com a hidrólise enzimática aumenta a conversão do bagaço de malte, reforçando sua aplicabilidade como matéria-prima para bioetanol e outros bioprodutos de alto valor agregado.

Palavras-chave: Hidrólise enzimática; Bagaço de Malte (BSG); Biomassa Lignocelulósica; Otimização de Processos; Pré-tratamento Ácido-Alcalino.

INTRODUCTION

The brewing industry plays a crucial role in the Brazilian economy, contributing approximately 2% of the Gross Domestic Product (GDP) and generating more than 2.5 million jobs throughout its production chain, with production exceeding 15 billion liters in 2023 (1,2). Such economic relevance is accompanied by the generation of large volumes of residues, among which brewer's spent grain (BSG) stands out, representing about 85% of the solid by-products of breweries and corresponding to approximately 31% of the original malt weight. Traditionally used as animal feed or fertilizer, this residue has a composition rich in fibers and proteins, which has drawn interest for higher value-added applications, such as bioethanol production (3).





Brewer's spent grain is a lignocellulosic biomass mainly composed of cellulose, hemicellulose, and lignin, with cellulose being one of its major components. For this reason, the residue represents a promising alternative source for cellulose recovery. Considering that researchers have been focusing efforts on the development of green and biodegradable materials, cellulose stands out as the main plant polysaccharide to be modified and applied in different studies (4,5).

A promising alternative for the reuse of brewer's spent grain is the application of enzymatic hydrolysis. The enzymes responsible for this process are mainly produced by microorganisms such as filamentous fungi (*Trichoderma reesei*, *Aspergillus niger*, among others) and bacteria (*Cellulomonas fimi* and *Clostridium thermocellum*) (6). However, lignocellulosic materials exhibit high resistance to enzymatic hydrolysis, since cellulose fibers are covered by a complex lignin–polysaccharide matrix. Therefore, pretreatment is required to increase enzymatic accessibility (7,8).

Pretreatment with acidic or alkaline solutions is widely used, as it reduces cellulose crystallinity and promotes the partial removal of lignin and hemicellulose. This process must be carefully controlled to avoid carbohydrate degradation, making cellulose more susceptible to cellulase action (9,10). Once pretreatment is carried out under appropriate conditions, enzymatic hydrolysis occurs in successive steps: initially, cellulases are added to the pretreated substrate under optimized temperature ($\approx 50\text{ }^{\circ}\text{C}$) and pH (≈ 4.8) conditions; then, they cleave cellulose chains, generating smaller oligosaccharides that are subsequently converted into glucose. This glucose can be used in fermentative processes, such as second-generation ethanol or methane production. One of the main advantages of enzymatic hydrolysis is its high selectivity, since cellulolytic enzymes act specifically on cellulose bonds, preserving other structural components and reducing the formation of undesirable by-products that may inhibit subsequent fermentation steps (11).

The product obtained is a hydrolysate rich in glucose, which can be used in fermentative processes for the production of second-generation ethanol. Several authors have studied optimizations for this production by analyzing improvement parameters in both



pretreatments and enzymatic hydrolysis. In the case of pretreatments, these variables include volume, acid or base concentration, and autoclaving time; while in enzymatic hydrolysis, factors such as pH, enzyme loading, reaction time, and temperature are considered (3). In this context, this study aims to optimize the enzymatic hydrolysis of brewer's spent grain for second-generation ethanol production, contributing to the development of strategies for the valorization of this residue.

MATERIALS AND METHODS

ACQUISITION, PREPARATION AND CONDITIONING OF BREWER'S SPENT GRAIN

Approximately 5 kg of brewer's spent grain was initially subjected to drying in an oven at 90 ± 2 °C for 72 h. Subsequently, the dried material was ground in a household blender for approximately 5 min, resulting in a more homogeneous powder suitable for the subsequent pretreatment and hydrolysis steps.

DILUTE ACID PRETREATMENT OF BREWER'S SPENT GRAIN

Brewer's spent grain (BSG) was subjected to dilute acid pretreatment using a 0.7% (v/v) H_2SO_4 solution ($\approx 0.13 \text{ mol}\cdot\text{L}^{-1}$). The biomass was mixed with the acid solution at a 1:8 (w/v) ratio and treated in sealed 2 L flasks in an autoclave at 120 °C for 30 min. After treatment, the material was filtered to separate the liquid hydrolysate, stored for further analyses, from the solid fraction. The solid residue was washed with distilled water until neutral pH, oven-dried at 105 °C for 72 h to constant weight, and ground to a fine powder. The pretreated biomass was stored in airtight containers for subsequent alkaline pretreatment and enzymatic hydrolysis.





