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The origin of mannans found in submerged culture of basidiomycetes

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ABSTRACT

Submerged cultures of basidiomycetes can be used to obtain polysaccharides in a shorter time, when compared to the production and extraction process of mushroom fruiting bodies. This encouraged many authors to develop a variety of culture media to optimize the production of these polymers. In this paper it was observed that the mannans isolated from submerged culture of mushrooms (*Ganoderma lucidum* and *Pleurotus* spp.) are very similar to yeast mannan, suggesting that these polymers could have arisen from yeast extract used in the culture media, and were not being produced by the basidiomycetes. Therefore, when yeast extract is used in the culture media in the submerged culture of basidiomycetes, a part of the fraction assumed as EPS is composed of yeast extract polysaccharides.

1. Introduction

Mushrooms have received much attention as a nutritionally functional food and a source of different compounds that can modify biological response. Polysaccharides have been studied especially because of their antitumor and immunomodulating properties (Wasser, 2002).

It is also possible to obtain these polymers from submerged culture of basidiomycetes (extracellular and mycelial polysaccharides), on selection of appropriate culture conditions. Molecules can be obtained over a short period of time, since mushroom cultivation requires 2–6 months to complete fruiting body development in a solid-state fermentation (Elisashvili, Wasser, Tan, Chichua, & Kachlishvili, 2004).

Many publications have described different culture media for submerged culture and often report the use of yeast extract, because of its stimulatory effect of its protein, amino acids, and vitamins content (Bolton & Blair, 1982). Glucose is usually used as a carbon source.

The optimization of exopolysaccharide production and mycelial biomass of mushrooms involve the study of nutritional factors, such as carbon and nitrogen source and environmental conditions, such as pH, temperature, aeration, pre-inoculum, and so on.

Besides the cultivation of mushrooms for optimization of biomass and exopolysaccharide production, there are many reports describing structural features of the polysaccharides obtained from submerged cultures. In relation to *Ganoderma lucidum* exopolysaccharides (EPS) were described a (1→3)-linked β-D-glucan (Sone, Okuda, Wada, Kishida, & Misaki, 1985; Wagner, Mitchell, Sassaki, & Amazonas, 2004) and an α-D-mannan with a (1→6)-linked main-chain substituted at O-2 with side-chains of different lenghts (Sone et al., 1985). Similar structures were also described for EPS from *Phomopsis foeniculi* (Corsaro et al., 1998) and *Pleurotus ostreatoroseus* (Rosado et al., 2002), while galactomannans were characterized from the EPS of *Ganoderma tsugae* (Peng, Zhang, Zeng, & Xu, 2003) and *Collybia maculat* (Lim et al., 2005).

In order to improve the knowledge regarding the relationship between the culture media and structure of mushroom polysaccharides, the present objective was the structural characterization of mannans isolated from culture media from *G. lucidum* and *Pleurotus* spp., as well as the implication of the isolation of these structures.

2. Materials and methods

2.1. Microorganism and culture conditions

Pure cultures of *G. lucidum* and *Pleurotus ostreatus* var. *H1* (CC 162) were donated by EMBRAPA/CENARGEM, Brasília, DF, Brazil and another of *Pleurotus citrinopileatus* was donated by BRAMICEL, Suzano, SP, Brazil. These were maintained in Petri dishes with potato dextrose agar (PDA) medium. In addition, *Pleurotus eryngii* and *P. ostreatus* var. *florida* were isolated from fragments of the basidiocarps and maintained under the same conditions described above.

The pre-inocula were prepared using a PDA culture medium, while potato filtrate plus dextrose (PD) was used for preparation of liquid inoculum.

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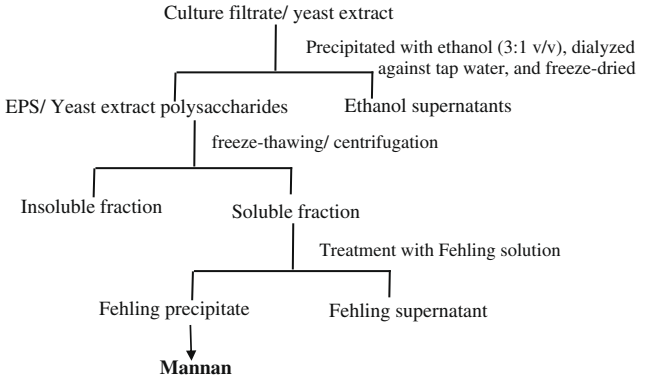

Fig. 1. Purification scheme of mannan fractions.

