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MICROBIOLOGY

β(1,3) β(1,6) glucogalactan from *Rhizopus microsporus* var. *oligosporus*: extraction, characterization, antioxidant and α-amylase inhibitory activities

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Abstract: In this study, the Box-Behnken experimental planning was used to optimize the extraction of polysaccharides from the cell wall of *Rhizopus microspore* var. *oligosporus*, with analysis of the quantitative effects of parameters pH, temperature and extraction time for polysaccharide yield. The optimal conditions for extraction were determined by the regression equation and evaluation of the response surface graphs, which indicated: pH 13, temperature of 120ºC and time of 60 min, with maximum yield around 18.5%. Fourier transform infrared spectroscopy analysis indicated typical polysaccharide signals. Nuclear magnetic resonance spectroscopy and monosaccharide analysis indicated a β(1,3) β(1,6) glucogalactan. The polysaccharide exhibited an average molecular weight of 120 kDa and a polymerization degree of 741. Antioxidant assays *in vitro* revealed the potential of polysaccharide in elimination of ABTS+ radical and hydroxyl radicals. EC_{50} values for free radical elimination were 7.69 and 17.8 mg/mL, for ABTS+ and hydroxyls, respectively. The polysaccharides showed potential for α-amylase inhibition with an EC_{50} of 1.66 mg/mL. The results suggest that β(1,3) β(1,6) glucogalactan from *Rhizopus microsporus* var. *oligosporus* can be used in biotechnological applications.

Key words: *α-amylase, antioxidant,* glucogalactan, polysaccharides, *Rhizopus microsporus* var. *oligosporus*.

INTRODUCTION

Filamentous fungi attract considerable commercial interest as they act as factories for a several of products. Rhizopus microsporus var. oligosporus is a filamentous fungus of economic importance in Indonesia, as it is used as an inoculum for the production of tempeh (Hyde et al. 2019). Tempeh is a protein-rich food, obtained from the fermentation of soybeans by species of Rhizopus sp. (Voidarou et al. 2021). The nature and structural characteristics of fungal biology encourage its exploration from an economic point of view.

The fungal cell wall has structures such as proteins, lipids and polysaccharides that play fundamental roles in the survival of the fungus. Cell wall polysaccharides have shown biological activities such as antioxidant, hypoglycemic, antimicrobial, anti-inflammatory, antitumour, among others (He et al. 2017).

Studies that seek to investigate potential antioxidant polysaccharides are important, considering that free radicals are molecules capable of causing cellular changes that culminate in a series of pathological conditions such as inflammation, ageing, cancer and

various diseases that impact human health (Kiokias et al. 2018). In this sense, combating the excessive generation of reactive oxygen species (ROS) is essential to prevent oxidative stress and consequent physiological injuries.

Many efforts have focused on the search for carbohydrate hydrolases inhibitors from natural sources, because of the unpleasant side effects of the inhibitors currently on the market. Kumar et al. (2018) and Mendes et al. (2023) found considerable hypoglycemic activity in polysaccharides extracted from *Agaricus Bisporus* and *Aspergillus niger*, respectively, when performing *in vitro* α-amylase enzyme inhibition assays.

The biological potential of polysaccharides is associated with their molecular characteristics, structure and solubility (Zhang et al. 2023). Therefore, characterization studies are relevant. In this study, polysaccharides were extracted from the cell wall of *Rhizopus microsporus* var. *oligosporus* and the chemical and structural characteristics were defined using Fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance (RMN) and gas chromatography coupled with mass spectrometry (CG-MS). The antioxidant and α-amylase inhibitory activities of the polysaccharide from *Rhizopus microspores* var. *oligosporus* (RMOP) were evaluated *in vitro*.

MATERIALS AND METHODS

Cultivation conditions

R. microsporus var. *oligosporus* was inoculated in PDA for 5 days at 30ºC in incubator for biochemical oxygen demand (BOD SL200/90 Incubator - SOLAB). After growth, approximately 1.0 x 10^7 spores/mL was inoculated into liquid medium containing 10 g/L of glucose, 5 g/L of peptone, 3 g/L of yeast extract and 3 g/L of malt extract diluted in distilled water (Chang et al. 2018). Submerged fermentation was carried out under constant agitation at 150 rpm in a shaker incubator (SL 222, SOLAB) at 28ºC for 120 h. Biomass was obtained from filtration and used for extraction of polysaccharides.

Polysaccharide extraction

From dry biomass (1 g), polysaccharides were extracted using 50 mL sodium hydroxide (0.1 mol.L^{-1}) at different pH values (11, 12 and 13), temperature (80, 100 and 120ºC) and extraction time (20, 40 and 60 min), following the Box-Behnken planning that totalled 15 experiments. The volume obtained was centrifuged at 8.000 g for 15 min (Model 206 BL-EXCELSA). Three volumes of absolute ethyl alcohol were added to the supernatant to precipitate the polysaccharides (Fan & Huang 2023). The mixture was stirred and kept for 12 h at 4 ºC. Polysaccharides were collected after centrifugation (Baby® I Model 206-BL FANEM centrifuge) at 8.000 g for 20 min. The precipitate containing the crude polysaccharides was lyophilized (Liotop K105). The polysaccharide percentage was determined by the phenol-sulfuric acid method (Dubois et al. 1956) and the product yield was measured in percentage of polysaccharides per unit of fungal biomass. Treatments were carried out randomly and the significance of the parameters on extraction performance was optimized using the Response Surface Methodology (RSM). Statistica® software version 10.0 and Analysis of Variance (ANOVA) were used for data analysis.

