Structural influence of planetary ball milling pre-treatment in kernel cake of macauba probed by ^{13}C CPMAS NMR spectroscopy

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ABSTRACT

The macauba is a palm tree mainly found in Brazil and it has garnered commercial interest due to the oils from its fruit pulp and kernel, which are rich in fatty acids suitable for the food, cosmetic, pharmaceutical, chemical, and energy industries. Oil extraction produces coproducts rich in carbohydrates, such as pulp cake and kernel cake. However, these carbohydrates are in recalcitrant and crystalline forms, requiring pretreatment to access them. Mechanochemical pretreatment using a planetary ball mill offers several advantages. This study evaluates the ball milling pretreatment of macauba kernel cake at 400 rpm for 30 to 240 minutes using ^{13}C CPMAS NMR spectroscopy, followed by enzymatic hydrolysis. Although enzymatic hydrolysis trials are ongoing, it has been observed that ball milling pretreatment increases amorphization of biomass's main structural constituent from 60 minutes. ^{13}C ssNMR has proven effective in characterizing PBM (planetary ball mil) development, with results not previously reported for macauba biomass.

Keywords: *Acrocomia aculeata*, macauba, ^{13}C CPMAS NMR, mannan, ball milling.

1 INTRODUCTION

Mechanochemical biomass pretreatment processes are gaining increasing attention to meet the demands of Greener Chemistry and Bioeconomy. Mechanochemical pretreatment has several advantages, such as processing a wide range of material types, reducing particle size, removing recalcitrance, and decreasing the crystallinity of polysaccharides in biomass. It offers a more sustainable approach due to low water consumption, no losses of fractions of interest, without generation of inhibitors and corrosives, and operation at room temperature. Similarly, using Bioeconomy and Circular Economy concepts based on the valorization of renewable resources can increase the added value of agribusiness products, promote sustainability in the sector, and reduce environmental impacts. In previous work[1] dry grinding ball milling mechanochemical pretreatment was used to analyze lignocellulosic biomass processing. This method altered the structure of the biomass components and reduced the degree of cellulose crystallinity[2]. The resulting changes were evaluated by ^{13}C solid-state nuclear magnetic resonance (ssNMR). It was possible to correlate these changes with the increase in enzymatic hydrolysis yield, measured in terms of glucose and xylose. In this context, coproducts from macauba biomass (*Acrocomia aculeata (Jacq.) Lodd. ex Mart.*) can also be studied using ball milling to reduce their recalcitrance and facilitate the next steps towards the production of biofuels and bioproducts.

The macauba is a species of evergreen palm tree native to tropical regions. The natural occurrence of macauba species is in the Central-South American continent, mostly in the Brazilian territory. The advantages of macauba over other oilseeds are related to its oil production potential, high rusticity, lower water requirements, occurrence in acidic soils, drought tolerance, and adaptable to cultivation in open areas and pastures, along with an easy harvesting system and logistics. These characteristics make it an interesting alternative to the cultivation of African oil palm (*Elaeis guineenses*), in addition to the significant generation of coproducts. The coproduct of macauba explored in this work is the endosperm cake (kernel cake) with part of the endocarp. The endosperm has characteristics similar to those of other palm species in that it is considered non-cellulosic, non-lignified, and rich in lipids, proteins and polysaccharides stored in the cell wall[3]. Aromatic lignin structures are also found due to the presence of the endocarp lignocellulosic during fruit processing[4].

This work aims to characterize the coproduct endosperm cake (kernel cake) of macauba by ^{13}C ssNMR in terms of their primary constituents *in natura* and pretreated forms in planetary ball milling (PBM) and to understand the effects of this pretreatment. Solid-state ^{13}C CPMAS NMR allows for the characterization of the form of mannan to support the preparation of enzyme formulations.

2 MATERIAL & METHODS

The macauba endosperm cake was donated by the company INOCAS® (Minas Gerais, Brazil) for the 2022 harvest. The batch of biomass was initially milled in a cutting mill model Pulverissette 19 (Fritsch, Germany) equipped with a sieve of 1.5 mm screen for particle size reduction. Then, 6.5g samples underwent lipid removal using the Soxhlet system in 6 hours with hexane solvent. After oil removal, the samples underwent a mechanochemical pre-treatment in a planetary ball milling room temperature in PBM (Retsch PM 400) with a rotation frequency of 400rpm for 30-, 60-,120-, 180-, and 240-minutes. The pre-treatment conditions chosen are exploratory and follow the protocols adopted by Bioethanol Laboratory (IQ/UFRJ). After pre-treatment the samples *in natura* and treated were analyzed by ^{13}C CPMAS NMR. Solid-state NMR analysis was performed using a Bruker Avance III 400MHz (9.4T) spectrometer operating at 100.63MHz (ν_{Larmor}[13]C), 4mm probe, pulse sequence of cross-polarization under magic-angle spinning (CPMAS), MAS rate of 10kHz, 0.5 ms contact time, recycle delay of 5s and 1000 scans. All NMR spectra obtained

were processed using TopSpin® software, version 4.1.4, with the use of 30 Hz line broadening and automatic baseline adjustment and manual phase adjustment to minimize baseline noise with first-order polynomial model baseline used in the processing. For all experimental spectra obtained, the deconvolution procedure was applied in the working window of 104-60ppm using the Gaussian/Lorentzian function model in TopSpin® software to obtain the width values at half height of the characteristic signals of mannan found in samples studied. Furthermore, the Ivory nut standard was used as a mannan reference.

