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Carbohydrate Research 343 (2008) 793–798
Carbohydrate RESEARCH

Note

Structural characterization of the cell wall D-glucans isolated from the mycelium of *Botryosphaeria rhodina* MAMB-05

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Received 26 October 2007; received in revised form 18 December 2007; accepted 22 December 2007
Available online 11 January 2008

Abstract—Three D-glucans were isolated from the mycelium of the fungus *Botryosphaeria rhodina* MAMB-05 by sequential extraction with hot-water and hot aqueous KOH (2% w/v) followed by ethanol precipitation. Following their purification by gel permeation chromatography on Sepharose CL-4B, the structural characteristics of the D-glucans were determined by FT-IR and ^{13}C NMR spectroscopy and, after methylation, by GC–MS. The hot-water extract produced a fraction designated Q_{1A} that was a β-(1→6)-D-glucan with the following structure:

$$\left[\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\right]_n$$

The alkaline extract, when subjected to repeated freeze–thawing, yielded two fractions: K_1P (insoluble) that comprised a β-(1→3)-D-glucan with β-D-glucose branches at C-6 with the structure:

$$\left[\begin{array}{l}\beta\text{-D-Glc}p\\ \quad 1\\ \quad\downarrow\\ \quad 6\\ \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\end{array}\right]_n$$

and K_1S_A (soluble) consisting of a backbone chain of α-(1→4)-linked D-glucopyranosyl residues substituted at O-6 with α-D-glucopyranosyl residues:

$$\left[\begin{array}{l}\alpha\text{-D-Glc}p\\ \quad 1\\ \quad\downarrow\\ \quad 6\\ \rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\left[\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\right]_6\end{array}\right]_n$$

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doi:10.1016/j.carres.2007.12.021

Keywords: *Botryosphaeria rhodina* MAMB-05; Fungal cell wall polysaccharides; D-Glucans

The fungal kingdom estimated at 1.5 million species contains a diverse range of species presenting polysaccharides that possess various biological activities and chemical properties exploitable for commercial applications. Numerous studies have reported the potential of fungal polysaccharides as antioxidants, as well as antimutagenic, anticoagulant, antithrombotic and immunomodulatory agents.[1] Fungal polysaccharides have been isolated from the fruiting body (mushroom), the mycelium, spores and sclerotia and the extracellular medium of fungi grown in culture.[2,3] The composition and chemical structure of the polysaccharides of the cell wall varies among the different fungal species. Among the polysaccharides found in nature, the homopolymer group of D-glucans are ubiquitous and comprise a major proportion of the cell walls of fungi and yeasts, mainly as (1→3)- and (1→6)-linked β-D-glucans.[4,5] These polysaccharides can also exist in the free form as exopolysaccharides, and when structurally bound to proteins, lipids and other polysaccharides occur in the fungal and yeast cell wall.[4,6]

The endophytic ascomycete *Botryosphaeria rhodina* MAMB-05 produces a family of exopolysaccharides, botryosphaerans,[7,8] of the β-(1→3),(1→6)-D-glucan type that vary in their degrees of branching at C-6 depending upon the nature of the carbohydrate substrates[9] used as carbon sources during fermentation. The fungal mycelium can constitute an important resource for chemicals including polysaccharides in the large-scale production of exopolysaccharides by fermentation such as botryosphaeran. Knowledge of the structure and biological activities of such polysaccharides may thus contribute to the practical and rational utilization of this biomass resource. In the present study, we report for the first time the D-glucans constituting the cell wall of *B. rhodina* MAMB-05 with a view to obtaining information on their chemical composition and structure that may hopefully lead to their biotechnological applications.

