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Characterization of Metal-Binding Bioflocculants Produced by the Cyanobacterial Component of Mixed Microbial Mats

JUDITH BENDER,* SUSANA RODRIGUEZ-EATON, UDOUDO M. EKANEMESANG, AND PETER PHILLIPS

Research Center for Science and Technology, Clark Atlanta University, Atlanta, Georgia 30314

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Mixed-species microbial mats that were dominated by the cyanobacterium *Oscillatoria* sp. and contained heterotrophic and purple autotrophic bacteria were constructed for specific bioremediation applications. When the mats were challenged with metals, production and secretion of metal-binding extracellular polysaccharide bioflocculants were observed. The concentration of these negatively charged polysaccharides was correlated with the removal of manganese from the water column beneath a surface microbial mat. Bioflocculants from an *Oscillatoria* sp. that was isolated from the mat were collected and concentrated for characterization. A chromatographic analysis revealed a heterogeneous population of polysaccharides with respect to charge density and molecular size. The subpopulation of polysaccharides which exhibited the highest level of flocculating activity was polyanionic and had a molecular weight of more than 200,000. A glycosyl analysis of the bioflocculants revealed the presence of galacturonic acid (2.2%) and glucuronic acid (1.86%). The presence of these components, which were negatively charged at the pH levels generated by the mats during photosynthesis (pH > 7.5), may account for the metal-binding properties of the mats.

Bioflocculants are extracellular macromolecules that are known for their ability to clarify turbid water. The production of these molecules has been observed in green algae (10), soil bacteria (11), and cyanobacteria (7). Bar-Or and Shilo (1) characterized the macromolecular bioflocculants released by the cyanobacterium *Phormidium* sp. strain J-1 as sulfated heteropolysaccharides to which fatty acids and proteins were bound. These bioflocculants were capable of binding to the cationic dye alcian blue and effectively precipitated bentonite particles from suspensions.

The environmental changes induced by bioflocculants and the ecological advantages of these alterations for the resident microbes have been well documented (8, 13). Since the benthic photoautotrophic cyanobacteria occupy a low-light zone, water-clarifying bioflocculants are important to the photosynthetic activity of these organisms. Because of their agglutinating properties, the bioflocculants may also remove soluble nutrients from the water column, resulting in heterotrophic activity in the sediment region. In addition, if the bioflocculants have numerous negatively charged binding sites, they may remove cations from the environment, thereby protecting cells from the toxic effects of heavy metals (10, 11).

Mixed microbial communities, or mats, were developed for bioremediation by stimulating the formation of a microbial consortium with specific substrates (ensiled grass) and inocula composed of *Oscillatoria* sp. and purple bacteria (identification in progress) and allowing volunteer heterotrophic groups from the soil bed to colonize the preparation along with the photosynthetic species (2, 5). The resulting mats were used for the bioflocculant research described below.

In this study we characterized the bioflocculants produced by the cyanobacterium *Oscillatoria* sp., and in this paper we describe the potential role of these bioflocculants in removing metals from water. Previous studies performed by workers in our laboratory showed that microbial mats that were dominated by an *Oscillatoria* sp. were effective in removing Pb, Cd, Cu, Zn, Co, Cr, Fe, and Mn from water (2–4, 6). Although the mechanisms of metal removal are complex, the production of bioflocculants by cyanobacteria may be central to the initial detoxification of metal-contaminated media by these organisms. In this paper we discuss correlations between bioflocculant production and metal removal from the water column, binding of metals to purified bioflocculants and to crude extracts prepared from the water column beneath a floating microbial mat, and several bioflocculant characteristics, including molecular weight, charge density, and glycosyl composition.

MATERIALS AND METHODS

Overview of research plan. Mats developed for previous metal-sequestering experiments were immobilized on glass wool floaters. Mature floater mats (FM) were placed on Mn-contaminated water. Metal removal from the water column was correlated with flocculant concentration. Spiking experiments were performed to assess the impact of multiple metal additions on the production of bioflocculants. The significance of differences was determined by a one-way analysis of variance between pairs (InStat; GraphPad Software, Inc., San Diego, Calif.).

Water was collected from under the FM, and this water provided the bioflocculant material which was purified and characterized to determine the molecular weight, charge density, and glycosyl composition of the bioflocculant.