Polysaccharide separation and determination of molecular weight and degree of polymerization

RMOP (10 mg/mL) was applied to a Sephadex-G100 column (Sigma, EUA), which was previously conditioned with Citrate-Phosphate buffer (0.05 mol.L^{-1}, pH 7). Fractions of 2 mL were collected and submitted to determination of

total polysaccharide content by the phenol-sulfuric acid method (Dubois et al. 1956) and reducing sugars by the DNS (3,5-dinitrosalicylic) method (Miller 1956). The results were used to estimate the average molecular weight (MWn) and the degree of polymerization (DPn). The values were applied to the formula of Vettori et al. (2012) where:

$$DPn = \frac{\text{total carbohhydrates in µg of D-glucose}}{\text{reduction value in µg of maltose}} \times 1.9$$

$$MWn = [DPn \times 162] + 18$$

FT-IR Spectroscopy

RMOP was analysed by FT-IR spectroscopy (Spectrometer FT-IR/Varian Inova 500) covering the region from 4000 to 500 cm^{-1}, with 20 scans.

Monosaccharide composition

For the analysis of monosaccharide composition in GC-MS, the RMOP sample was hydrolysed with 3 mol/L trifluoroacetic acid (TFA) at 120ºC for 1 h according to the methodology of Yan et al. (2019) with adaptations. The sample was subjected to derivatization by silylation, from a reaction mixture of N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Sigma-Aldrich®) and pyridine. The mixture was heated at 70°C for 30 min (Sjöström & Alén 1998) and injected into the GC-MS (GCMS-QP2010 SE). The mass detector operated with electron impact ionization (70 eV) and mass scanning in the range of 30 to 600 Da.

RMN spectroscopy

The RMOP sample was dissolved in deuterated water and inserted in a spectrometer (Varian Inova 500) operating at 11.7 T. The analysis was carried out observing the ^{1}H at 500 MHz and the ^{13}C at 125 MHz with a direct detection probe. Spectra were recorded at 340 K. Tetramethyl silane (TMS) was used as chemical shift reference.

Antioxidant activity of RMOP

Scavenging of $ABTS^{+}$ radical

In the $ABTS^{+}$ radical scavenging assay (Re et al. 1999) 30µL of the RMOP sample at different concentrations (0.5, 1.0, 2.0, 4.0, 8.0, 10.0, 20.0, 30.0, 40.0, 50.0 and 60.0 mg/mL) and 3 mL of the $ABTS^{+}$ solution (7 mM) was allowed to react for 5 min under the protection of light. The Reading was carried out in a spectrophotometer (Marte spectro 560) at 734 nm. Ascorbic acid was used as a standard. Antioxidant activity rates were expressed as a percentage:

$$\text{Scavenging rate (\%)} = \frac{(A0 - A1)}{A0} \times 100$$

Where A1 is the sample absorbance and A0 is the control absorbance.

Scavenging of hydroxyl radical (OH•)

In the hydroxyl radical (OH·) scavenging assay, the reaction took place using a mixture containing 1 mL of ferrous sulphate (9 mmol/L), 1 mL of salicylic acid (9 mmol/L) diluted in absolute ethanol, 1 mL of RMOP sample in different concentrations (0.5, 1.0, 2.0, 4.0, 8.0, 10.0, 20.0, 30.0, 40.0, 50.0 and 60.0 mg/mL) and 1 mL of hydrogen peroxide (8.8 mmol/L). The mixture was incubated at 37°C for 30 min and the reading was carried out at 510 nm (Lin et al. 2023). Ascorbic acid was used as a standard. Antioxidant activity was expressed as:

$$\text{Scavenging rate (\%)} = \frac{(A0 - A1)}{A0} \times 100$$

Where A1 is the sample absorbance and A0 is the control absorbance.

The values found in the $ABTS^{+}$ and hydroxyl radical scavenging test were interpreted as the sample concentration that was capable of causing a 50% reduction of free radicals (EC_{50}).

Determination of α-amylase inhibition capacity

The assay to determine the α-amylase inhibition capacity was performed following the methodology of Gulati et al. (2012) with modifications. One hundred microliters of porcine pancreatic α-amylase solution (2 mg/mL) in phosphate buffer (pH 6.9), 100 µL of the polysaccharide sample at different concentrations (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1 and 2 mg/mL) and 100 µL of starch solution (1%). The mixture was incubated at 50ºC for 10 min and after this period it was interrupted with the addition of 200 µL of DNS reagent (3,5-dinitrosalicyl) and incubated at 100ºC for 5 min. Two milliliters of distilled water were added to dilute the reaction mixture. The reading was performed at 540 nm. Acarbose was used as a standard. The α-amylase inhibition activity was expressed as:

$$\text{Inhibition rate (\%)} = \frac{(A0 - A1)}{A0} \times 100$$

Where A1 is the absorbance of the sample and A0 is the absorbance of the control.