OPTIMIZATION OF ALKALINE PRETREATMENT OF BREWER'S SPENT GRAIN

The alkaline pretreatment of brewer's spent grain (BSG), previously subjected to dilute acid pretreatment, was optimized to promote partial lignin removal and improve cellulose and hemicellulose accessibility. A Central Composite Rotatable Design (CCRD, 2^3) was carried out using Statgraphics Centurion, considering three independent variables: solid content (% w/v), NaOH concentration (% w/v), and reaction time (min), each coded at five levels ($-\alpha$, -1 , 0 , $+1$, $+\alpha$; $\alpha = 1.68$). The design comprised 17 experiments (8 factorial points, 6 axial points, and 3 central replicates), covering experimental ranges of 6.64–13.36% (solids), 1.96–12.04% (NaOH), and 19.8–70.2 min (reaction time).

After each run, the solid fraction was separated, washed with distilled water to neutral pH, and oven-dried at 100 °C for 72 h to constant weight, while the liquid fraction was collected for disposal or complementary analysis. Response variables included total phenolic content, determined by the Folin–Ciocalteu method (mg GAE/g biomass), and reducing sugars, quantified by the DNS method (g glucose equivalent/L).

Experimental results were analyzed by ANOVA, Pareto and main effect plots, and response surface methodology in Statgraphics Centurion. Model adequacy was evaluated by significance, R^2 , and lack-of-fit tests. Optimal conditions identified were validated experimentally by comparing predicted and observed values.

OPTIMIZATION OF ENZYMATIC HYDROLYSIS OF THE CELLULOSIC FRACTION OF BREWER'S SPENT GRAIN

The enzymatic hydrolysis of the cellulosic fraction of brewer's spent grain (BSG), previously subjected to dilute acid and alkaline pretreatments, was optimized through a Central Composite Rotatable Design (CCRD, 2^3) using Statgraphics Centurion. Independent variables included solid content (2.0–12.0 % w/v), enzyme loading (6.4–74.0 FPU/g





substrate), and reaction time (8–90 h), coded at five levels ($-\alpha$, -1 , 0 , $+1$, $+\alpha$; $\alpha = 1.68$). The design comprised 17 experiments (8 factorial, 6 axial, and 3 central replicates).

Experiments were performed in 125 mL Erlenmeyer flasks containing 25 mL of 50 mM sodium acetate buffer (pH 4.8), under orbital shaking at 180 rpm and 50 °C. The enzyme used was Cellulase from *Trichoderma longibrachiatum* (Sigma Aldrich), dosed in FPU/g dry substrate. Reducing sugar release was monitored as the response variable, quantified by the DNS method and expressed as g/L glucose equivalent.

Data were fitted to a quadratic response surface model (RSM), allowing evaluation of linear, quadratic, and interaction effects. Statistical analysis included ANOVA, Pareto plots, response surfaces, and validation of the predictive model, with significance considered at $p < 0.05$. Model adequacy was assessed by R^2 , adjusted R^2 , and lack-of-fit tests.

Optimal conditions predicted by the model were validated experimentally in triplicate, comparing predicted and observed values. Control assays included hydrolysis of untreated BSG and BSG subjected only to acid pretreatment, confirming the improvements achieved by sequential acid–alkaline pretreatment followed by enzymatic hydrolysis.

QUANTIFICATION OF REDUCING SUGARS BY THE DNS METHOD

Reducing sugars were quantified using the 3,5-dinitrosalicylic acid (DNS) method, in which reducing sugars reduce DNS to a reddish compound measurable by spectrophotometry. Samples were diluted (1:50), mixed with 1.5 mL of DNS reagent, and heated in a boiling water bath at 100 °C for 5 min. After cooling in an ice bath for 5 min, 7.5 mL of distilled water was added, resulting in a final volume of 10 mL. Absorbance was read at 540 nm using distilled water as blank. Total reducing sugar concentration was calculated from a glucose standard curve (0.1–0.9 g/L) (12).





DETERMINATION OF TOTAL PHENOLICS (FOLIN–CIOCALTEU METHOD)

Total phenolic content was determined using the Folin–Ciocalteu colorimetric method. Diluted samples (1:500) were mixed with 200 μ L of Folin–Ciocalteu reagent and, after 5 min, 600 μ L of 20% (w/v) sodium carbonate solution was added. The mixtures were incubated at room temperature, in the dark, for 30 min, and absorbance was measured at 760 nm using a spectrophotometer. A calibration curve was prepared with gallic acid (1–10 mg/L), and results were expressed as milligrams of gallic acid equivalents per gram of brewer's spent grain (mg GAE/g) (12).

RESULTS AND DISCUSSION

DILUTE ACID HYDROLYSIS

Dilute acid pretreatment resulted in significant solubilization of hemicellulose, as demonstrated by the concentration of 10.16 g/L of reducing sugars in the hydrolysate. This highlights the selectivity of the process towards hemicellulose, consistent with values reported for other lignocellulosic residues such as wheat straw and sugarcane bagasse. In addition, the total phenolic content reached 1.62 g/L, indicating the release of lignin derivatives and degradation by-products.