Pleurotus ostreatus var. *H1* and *P. eryngii* were cultivated in POL liquid medium (Maziero, Cavazzoni, & Boroni, 1999) that consisted of: peptone 1.0 g l^{-1}, yeast extract 2.0 g l^{-1}, K_2HPO_4 1.0 g l^{-1}, $MgSO_4.7H_2O$ 0.2 g l^{-1}, $(NH_4)_2SO_4$ 5.0 g l^{-1}, $CaCO_3$ 1.0 g l^{-1}, and glucose 60.0 g l^{-1}.

Pleurotus citrinopileatus was cultivated in Mushroom Complete Medium (MCM) liquid medium (Kim et al., 2005), that contained peptone 2.0 g l^{-1}, yeast extract 2.0 g l^{-1}, K_2HPO_4 1.0 g l^{-1}, KH_2PO_4 0.46 g l^{-1}, $MgSO_4.7H_2O$ 0.5 g l^{-1}, and glucose 60.0 g l^{-1}.

Pleurotus ostreatus var. *florida* was cultivated in POL and also in CZAPECK liquid medium, which consisted of: $NaNO_3$ 3.0 g l^{-1}, KH_2PO_4 1.0 g l^{-1}, $MgSO_4.7H_2O$ 1.0 g l^{-1}, $FeSO_4.7H_2O$ 0.18 g l^{-1}, KCl 0.5 g l^{-1}, yeast extract 1.0 g l^{-1}, and glucose 10 g l^{-1}. While *G. lucidum* was cultivated in GL liquid medium (Peng et al.; 2003) which contained peptone 10.0 g l^{-1}, yeast extract 10.0 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, KH_2PO_4 0.5 g l^{-1}, $MgSO_4$ 0.5 g l^{-1}, and glucose 150.0 g l^{-1}.

The pre-inocula were grown in 250 ml Erlenmeyer flasks containing 100 ml of PD liquid medium, at 28 °C on a rotary shaker at 143 rpm. After 10 days, the pre-inocula were inoculated in 2000 ml Erlenmeyer flasks containing 1000 ml of POL, CZAPECK, MCM, or GL liquid medium, as described previously, and incubated for 10 days more under the same conditions as the pre-inocula.

The mycelia were separated by centrifugation and the culture filtrates had their volume reduced and were then treated with ethanol (3:1, v/v), giving rise to precipitates. These were dialyzed against tap water, and freeze-dried to obtain extracellular polysaccharide (EPS) fractions.

2.2. Purification of exocellular polysaccharide fractions

EPSs obtained from submerged culture of *G. lucidum* and *Pleurotus* spp. were submitted to a purification process (Fig. 1). These were firstly treated with water at 100 °C for 3 h, and the water-soluble fractions were freeze–thawed. This process gave rise to insoluble and soluble fractions. The latter were treated with Fehling solution (Jones & Stoodley, 1965) and the resulting insoluble Cu^{2+} complexes were isolated by centrifugation. Both the complex and supernatant were neutralized with HOAc, dialyzed against tap water, deionized with mixed ion-exchange resins, and then freeze-dried. Studies on the structural features of the Fehling precipitates were then carried out.

2.3. Isolation of mannan from yeast extract

Commercial yeast extract from Vetec (Brasil) and Usb (Affymetrix Inc., USA) were used to isolate yeast mannan, 10 g of each being treated with ethanol (3:1, v/v), giving rise to precipitates that were dialyzed against tap water, and freeze-dried to obtain fractions without low molecular mass compounds. The mannan isolation was carried out as describe in item 2.2 for purification of EPS fractions.

2.4. Determination of homogeneity and molar mass (M_w) of mannans

A Waters size exclusion chromatography (SEC) apparatus coupled to a differential refractometer (RI), and a Wyatt Tecnology Dawn-F multi-angle laser light scattering (MALLS) detector was used for examination of fractions, during the process of purification. Four Waters Ultrahydrogel 2000/500/250/120 were connected in series and coupled with a multi-detection equipment. Samples, previously filtered through a membrane (0.22 μm; Millipore), were injected (250 μl loop) at 1 mg ml^{-1}concentration. The eluent was 0.1 mol l^{-1} aq. $NaNO_3$ with 200 ppm aq. NaN^3. The specific refractive index increment (d*n*/d*c*) was determined using a Waters 2410 detector. All experiments were carried out at 25 °C.