Statistical analysis

Antioxidant activity and alpha-amylase inhibition experiments were performed in triplicate and data were presented as mean ± standard deviation (SD) and EC_{50}. Statistical comparisons were performed using the Mann Whitney test, with a significance level of $p \leq 0.01$ using the GraphPad Prism 5.0 Software.

RESULTS AND DISCUSSION

Optimization of RMOP extraction

The Box-Behnken experimental planning allowed the optimization of RMOP extraction conditions, based on the analysis of three variables at three levels that totalled 15 experiments, including the triplicate at the central point. The results of polysaccharide yield from 1g of biomass are shown in Table I. The contrast between observed and predicted values demonstrates

Table I. Experimental planning Box-Behnken applied to the optimization of polysaccharide production from RMOP. (C): Central point; Coded values are show in parentheses.

Experiment	pH	Temperature (°C)	Time (min)	Observed values Mass obtained (g)	Predicted values (g)
1	13 (+1)	80 (-1)	40 (0)	0,1413	0,1233
2	13 (+1)	100 (0)	20 (-1)	0,0737	0,0829
3	13 (+1)	100 (0)	60 (+1)	0,1749	0,1744
4	13 (+1)	120 (1)	40 (0)	0,1415	0,1508
5	12 (0)	80 (-1)	20 (-1)	0,0218	0,0306
6	12 (0)	80 (-1)	60 (+1)	0,0084	0,0269
7C	12 (0)	100 (0)	40 (0)	0,0698	0,0703
7C	12 (0)	100 (0)	40 (0)	0,0623	0,0703
7C	12 (0)	100 (0)	40 (0)	0,0789	0,0703
8	12 (0)	120 (1)	20 (-1)	0,0320	0,0135
9	12 (0)	120 (1)	60 (+1)	0,1073	0,0985
10	11 (-1)	80 (-1)	40 (0)	0,0003	0,0000
11	11 (-1)	100 (0)	20 (-1)	0,0007	0,0012
12	11 (-1)	100 (0)	60 (+1)	0,0003	0,0000
13	11 (-1)	120 (1)	40 (0)	0,0002	0,0182

that the residual variance is sufficiently small in the applied model.

MSR is a technique that can be used to optimize and determine the optimal conditions for the extraction of polysaccharides (Tang et al. 2023). The inspection of the MSR (Figure 1) revealed that the ideal conditions for the highest yield of polysaccharides within the experimental region studied tend to be higher, including pH 13, temperature of 120ºC and time of 60 min. The maximum yield of polysaccharides under optimal extraction conditions was approximately 18.5%, with mass of 0.1848 g. Higher extraction temperatures were used in studies conducted by Yang et al. (2013) equivalent to 210ºC for 43.6 min to extract polysaccharides from *Grifola frondosa* and obtained a yield of 25.1%. Yield of 17.28% was obtained by Chen et al. (2014) from the optimal conditions using NaOH solution at 0.3 M and temperature of 80ºC for 4 h.

The behavior of the RMOP extraction response is explained by the quadratic equation given below.

$$\text{Polysaccharide Mass (g)} = 1.1626 (\pm 0.6955) + 0.0033(T) (\pm 0.0033) - 0.00002(T)^2 (\pm 0.00001) - 0.2311(pH) (\pm 0.1062) + 0.0103(pH)^2 (\pm 0.0043) - 0.01613(t) (0.0028) - 0.00004(t)^2 (\pm 0.00001) + 0.000004(T)(pH) (\pm 0.0002) + 0.00005(T)(t) (\pm 0.00001) + 0.001267 (pH)(t) (\pm 0.0002).$$

Where, (T) = Temperature in ºC and (t) = Time in minutes.

The analysis of the Pareto diagram (Figure 2) reveals that linearly (L) the pH, time and temperature variables were significant for the extraction of polysaccharides, as well as the relationship between the pH and time and temperature and time variables. Quadratic (Q) the relationship between the variables did not have a p value greater than 0.05.

The analysis of variance (ANOVA) (Table II) evaluates the quadratic model in terms of its ability to explain the behavior of the variables and the influence on the result obtained, discarding random errors. The R^2 value of 0.96 found for the quadratic model infers that 96% of the results found are explained by the proposed experimental model, with a confidence interval of 95%. For lack of adjustment, the $F_{calculated}$ value is smaller than the $F_{tabulated}$, indicating that the model was satisfactory and there is no lack of adjustment. The results show that the studied region can be applied for RMOP extraction.

In comparison, Table III presents the ANOVA result for the linear model in the optimization of RMOP extraction. Although the linear model indicates a significant regression and non-significant lack of adjustment, the quadratic model better represents the behavior of the variables.

