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Oxidation and *N*-Alkylation at the C-6 Position of Galactomannan Extracted from *Caesalpinia ferrea var. ferrea* Seeds

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Summary: Carboxyl (GMOX) and carboxyl-aminoalkylated derivatives (GMOXD) of galactomannan (GM), extracted from the seeds of *Caesalpinia ferrea* (Pau-ferro; Brazilian Ironwood), were synthesised by selective modification of the polysaccharide at the C-6 position in the presence of TEMPO reagent (2,2,6,6-tetramethylpiperidine-1-oxyl) and dodecylamine, with high yields (77 and 94% for GMOX and GMOXD, respectively). The chemical modifications were confirmed by Fourier-transform infrared (FTIR) and nuclear magnetic resonance (^{13}C-NMR) spectroscopy. Analysis by gel permeation chromatography (GPC) coupled to refractive index (RI), light-scattering (LS) and viscometer detectors revealed a decrease in molar-mass values and the intrinsic viscosity and an increase in polydispersity. Modification of galactomannan with a primary amine led to an interesting anionic water-soluble polymeric surfactant, as determined by interfacial tension values at 24 °C. The critical aggregation and micellar concentration values of amphiphilic GMOXD were determined to be 0.48 and 0.89% (w/v), respectively. The development of polyelectrolyte and amphiphilic derivatives of GM and their physicochemical characterisation can contribute to numerous product applications such as nano-structured films, cosmetics and pharmaceuticals.

Keywords: amphiphilic biopolymer; modification; polyelectrolites; polysaccharides; surface tension

Introduction

Galactomannans (GMs) are neutral polysaccharides deposited in the seed endosperm of many plants of the Leguminosae family, where they function as an energy-storage reservoir. The chemical structure is a linear main chain consisting of (1→4)-linked β-D-mannopyranosyl (β-D-Man*p*) units partially substituted at *O*-6 with α-D-galactopyranosyl (α-D-Gal*p*) (Figure 1). The degree of substitution is dependent on the origin of the biopolymer.[1]

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Like other polysaccharide gums extracted from plant materials, GMs have potentially important commercial applications, particularly in the pharmaceutical[2] and food industries[3,4] for controlling drug release and modifying texture, respectively. Chemical and/or enzymatic modifications of polysaccharides, in general, are useful for altering their physicochemical-functional properties for diversified applications, for example, certain types of derivatives can be used to improve drug-release specificity.[5] Nooy et al.[6,7] described a derivatisation method via the selective oxidation of the primary alcohols found in polysaccharides using the 2,2,6,6-tetramethylpiperidine-1-oxyl reagent (TEMPO) in catalytic concentration and a hypochlorite/bromide system.

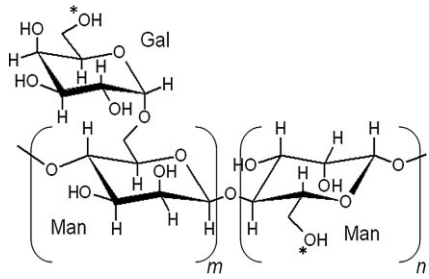

Figure 1.
Partial structure of galactomannan. *position available for chemical modification.

Through the aldehydic intermediary groups and the grafting of dodecylamine, Sierakowski et al.[8] generated an amphiphilic polymer product.

Considering that amphiphilic polymers derived from polysaccharides are attractive materials because they exhibit biocompatible and biodegradable characteristics, the aim of this study was the synthesis and physicochemical characterisation of carboxyl (polyelectrolyte, GMOX) and carboxyl-aminoalkylated (amphiphilic, GMOXD) derivatives of the galactomannan (GM) extracted from *Caesalpinia ferrea* seeds (Pau-ferro; Brazilian Ironwood), a Brazilian native specie, whose chemical structure is being determined in our laboratory. For derivatives characterization, was used gel permeation chromatography (GPC), Fourier-transform infrared (FTIR), nuclear magnetic resonance (^{13}C-NMR) spectroscopy and surface-tension analysis, this latter not commonly used for the polysaccharides characterization.