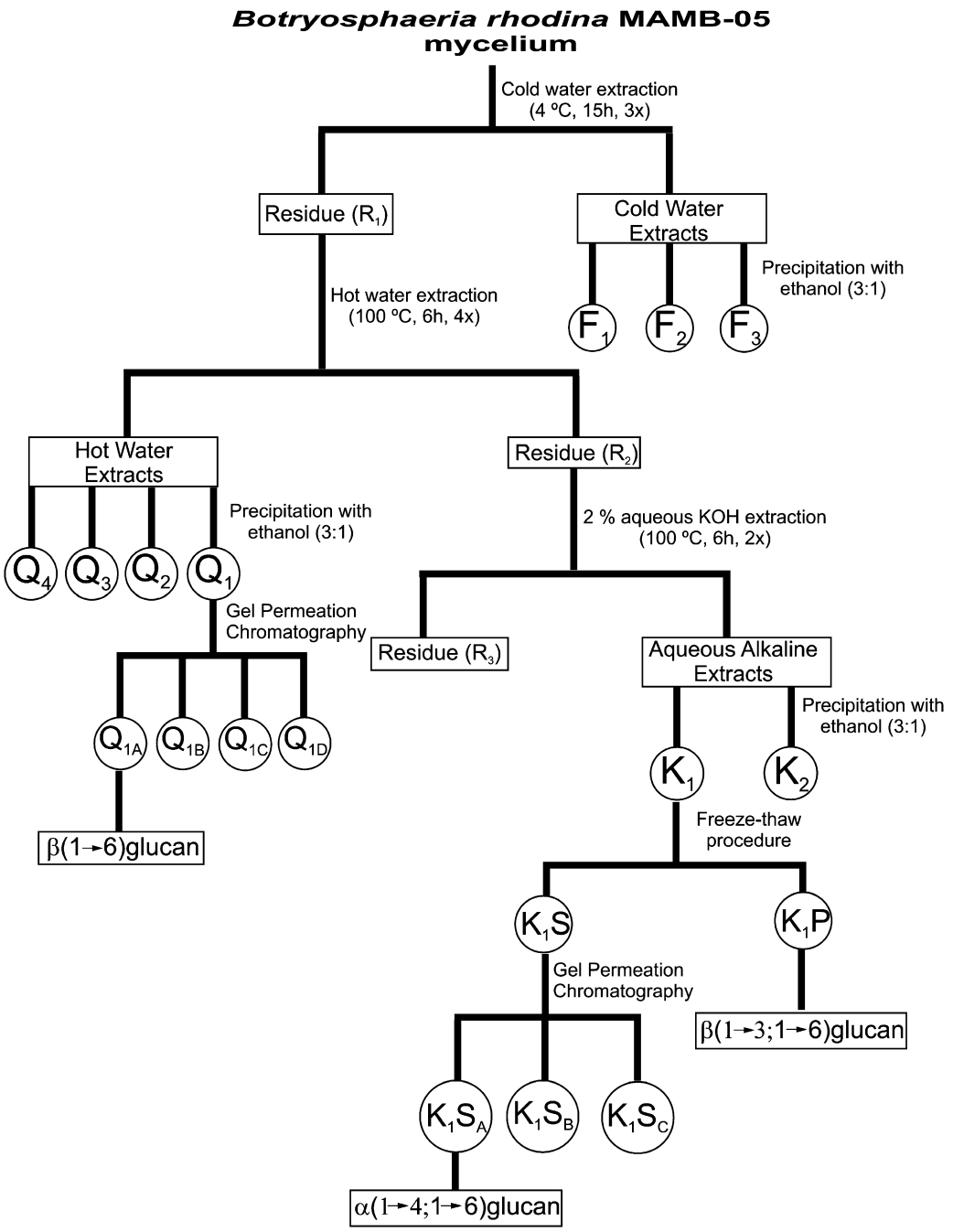
In the study reported herein, the dried mycelium from *B. rhodina* MAMB-05 freed of the exopolysaccharide botryosphaeran was subjected to sequential extractions with cold-water, hot-water and 2% hot aqueous KOH as indicated in Scheme 1. Crude polysaccharide fractions obtained from hot-water and aqueous KOH extractions, designated Q_1 and K_1, respectively, were investigated in this work.