2.5. Monosaccharide analysis

Monosaccharide compositions were determined by hydrolysis of the polysaccharide samples with 2 M TFA at 100 °C for 8 h. The hydrolyzates were evaporated to dryness, followed by successive reduction of the residues with $NaBH_4$ and acetylation with Ac_2O-pyridine (1:1, v/v; 2 ml) at ~25 °C (Wolfrom & Thompson, 1963a, 1963b). The resulting alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS) using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer, with a DB-225 capilary column (30 m × 0.25 mm i.d.), with helium as carrier gas. The analysis was carried out from 50 to 220 °C at 40 °C min^{-1}, maintaining the temperature constant to the end of analysis (18 min). The products were identified by their typical retention times and electron impact profiles (Sassaki et al., 2008).

2.6. Methylation analysis of polysaccharide

Per-*O*-methylation of the mannan fraction (M-PFP) was carried out by the method of Ciucanu and Kerek (1984). The sample (10 mg) was dissolved in dimethyl sulfoxide (1 ml), and powdered NaOH (20 mg) and iodomethane (CH_3I) (1 ml) were added. After 30 min at 25 °C with vigorous stirring, the mixture was maintained overnight at 25 °C. The reaction was interrupted by addition of water, neutralization with HOAc, dialysis against distilled water

Table 1
Extracellular polysaccharide production and relative yield of mannan.

Species	Culture medium	[Yeast extract] (g l^{-1})	[EPS][a] (mg l^{-1})	Relative yield of mannan (%)[b]
Ganoderma lucidum	GL	10	912	19
Pleurotus ostreatus var. *H1*	POL	2	275	3.6
Pleurotus eryngii	POL	2	241	9.5
Pleurotus citrinopileatus	MCM	2	677	3.2
Pleurotus ostreatus var. *florida*	CZAPECK	1	530	2.8
Pleurotus ostreatus var. *florida*	POL	2	464	2.0

[a] Percentage of mannan in relation of yeast polysaccharide yield.
[b] Percentage of mannan in relation of total EPS yield.

and freeze-drying. The products were submitted to one more cycle of methylation, and the products were isolated by partition between $CHCl_3$ and water. The per-*O*-methylated derivatives from the lower layer (2 mg) were hydrolyzed with H_2SO_4 72% at 100° for 12 h (Seaman, Moore, Mitchell, & Millet, 1954), followed by $NaBH_4$ reduction and acetylation as above (item 2.4), to give a mixture of partially *O*-methylated alditol acetates, which were analyzed by GC–MS using a DB-225 capillary column (30 m × 0.25 mm i.d.), programmed from 50 to 210 °C at 40 °C min^{-1}, the total analysis time being 36 min.

2.7. Nuclear magnetic resonance spectroscopy

NMR spectra (^1H, ^{13}C, DEPT, HMQC) were obtained using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. ^{13}C NMR (100.6 MHz) analyzes were performed at 70 °C, with the samples being dissolved in D_2O and their chemical shifts are expressed in ppm (δ) relative to acetone at δ 30.20 and δ 2.22 for ^{13}C and ^1H signals, respectively.

3. Results

3.1. Structural characterization of the mannans obtained from submerged culture

The submerged culture of the basidiomycetes gave different yields for EPSs, varying from 241 to 912 mg l^{-1}, for *P. eryngii* and *G. lucidum*, respectively (Table 1).

Each EPS fraction was submitted to the same purification process (Fig. 1). Water-soluble fractions were treated with Fehling solution, which gave rise to an insoluble copper complex, containing mannose as the major monosaccharide component, according to derived GC–MS alditol acetates. When these fractions were analyzed by HPSEC-MALLS, the elution profile was a symmetrical peak, which represents a mannan homogeneous fraction and its molecular mass varied from 32 to 46 kDa. The relative yields of mannans were different for: *G. lucidum* (M-GL), *P. ostreatus* var. *H1* (M-POH1), *P. citrinopileatus* (M-PC), *P. eryngii* (M-PE), *P. ostreatus* var. *florida* cultivated in CZAPECK liquid medium (M-PFC) and POL liquid medium (M-PFP) (Table 1).