Experimental Part

Materials

The seeds of *Caesalpinia ferrea* var. *ferrea* were provided by St. Helena Agricultural Enterprises Ltd. - Native Plant Project, Itatinga, São Paulo, Brazil. First, the ground seeds were submitted to an extraction of pigments and lipids with ethyl ether under reflux in a Soxhlet system. The isolation of the galactomannan (GM) was done as described by Lucyszyn et al.[9] The GM had a Manannose:Galactose (Man:Gal) ratio of 2.1, determined by alditol acetate composition[10] and confirmed by ^{1}H and ^{13}C NMR, in D_2O at 75 °C, from the integrals of the anomeric regions (data not shown). 2,2,6,6-tetramethylpiperidine-1-oxyl reagent (TEMPO; $C_{29}H_{18}NO$) was purchased from Acros Organics with 98% purity. The other materials used were of commercial grade and were used without purification.

Oxidation and *N*-Alkylation of Galactomannan

Galactomannan from *C. ferrea* seeds was oxidised as previously reported by Sierakowski et al.[8] The GM (1.5 g polysaccharide or 10.2×10^{-3} mmol (expressed in monosaccharide Man and Gal units) was dissolved in 1 L of distilled water. Under stirring, a 10% sodium hypochlorite solution (10.71 mL or 14.36 mmol) was added, the pH was adjusted to 9.3 with 2 mol · L^{-1} HCl solution and the polymer solution was cooled and the reaction was performed at a temperature of 3 ± 1 °C. TEMPO (0.023 g or 0.149 mmol) and NaBr (0.115 g or 1.09 mmol) were added, and the pH was maintained at 9.3 by addition of a 0.05 mol · L^{-1} aqueous NaOH solution. After the oxidation process, the reaction was stopped by addition of $NaBH_4$ (0.030 g or 0.795 mmol) and 20 mL of ethanol under stirring for 45 min. Then, the pH of the mixture was adjusted to 7 by addition of HCl solution and the modified polysaccharide was precipitated with two volumes of commercial ethanol in the presence of NaCl (up to 0.1 mol · L^{-1}). The oxidised polymer (GMOX) was isolated by centrifugation, washed several times with EtOH, filtered, dried and the yield was calculated. These conditions concern the process yielding the higher degree of oxidation (100% of free OH units presents in the C-6 of Man and Gal units). The alcohol-to-acid conversion was estimated by titration of the mixture with an aqueous NaOH solution (0.05 mol · L^{-1}), also used to maintain

the pH around 9.3 during the synthesis. Therefore, the formation of carboxyl groups corresponded to the amount of titrate NaOH.

For N-alkylation, a primary amine with a C_{12} chain was introduced as the reagent under the following conditions: at the beginning of the synthesis, TEMPO-oxidation was performed as previously described but without the NaBr, resulting in a lower degree of oxidation. After a period of oxidation reaction at $3 \pm 1\,^{\circ}C$, the temperature was adjusted to 25 °C and dodecylamine (0.1073 g or 0.58 mmol) and sodium cyanoborohydride ($NaCNBH_3$, 0.087 g or 1.734 mmol) were added to obtain a final product with around 7% alkylation. The reaction was conducted for four hours. After the alkylation/oxidation process, the reaction was stopped by the addition of $NaBH_4$ (0.030 g) and 20 mL of ethanol under stirring for 60 min. The pH was adjusted to 7 and the modified polymer (GMOXD) was precipitated with two volumes of EtOH, centrifuged, washed with EtOH and dried.

Characterisation of Modified Galactomannans

The Fourier-transform infrared (FTIR) spectra (Bio-Rad Laboratories, Excalibur series FTS 3500GX, Cambridge, USA) were determined in the wavenumber range of 400 to 4,000 cm^{-1} from samples in KBr pellets. ^{13}C-NMR (100 MHz) analyses were performed using a Bruker Avance DRX400 MHz NMR spectrometer with a 10 mm probe at 30 °C. The samples were dissolved in D_2O ($25\,mg \cdot mL^{-1}$) and chemical shifts were referred, in ppm (δ), to the corresponding acetone (δ_C 30.20) signal. For GPC analysis, the experiments were carried out at 30 °C using a Viscotek-GPC Multi-detector with refractive index (RI), light scattering (LS) and viscometer systems (Malvern Co., USA), with TSK PWxl (Tosoh, Japan) columns models 6000, 4000 and 2500, sequentially coupled, with size-exclusion limits of 8×10^6, 3×10^5 and 3×10^3 Da, respectively. The samples ($1\,mg \cdot mL^{-1}$) were solubilised in aqueous $0.1\,mol \cdot L^{-1}$ sodium nitrate containing 0.02% (w/w) sodium azide and filtered through a 0.45-μm pore-diameter membrane. The solutions (100 μL) were measured using GPC to calculate the polydispersity index ($\overline{M}_w/\overline{M}_n$) and the average molar mass, $\overline{M}_w$, relative to PEO 22k (polyethylene oxide), which was used as standard to determination of instrument constants (calibration). The results were compiled in OmniSEC Software (Malvern Co., USA) using a refractive-index increment (*dn/dc*) of 0.146. The surface-tension measurements were performed at 24 °C using a DataPhysics OCA15 plus tensiometer in Milli-Q-purified water at concentrations of 0.2 - 1.0% (w/v).