Fraction Q_1 contained carbohydrate (99%) and protein (1%), and compositional analysis on crude Q_1 following acid hydrolysis revealed the presence of galactose (56%), glucose (25%), mannose (16%) and fucose (3%). Following gel permeation chromatography on Sepharose CL 4B, one of the resulting peaks, subfraction Q_{1A}, showed glucose as the main monosaccharide component, consistent with a glucan. Methylation of Q_{1A} followed by acid hydrolysis, $NaBH_4$ reduction and acetylation and GC–MS analysis (Table 1) indicated that Q_{1A} was a (1→6)-linked glucan. The FT-IR spectrum (Fig. 1) showed bands at 920, 1040, 1110, 1150 and 1558 cm^{-1}, characteristic of glucans. The β-anomeric configuration was revealed by absorption at 890 and 1371 cm^{-1}, and was further supported by the chemical shift of the C-1 signal by ^{13}C NMR. As shown in Table 2, Q_{1A} contained six signals at 102.96, 75.89, 75.06, 73.22, 69.93 and 69.01 ppm that arise from C-1, C-3, C-5, C-2, C-4 and C-6, respectively, and are attributable to a β-(1→6)-D-glucan with a possible structure as shown below:

$$\left[\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\right]_n$$

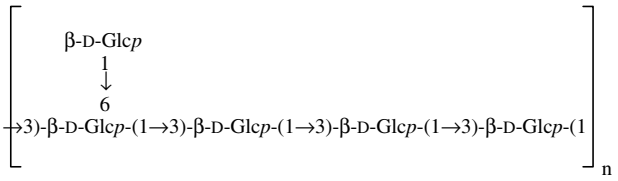
These data are in agreement with those reported for cell wall glucans from *Schizosaccharomyces pombe*[10] and *A. blazei* Murril.[11]

Fraction K_1 obtained from 2% aqueous KOH extraction at 100 °C was submitted to repeated freeze–thaw cycles until no further precipitation occurred (Scheme 1). After centrifugation, sub-fractions K_1S (soluble) and K_1P (insoluble) were isolated. K_1P contained mainly glucose (93%), consistent of a glucan. To elucidate the linkage type, the glucan was subjected to methylation analysis (Table 1), which showed alditol acetates of 2,3,4,6-Me_4Glc (20%), 2,4,6-Me_3Glc (62%) and 2,4-Me_2Glc (18%) indicating the presence of a branched (1→3),(1→6)-linked glucan. ^{13}C NMR showed signals attributable to a β(1→3)-glucan O-substituted on C-6 (Table 2) in accordance with literature reports.[12–15] Peaks visible at δ 102.98 and 102.54 ppm strongly indicated that only β-anomeric carbons were present, and this was confirmed by FT-IR spectroscopy; bands at 890 and 1371 cm^{-1} being typical of β-glucans (Fig. 1). The signal at δ 102.98 ppm was attributable to the C-1 of 3-O-substituted glucopyranosyl units, while δ 102.54 ppm was due to 3-O-substituted glucose units of the main chain containing branched residues on C-6, in agreement with the general rule for β-anomeric carbon glycosylation and explained by an upfield chemical shift. The 3-O-substituted carbon atoms from the main chain were detected at δ 84.80 and 84.54 ppm. The most intensive signal, δ 84.80, was attributed to the main glucan chain C-3 devoid of substitutions. A signal at 84.54 ppm was attributed to 3,6-di-O-substituted by β-glucopyranosyl residues, and signals at δ 69.97 and 60.99 ppm were attributable to substituted C-6 and free C-6, respectively. Other signals, δ 75.93/75.06, 73.20 and 68.98/68.32 ppm, arise from C-5, C-2 and C-4 glucopyr-



Scheme 1. Diagrammatic scheme outlining the protocol for the extraction and purification of the mycelial polysaccharides from *Botryosphaeria rhodina* MAMB-05.

