ENHANCED ENZYMATIC HYDROLYSIS OF CORN STARCH VIA SHORT-TIME BALL MILLING: CHEMICAL AND STRUCTURAL CHANGES

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ABSTRACT

This study explores short-time (4 minutes) ball milling as a sustainable pretreatment method for processing corn grain to produce glucose and maltose syrup via enzymatic hydrolysis, offering an alternative to the traditional jet cooking. The pretreatment effects on the corn grains were determined from the X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), Scanning electron microscopy (SEM), water absorption and solubility indices, and enzymatic hydrolysis data before and after ball milling. Ball milling slightly altered the starch crystallinity and chemical composition of the corn grains. FTIR analysis of the ball-milled corn indicates an increase in free hydroxyl groups, potentially enhancing starch hydrophilicity, corroborated by a 43% increase in its water absorption and a 27% increase in its water solubility. Enzymatic hydrolysis also reveals a 119.5% increase in glucose plus maltose yield after ball milling pretreatment. These findings suggest ball milling as a promising, eco-friendly approach for corn processing, suitable for glucose and maltose syrup production, with potential application in the bioethanol industry.

Keywords: Ball-milling. Corn. Starch. Hydrolysis.

1 INTRODUCTION

The net increase in carbon dioxide emissions due to rise in global energy consumption with a contribution of 85% from fossil fuels and the increase in greenhouse gas emissions have been deepening environmental problems. As such, transitioning to biofuels is of paramount importance. Despite being the second-largest ethanol producer globally, Brazil's ethanol production, mainly produced from sugarcane, struggles to meet domestic demands and environmental commitments from COP21[(1)]. Declining sugarcane productivity and reduced investments alongside idle periods of up to four months during the off-season [(2)] have called for options such as corn ethanol production that has been thriving. Corn, Brazil's second most cultivated grain, has seen a 68% production increase over the past seven years, with the 2023/24 harvest estimated at 111,635.8 million tons. CONAB estimates corn ethanol production for this period to be 5.64 billion liters, a 42% increase from the previous harvest, with total ethanol production expected to reach 33.17 billion liters, significantly boosted by corn[(3)].

Corn starch, composed of amylose and amylopectin, presents processing challenges due to its semicrystalline structure and low solubility in cold water. Therefore, corn ethanol production involves a high temperature wet liquefaction step before the enzymatic saccharification to produce the glucose syrups. The entire liquefaction–saccharification step can take up to 48 hours. Currently, enzymatic methods are the primary approach for the hydrolysis of starch, but these reactions occur slowly due to the semicrystalline nature of starch [(4)].

To aid the enzymatic hydrolysis process, the conventional pretreatment technology in the ethanol industry is jet-cooking, a hydrothermal process operating at high temperatures, which can be energy-intensive. A promising alternative to jet-cooking is ball milling, an effluent free environmentally friendly and safe technology that modifies starch properties towards enzymatic hydrolysis. However, ball milling is also energy-intensive, making shorter grinding times more viable [(5)].

This work aims to evaluate ball milling to process corn aiming starch ethanol production, contributing to sustainable energy practices and exploring alternative methods for processing agricultural commodities.

2 MATERIAL & METHODS

The corn grains utilized in this study were generously provided by Embrapa Agroindústria de Alimentos (Rio de Janeiro, Brazil). Initially, the corn grains were milled using a knife mill (Pulverisette 19 Fritsch®), sieved through a 1 mm screen, and stored at room temperature. For pretreatment, the milled corn underwent ball milling (MM 400, Retsch, Germany). Samples were milled at 30 Hz in a 45 mL milling pot containing a single 15 mm sphere, for a duration of 4 minutes at room temperature.

To characterize the starch before and after pretreatment, several analyses were conducted. The amylose/amylopectin ratio was determined using a commercial kit from Megazyme, following the manufacturer's instructions. X-ray diffraction patterns of the milled and ball-milled corn were obtained using an X-ray diffractometer (MiniFlex II, Rigaku Americas Corporation, USA) with Cu Kα radiation at 40 kV and 15 mA. Water absorption index (WAI) and water solubility index (WSI) were measured according to Faushen et al. (2022). FTIR spectra were recorded using an FTIR spectrophotometer (Thermo Nicolet NEXUS 470) across the range of 4000–400 cm^{-1} at a resolution of 2 cm^{-1} from 32 scans. The spectral data were processed using Origin 9.5 software

(OriginLab Corporation, USA), noting absorbance amplitudes at 995 cm^{-1} and 1022 cm^{-1} for each sample spectrum. The changes in the morphology of the starch were observed using a Scanning Electron Microscope (SEM) at different magnifications, utilizing an Inspect S50-FEI microscope.