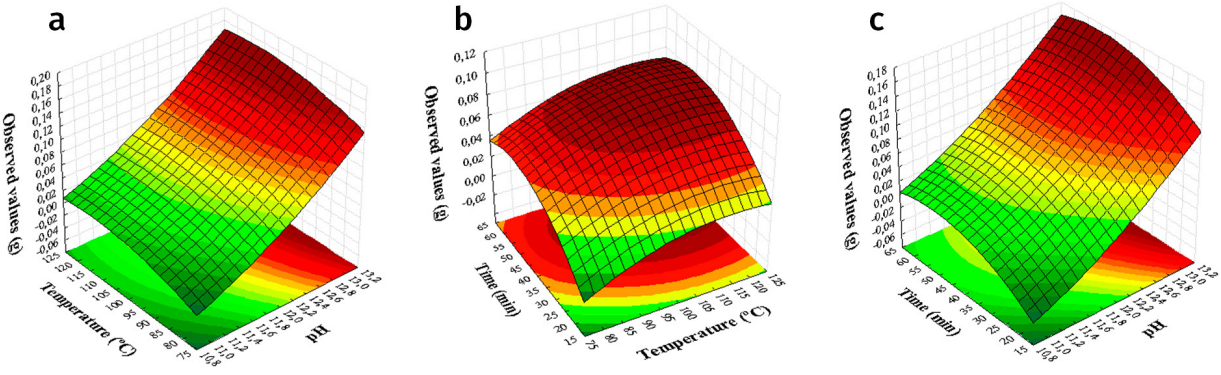

Figure 1. Surface plot for optimizing the extraction of RMOP (a) pH and Temperature (b) Time and Temperature (c) pH and Time.

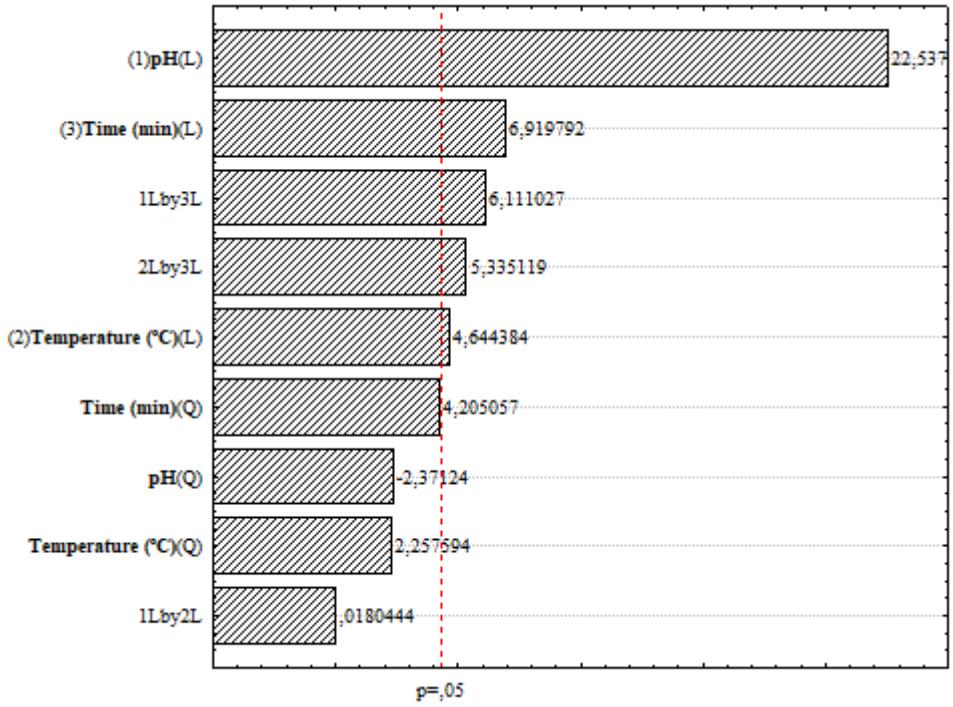

Figure 2. Pareto diagram with the significance of the variables in the RMOP extraction. (L) Linear (Q) Quadratic.

Table II. Analysis of variance – ANOVA for the quadratic model obtained in the optimization of polysaccharide extraction from RMOP. QS: Quadratic sum; df: Degrees of freedom; MS: Mean square. R^2 = 0.96

	QS	df	MS	$F_{calculated}$	$F_{tabulated}$
Regression	0,046489	9	0,005165	13,16	4,77
Residue	0,001963	5	0,000393		
Lack of fit	0,001825	3	0,000608	8,80	19,16
Pure error	0,000138	2	0,000069		
Total SS	0,048452	14			

Table III. Analysis of variance – ANOVA for the linear model obtained in the optimization of polysaccharide extraction from RMOP. QS: Quadratic sum; df: Degrees of freedom; MS: Mean square. R^2 = 0.91.