anosyl 3-O or 3,6-di-O-substituted residues, respectively. A possible structure for the branched (1→3),(1→6)-linked glucan is shown below:



Compositional analysis of K_1S by HPAEC indicated galactose (30%), glucose (65%) and mannose (5%). After gel permeation chromatography on Sepharose CL 4B, the resulting sub-fraction, K_1S_A, showed glucose as the main monosaccharide component (92%), indicating a glucan. Methylation analysis of K_1S_A revealed the presence of three types of glucose derivatives as partially methylated alditol acetates: 2,3,4,6-Me_4- (8%), 2,3,6-Me_3- (87%) and 2,3-Me_2- (5%) derivatives (Table 1). The results are consistent with a (1→4)-linked glucan with (1→6)-linked branches. The IR band at 850 cm^{-1}

Table 1. GC–MS data arising from the methylation analyses of the purified D-glucans from sub-fractions isolated from the mycelium of *Botryosphaeria rhodina* MAMB-05

Sub-fraction	Components[a]	T_R[b]	Molar percent (%)	Linkage
Q_{1A}	2,3,4-Me_3-Glc	2.20	100	→6)-Glc*p*-(1→
K_1P	2,3,4,6-Me_4-Glc	1.00	20	Glc*p*-(1→
	2,4,6-Me_3-Glc	1.82	62	→3)-Glc*p*-(1→
	2,4-Me_2-Glc	4.20	18	→3,6)-Glc*p*-(1→
K_1S_A	2,3,4,6-Me_4-Glc*p*	1.00	8	Glc*p*-(1→
	2,3,6-Me_3-Glc*p*	2.32	87	→4)-Glc*p*-(1→
	2,3-Me_2-Glc*p*	4.50	5	→4,6)-Glc*p*-(1→

[a] 2,3,4,6-Tetra-*O*-Me-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucose, etc.
[b] Retention times of the corresponding alditol acetates compared to 2,3,4,6-tetra-*O*-methyl-glucose.

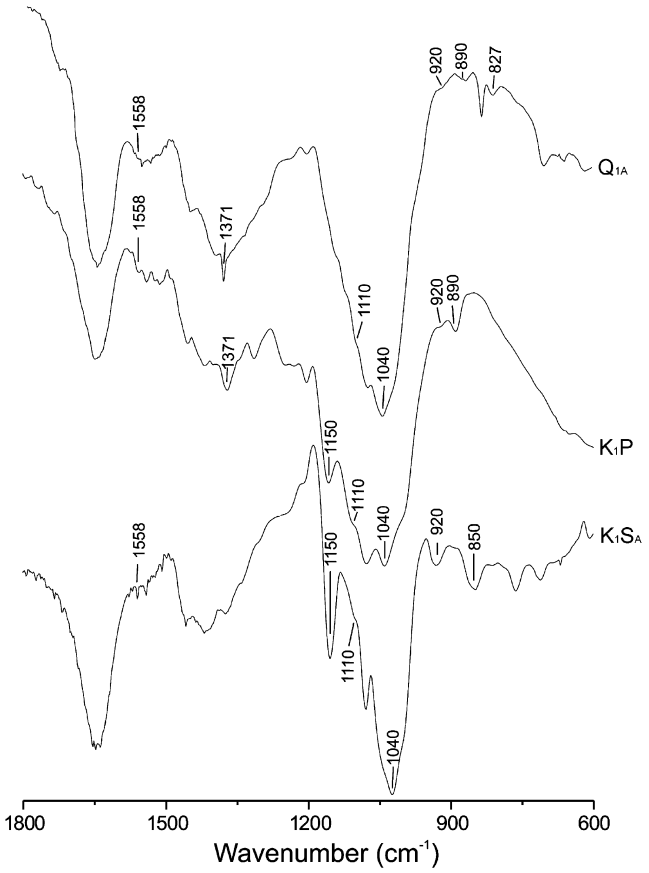
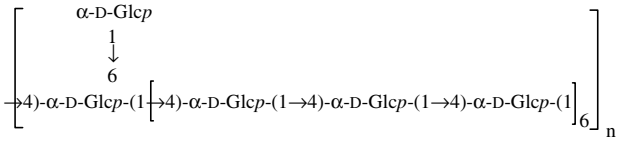