For enzymatic hydrolysis, 2 g (dry mass) of both ball-milled and milled corn at 10% solids loading were mixed with 131.5 IU/g of Liquozyme ZpH (alpha-amylase) and 16.17 IU/g of Spirizyme Ultra XHS per gram of starch. The mixture was prepared in a 50 mM citrate buffer at pH 4.8, resulting in a total assay mass of 20 g. The mixture was incubated at 65 °C and 200 rpm for 0, 3, 6, and 24 hours. Post-hydrolysis, glucose and maltose levels were measured using high-performance liquid chromatography (HPLC) (ULTIMATE 3000, Thermo Scientific) with a refractive index detector (RefractoMax 521, ERC Inc.), as described by Faushen et al. (2022)[6].

3 RESULTS & DISCUSSION

The crystalline structure of milled corn and ball-milled corn was investigated using XRD analysis. Starch granules exhibit three distinct crystalline polymorphic structures (A, B, and C), which are determined by their botanical origin, amylose concentration, and arrangement of the amylopectin double helices[7,8]. The milled corn exhibited a distinctive A-type crystallinity, characterized by significant peaks at 15°, 17°, 18°, and 23°, with a crystallinity index of 18%, consistent with classifications for corn starch. Before pretreatment, the peaks at 15°, 17°, 18°, and 23° remained unaltered, and, contrary to expectations, the ball milling pretreatment applied in this study did not significantly alter the crystalline profile. Typically, ball milling pretreatment disrupts biomass structure through mechanical energy from impact, compression, attrition, and shear between balls, biomass, and the reactor surface. However, in this work, ball milling was conducted with a single sphere, primarily impacting the biomass against the reactor surface, possibly preserving biomass crystallinity.

FTIR spectral analysis elucidated how ball milling affected the properties of milled corn. Absorption bands around 3300-3600, 2900, 1150, and 1000-1100 cm^{-1} indicated the presence of OH, C-H, C-O-C, and C-O functional groups, respectively[9]. Comparing milled and ball-milled corn spectra revealed no new chemical bonds or functional groups formed during ball milling, suggesting minimal alteration in chemical composition (Figure 1a). However, ball milling intensified the main FTIR absorption bands, particularly those related to starch, such as 3650-3000 cm^{-1} and 1200-800 cm^{-1}[8]. The band around 3400 cm^{-1} corresponds to O–H stretching vibrations, indicating increased hydroxyl group availability in milled corn, potentially enhancing its hydrophilicity and paste-forming ability in cold water. The region from 930-400 cm^{-1} specifically reflects the helical secondary structure of starch. Ball milling increased band intensities in this region, indicating greater α-glucan polymer conformations and reduced helical content, suggesting lower starch order and crystallinity[10]. Ball-milled corn exhibited higher disorderliness in SEM images, showing reduced granule size and rougher, irregular surfaces compared to untreated starch granules (Figure 1b). This polymer disorganization facilitates enzymatic access, potentially enhancing glucose production during bioethanol fermentation.

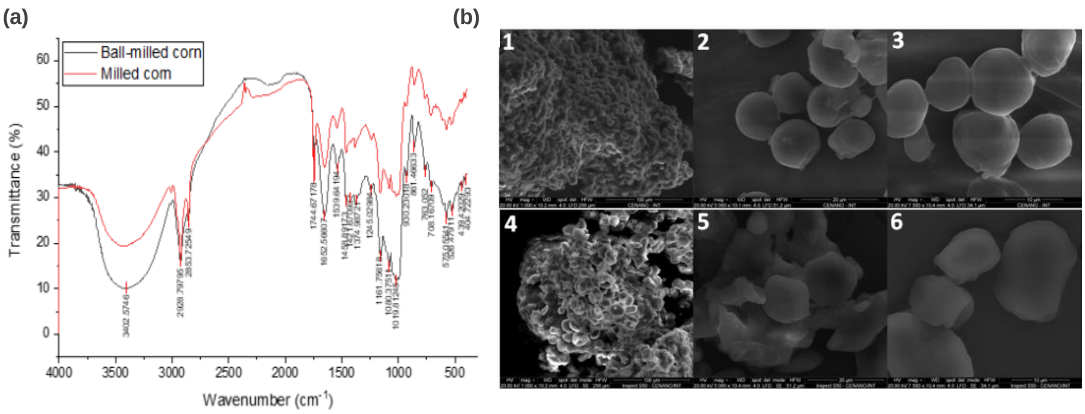

Figure 1: (a) FTIR spectra of milled and ball-milled corn. **(b)** SEM micrographs of milled corn (1, 2, and 3) and ball-milled corn (4, 5, and 6) at different magnifications.