Results and Discussion

The oxidation degrees (OD) of the GMOX and GMOXD derivatives are shown in Table 1; both products were obtained in good yield (77 and 94%, respectively). Using the same process, a similar derivatisation efficiency has been obtained for galactomannan from *Leucaena leucocephala*,[8] xyloglucan from *Hymenaea courbaril* [11] and gum exudates of *Anarcadium occidentale*.[12]

Table 1.
Yield (% w/w) and oxidation degree (OD, %) of oxidised and oxidised-N-alkylated galactomannan from *C. ferrea* seeds.

Derivative	NaOH consumption (mL)	Yield (% w/w)	OD[a)] (%)
GMOX	58	77.0	62.8
GMOXD[b)]	50	94.0	45.1

[a)]By consumption of NaOH solution at the end of the reaction.
[b)]Oxidation after N-alkylation.

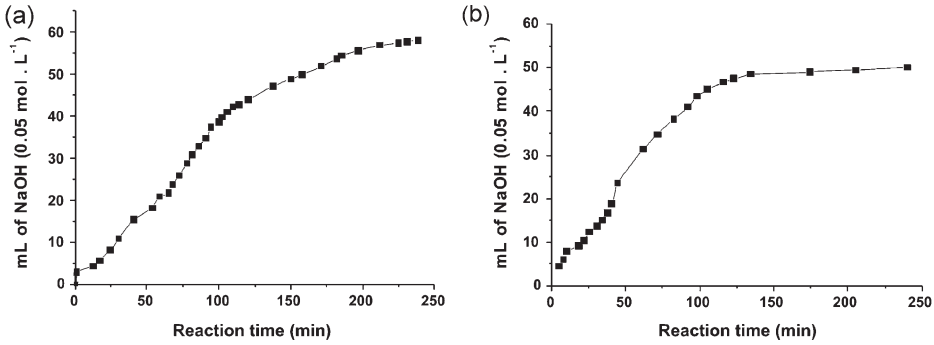

Figure 2.
Oxidation kinetics of galactomannan by TEMPO as represented by NaOH (aq) consumption as a function of time; (a) oxidation reaction, (b) oxidation after N-alkylation reaction.

In the TEMPO-mediated reaction, the carboxyl group formation at C-6 decreased the pH of the system. Thus, the time and volume of aqueous NaOH consumed were used to control the oxidation process. The conversion kinetics of the hydroxyl to acid groups is shown in Figure 2.

Compared with direct oxidation synthesis (Figure 2a), a lower consumption of base was observed for the N-alkylation process (Figure 2b); this performance was due to the absence of bromide ions in the latter, which decreased the rate of carboxyl-group formation.[13]

The aim of the oxidation process was to generate negative charges along the polysaccharide chain, giving it an electrolyte character, thus diversifying and promoting its use in systems that specifically need to stabilise and interact with positively charged compounds.[14] By NaBr/NaClO presence, the reagent TEMPO generates *in situ* the N-oxoammonium ion, responsible to primary alcohol oxidation in high conversion rate.[7,15]

The proposed mechanism of modifications was based on previous reports in the literature.[8,13,15] Under alkaline conditions, after a hydrogen abstraction by bromide an alcoholic substrate and an N-oxoammonium ion combine through a nucleophilic addition followed by a cyclic elimination, generating acid groups at the end of reaction (Figure 3a).[7,16] In the same conditions but without NaBr, the N-alkylation reaction was performed in two successive steps (Figure 3b). First, an OH^- ion abstracts a hydrogen atom from the amine and the nitrogen receives a lone pair of electrons; then, during the formation of aldehydic groups on GM, the addition of the hydrophobic chain occurs by the reductive amination of dodecylamine via $NCBH_3^-$.