	QS	df	MS	$F_{calculated}$	$F_{tabulated}$
Regression	0,044446	6	0,007408	14,79	3,58
Residue	0,004006	8	0,000501		
Lack of fit	0,003867	6	0,000645	9,33	19,33
Pure error	0,000138	2	0,000069		
Total SS	0,048452	14			

Molecular weight and Degree of polymerization

Separation by exclusion chromatography allowed for the separation of the particular fraction of the RMOP (Figure 3). The mean MWn found was around 120 kDa and DPn of 741 according to the equation by Vettori et al. (2012). Molecular weights are directly involved with the biological activities of polysaccharides (Soltani et al. 2013). Huang et al. (2016) showed that polysaccharides with sizes greater than 100 kDa generally have biological properties.

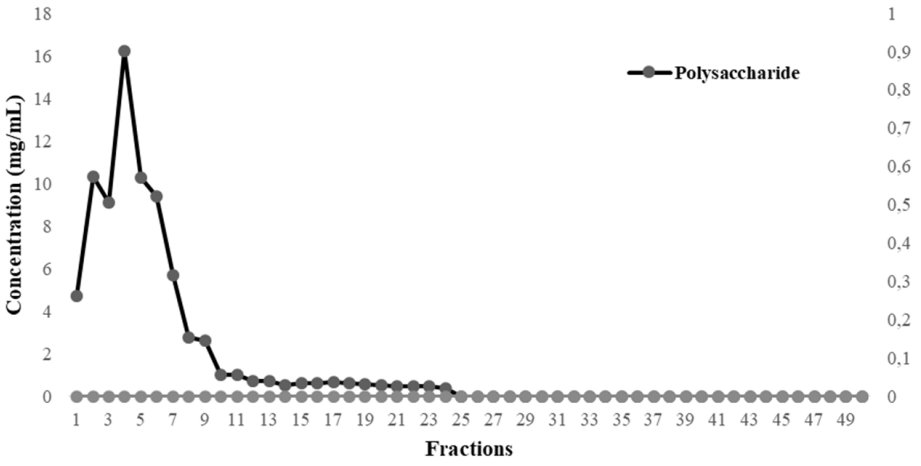

Figure 3. Separation of RMOP on Sephadex G-100 by Size Exclusion Chromatography.

FT-IR Spectroscopy

FT-IR spectroscopy was used to identify the main functional groups present in the sample. Figure 4 shows the absorption bands that represent the vibration of chemical bonds. The signal at position 3241 cm^{-1} corresponds to the stretch vibration of the OH, linked to the rings of the monosaccharides, while the signals 2926,2 cm^{-1} and 2854,6 cm^{-1} equivalent to the bonds between hydrogens and carbons of the rings of the sugars (CH). The bands at 1434 cm^{-1} and 1405 cm^{-1} may be equivalent to CH_2 bending vibration and the presence of carbonyl compounds (Venkatesan et al. 2012). Band of absorption in the 1025 cm^{-1} may indicate the skeleton of the pyranose ring (Hu et al. 2017). Bands in 865, 683 and 554 cm^{-1} are characteristics of carbohydrates, principally glucans (Mateus et al. 2017, Synytsya & Novak 2014). Carbohydrates exhibit strong absorption in the region of 1200-950 cm^{-1}, known as the fingerprint region. In this position, the intensity of the bands is typical of polysaccharides, allowing their identification (Moghannem et al. 2018). The RMOP sample indicates fragments compatible with the polysaccharide structure.

Monosaccharide composition

The GC-MS chromatogram of the hydrolysed RMOP samples shows 7 substances that were identified by comparison with library records (Figure 5). It was possible to detect the presence of glucose (96% similarity) and galactose (90% similarity) and the other substances mentioned are products pf the derivatization process. Glucose and galactose were also detected in polysaccharide samples from *Russula seneci*, in addition xylose, rhamnose and mannose in smaller proportions (Khatua & Acharya 2018). Derivatization by silylation allowed the replacement of hydrogen from polar groups such as OH by silyl groups, significantly contributing to the volatilization of the sample required for GC-MS (Su et al. 2017).

NMR Spectroscopy

Configurations of β-type appear mainly in the 101 to 105 ppm region of the NMR spectrum (Lin & Yang 2019, Pomin 2012). Thus, we can infer that the signals δ 105.19 and 105,69 in the ^{13}C spectrum (Figure 6a) corresponding to the anomeric carbon (C1) of two apparent carbohydrates, present configurations of β-type. Protons appearing in the region between 4-5 ppm in the ^{1}H spectrum (Figure 6b) are indicative of proton bound to the β-anomeric carbon. C2-C5 carbons and H2-H6 protons are generally identified between 65-87 ppm and 3.2-4.5 ppm, respectively, while C6 are generally