Development and application of FM. FM were developed by enriching a water surface with ensiled grass clippings (14) at a concentration of 7 g/liter and adding an *Oscillatoria* sp. that was isolated in Florida (5). Nonsterile soil (sandy loam from a variety of environments) provided a mixed heterotrophic population, the components of which were expected to spontaneously integrate with the cyanobacteria to provide a mixed autotrophic-heterotrophic community. The presence of a multispecies consortium was verified by electron microscopy and by isolating various microbial species from mature mats (unpublished data). Ensiled grass provided a surface substrate for

* Corresponding author. Phone: (404) 880-8809. Fax: (404) 880-8360.

microbial attachment and an initial supply of nutrients. A mature mat was attached to glass wool floaters (4 g of wool shaped into irregular rectangles that were approximately 6 by 3 by 3 cm), and the floaters were placed on top of 1-liter portions of an Mn solution (38 mg/liter) in glass trays (21 by 12 by 7.5 cm). The water columns beneath the floaters were sampled immediately after the FM were added and daily thereafter for 10 days. Samples were assessed for flocculant levels and Mn concentrations.

In separate experiments in which identical FM were used mass balance determinations were used to define the locations of deposited Mn. The following portions of the FM preparations were separated and analyzed for metal content: mats and associated organic material, water column, and residue at the tray bottom. The total amount of Mn recovered and the percentage of Mn in each location were calculated.

Metal and bioflocculant determinations. Samples collected for metal analysis were hydrolyzed by microwave digestion (model MDS-200 instrument; CEM Corp., Matthews, N.C.) and were analyzed by atomic absorption (double-beam model Spectra AA-20 BQ instrument; Varian, Palo Alto, Calif.).

Levels of bioflocculant activity were determined by the alcian blue binding assay described by Bar-Or and Shilo (1). Alcian blue binds to charged organic materials (bioflocculants), forming dense complexes which precipitate, thereby clarifying the water column. The resulting decrease in the blue color of the alcian blue solution is correlated with the concentration of the bioflocculant present in the test solution. Alcian blue 8NGX (Sigma Chemical Co., St. Louis, Mo.) was dissolved at a concentration of 1 mg/ml in 0.5 N acetic acid. Water column samples (0.5 ml) collected from below the mats were diluted in 4.25 ml of 0.5 N acetic acid and added to 0.25 ml of the alcian blue dye preparation. After overnight incubation at room temperature, each solution was centrifuged at 48,000 × *g* for 20 min, and the supernatant optical density at 610 nm was determined. Flocculant-bound dye precipitated during incubation, resulting in a loss of color in the solution.

Multiple metal additions. For separate experiments that were designed to study the effect of multiple Mn additions on bioflocculant activity, we used circular FM (diameter, 9.5 cm; depth, 2 cm). Mn ($MnCl_2$; final concentration, 19 mg/liter) was added to the water column, and as the Mn concentration in the water column decreased to <5 mg/liter (on days 6 and 12), Mn was added again to a final concentration of 19 mg/liter. The mats were harvested on day 15. Bioflocculant activity was measured daily. The bioflocculant activity after multiple metal additions was compared with the activity after a single metal addition and with the activity in controls that received no metal.

All metal-binding experiments were carried out by performing two identical trials, using triplicate experimental and control tanks for each trial.

Purification of bioflocculant and uronic acid analysis. A non-axenic *Oscillatoria* sp. isolated from the mats used in the metal experiments provided the microbial material used to isolate extracellular bioflocculants (7). Cyanobacteria (28-day cultures) were harvested by centrifugation at 12,000 × *g* for 15 min. The supernatant was concentrated to a volume of 0.1 ml with a flask evaporator at 45°C. The concentrate was treated with pronase (100 μg/ml; (Sigma) for 1 h at 37°C and was dialyzed overnight against distilled water at 4°C. Then, 2 volumes of cold ethanol was added to the dialysate, and the precipitate was removed by centrifugation at 12,000 × g for 15 min and vacuum dried. The uronic acid (including glucuronic acid and galacturonic acid) content of the purified bioflocculant was quantified by the acid borate-carbazole method as modified by Taylor and Buchanan-Smith (15).