While these compounds reflect the partial effectiveness of the pretreatment in disrupting the lignin–hemicellulose matrix, they also point to the generation of potential fermentation inhibitors, including phenolic acids and aromatic aldehydes. Therefore, the results emphasize the need for subsequent alkaline pretreatment, which aims to further reduce the lignin fraction and decrease the concentration of inhibitory compounds, thus improving the efficiency of the enzymatic hydrolysis stage.





OPTIMIZATION OF ALKALINE PRETREATMENT OF BREWER'S SPENT GRAIN

The alkaline pretreatment was carried out in 17 runs, according to a Central Composite Rotatable Design (CCRD), varying solid content (% w/v), NaOH concentration (% w/v), and reaction time (min). After reaction, the hydrolysates were separated and analyzed for total phenolics, as an indicator of partial lignin solubilization, and reducing sugars, which reflect undesirable degradation of hemicellulose and cellulose.

Total phenolics ranged from 2.9 g/L (with low NaOH concentration) to 5.6 g/L (with high solids, high base, and long reaction time). Reducing sugars varied from 0.50 g/L (low NaOH) to 1.32 g/L, showing that harsher conditions increase both lignin removal and carbohydrate loss. This relationship indicates that the more severe the pretreatment, the higher the release of desirable phenolics, but also the greater the solubilization of fermentable sugars.

The results were statistically analyzed by ANOVA, separately for total phenolics and reducing sugars. For total phenolics, only solid content ($p = 0.0025$) was significant, explaining most of the variation observed. NaOH concentration and time were not statistically relevant ($p > 0.05$). This behavior is consistent with the process logic: the higher the biomass load, the greater the lignin availability for solubilization. For reducing sugars, two factors were significant: solid content ($p = 0.0056$) and NaOH concentration ($p = 0.0359$). This demonstrates that, in addition to the amount of solids, base concentration is a determining factor for carbohydrate release. Time was not significant ($p = 0.3514$), suggesting that the reaction is relatively fast and prolonged durations do not substantially increase sugar release.

The ANOVA for total phenolics (Table 1) confirmed significant effect only of solid content ($p = 0.0025$), while NaOH concentration ($p = 0.1401$) and time ($p = 0.8458$) showed no statistical influence. The adjusted model showed $R^2 = 80.63\%$ and adjusted $R^2 = 55.73\%$, with good explanatory capacity despite lack-of-fit due to system complexity. The linear model obtained was (Eq. 1):



$$\text{Total phenolics} = -0,0418 + 0,3403 * A + 0,0783 * B + 0,00226 * C \quad (\text{Eq. 1})$$

Where:

A = Solid content

B = NaOH concentration

C = Time

TABLE 1: Statistical data (ANOVA) based on the results obtained and the variables used for the response variable total phenolics.

SOURCE OF VARIATION	TOTAL PHENOLICS				
	Sum of squares	Degrees of freedom	Mean square	F-ratio	P-Value
A: Total solids	0,365843	1	0,365843	21,19	0,0025
B: NaOH concentration	0,0477834	1	0,0477834	2,77	0,1401
C: Time	0,000702843	1	0,000702843	0,04	0,8458
AA	0,0353919	1	0,0353919	2,05	0,1953
AB	0,00177426	1	0,00177426	0,10	0,7579
AC	0,000322022	1	0,000322022	0,02	0,8952
BB	0,000727679	1	0,000727679	0,04	0,8432
BC	0,00152523	1	0,00152523	0,09	0,7749
CC	0,0518786	1	0,0518786	3,01	0,1266
Total error	0,120833	7	0,0172619	-	-
Total (corr.)	0,623813	16	-	-	-

Source: (Author's own work, 2025).

For reducing sugars (Table 2), solid content ($p = 0.0056$) and NaOH concentration ($p = 0.0359$) were significant, while time ($p = 0.3514$) was not, suggesting sugar release occurs at early stages and does not intensify with longer reaction times. The adjusted model presented $R^2 = 77.65\%$ and adjusted $R^2 = 48.92\%$, and was described by (Eq. 2):



$$\text{Reducing sugars} = -0,5101 + 0,0916 * A + 0,0396 * B + 0,00330 * C \quad (\text{Eq. 2})$$

Where:

A = Solid content

B = NaOH concentration

C = Time

TABLE 2: Statistical data (ANOVA) based on the results obtained and the variables used for the response variable reducing sugars.