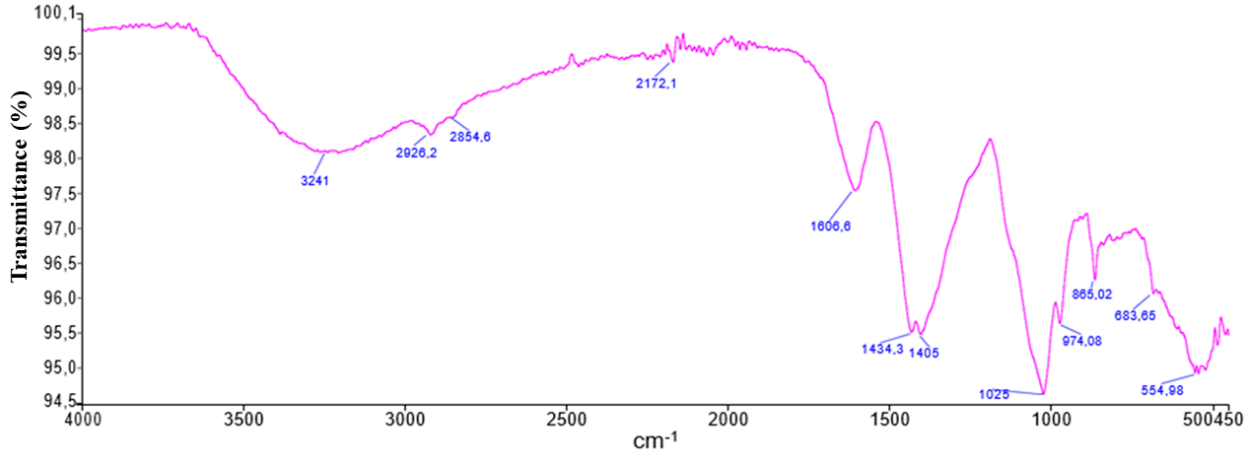

Figure 4. FT-IR spectrum of RMOP.

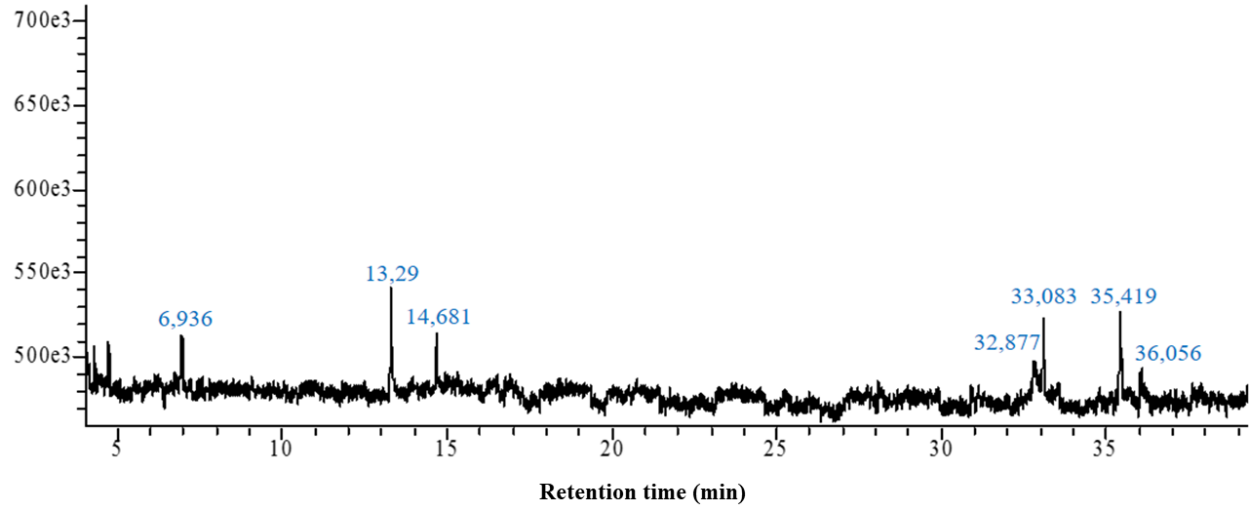

Figure 5. Monosaccharide composition of RMOP obtained by GC-MS.

rated between 55.7-64.7 ppm (Lin & Yang 2019). In this study, δ 74.56 was comprehended as bound C6 from one of the sugars, while δ 63.76 is associated with unbound C6 from another sugar. The ^{1}H and ^{13}C spectrum showed signals characteristic of polysaccharides that indicate β (1→3) bonds with β (1→6) branches. Signals displayed on GC-MS and NMR spectrum indicate that RMOP is a β(1,3) β(1,6) glucogalactan.

Antioxidant activity of RMOP

The antioxidant capacity of RMOP was determined in this study through its ability to scavenge $ABTS^{+}$ and OH^{-} radicals (Figure 7).