The specific modifications of the biopolymer were characterised by spectroscopic and chromatographic methods. FTIR analysis showed a symmetric C–H stretching appearing at 2,900 cm^{-1}, and there was an increment in the absorption due to carbon–hydrogen (C–H) bending in GMOXD related to the alkyl chain of dodecylamine (Figure 4).

The oxidised galactomannan spectra indicated the introduction of substituent groups (C = O) with an absorption around 1,730–1,750 cm^{-1} and a complementary band at 1,640 cm^{-1} corresponding to the deformation of carboxylic acid groups. A stretching was observed at 1,650 cm^{-1}, corresponding to the symmetrical angular deformation of C–N, and at 1,620 cm^{-1}, attributed to the angular deformation of N–H. The bands around 700-1,300 cm^{-1}, common to the GMOX and GMOXD samples, were attributed to the polysaccharide backbone as observed on unmodified galactomannan.

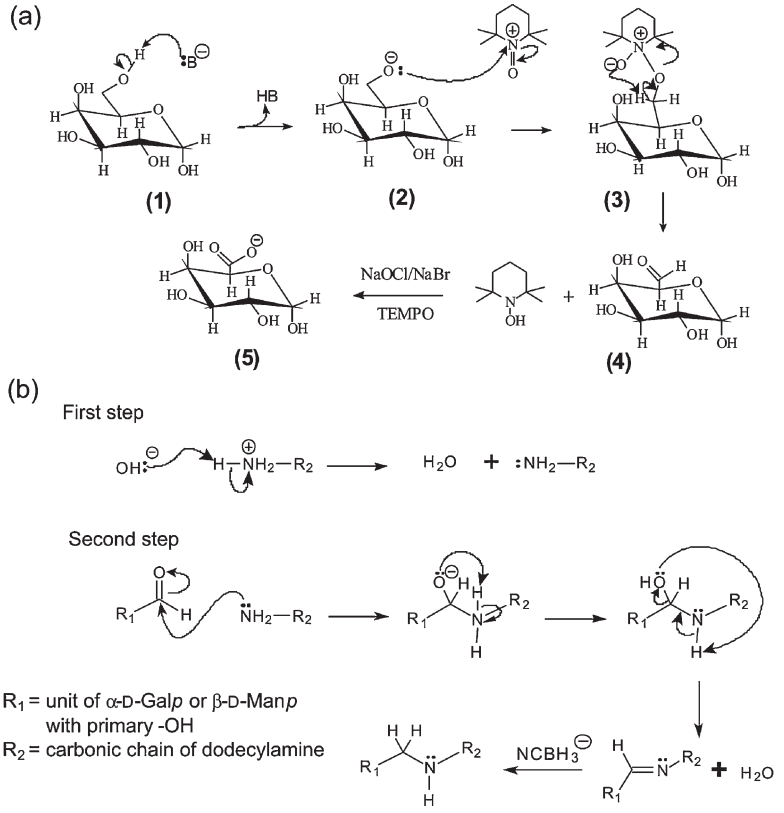

Figure 3.
Schematic representation of the oxidation mechanism (a) and N-alkylation mechanism (b) of galactomannan based on TEMPO-mediated reactions.

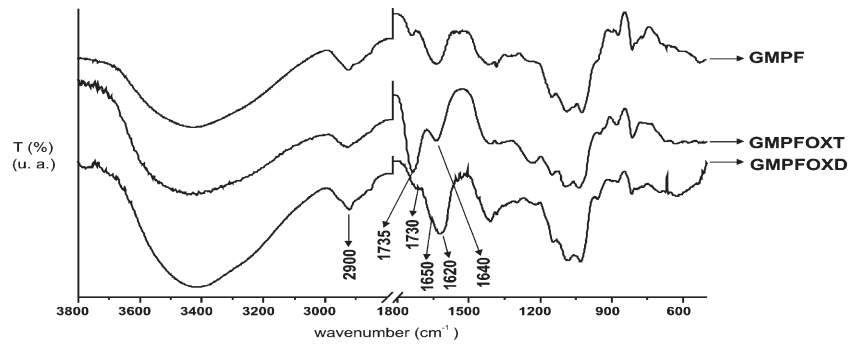

Figure 4.
FTIR spectra of unmodified (GM), oxidised (GMOX) and oxidised-N-alkylated (GMOXD) galactomannan; (u. a. = arbitrary units).