3 RESULTS & DISCUSSION

After removing almost 100% of lipids without changing the study matrix, the results of ^{13}C CPMAS NMR spectra and chemical shift assignments for the hemicellulose (H), lignin (L), and protein (PT) from the studied biomasses are shown in Figure 1. Hemicellulose spectral signals are found in acetyl groups (CH_3 at 21 ppm and C=O at 173 ppm). Signs characteristic of lignin are found in the methoxyl group at 56 ppm. The aromatic region of aryl C is extensive and of lesser intensity between 160 and 110 ppm, in which typical signs of wood are found at 153 ppm (syringyl), 145 ppm (guaiacil and syringyl) and 115 ppm (p-hydroxyphenyl) according to the detailed study for macauba endocarp[5] and other studies to characterize lignocellulosic biomass[1] which is why less intense cellulose I signals can be seen in the samples studied, especially in the region close to 105-104ppm. However, very fine spectral signals of polymorph homomannan-I are found dominant in C1-101.7ppm, C4-81ppm, C2-75.9ppm, C3-72ppm, C5-69.8ppm and C6-61.9ppm[6,7]. Moura et al. (2010)[3] reports that the endosperm has a thicker cell wall due to the deposition of storage polysaccharides from the post-germination period, mainly hemicelluloses and mannan, which dominate the composition of the endosperm cell walls. The mannans found are of the "pure mannan" type and polysaccharides that contain less than 10% sugar residues other than mannose. Also, the high content of proteins such as arginine and glutamate stand out[3]. In addition to the characteristic domains of arginine and glutamate proteins as reported in work carried out in the macauba kernel[8]. Typical arginine signals can be found near C-157.7ppm, C_α-56.4-53.9ppm, C_δ-41.1ppm, C_β-28.7ppm and C_γ-25.3ppm. Typical glutamate signals can be found near C_δ-178.2ppm, C_α-56.4-53.9ppm, C_γ-32-30.9ppm and C_β-28.7ppm[9].
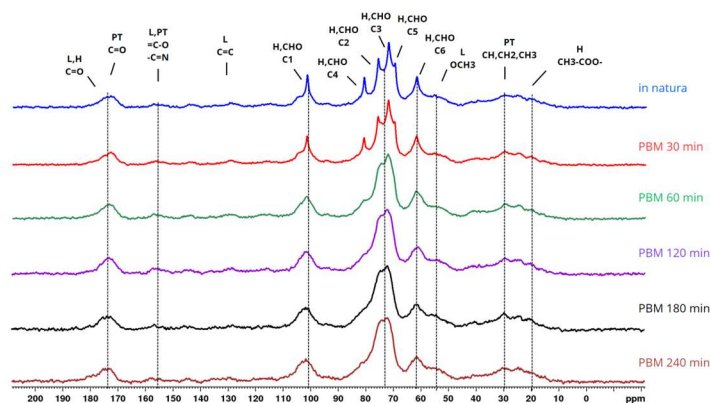

Figure 1 ^{13}C CPMAS NMR spectra of kernel cake of macauba obtained after different milling times (H-hemicellulose; L-lignin; PT- protein).

Results of ^{13}C CPMAS NMR spectra and chemical shift assignments for mannan carbon from the studied biomasses are shown in Figure 2. The chemical shifts of the Ivory nut standard are compatible with those found *in natura* samples, and the effects of pre-treatment in a ball mill can be monitored as a function of grinding time, as shown in Table 1. The six signs of the Ivory nut standard correspond to the six carbons of the β-(1→4)-linked D-mannosyl residues of the mannan-I crystalline or mannanopyranose structure or pure mannan as found in Ivory nut through C1-102ppm, C4-81.4ppm, C2-76.3ppm, C3-72.5ppm, C5-70.3ppm and C6-62.4ppm, as studied by for ivory nut mannans samples[6,10], comparable to the signals found in the *in natura* sample studied. Pure mannans are typically found in the endosperm of higher plants, which provide mechanical resistance and insolubility in water. Furthermore, pure mannan presents crystalline polymorphism in which mannan I and mannan II are often found together[11]. The suspicion that mannan II is present in this work is confirmed due to the observation of the minimum spectral signals present in C1-102.8ppm, C4-82.7ppm, C2-74.9ppm, C3-72.5ppm; C5-70.9ppm and C6-62.9ppm of the in the *in natura* sample studied. Despite that, the six signs allowed us to confirm that the polymorph mannan-I is the main polysaccharide present in the chemical constitution of the studied biomass.
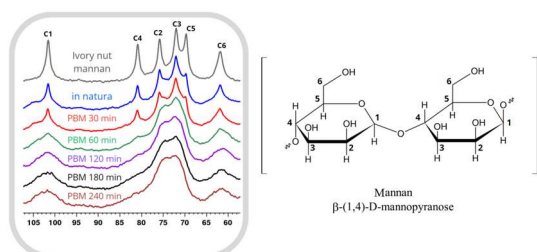

Figure 2 Comparison of ^{13}C CPMAS spectra (105-60 ppm region) of mannan samples with the Ivory nut mannan standard.