Molecular weight determination. Bioflocculants were dissolved in a 1% solution of trifluoroacetic acid. The bioflocculant solution was loaded onto a Sephadex G-200 column (1.5 by 120 cm; Sigma) that had been previously equilibrated with 50 mM NaCl and was eluted with the same saline solution. The column was calibrated with blue dextran (molecular weight, 2,000,000). The fractions were analyzed for total sugar content, flocculating activity, and A_{206}.

Charge density determinations. Charge density analyses were performed with a DEAE Bio-Gel anion-exchange column (1.5 by 120 cm; Bio-Rad Co., Richmond, Calif.). The crude bioflocculant in a 1% trifluoroacetic acid solution was loaded onto a column which had been equilibrated with 50 mM NaCl. Then the sample was serially eluted with 50-ml portions of 0.5, 0.1, 2.5, and 5.0 M NaCl and 6.0 M urea. The fractions were analyzed for total sugar content and flocculating activity. The fractions that exhibited flocculating activity were pooled and dialyzed against distilled water at 4°C overnight.

Glycosyl composition. Glycosyl composition was determined by performing a gas-liquid chromatography analysis of methylated alditols and trimethylsilyl derivatives. Each sample (0.5 to 1.0 mg) contained 20 μg of inositol as an internal standard and was hydrolyzed in 0.5 ml of 2 M trifluoroacetic acid for 2 h at 121°C. The acid was removed by evaporation with nitrogen. The sample was reduced in 0.25 ml of a 1 M ammonium hydroxide solution containing 10 mg of sodium borodeuteride per ml at room temperature for 1 h. Excess borohydride was removed with acidic methanol, and the solution was evaporated under nitrogen at room temperature. O acetylation was carried out in the presence of acetic anhydride (0.1 ml) and pyridine (0.1 ml) at 121°C for 20 min. The sample was dried in nitrogen and dissolved in 0.5 ml of chloroform. Then, 0.5 ml of water was added, and the solution was vortexed for 2 min. The organic layer was extracted and dried in nitrogen. The residue was dissolved in 50 to 100 μl of acetone for the gas-liquid chromatography analysis. The carboxyl groups of uronic acids were reduced by the method of Taylor and Conrad (16).

RESULTS

Bioflocculants and manganese removal. Figure 1 shows the correlation between Mn removal and bioflocculant concentration. Bioflocculant concentrations less than 0.9 (1/optical density at 610 nm) had little effect on Mn removal. Although some bioflocculant was present in the controls (without Mn), the higher levels in the presence of Mn indicated that the metal stimulated bioflocculant production. Since the mats were immobilized on glass wool floaters (and grown to the stationary phase before the metal-bioflocculant experiment), the possibility that there would be continued growth and corresponding increases in bioflocculant levels was eliminated.

The final metal concentrations in the water column (day 15) were 1.9 mg/liter in the single-Mn-addition experiment and 0.3 mg/liter in the multiple-metal-addition experiment. Separate mass balance experiments revealed that 99.7% of the Mn was recovered. The locations of the Mn were as follows: 77% of the Mn was in the surface mat and associated organic material, 14% was in the residue at the bottom of the tray, and 9% was in the water column. In control experiments performed with only glass wool (and no mat) all of the Mn remained in the water column and no binding to the glass wool floater occurred.

The levels of bioflocculant production in systems that re-

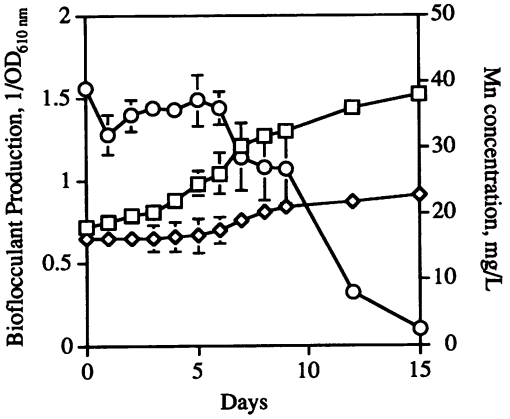

FIG. 1. Relationship between bioflocculant production and manganese removal. We measured bioflocculant production in experimental (□) and control (◇) tanks and manganese concentration in experimental tanks (○). $OD_{610\ nm}$, optical density at 610 nm.