SOURCE OF VARIATION	REDUCING SUGARS				
	Sum of squares	Degrees of freedom	Mean square	F-ratio	P-value
A: Total solids	0,639427	1	0,639427	15,57	0,0056
B: NaOH concentration	0,27556	1	0,27556	6,71	0,0359
C: Time	0,0409324	1	0,0409324	1,00	0,3514
AA	0,00127887	1	0,00127887	0,03	0,8649
AB	0,000106132	1	0,000106132	0,00	0,9609
AC	0,0230763	1	0,0230763	0,56	0,4780
BB	0,00176019	1	0,00176019	0,04	0,8419
BC	0,00000327702	1	0,00000327702	0,00	0,9931
CC	0,0113685	1	0,0113685	0,28	0,6151
Total error	0,287567	7	0,041081	-	-
Total (corr.)	1,2869	16	-	-	-

Source: (Author's own work, 2025).

The determination coefficients were satisfactory in both models ($R^2 > 80\%$), confirming that the investigated factors consistently explained the experimental variability. Pareto charts (Figures 1 and 2) reinforced the ANOVA conclusions. For total phenolics, solid





content (A) was the only factor above the significance threshold, confirming its predominance. For reducing sugars, both solids (A) and NaOH (B) were significant, with positive effect magnitudes.

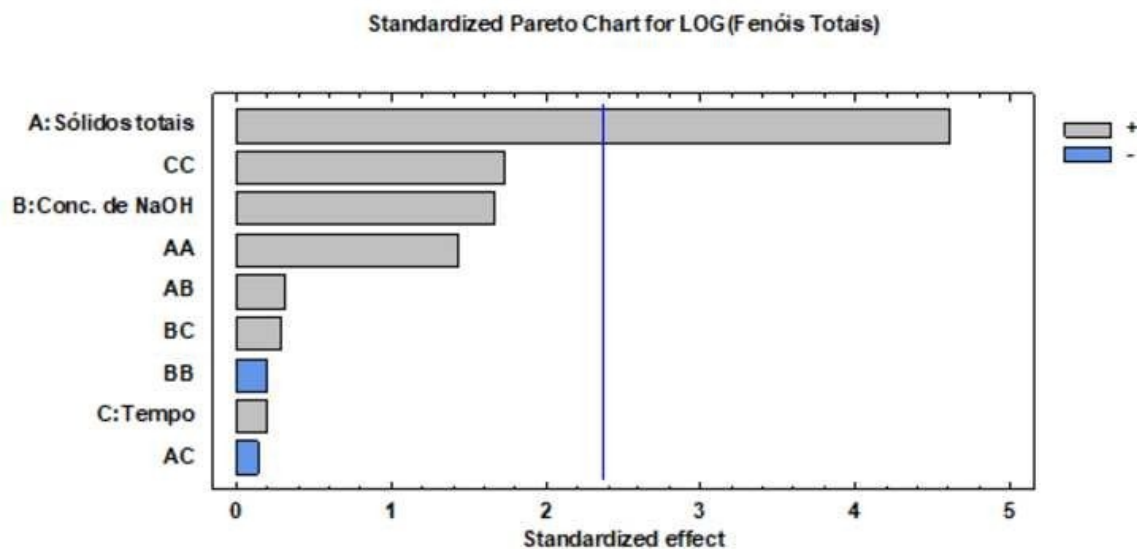


Figure 1. Pareto chart for total phenolics concentration

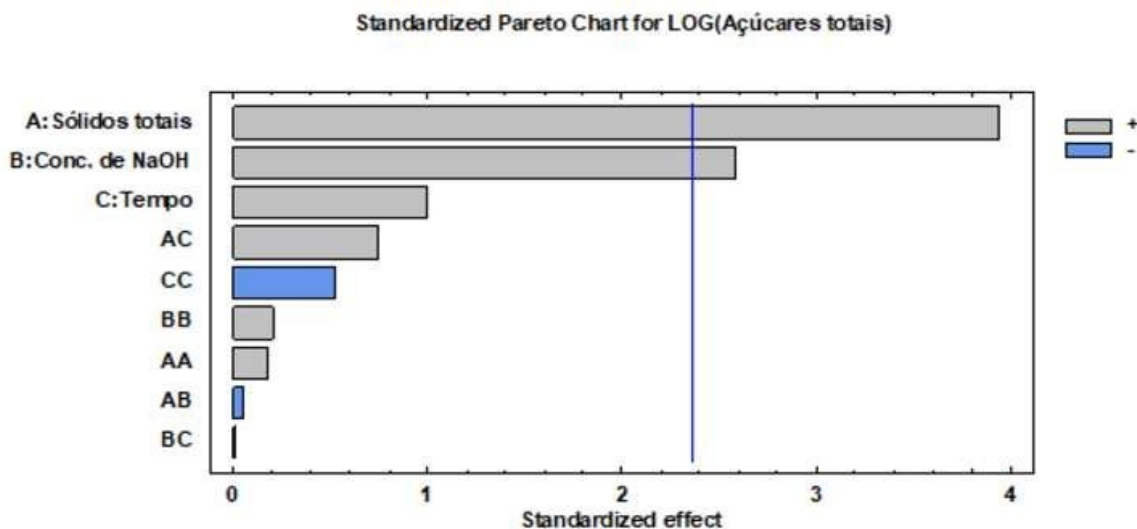


Figure 2. Pareto chart for reducing sugars concentration

A simultaneous analysis of the responses was carried out through a desirability surface (Figure 3). The model aimed to maximize phenolics and minimize sugars, identifying the



optimal process region. The red/orange areas indicate favorable conditions, while blue areas represent low desirability.