It is notable that the changes in the spectra profiles correspond to greater disorganization of the structure and its amorphization process. Small changes were identified at a grinding time of 30min. However, from 60 minutes onwards, amorphization becomes more evident, and structural changes are maintained until 240 minutes. When mixing times exceed 120 minutes, it is suggested that the structure is already very disorganized or amorphous with conditions favorable to enzymatic attack. In other words, since the chemical shifts of the six carbons of mannose skeletal did not vary mainly under the grinding times studied, the effects of PBM on biomass were followed through the width at half-height parameter ($\Delta v\frac{1}{2}$) after deconvolutions of mannan carbons. The results are listed in Table 1.

Table 1 Variation of width at half-height ($\Delta v\frac{1}{2}$, Hz) of mannan domains measured by ^{13}C CPMAS NMR.

| SAMPLE | $\Delta v\frac{1}{2}$ (Hz) | | | | | |
| | C1 | C4 | C2 | C3 | C5 | C6 |
	101.7ppm	81ppm	76-74ppm	72ppm	71-70ppm	62-61ppm
In natura	171	220	222	344	96	453
PBM 30min	196	584	299	395	84	360
PBM 60min	492	263	710	227	70	425
PBM 120min	585	311	633	410	267	593
PBM 180min	626	568	739	249	134	552
PBM 240min	662	184	626	398	317	558

The increase of $\Delta v\frac{1}{2}$ in the spectral signals expresses the loss of crystallinity of the main polysaccharide studied. The $\Delta v\frac{1}{2}$ values measured for the six signals of the mannose skeletal showed an increase with the grinding times, mainly for carbons C1 and C6. Thus, this evidences an increase in amorphization of the mannan portion under the influence of grinding times, with a greater impact from 60 minutes for C1, the anomeric carbon, from 171 Hz to 492 Hz. The other signals also suffer the effect of grinding treatment, although the values measured were not as straightforward as in the case of C1 carbon.

4 CONCLUSION

The mechanochemical pre-treatment by ball milling has several advantages and is part of the biorefinery's new approach. PBM modified the crystalline mannan distribution for the biomass evaluated, increasing amorphization. That result is not shown in the literature so far for macauba biomass. Also, ^{13}C ssNMR has proven useful in the PBM's development characterization protocol.

REFERENCES

[1] FORTUNATO, M.R.C.; SAN GIL, R.A.S.; BORRE, L.B.; BARROS, R.R.O.; FERREIRA-LEITÃO, V.S.; TEIXEIRA, R.S.S. 2023. BioEnergy Research 16, 2068–2080.
[2] LIU, H.; CHEN, X.; JI, G.; YU, H.; GAO, C.; HAN, L.; XIAO, W. 2019. Bioresource Technology 286, 121-364.
[3] MOURA, E.F.; VENTRELLA, M.C.; MOTOIKE, S.Y. 2010. Sci. Agric. (Piracicaba, Braz.), v.67, n.4, 399-407.
[4] ALVES, J.L.F.; SILVA, J.C.G.; MUMBACH, G.D.; ALVES, R.F.; SENA, R.F.; MACHADO, R.A.F.; MARANGONI, C. 2022. Thermochimica Acta 708, 179-134.
[5] LEÓN-OVELAR, R.; FERNÁNDEZ-BOY, M.E.; KNICKER, H. 2022. Horticulturae 8, 739.
[6] MARCHESSAULT, R.H. TAYLOR, M.G. 1990. Can. J. Chem. 68, 1192.
[7] JARVIS, M.C. 1990. Carbohydrate Research, 197, 276280.
[8] LESSA, V.L.; OMURA, M.H.; PACHECO, S.; OLIVEIRA, E. B.; BARROS, F.A.R. 2022. Food Research International 161, 111-848.
[9] BREITMAIER, E.; VOELTER, W. 1990. Carbon-13 NMR Spectroscopy. High-Resolution Methods and Applications in Organic Chamistry and Biochemistry. Third edition.
[10] GIDLEY, M.J.; McARTHUR, A.J.; UNDERWOOD, D.R. 1991. Food Hydrocolloids Vol.5 no.112 pp.129-140.
[11] PETKOWICZ, C.L.O.; REICHER, F.; CHANZY, H.; TARAVEL, F.R.; VUONG, R. 2001. Carbohydrate Polymers 44, 107–112.

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