^{13}C NMR spectra of the mannans from the basidiomycetes were very similar (Fig. 2), so that further analyses were carried out only on that of *P. ostreatus* var. *florida*, obtained from submerged culture from CZAPECK (M-PFC) liquid medium (Table 2).

Its HMQC spectrum contained signals in the anomeric region of C-1/H-1, which can be assigned to non-reducing end (δ 102.0/ 5.18), 3-*O*- (δ 102.0/5.08), 2-*O*- (δ 100.5/5.25), and 2,6-di-*O*-substituted α-D-Man*p* units (δ 98.3/ 5.08). The (1→6)-glycosidic linkages of the mannan were shown by the presence of *O*-substituted-CH_2-6 signal at δ 65.8, while –CH_2 without substitution at δ 61.1, was confirmed by the inversion of the signals in its DEPT spectrum (data not shown).

In order to confirm the linkage type of this polymer, M-PFC fraction was submitted to methylation analysis, which showed it to be composed of non-reducing end- (29.0%), 2-*O*- (30.2%), 3-*O*- (1.8%) and 6-*O*- (5.0%), and 2,6-di-*O*-substitited Man*p* units (34.0%).

These data, when compared with those of the literature (Corsaro et al., 1998; Gorin, 1973; Rosado et al., 2002) suggest a structure with a main-chain of (1→6)-linked α-D-mannopyranosy units, mainly branched at O-2 with side-chains of different lengths with 2-*O*- and 3-*O*- substituted mannopyranosyl units.

3.2. Structural characterization of the mannans obtained from yeast extract

The commercial yeast extracts (Vetec and Usb) (Table 3) were solubilized in distilled water, treated with excess ethanol (3:1, v/v), and the precipitate isolated, dialyzed and lyophilized. The water-soluble fractions obtained after freeze–thawing were submitted to a Fehling solution treatment which gave rise to soluble and insoluble copper