$ABTS^{+}$ is a free radical widely applied in assays to determine antioxidant activity (Hu et al. 2017). The ability of RMOP to scavenge $ABTS^{+}$ radicals was concentration dependent. At the concentration of 60 mg/mL the activity was equivalent to 92 ± 0.41%, while at the concentration of 1 mg/mL the activity was 50.85 ± 0.74% (Figure 7a). This finding is encouraging compared to those reported by Yuan et al. (2017) with polysaccharides from *Russula griseocarnosa* that exhibited activity of approximately 10% at a concentration of 1 mg/mL. Antioxidant compounds act by capturing $ABTS^{+}$ cation, promoting the stability of the molecule. The

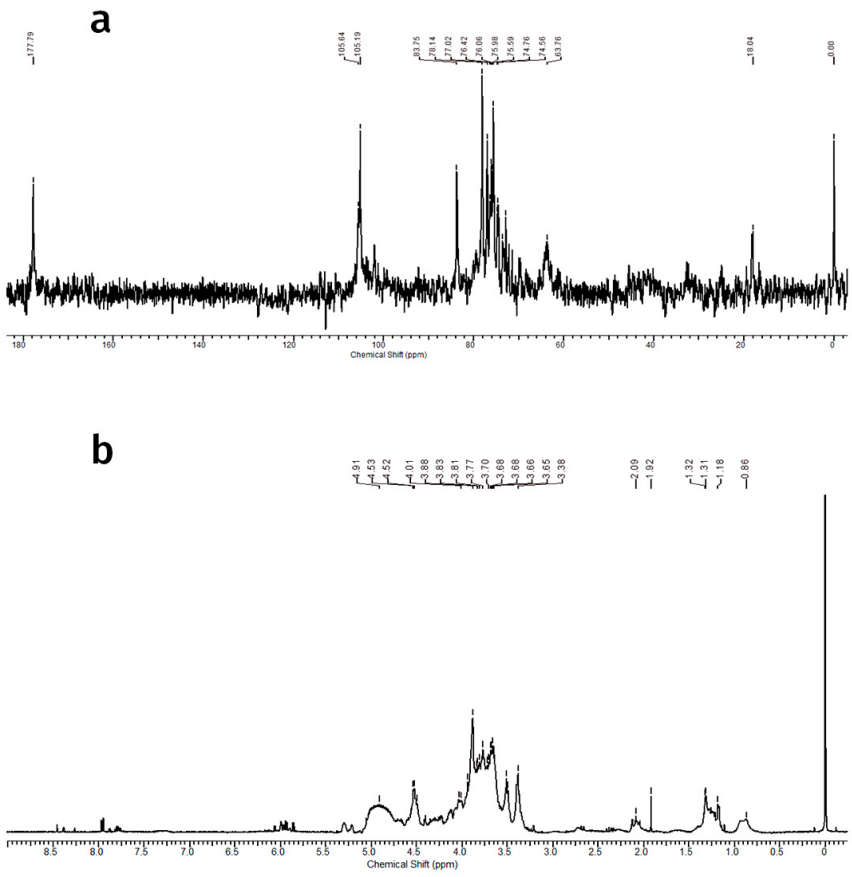

Figure 6. ^{13}C NMR spectrum (a) and ^{1}H NMR spectrum (b) of RMOP.

hydroxyl groups of polysaccharides are strongly associated with reported antioxidant activities (Hu et al. 2017).

The hydroxyls radicals have been considered the most responsible for oxidative damage to most biomolecules, such as lipids, proteins, carbohydrates and DNA (Wu et al. 2017). The antioxidant activity in the scavenging of hydroxyl from RMOP at a concentration of 60 mg/mL was 98.8 ± 0.03%, a value close to the activity of ascorbic acid used as a control (Figure 7b). The elimination of OH• is essential for the protection of living systems due to its strong reactivity, since, when uncontrolled, it can lead to numerous pathologies.

The EC_{50} was calculated by plotting the percentage of inhibition against the different concentrations of the antioxidant sample. For the $ABTS^{+}$ scavenging assay the calculated EC_{50} was 7.69 mg/mL and for hydroxyl scavenging assay was 17.8 mg/mL. The EC_{50} values indicate the concentration of the sample that leads to a 50% reduction of the initial radical concentration.

α-amylase inhibition activity

Diabetes Mellitus is characterized by an increase in blood sugar levels, which can cause numerous complications to the individual. Therapeutic approaches mainly seek to reduce fluctuations in sugar levels. To delay glucose absorption and decrease postprandial hyperglycemia, the use of carbohydrate-hydrolyzing enzyme inhibitors, such as α-amylase, is required (Thilagam et al. 2013).

RMOP showed a capacity to inhibit the α-amylase enzyme equivalent to 48.5 ± 0.07% at a concentration of 0.05 mg/mL reaching 61.1 ± 0.37% at the highest concentration evaluated in the study (2 mg/mL). The calculated EC_{50}