Figure 1. FT-IR spectra of sub-fraction Q_{1A} present in hot-water extract Q_1, and sub-fractions K_1P and K_1S_A from the hot aqueous alkaline extract (K_1) of the mycelium of *Botryosphaeria rhodina* MAMB-05.

(Fig. 1) suggested an α-configuration of the glucosyl motif, and ^{13}C NMR presented 6 signals of higher intensities corresponding to glucopyranosyl residues linked α-(1→4) (Table 2). The chemical shift at 100.39 ppm was attributable to an anomeric carbon in the α-configuration, and the shift at 78.26 ppm represents a signal typical of a O-4. The chemical shifts at 73.95, 72.32, 72.07 and 61.41 ppm arise from C-3, C-2, C-5 and C-6, respectively.[11,16] Two signals of lower intensity, δ 99.26 and 70.33 ppm, were respectively attributable to the C-1 and substituted C-6 of the 4,6-di-O-substituted glucopyranosyl units. These signals agreed with the methylation analysis, indicating that fraction K_1S_A was an α-(1→4)-D-glucan with 5% glucosyl residues on C-6 with a possible structure as shown below:



A similar polysaccharide was isolated from the mycelium of *C. sinensis*.[17]

1. Experimental

1.1. Mycelium production

Mycelium was produced according to the method for producing botryosphaeran by *B. rhodina* MAMB-05 using glucose (50 g/L) as sole carbon source.[8] The mycelium recovered by centrifugation was separated from residual exopolysaccharides by exhaustively washing with distilled water until no sugars were detectable in the washings by the phenol–sulfuric acid method.[18]

1.2. Analytical techniques

Total sugars were determined by the phenol–sulfuric acid method,[18] and reducing sugars by the cupro-arsenate method of Somogyi,[19] with D-glucose as the standard. Protein was measured by the method of Bradford[20] using bovine serum albumin as standard.

1.3. Extraction of cell wall polysaccharides

Mycelium from *B. rhodina* MAMB 05 resulting from multiple water washings was freeze-dried and ground to a powder, and submitted to sequential extractions as follows: 70 g of mycelial powder was extracted 3 times

Table 2. ^{13}C NMR spectral assignments of the purified D-glucans from sub-fractions isolated from the mycelium of *Botryosphaeria rhodina* MAMB-05 measured at 30 °C

Sub-fraction	Linked-glucose residue	Chemical shifts (δ, ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
Q_{1A}	→6)-D-Glc*p*-(1	102.96	73.22	75.89	69.93	75.06	69.01
K_1P	→3)-D-Glc*p*-(1	102.98	73.20	84.80	68.98	75.93	60.99
	→3,6)-D-Glc*p*-(1	102.54	73.20	84.58	68.32	75.06	69.97
K_1S_A	→4)-D-Glc*p*-(1	100.39	72.32	73.95	78.26	72.07	61.41
	→4,6)-D-Glc*p*-(1	99.26	72.55	73.44	77.60	71.22	70.33

with water (4 °C, overnight, 1 L). After centrifugation (8000*g*/15 min/4 °C), the supernatant solutions (designated F_1, F_2, F_3) were concentrated and the polysaccharides precipitated by the addition of three volumes of EtOH. A similar procedure was used with hot-water (100 °C, 6 h, 1 L, 4×), and 2% (w/v) aqueous KOH (100 °C, 4 h, 1 L, 2×). The fractions obtained were labelled Q_1, Q_2, Q_3, Q_4; K_1, K_2, respectively (Scheme 1). The alkaline extracts were neutralized with acetic acid prior to ethanolic precipitation. All fractions (F_1, F_2, F_3; Q_1, Q_2, Q_3, Q_4; and K_1 and K_2) obtained were dialyzed against distilled water for 48 h, concentrated under reduced pressure and lyophilized.