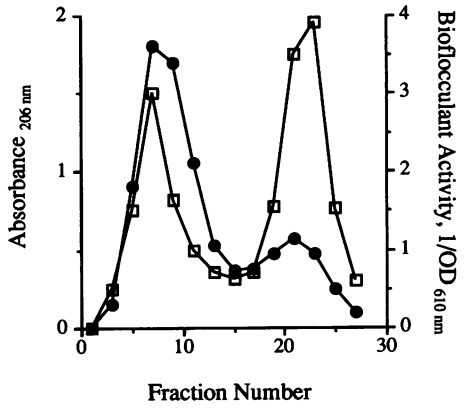

FIG. 3. Elution profile of bioflocculants on Sephadex G-200. Symbols: □, A_{206}; ●, bioflocculant activity. $OD_{610\ nm}$, optical density at 610 nm.

ceived single metal additions and multiple metal additions are shown in Fig. 2. There was no significant difference between the results obtained after the two types of metal addition ($P = 0.1368$), whereas both values were significantly different from the values obtained for the controls that received no metal (single addition versus control, $P = 0.0081$; multiple addition versus control, $P < 0.0001$).

Molecular weight of the bioflocculant. The molecular weight of bioflocculant that was isolated by the procedures described above was determined (Fig. 3). The macromolecule that exhibited the greatest flocculating activity eluted in the void volume. This molecule had a molecular weight of 200,000 or more. The second absorbance peak exhibited only slight flocculating activity.

Charge density. The elution profile obtained from the anion-exchange column (Fig. 4) revealed that the bioflocculants were heterogeneous polymers. Neutral polysaccharides eluted in distilled water and 0.5 M NaCl. Acidic polysaccharides eluted in the following solutions: 1.0, 2.5, and 5.0 M NaCl and 6.0 M urea. The neutral polysaccharides exhibited low levels of flocculating activity, whereas the flocculating activities of the acidic fractions were comparatively high.

Glycosyl composition. Two analytical methods were used to confirm the presence of uronic acids as glycosyl components in the bioflocculant. The results of colorimetric and gas chromatography-mass spectrometry (GC-MS) analyses indicated that the uronic acid contents were 25 and 4%, respectively. The insolubility of the carbohydrate may explain the different values, suggesting that the value obtained by GC-MS analysis is abnormally low. The bioflocculant was sparingly soluble in dimethyl sulfoxide (the standard solvent used for the GC-MS analysis), and the uronic acid component, specifically, contributes to the insoluble character of the carbohydrate (12). Thus, the value of 4% is a minimal value for the uronic acid content determined by this method. The method used for the colorimetric analysis, a destructive process, increased the solubility but precluded further GC-MS analysis. More systematic methods are currently being used to study the bioflocculant structure.

Because uronic acids are negatively charged in basic pH solutions and the mats generate and maintain pH levels generally between 7.5 and 9.5, these acids are presumed to be important components that contribute to metal binding in water containing cyanobacterial mats. Figure 5 shows the glucuronic and galacturonic acid GC-MS chromatograms which we obtained.

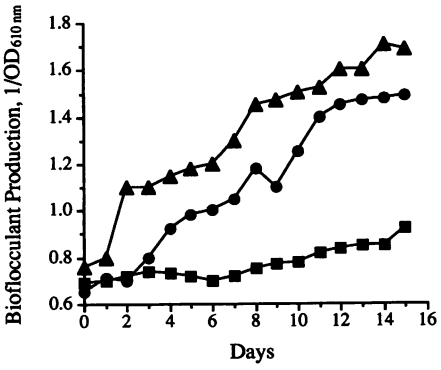

FIG. 2. Relationship between bioflocculant production and metal addition. Symbols: ●, single metal addition; ▲, multiple metal additions; ■, control. $OD_{610\ nm}$, optical density at 610 nm.