WAI measures the amount of water taken up by the sample when immersed in excess water, which depends on the availability of hydrophilic groups that bind to water molecules[11]. The value of WSI indicates the solubility of samples in water and evaluates how much soluble polysaccharides are liberated from the starch granule in excess water[12]. After ball milling pretreatment, an increase of 43% and 27% in the WAI and WSI values were observed, respectively. The higher WAI and WSI values for the pretreated corn indicate that the starch structure is more exposed in that material, which favors water absorption and the solubilization of previously inaccessible components.

In relation to the ratio of amylose and amylopectin, a higher amylose content indicates a more resistant starch, which is more challenging to break down. However, several studies have shown that ball milling pretreatment increases the amylose portion due to the disruption of amylopectin chain branches, which occurs over time[5], and an elevated ratio of amylose content may culminate in the formation of amylose-lipid complexes, diminishing the efficacy of enzymatic hydrolysis[13]. In this study, the starch was composed of 22.9% of amylose and 50.5% of amylopectin and, after pretreatment, the amylose and amylopectin content remained unchanged.

The results of enzymatic hydrolysis of milled corn and ball-milled corn indicate that the pretreatment led to a higher sugar release, with concentrations of 68.7 g/L of glucose plus maltose, whereas the control concentrations were 31.2 g/L of glucose plus maltose, both measured after 3 hours of hydrolysis. Regarding yield, following a three-hour hydrolysis period, a starch yield of 79.8% was attained, demonstrating a productivity of 26.6 g/L/h. This achievement corresponds to a noteworthy 119.5% escalation in yield when compared to untreated corn (control).

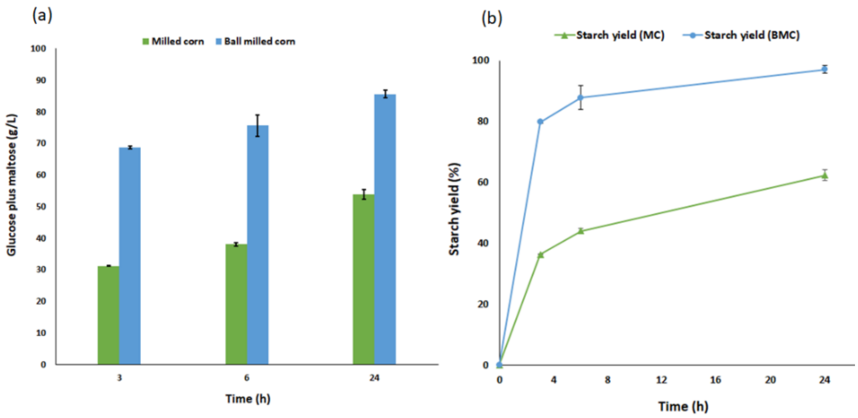

Figure 2: Glucose plus maltose concentration (g/L) **(a)** and starch yield (%) for enzymatic hydrolysis of milled corn (MC) and ball-milled corn (BMC) with 10% of solid loading **(b)** *The bars represent the glucose plus maltose concentration and the lines represent the starch yield.

In ethanol production from starchy biomass, effective starch hydrolysis is crucial as it converts starch into glucose, the substrate for fermentation. Typically, this involves gelatinization, liquefaction, and saccharification stages, with gelatinization disrupting starch granules to form a viscous solution. However, this study demonstrates that ball milling can bypass the gelatinization step by mechanically breaking down the starch granules, enhancing enzymatic hydrolysis efficiency. This dry milling process conserves water and avoids using chemicals, making it a more environmentally friendly and potentially cost-effective approach for industrial applications.

4 CONCLUSION

The study shows the potential of ball milling as a clean, efficient, and environmentally friendly method for starch processing to produce high yields of glucose syrups. Despite the well-known milling high energy consumption, the results regarding the short milling time of four minutes coupled with the short enzymatic hydrolysis time of six hours could counterbalance the overall energy consumption of the process. It is noteworthy that this pretreatment leads to significant improvements in enzymatic hydrolysis, attributed to an increase in free OH groups, disorganization of starch granules, an increase in water absorption index (WAI) and water solubility index (WSI), and a slight reduction in crystallinity, rendering the starch more accessible for enzymatic action.

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ACKNOWLEDGEMENTS

The authors gratefully acknowledge Sinochem Petróleo Brasil LTDA and the Agência Nacional de Petróleo, Gás Natural e Biocombustíveis (ANP) for financing this project under the obligation to carry out investments in research, development, and innovation (RD&I) - Project SI-22-002. The authors also thank the National System of Nanotechnology Laboratories (MCTI/SisNANO/INT-CENANO-CNPq - Process number 442604/2019-0) for analytical support. This work was supported by the Brazilian National Council for Scientific and Technological Development (CNPq - grant numbers 429529/2018-0 and 306978/2022-9).