MONITORING THE MANNAN-OLIGOSACCHARIDES PRODUCTION FROM AÇAÍ SEEDS HYDROLYSIS USING ORGANIC ACIDS

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ABSTRACT

Oligosaccharides are the main substances among the commercially available products with prebiotic effects, i.e., substances that promote the selective growth of beneficial microorganisms. Among these, mannan-oligosaccharides (MOS) are non-digestible carbohydrates consisting of 2-7 mannose units, recognized as emerging prebiotics. MOS can be of two types: α-MOS, derived from *Saccharomyces cerevisiae* cell walls, and β-MOS, produced from plant cell walls rich in mannan. In this context, açaí seeds could be used to produce β-MOS, because they are rich in mannan (~50%). However, due to the difficulty in hydrolyzing the mannan from açaí seeds and the need to control conditions to favor the accumulation of β-MOS, a method to monitor reaction conditions is necessary for a faster screening. Among the possible techniques for MOS detection, thin layer chromatography (TLC) is a fast and easy technique and could be useful in screening kinetic conditions to produce β-MOS. Thus, the objective of this study was to evaluate the TLC as a technique for monitoring the production of β-MOS from açaí seeds, using dicarboxylic acids hydrolysis. This study is expected to contribute to the establishment of a technique that can assist in the transformation process of açaí seeds into a bioproduct of interest.

Keywords: mannan-oligosaccharides. prebiotics. organic acid hydrolysis. açaí seeds. agro-industrial residues.

1 INTRODUCTION

According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), prebiotics are defined as "a substrate that is selectively utilized by host microorganisms, conferring a health benefit"[1]. Despite the increase in the number of prebiotic studies, there are still few commercial products with effects proven by randomized clinical studies. Currently, this market is mainly represented by oligosaccharides[2,3]. In this context, plant agro-industrial residues represent raw materials for obtaining oligosaccharides with prebiotic potential, aligning with the concepts of the Circular Economy. Among these oligosaccharides derived plant biomass are the mannan-oligosaccharides (MOS). MOS are oligosaccharides composed of 2-7 units of D-mannose and may or may not contain other sugars in branching positions of the main chain. They are already widely used in animal feed due to their claimed prebiotic potential[4]. Although widely used in practice, mainly as feed additives, MOS are considered emerging prebiotics by ISAPP, as their prebiotic efficacy is still under scientific investigation. There are two types of MOS: α-MOS obtained from the thermal treatment of the yeast cell wall, usually from *Saccharomyces cerevisiae*, with α-1,6 linkages, and being more studied and currently the most commercialized; and β-MOS, produced from the plant cell wall of feedstocks rich in mannan, consisting of β-1,4 linkages[5,6]. Among the potential residues for producing β-MOS, açaí seeds stand out due to their high content of mannan (about 50% of its dry mass) and high availability[7].

Açaí seeds (*Euterpe oleracea* Mart.) accumulate in high quantities in the Amazon region, due to the growth of açaí production in the region and are therefore considered residue. In 2022, the production of açaí exceeded 1.9 million tons (https://sidra.ibge.gov.br/), with seeds representing 85% of the fruit mass, leading to the generation of more than 1.65 million tons of seeds annually. This accumulation results in environmental, and public health problems. Therefore, finding ways to better utilize these seeds is highly desirable. Obtaining of β-MOS from agro-industrial residues, such as açaí seeds, can be achieved through the partial hydrolysis of its mannan. However, according to data from our group, the linear mannan present in açaí seeds has a crystalline structure[7,8], which makes it difficult to break and consequently to obtain β-MOS. In an attempt to overcome this recalcitrance, oxalic and maleic acids have been evaluated as an alternative for the release of β-MOS. These organic acids are weaker than the usual inorganic acids and have been proven to be adequate for açaí seed mannan hydrolysis[9]. The goal is to achieve a controlled partial hydrolysis of mannan. However, it is essential to monitor the process conditions to optimize and improve the yield of the product of interest, as uncontrolled processes conditions may lead to the production monosaccharides instead of the accumulation of oligosaccharides. Therefore, rapid detection and monitoring techniques are required and commonly adopted to understand how the reaction kinetics are progressing.

Several techniques are available for detecting MOS, including electrophoretic gels and thin layer chromatography (TLC)[10]. TLC is one of the most common methods to monitor the degree of polymerization (DP) of the oligosaccharides. It utilizes silica gel, alumina or cellulose coated plates as stationary chromatographic phase and a solvent as the mobile phase for the separation of oligosaccharides from different DPs[10]. Hydrolysis products are visually as spots on the plate that migrate at different rates based on their size and polarity; for example, low DP MOS move faster and appear higher above the plate[10]. TLC also allows for the visualization of different samples on a single plate, allowing direct comparison between them[10]. TLC is a fast, easy, and low-cost method for analyzing oligosaccharides and monitoring reaction kinetic almost in real time. Therefore, this work aims to evaluate the TLC as a technique for monitoring the production of β-MOS from the hydrolysis of açaí seeds using dicarboxylic acids.