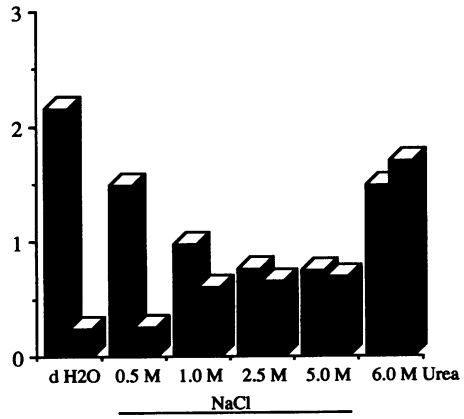

FIG. 4. Relationship between bioflocculant activity and anionic charge distribution as determined by anion-exchange chromatography. Solid bars, A_{206}; cross-hatched bars, bioflocculant activity. d H2O, distilled water.

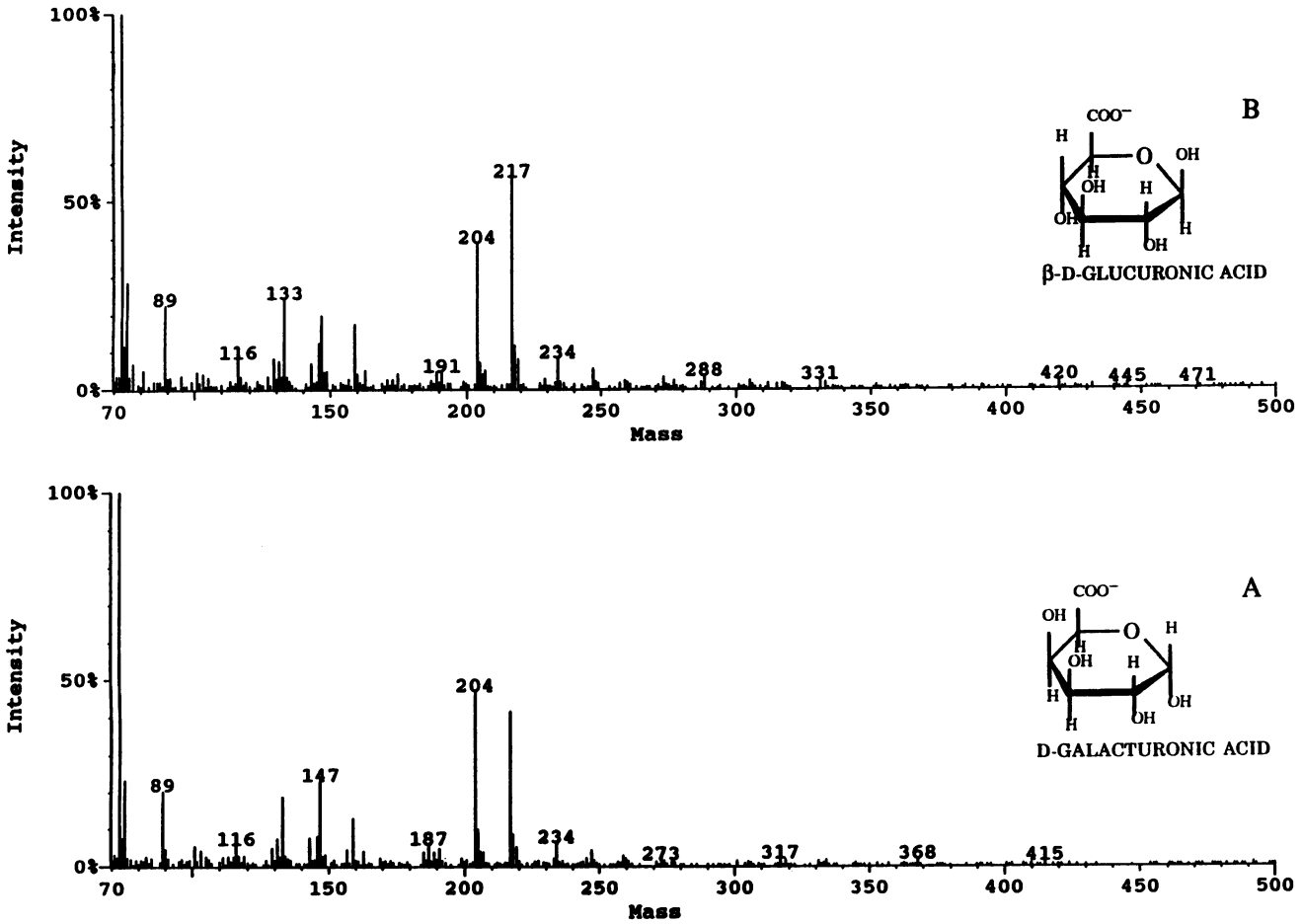

FIG. 5. Chromatograms for glucuronic acid components of the bioflocculant.