A number of ^{13}C signals in the NMR spectra of the derivatives (Figure 5) were assigned and are shown in Table 2.

After oxidation, the methylene groups at position 6 of the native polymer (δ_{Man} 61.7 and δ_{Gal} 61.1; data not shown) disappeared in the spectrum of GMOX (Figure 5a), and at high field the signal at δ 175.0 related to the carbonyl group appeared, indicating a complete oxidation of the primary hydroxyl

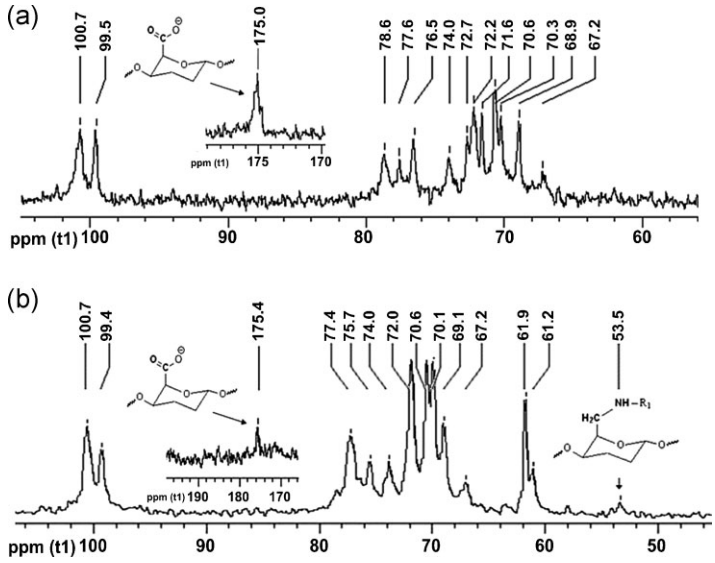

Figure 5.
^{13}C-NMR spectra of oxidised GMOX (a) and oxidised-N-alkylated GMOXD (b) GM derivatives in D_2O (25 mg · mL^{-1}) at 30 °C; chemical shifts (δ) are in ppm.

groups. In the GMOXD spectrum only one peak was found at δ_c 51.3, related to a secondary amine (see Figure 5b); thus, regioselective N-alkylation at the C-6 position occurred.

The low acquisition of signal was due to limiting the addition of dodecylamine during the N-alkylation process to only 7%. The relative signals of the unsubstituted C-6 (δ_c 61.9 - 61.2) of GMOXD compared with the others and a signal at δ_c 175.4 showed the existence of C-6 carboxyl oxidation products after the N-alkylation reaction. The spectra were similar to those obtained by Sierakowski et al.[8] in the modification of galactomannan from *L. leucocephala* seeds.

Gel permeation chromatography (GPC) was performed to determine the molar masses of the derivatives compared to the neutral polysaccharide. The results of the GPC analyses of both products are shown in Table 3. The derivatives showed homogeneous profiles as seen by the single right-angle laser-light scattering (RALLS), but higher polydispersity profiles as calculated by the $\overline{M}_w/\overline{M}_n$ indices when compared with the value of 1.44 obtained for the native GM. Furthermore, the modified GMs showed molar mass reductions during the derivatisation process, as native GM presented an $\overline{M}_w$value of 8.99×10^5 Da. These values suggested that the occurrence of some chain scission during oxidation

Table 2.
^{13}C-NMR chemical shifts (ppm) for oxidised (GMOX) and oxidised-N-alkylated (GMOXD) galactomannan from *Caesalpinia ferrea* seeds.

Sample	C-1	(C-2 – C-5)[a)]	C-6	C-6_{ox}	C-$6_{N\text{-}alk}$
GMOX	100.7-99.5	78.6 – 67.2	-	175.0	-
GMOXD	100.7-99.4	77.4 – 67.2	61.9 – 61.2	175.4	53.5

[a)]No attempts were made to assign the four ring carbon signals.