2 MATERIAL & METHODS

The acid hydrolysis of açaí seeds were carried out using diluted oxalic or maleic acids under various conditions to evaluate reaction parameters such as time, temperature, and acid concentration (**Table 1**). Each parameter range was chosen according to the literature and previous studies of the group. These experiments were intended to assess preliminary conditions to be used as basis for an experimental design aiming at optimizing the reaction conditions for MOS yield in the subsequent steps of this work.

Table 1. Description of the different conditions evaluated in the hydrolysis of organic acids.

Oxalic acid assays				
Assay	**Initial CSF**	**organic acid concentration (% m/v)**	**Temperature (ºC)**	**Time (min)**
TO1	0.93	4	115	25
TO2	1.03	2	115	40
TO3	1.33	1.32	130	32.5
TO4	1.39	3	121	60
Maleic acid assays				
Assay	**Initial CSF**	**organic acid concentration (% m/v)**	**Temperature (ºC)**	**Time (min)**
TM1	0.98	0.8	150	30
TM2	0.99	3	121	60
TM3	1.08	4	121	60
TM4	1.15	5	121	60

Initial CSF: initial combined severity factor; SF: solid fraction. TO1, TO2, TO3 and TO4: test 1, 2, 3 and 4, respectively, with acid oxalic. TO1- TO4: tests with acid oxalic. TM-TM4: tests with maleic oxalic. The conditions of the tests TO4 and TM4 were based on a previous patent of the group[11]

Following these procedures, samples from the liquid fractions of the organic acid hydrolysis assays were characterized according to the methodology described by the Analytical Procedure of the National Renewable Energy Laboratory (NREL)[12], based on the total hydrolysis using sulfuric acid, and analyzed for their sugar content by HPLC. The amount of MOS was determined by the difference between the amount of mannose before and after the post-total hydrolysis, which was conducted during the characterization of the liquid fractions. To monitor the process and detect MOS, the same liquid fractions were qualitatively analyzed by TLC, performed on silica gel plates $60F_{254}$ (Merck Art 20 x 20 cm, Darmstadt, Germany). Standard solutions (10 µg/mL) and samples were applied (spot) in equal volume (1 µl) and then placed in a chromatographic tank for elution with a solvent mixture consisting of n-butanol:acetic acid:water (volume 2:1:1)[13]. Subsequently, the completely eluted plate was subjected to color development by spraying a solution composed of 0.2 g of diphenylamine, 0.2 mL of aniline, 10 mL of acetone, 1.5 mL of phosphate acid, followed by heating at 120 ºC for approximately 5 min.

3 RESULTS & DISCUSSION

First, the liquid fractions from the each of the four hydrolysis conditions, evaluated for either oxalic or maleic acid, were analyzed using the TLC method. These conditions were named TO1-TO4 for oxalic acid and TM1-TM4 for maleic acid. Results are shown in **Figure 1.**

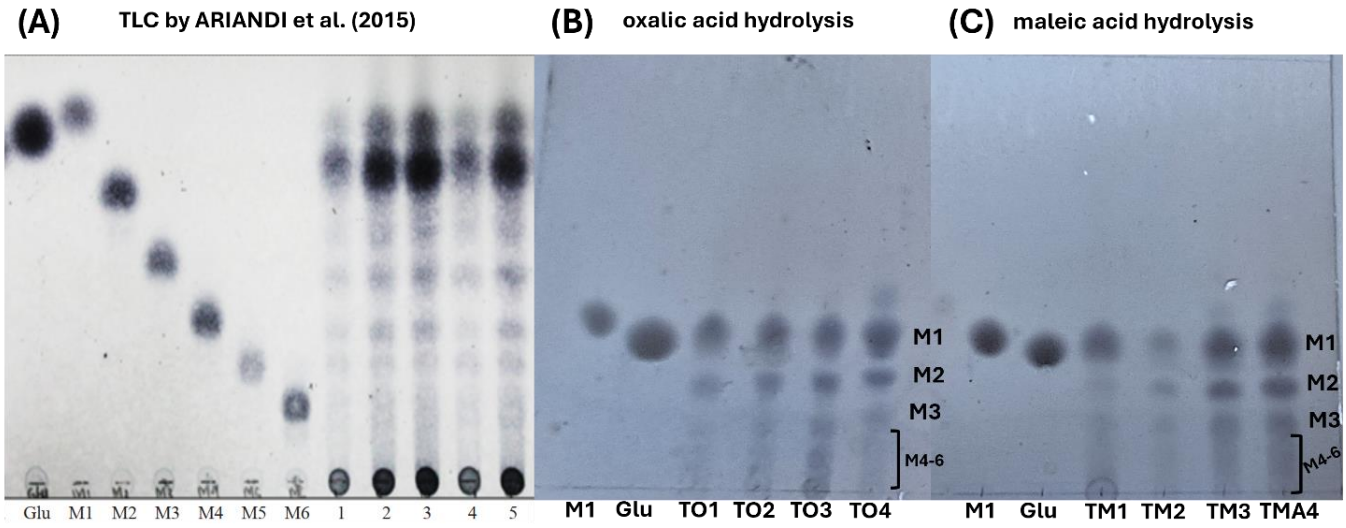

Figure 1. Analysis of MOS by TLC. First figure (A) was TLC analysis performed by ARIANDI et al. (2015)[13] and the other two figures (B and C) was TLC analysis of liquid fraction obtained in dicarboxylic acids hydrolysis, oxalic acid and maleic acid, respectively. Glu: glucose; M1: mannose; M2: mannobiose; M3: mannotriose; M4: mannotetrose; M5: mannopentose; M6: mannohexaose. TO1- TO4: tests with acid oxalic. TM-TM4: tests with maleic oxalic. The conditions of the tests TO4 and TM4 were based on a previous patent of the group[11].