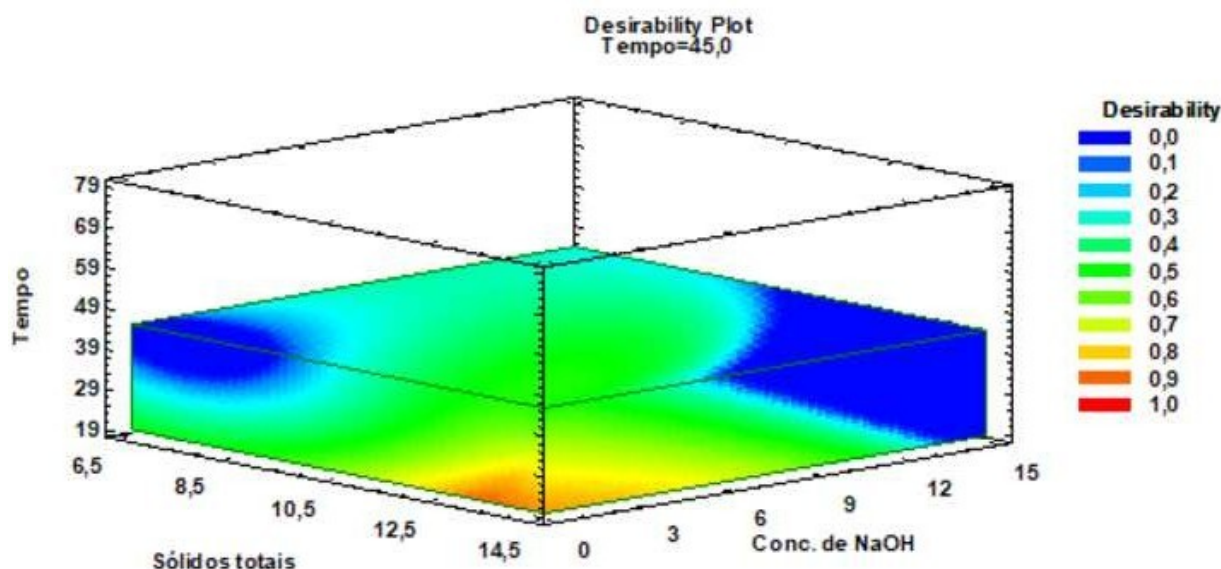


Figure 3. Response surface of the optimal conditions of alkaline hydrolysis

Predicted by the model:

- Total phenolics = 5.6 g/L (95% CI: 2.87–10.94; desirability = 0.9999)
- Reducing sugars = 0.69 g/L (95% CI: 0.24–1.93; desirability = 0.7736)

The global desirability was 87.95%, considered excellent for a multifactorial system.

Finally, the process was optimized under the conditions shown: approximately 13% solids, low NaOH concentration (~1.95%), and a relatively short reaction time (~20 min). The results demonstrate that alkaline pretreatment can be efficiently performed under mild conditions, with low base concentration and short time, reducing operational costs and effluent generation. The predominance of solid content as a key factor for phenolic release aligns with recent studies highlighting biomass load as a determinant for alkaline delignification efficiency (13,14). On the other hand, the influence of NaOH concentration on



sugar release confirms that excessive base promotes undesirable hemicellulose solubilization (15).

OPTIMIZATION OF ENZYMATIC HYDROLYSIS OF BREWER'S SPENT GRAIN

Reducing sugar concentrations ranged from 1.83 g/L (condition with 2% solids, 40 FPU/g, and 48 h) to 24.7 g/L (condition with 10% solids, 60 FPU/g, and 72 h), evidencing the strong influence of process parameters on hydrolysis efficiency. In general, higher solid concentrations and longer reaction times were associated with greater sugar release.

Analysis of variance revealed that solid content (A) was the most significant variable for reducing sugar release, with high statistical significance ($p = 0.0001$) and the highest F-ratio (62.12). This result confirms that substrate availability plays a decisive role in overall enzymatic hydrolysis efficiency. Reaction time (C) was also highly significant ($p = 0.0051$), indicating that extending hydrolysis favored cellulose conversion, although its contribution was lower than that of solids. Enzyme loading (B) showed a positive and significant effect, but of lower magnitude ($p = 0.0402$), suggesting that moderate increases in enzyme dosage contributed to sugar release, though to a lesser extent compared with solids and time.