1.4. Purification of cell wall polysaccharide fractions Q_1 and K_1

Ten milligrams of hot-water extract fraction Q_1 was dissolved in water (2 mL) and fractionated by gel filtration chromatography on a column of Sepharose CL-4B (50.0 × 2.5 cm) eluted with water (1.2 mL/min). Fractions (5.0 mL) were collected and analyzed for carbohydrate by the phenol–sulfuric acid method and protein. Fractions corresponding to peaks were pooled and freeze-dried. Alkaline extract fraction K_1 was dissolved in water and frozen, and then subjected to repeated freeze–thaw cycles (four times) with thawing conducted at 20 ± 2 °C. This procedure resulted in two sub-fractions: one soluble, the other insoluble. The soluble sub-fractions (K_1S) were combined, concentrated and the polysaccharides precipitated with EtOH (3:1). The insoluble material was combined and labelled K_1P. A similar procedure as described for extract Q_1 was used to purify fraction K_1S by gel permeation chromatography on Sepharose CL-4B.

1.5. Monosaccharide composition

Each polysaccharide fraction and sub-fraction (0.05 mg total sugar) were hydrolyzed[8] with 5 M TFA (0.3 mL) in a sealed tube for 16 h at 100 °C, and the hydrolysates analyzed by high performance anionic exchange chromatography with pulsed amperometric detection (HPAEC/PAD) on a Dionex Chromatograph DX 500 using a CarboPac PA-1 (Dionex Chromatography) column (4 × 250 mm) equipped with a PA-1 guard-column at a flow rate of 1.0 mL/min.[9,21]

1.6. Methylation-acid hydrolysis–reduction–acetylation

Ten milligrams of each purified sample(s) (Q_{1A}, K_1P and K_1S_A) was per-O-methylated using powdered NaOH in Me_2SO–MeI.[22] The per-O-methylated derivatives were solubilized in 50% (v/v) H_2SO_4 (0.5 mL) for 1 h, followed by dilution with water (4 mL), and refluxed for 18 h at 100 °C.[23,24] The resulting partially methylated sugar hydrolysates were reduced with $NaBH_4$, acetylated, and analyzed by GC–MS on a Varian Saturn 2000R gas chromatograph linked to a Finnigan ion-trap on an 810 R-12 mass spectrometer using He as carrier gas. A DB-225 capillary column (30 m × 0.25 mm i.d.) held at 50 °C during injection and programmed at 40 °C/min to 210 °C (constant temperature) was used for analysis of the partially O-methylated alditol acetates.[25]

1.7. FT-IR and ^{13}C NMR spectroscopy

FT-IR spectroscopy was performed on a Bruker Vector 22 spectrometer on 1 mg freeze-dried samples (Q_{1A}, K_1P and K_1S_A) in 250 mg KBr discs, and scans conducted within 1800–600 cm^{-1} at a resolution of 1 cm^{-1}. ^{13}C NMR spectra were recorded at 400 MHz using a Bruker Avance DRX spectrometer. Samples (Q_{1A}, 15 mg; K_1P, 20 mg; and K_1S_A, 15 mg) were dissolved in Me_2SO and examined at 30 °C. Chemical shifts[26] were relative to resonance of Me_2SO-d_6 at δ 39.70 (^{13}C).

Acknowledgments

The authors are grateful to FAPESP (05/53879-3), Fundação Araucária (Projeto 5777), PROPPG-UEL and Pronex/Carboidratos for financial support. E. K. Fukuda, A. C. Matias and N. K. Monteiro gratefully acknowledge CAPES, FAPESP (06/61794-0) and CNPq-PIBIC, respectively, for scholarships.

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