Table 3.
Physicochemical characteristics of modified galactomannans from *C. ferrea* seeds as determined by GPC.[a)]

Sample	GM	GMOX	GMOXD
$\overline{M}_w$ (Da)	8.99×10^5 (± 0.61)	2.25×10^5 (± 0.07)	2.35×10^5 (± 0.11)
$\overline{M}_n$ (Da)	6.25×10^5 (± 0.091)	1.44×10^5 (± 0.023)	1.13×10^5 (± 0.10)
$\overline{M}_w/\overline{M}_n$	1.44 (± 0.11)	1.56 (± 0.05)	2.17 (± 0.10)
[η] (dL · g^{-1})	10.51 (± 0.53)	1.85 (± 0.08)	2.74 (± 0.11)

[a)]Viscotek Dual Multidetector (RI, LS and Viscometer) in 0.1 mol · L^{-1} $NaNO_3$ plus sodium azide (0.02%, w/w) with PWxl columns (models 2500, 4000 and 6000) at 30 °C.

was responsible for the polysaccharide degradation during the synthesis. Previous studies have indicated a pH dependence for specific modifications with TEMPO.[15] Therefore, in alkaline conditions C-6-oxidised pyranosides are susceptible to β-elimination reactions and subsequent depolymerisation of polysaccharides.[17] This phenomenon has been observed previously by Nooy et al.,[7] Sierakowski et al.,[8] Lucyszyn et al.[11] and Cunha et al.[12]

The effects of the charges incorporated into the GM structure by the syntheses were studied by surface tension measurements. As the concentration of polymers increases in a solution, the physical properties of water are changed and the surface tension (ST) decreases.[18] Figure 6 presents the results of a surface-tension analysis of GMOXD.

The ST of water in the presence of amphiphilic galactomannan showed sharp changes as a function of concentration; the critical aggregation concentration (CAC) and critical micelle concentration (CMC) were determined to be 0.48 and 0.89% (w/v), respectively. Thus, the N-alkylated galactomannan tends to adsorb in the surface solution, forming a monolayer and then form aggregates within the solution.[18,19]

Through the Gibbs adsorption isotherm ($d\gamma/d\ln C = -RT/\Gamma$),[18,19] which relates the excess surface concentration of the adsorbed species to the surface or interfacial tension of the system, the excess of GMOXD on the surface (Γ) was determined to be 3.31×10^{-4} mol · m^{-2} and the molecular area, calculated as $1/(N_A\ \Gamma)$, where N_A is Avogadro's number, was 0.501 Å^2. These results are in agreement

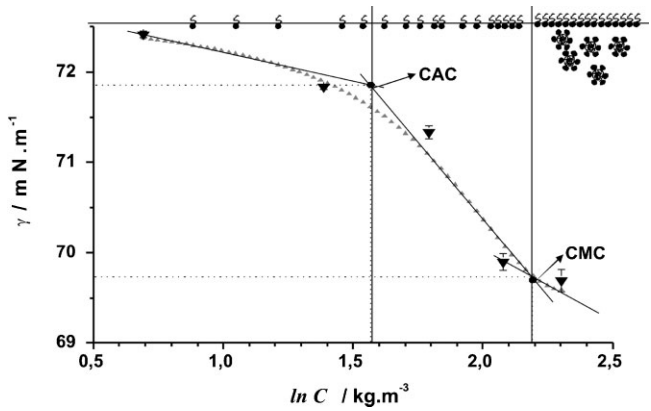

Figure 6.
Surface tension versus *ln C* determined for oxidised N-alkylated galactomannan (GMOXD) from *C. ferrea* seeds in Milli-Q water at 24 °C.

with that observed with surfactants and high-molecular-mass polymers[20] forming surface monolayer films.[18]

Conclusion

The route to specific modifications of galactomannans using the TEMPO reagent proved to be a good alternative allowing for changes in C-6, generating amphiphilic compounds with different ionic and hydrophobic properties and polymer-chain sizes in controlled conditions. The surfactant properties of the oxidised-N-alkylated derivative enable new applications for polysaccharides, which can be exploited in biotechnological studies.

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