Among quadratic and interaction terms, only the AA effect (solids²) was significant ($p = 0.0150$), indicating a non-linear behavior of this variable. This suggests the existence of an intermediate optimal solids concentration that maximizes sugar production; above this level, suspension viscosity may limit enzyme diffusion and reduce process efficiency, a behavior also reported in recent optimization studies (14,15). Other interactions (AB, AC, BC) were not statistically significant ($p > 0.05$), indicating that variable effects were predominantly additive.

The adjusted model presented $R^2 = 93.37\%$, indicating that the variables explained most of the observed variation. The adjusted $R^2 = 84.84\%$ reinforced the quality of the fit even after correction for degrees of freedom. The standard error of estimate (SEE) was 0.245, and the mean absolute error (MAE) was 0.120, both considered low for this type of





multifactorial system. These statistical indicators demonstrate the adequacy of the quadratic model to describe reducing sugar release in enzymatic hydrolysis of brewer's spent grain, ensuring reliability of predictions.

The Pareto chart (Figure 4) confirmed the ANOVA, highlighting solid content (A) as the most influential variable for sugar release, followed by reaction time (C), and, to a lesser extent, enzyme loading (B). The dominance of variable A evidences that substrate concentration is a determinant factor for process yield, as it provides more cellulose available for enzymatic attack. However, very high solid concentrations may increase medium viscosity and mixing difficulties, compromising enzyme diffusion, which explains the significant quadratic effect observed for AA.

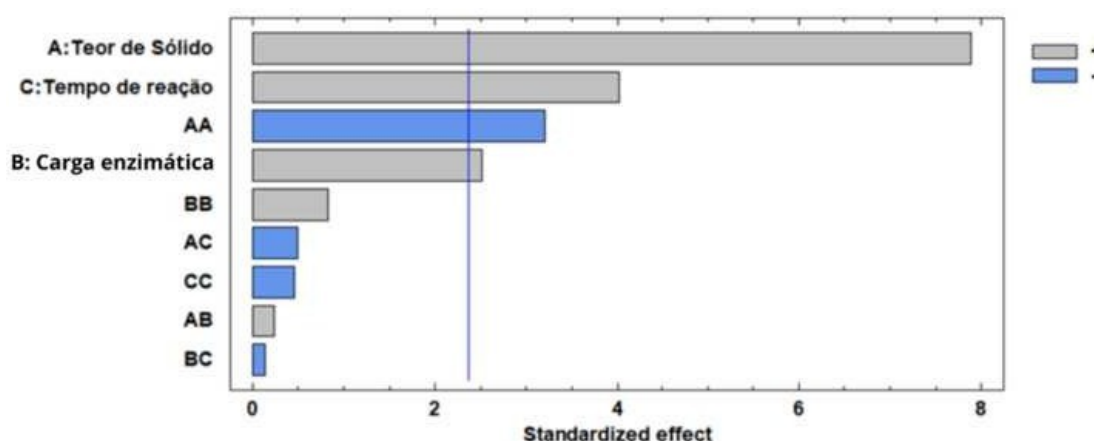


Figure 4. Pareto chart for the concentration of reducing sugars

Increasing solid content up to intermediate levels (~10% w/v) resulted in a sharp increase in sugar release, but beyond this point, the rate of gain decreased, indicating a plateau. Reaction time showed a positive linear effect, with longer hydrolysis periods producing higher sugar concentrations, though with a stabilization trend after 70–80 h, due to substrate depletion and product inhibition. Enzyme loading also showed a positive effect, though less pronounced: increases above 60 FPU/g resulted in limited gains, suggesting enzyme saturation of the system.



These results agree with recent literature, which identifies solid content as a critical variable for sugar yield in enzymatic hydrolysis, while enzyme loading should be adjusted only up to an economically viable level, avoiding unnecessary excess (13–15).

The fitted equation from the CCRD to predict reducing sugar release is shown in Eq. 3:

$$\text{Reducing sugars} = -8,8152 + 1,54832 * A + 0,102462 * B + 0,116255 * C \quad (\text{Eq. 3})$$

Where:

- A = Solid content (% w/v)
- B = Enzyme loading (FPU/g)
- C = Reaction time (h)

The model showed a good fit to the experimental data, indicating that the selected variables explained most of the observed variation in the response. The equation demonstrated that solid content (A) was the most significant positive contributor, followed by reaction time (C) and, to a lesser extent, enzyme loading (B). In addition to the linear effect, a significant quadratic effect was detected for solid content, showing that very low or very high concentrations reduce efficiency, whereas intermediate values maximize sugar release. The quadratic term for solids (AA) was significant ($p < 0.05$), confirming the existence of an intermediate optimum (~10% w/v). These results confirm the ANOVA and Pareto chart analyses, reinforcing that adequate substrate supply is critical for process yield.