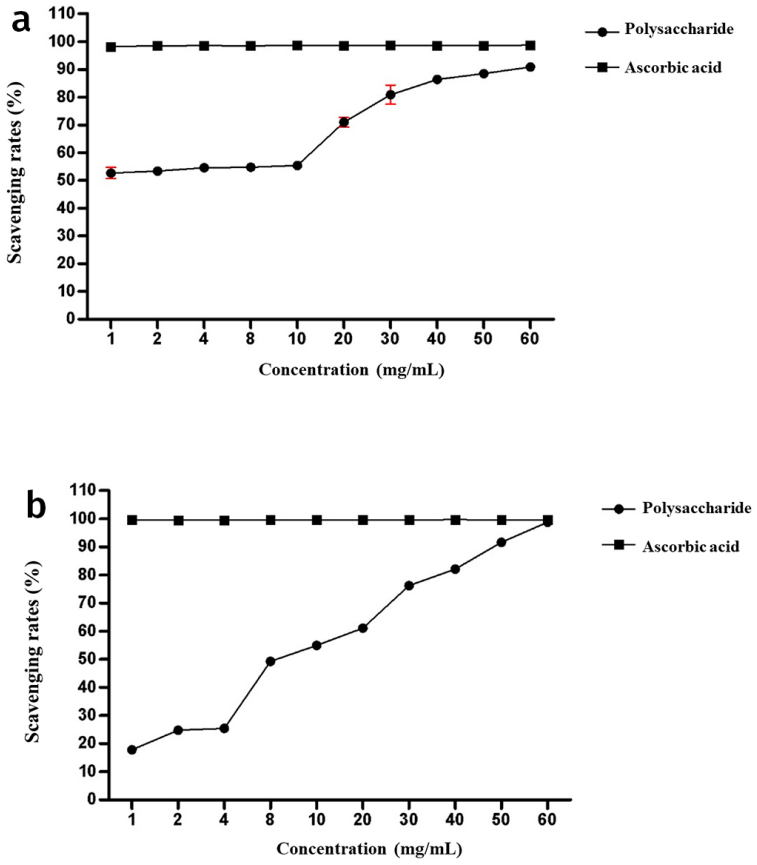

Figure 7. Antioxidant activity *in vitro* of RMOP (a) effect of elimination from ABTS^{+} radicals (b) effect of elimination from hidroxyl radicals. The results are expressed as mean ± SD (n= 3).

was 1.66 ± 0.35 mg/mL. Acarbose, used as a standard, indicated a hypoglycemic activity superior to that of the polysaccharide, with a rate of 85.69 ± 0.38% at a concentration of 2 mg/mL (Figure 8), with statistical difference ($p < 0.01$) between the groups that make up the sample and the standard. The ability of RMOP to inhibit α-amylase was superior to that of polysaccharides from *Inonotus obliquus*, which presented approximately 30% and close to that of its chromium (III) complex, with approximately 60% of inhibition rate at a concentration of 2.5 mg/ mL (Wang et al. 2017).

The inhibition of α-amylase contributes to the reduction of glucose absorption and is considered an effective strategy for the control of Diabetes Mellitus. However, the possibility of resistance, added to the adverse side effects of current inhibitors, permeates the need to search for new effective inhibitors with fewer undesirable side effects.

CONCLUSIONS

Exploring the characteristics of biomolecules such as polysaccharides is essential to understand their application possibilities. The optimization of RMOP extraction made it possible to identify as optimal conditions, which lead to a higher mass yield, at a temperature of 120ºC, time of 60 min and pH 13. FT-IR analysis indicated characteristic bands of polysaccharides. GC-MS and NMR indicated that RMOP is possible a β(1,3) β(1,6) glucogalactan. RMOP has shown potential to scavenge ABTS+ and hydroxyl free radicals and as α-amylase inhibitor, an important enzyme in carbohydrate digestion processes, both in a dose-dependent

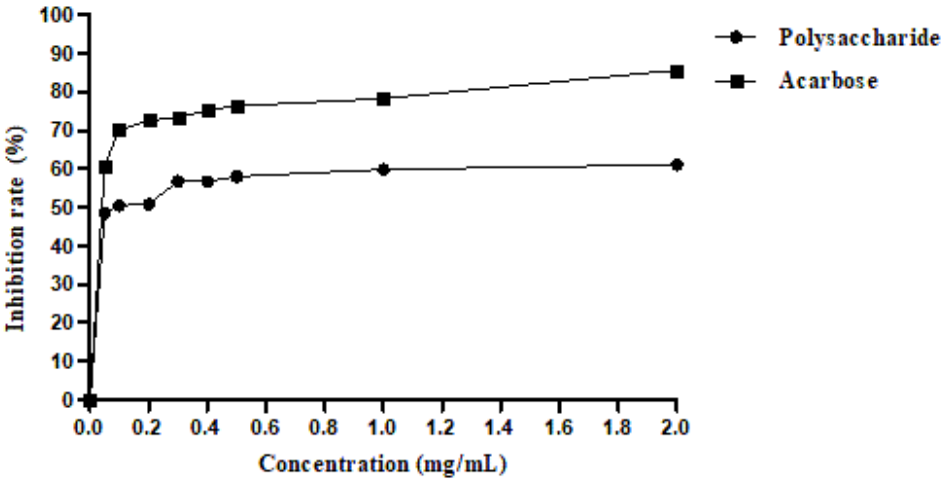

Figure 8. α-Amylase inhibition capacity by RMOP. The results are expressed as mean ± SD (n= 3).

manner. The results obtained so far provide evidence that RMOP can potentially be used in industrial applications.

Acknowledgments

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