Based on the TLC analysis, the hydrolysis of açaí seed with dicarboxylic acid produced mannose and a mixture of β-MOS with different DP, primarily low DP MOS, such as mannobiose and mannotriose. Additionally, in the TO4, TM3 and TM4 assays, a spot appeared above the mannose, corresponding to an unidentified compound. Through the qualitative analysis of the TLC spots, there were indications that the oxalic acid condition TO3 was the most favorable for β-MOS production, while TM4 and TM5 assays with maleic acid seem equally promising for β-MOS production, as evidenced by the darker spots compared to the

other tests. Then, to determine whether the qualitative analysis of the TLC plates corresponded to quantitative results, the same liquid fractions were characterized to quantify the mannose and β-MOS content. The results are shown at Table 2.

Table 2. Results of acid hydrolysis preliminary tests of açaí seeds for release of β-MOS.

Oxalic acid assays				
Assay	**Mannose (g/L)**	**β-MOS (g/L)**	**Mannose yield (%)**	**β-MOS yield (%)**
TO1	5.8	7.2	4.3	5.4
TO2	7.1	7.2	5.3	5.4
TO3	7.7	7.7	5.8	5.7
TO4	19.6	5.1	14.6	3.8
Maleic acid assays				
Assay	**Mannose (g/L)**	**β-MOS (g/L)**	**Mannose yield (%)**	**β-MOS yield (%)**
TM1	3.6	7.8	2.7	5.8
TM2	9.8	3.2	7.8	2.5
TM3	12.1	10.3	9.6	8.2
TM4	17.8	10.4	14.2	8.2

TO1- TO4: tests with acid oxalic. TM-TM4: tests with maleic oxalic. The conditions of the tests TO4 and TM4 were based on a previous patent of the group[11].

The concentration of β-MOS ranged from 5.1 to 7.7 g/L for reactions with oxalic acid and from 3.2 to 10.4 g/L for reactions with maleic acid. Interestingly, the conditions indicated by TLC as the most promising corresponded to those with the highest β-MOS concentrations. The findings reported by Murillo-Franco et al. in 2023 and 2024[14,15] obtained between 8-10 g/L and approximately 12 g/L under optimized conditions, respectively, of β-MOS of açaí seed with DPs between 2-5 and mannobiose being the predominant product in second study, which corroborates the data obtained with our study. For the maleic acid reactions, it was observed that the higher acid concentration and lower temperature led to higher β-MOS yields. This correlation was evident on the TLC plates, where the TM3 and TM4 assays showed darker spots compared to the other tests. For the oxalic acid reactions, the TO3 assay yielded the highest β-MOS concentration, which also reflected in the TLC plates, where the TO3 assay showed the darkest spots, indicating a higher β-MOS concentration. However, the results indicate relatively low concentrations and a yield of β-MOS, suggesting that further studies are needed to find more effective hydrolysis conditions. Given that characterizing the hydrolysate and performing HPLC analysis are time-consuming and expensive, using the TLC method to monitor conditions will be an asset for this study. TLC will allow for real-time monitoring of the process, enabling adjustments to acid concentration and temperature to increase β-MOS yields. Additionally, once appropriate conditions are defined, TLC will be crucial for monitoring the kinetics of the reaction and determining the optimal time to stop the reaction to favor β-MOS production over complete hydrolysis to mannose. Furthermore, TLC will be helpful in future steps when investigating chemoenzymatic routes to produce β-MOS, as it can provide insights into the cleavage patterns of different endo-β-1,4-mannanases.

4 CONCLUSION

According to the preliminary results obtained, the use of dicarboxylic acid is promising for obtaining β-MOS. However, the yields obtained using both oxalic and maleic acids are still very low. Therefore, additional acid hydrolysis assays must be conducted with appropriate adjustments to increase the yield of the desired product. In this context, the TLC technique has proven to be an excellent method for monitoring the reaction almost in real time, allowing for faster adjustments of the conditions. Additionally, TLC revealed that the main mannan-oligosaccharides obtained from acid hydrolysis of açaí seeds were mannobiose and mannotriose. Thus, this study will assist the future steps of hydrolysis, as well as contribute to the investigation of the breakdown of the mannan from açaí seeds by the action of the acid (chemical route) or combined with the enzymes (chemoenzymatic route) in an optimized process to achieve higher yields of β-MOS.

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