Response surface plots (Figure 5) enabled visualization of variable interactions and identification of regions of higher sugar production. They highlighted the critical process factors, with solid content as the main impact variable, followed by reaction time and enzyme loading. Sugar release increased progressively with higher solid content up to around 10%, combined with longer reaction times (>70 h). Regarding enzyme loading, increases up to ~60 FPU/g promoted relevant gains, but further increases provided only marginal improvements, suggesting enzyme system saturation.



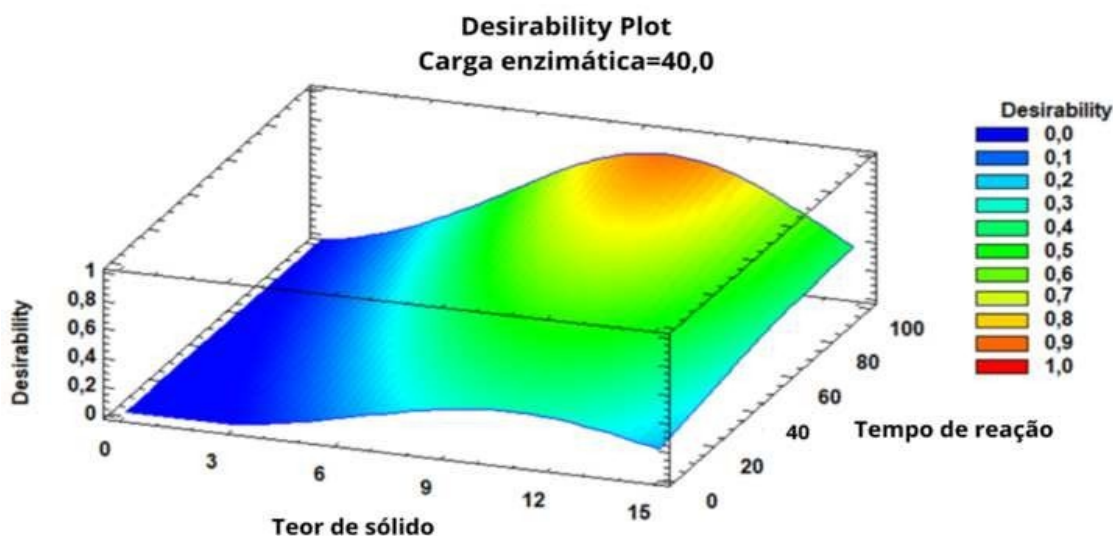


Figure 5. Response surface of the optimal conditions of enzymatic hydrolysis

The graphical analysis corroborated the quadratic effect detected for factor AA (solid content), showing that both very low and excessively high concentrations reduced yield. At low levels, substrate availability was insufficient to maximize enzymatic activity; at high concentrations, increased viscosity and mass transfer limitations hindered enzyme–substrate contact.

The model-predicted optimum condition for maximizing sugar release was:

- Solid content = 9.96% (w/v)
- Enzyme loading = 61.3 FPU/g
- Reaction time = 71.7 h

Under these conditions, the maximum sugar concentration predicted was approximately 25 g/L, consistent with the best experimental results obtained (run 6, with 24.7 g/L). This demonstrates the robustness of the fitted model and confirms that response surface methodology was effective in identifying the optimal process point.



The determination of optimal conditions highlighted the critical factors affecting enzymatic hydrolysis of brewer's spent grain. Solid content proved decisive but limited by rheological effects; reaction time strongly influenced sugar release, although it tended to stabilize at prolonged times; and enzyme loading had a positive effect but should be applied only up to the saturation point (~ 60 FPU/g) for economic viability. This optimum is consistent with the quadratic solids term identified in the model, which indicated maximum sugar release around 10% (w/v).

These results corroborate recent studies emphasizing the importance of balancing solid content and enzyme loading to maximize sugar release without compromising economic feasibility (13–15). Experimental validation of the optimal point confirmed the model predictions, showing the superiority of brewer's spent grain subjected to combined acid–alkaline pretreatment. While conditions without pretreatment (7.04 ± 0.29 g/L) and with only acid pretreatment (6.18 ± 0.49 g/L) yielded low sugar concentrations, the integrated process achieved significantly higher yield (19.27 ± 0.83 g/L). Although the value obtained was slightly lower than that predicted by the response surface (~ 25 g/L), the difference can be attributed to experimental variability and intrinsic hydrolysis factors, such as product inhibition and biomass structural heterogeneity. Nevertheless, the proximity between predicted and observed values confirms the adequacy of the model, considering the complexity inherent to enzymatic hydrolysis of heterogeneous biomasses. This validation reinforces the consistency of the mathematical model and demonstrates the efficiency of the dual pretreatment in increasing enzymatic digestibility of brewer's spent grain, consolidating its potential as a promising substrate for second-generation bioethanol and other bioproduct production routes.