DISCUSSION

In this paper we report that microbial mats that were dominated by cyanobacteria produced negatively charged polysaccharide bioflocculants. The relationship between metal removal and bioflocculant production is shown in Fig. 1. Once stimulated, bioflocculant production continued at an elevated level even after the metal concentration decreased. In the period between days 6 and 8 there was rapid production of the bioflocculant and a corresponding decrease in the Mn concentration. Multiple additions of Mn resulted in insignificant increases in bioflocculant production. Controls that contained no Mn revealed that Mn was necessary for stimulation of bioflocculant production.

Mass balances experiments, in which we identified the location of the Mn after it left the water column, revealed that most of the metal was eventually deposited within the mat and not at the bottom of the tray, as might be expected. Since the mat remained buoyant at all times and the system was quiescent (there was no mixing, flowing, or bubbling), the question of how the metal moved through the water column to the surface mat remains. In addition, the impact that the bioflocculants (which were present in the water column) had on the eventual migration of Mn to the surface is an interesting question. While we expected that carbohydrate and protein materials at the cell surface would bind most metals present in a continuously moving solution, the presence of aqueous bioflocculants that are separate from cells and are released into the water column raises several broader issues. The impact of bioflocculant-metal binding on motile microbes in the water column and on the ultimate transport of bioflocculant-metal complexes through the static water column to the surface mat remains a complex issue with significant remediation implications. While in our experiments we did not identify the processes by which the metals were deposited in the mat, certain hypotheses can be examined. Several factors may act in concert to determine the final metal accumulation at the surface. Previous research on mat removal of metals revealed that Pb deposits occur at a similar location (2). This research showed that there were substantial metal concentrations in the water column associated with materials retained by a 0.45-μm-pore-size filter. These components, which were probably bacterial cells, bound in some way to the metal. If the bacteria were protected by a bioflocculant-metal complex, the cells might be expected to remain viable. Motile species may have transported the metal to the surface mat. It has been demonstrated that members of several motile bacterial groups that are present in the water column respond chemotactically to a surface mat (5). Eventual migration of bacteria to a surface mat may transport metals to this region.

The elution profiles obtained by anion-exchange chromatography showed that the extracellular polysaccharides are heterogeneous, ranging from neutral molecules to highly acidic

TABLE 1. Glycosyl composition of cyanobacterial bioflocculants

Sugar	%
Ribose/rhamnose	7.11
Fucose	8.80
Xylose	8.29
Glucuronic acid	1.86
Mannose	14.46
Galactose	17.77
Galacturonic acid	2.20
Glucose	32.88
Unidentified sugar	5.56
Unknown amino sugars	1.07

molecules. Although bioflocculant activity was correlated with anionic charge density distribution, it was difficult to measure bioflocculant activity in the presence of 5 M NaCl and 6 M urea. High concentrations of salts tended to interfere with absorbance, and dialysis produced abnormally high concentrations of the bioflocculant. However, at NaCl concentrations of 1.0 and 2.5 M we observed elevated bioflocculant activity compared with the activity at an NaCl concentration of 0.5 M, while neutral polysaccharides exhibited low bioflocculant activity.

Table 1 shows the glycosyl composition of cyanobacterial bioflocculants. The glucuronic and galacturonic acid content (total, 4%) of the isolated bioflocculant suggests that negatively charged binding sites are available at pH values greater than 7. Using GC-MS analysis (Fig. 4), we positively identified the uronic acid component. At basic pH levels, these sites would be negatively charged, thereby providing numerous cation-binding sites. Microbial mats generate basic pH levels whose ranges are variable (pH 7.5 to 9.5) (2). Thus, the macro-environment, mediated by the mat, favors bioflocculant-metal binding with subsequent metal removal from the water column.

Our current investigations include studies of bioflocculant binding to cobalt and hexavalent and trivalent chromium, as well as studies of the structural properties of the bioflocculant that may have an impact on metal binding.

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Glycosyl composition research was performed in collaboration with the Carbohydrate Research Center, University of Georgia, Athens.

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