ACKNOWLEDGMENTS

This work was supported by CAPES (Coordination for the Improvement of Higher Education Personnel), FAPEMIG (Research Support Foundation of the State of Minas





Gerais), and UFSJ (Federal University of São João del-Rei). The author(s) also wish to thank the research group colleagues for their valuable contributions during discussions and laboratory support, as well as the professors and technical staff who provided guidance throughout the development of this study.

DECLARATION OF AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the author(s) used ChatGPT (OpenAI) in order to support English translation and refinement of the manuscript.. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

REFERENCES

1. Ministério da Agricultura e Pecuária & Secretaria de Defesa Agropecuária: Anuário da Cerveja 2024: ano de referência 2023. in (MAPA, 2024).
2. SINDICERV: O setor em números. (2023). at <<https://sindicerv.com.br/o-setor-em-numeros/>>
3. Albuquerque Garcia, G., Pereira Lôbo, F., Dos Santos Moraes, K., Barbalho Dantas Bezerra, M. & Aguiar de Oliveira, E.: Pré-tratamento termo-ácido e hidrólise enzimática de uma mistura de casca de cacau e bagaço de malte. Scientia Plena, 19, (2023).
4. Lima, V. H. de: Obtenção de nanocelulose via hidrólise ácida a partir dos resíduos da produção de cerveja. (2016).
5. Mussatto, S. & Roberto, I.: in Matérias-Primas dos Alimentos, (ed. Urgel de Almeida Lima) Cap. 11, 1–427 (Blucher, 2010).





6. Cândida Rabelo, S.: Avaliação e otimização de pré-tratamentos e hidrólise enzimática do bagaço de cana-de-açúcar para a produção de etanol de segunda geração. (2010). doi:10.47749/T/UNICAMP.2010.770600
7. Basera, P., Chakraborty, S. & Sharma, N.: Lignocellulosic biomass: insights into enzymatic hydrolysis, influential factors, and economic viability. *Discover Sustainability*, 5, 311 (2024).
8. Saha, B. C.: Hemicellulose bioconversion. in *J Ind Microbiol Biotechnol*, 30, 279–291 (2003).
9. Asem, M., Noraini Jimat, D., Huda Syazwani Jafri, N., Mohd Fazli Wan Nawawi, W., Fadhilah Mohamed Azmin, N. & Firdaus Abd Wahab, M.: Entangled cellulose nanofibers produced from sugarcane bagasse via alkaline treatment, mild acid hydrolysis assisted with ultrasonication. *Journal of King Saud University - Engineering Sciences*, 35, 24–31 (2023).
10. Avicenna, Jimat, D. N., Salleh, H. Mohd. & Anuar, H.: Effect of fungal pretreatment by *Aspergillus niger* assisted by ultrasonication on microfibrillated cellulose obtained from cocoa pod husk. in (2016). at <<https://api.semanticscholar.org/CorpusID:46800397>>
11. Woźniak, A., Kuligowski, K., Świerczek, L. & Cenian, A.: Review of Lignocellulosic Biomass Pretreatment Using Physical, Thermal and Chemical Methods for Higher Yields in Bioethanol Production. *Sustainability*, 17, 287 (2025).
12. Sarrouh, B., Rodrigues, D. de F., Cunha Filho, R. G. da, Rodrigues Júnior, A. F., Moraes, E. R. G. de & Lofrano, R. C. Z.: Study of Diluted Acid Hydrolysis of Coffee Husk Aiming At Obtaining Fermentable Sugars. *Revista de Gestão Social e Ambiental*, 18, e010373 (2024).
13. Baksi, S., Saha, D., Saha, S., Sarkar, U., Basu, D. & Kuniyal, J. C.: Pre-treatment of lignocellulosic biomass: review of various physico-chemical and biological methods influencing the extent of biomass depolymerization. *International Journal of Environmental Science and Technology*, 20, 13895–13922 (2023).
14. Zhang, H., Wu, J., Zhang, M. & Sun, Y.: Systematic optimization of cellulases production and extraction by *Sporotrichum thermophile* mutant strain TH3-9 and application in enzymatic hydrolysis of corn stover. *Environ Technol Innov*, 36, 103868 (2024).





15. Kululo, W. W., Habtu, N. G., Abera, M. K., Sendekie, Z. B., Fanta, S. W. & Yemata, T. A.: Advances in various pretreatment strategies of lignocellulosic substrates for the production of bioethanol: a comprehensive review. Discover Applied Sciences, 7, 476 (2025).

Recebido em: 13/08/2026

Aprovado em: 05/09/2026

Publicado em: 24/09/2026