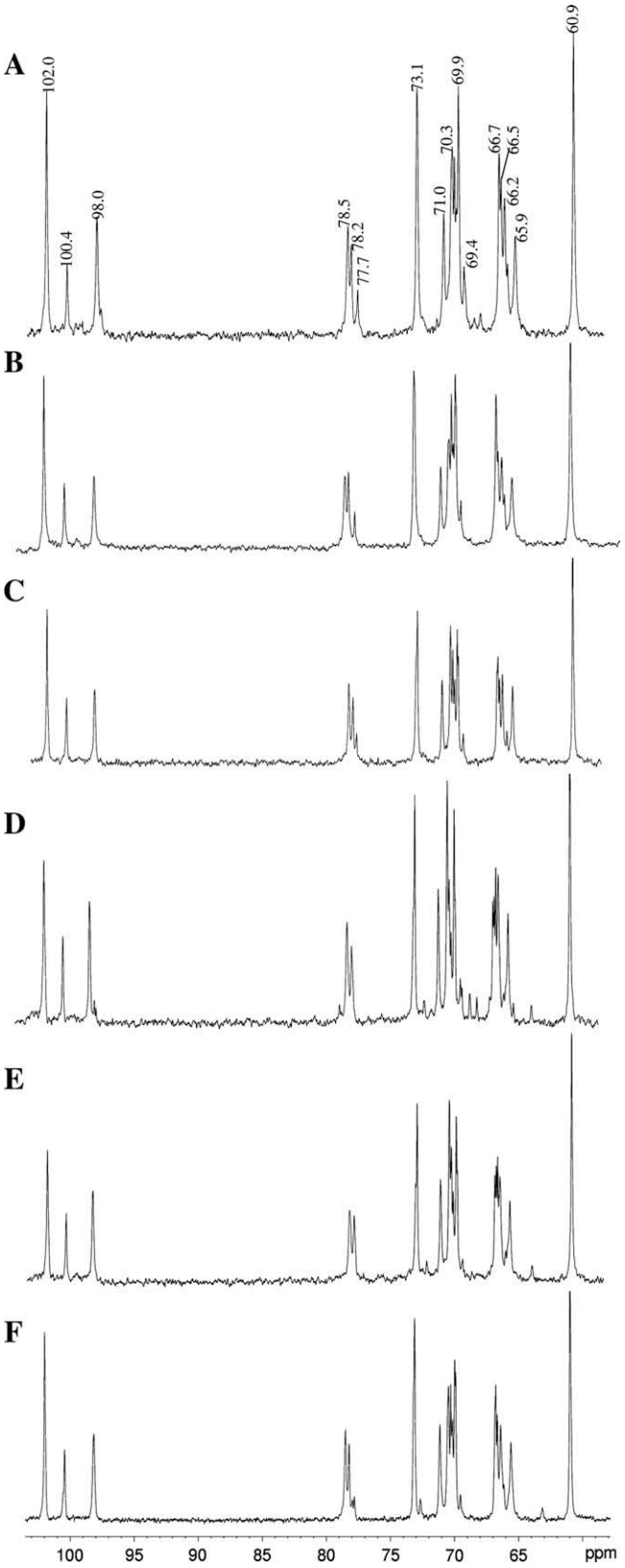

Fig. 2. ^{13}C NMR spectra of M-GL (A) M-POH1 (B), M-PE (C), M-PC (D), M-PFC (E), and M-PFP (F) in D_2O at 70 °C (chemical shifts are expressed in ppm).

Table 2
Analysis of partially *O*-methylated alditol acetates obtained on methylation analysis of mannan from *P. ostreatus* var. *florida* (M-PFC).

O-Methylalditol acetate	% Area of fragments	Linkage type[a]
2,3,4,6-Me_4-Man	29.0	Man*p*-(1→
3,4,6-Me_3-Man	30.2	2→)-Man*p*-(1→
2,4,6-Me_3-Man	1.8	3→)-Man*p*-(1→
2,3,4-Me_3-Man	5.0	6→)-Man*p*-(1→
3,4-Me_2-Man	34.0	2,6→)-Man*p*-(1→

[a] Based on derived *O*-methylalditol acetates.

Table 3
Relative yield of mannan from yeast extract polysaccharide.

Yeast extract	Yield of polysaccharide (g l^{-1})	Relative yield of mannan (%)[a]
Vetec	1.3	3.5
Usb	1.4	3.4

[a] Concentration of EPS in relation of dry weight.

complexes (Fig. 1). The latter contained mannose as the major monosaccharide component, according to GC–MS of derived alditol acetates. When analyzed by HPSEC-MALLS the molecular mass of both mannans was around 61 kDa.

Concerning their ^{13}C NMR spectra, the major signals present in these fractions have a great similarity to those of the ^{13}C NMR spectra from mannans of basidiomycetous EPS (Fig. 3).

4. Discussion

The mannans now described have a similar structure to those from *G. lucidum* (Sone et al., 1985), *P. foeniculi* (Corsaro et al., 1999) and *P. ostreatoroseus* (Rosado et al., 2002). Interestingly, the mannans are not described for the basidiocarps from *Pleurotus* species, neither from *Ganderma* sp., but only for the EPS fractions obtained in submerged cultures.

The mannans described above, as well as the ones now described, were found in submerged cultures which used commercial yeast extract on its media components. It seemed appropriate to investigate the extracts in order to determine if the presence of mannans was due to their use, since it is already known from previous publications that the mannan moiety has a branched structure resembling that of the yeast cell surface. Polysaccharides containing only mannose are typical for yeasts, such as the described for *Saccharomyces cerevisiae* (Gorin & Spencer, 1970; Kath & Kulicke, 1999), *Hansenula subpelliculosa*, *Endomycopsis fibuliger* (Gorin & Spencer, 1970).

The mannans isolated now were found in a media which used Vetec yeast extract. As a parameter Usb yeast extract was also analysed, verifying that the structure was common to both commercial yeast extracts. Thus, the possibility that these mannans originated from the culture medium cannot be ruled out. This fact was also suggested by Sone et al. (1985) that found in the culture medium of *G. lucidum*, a similar yeast mannan, but with shorter side-chains. The molecular mass detected by HPSEC-MALLS for the mannans isolated was around 32–46 kDa, while the mannans present in the commercial yeast extract had a mass of ~61 kDa, which is compatible to those found in *S. cerevisiae* (Wattendorf, Coullerez, Vörös, Textor, & Merkle, 2008).

Besides, we can observe that the yield of the mannan in the EPS fractions was very low, however the highest yield was observed for *G. lucidum* cultivated in liquid medium GL, which is composed of 10 g l^{-1} of yeast extract, while the other culture media are composed of 1–2 g l^{-1} of this compound. The highest yield of mannan came from the culture media with a high content of yeast extract, so that the use of yeast extract in culture media means that a certain proportion of the EPS obtained could be from the mannan present in yeast extract.

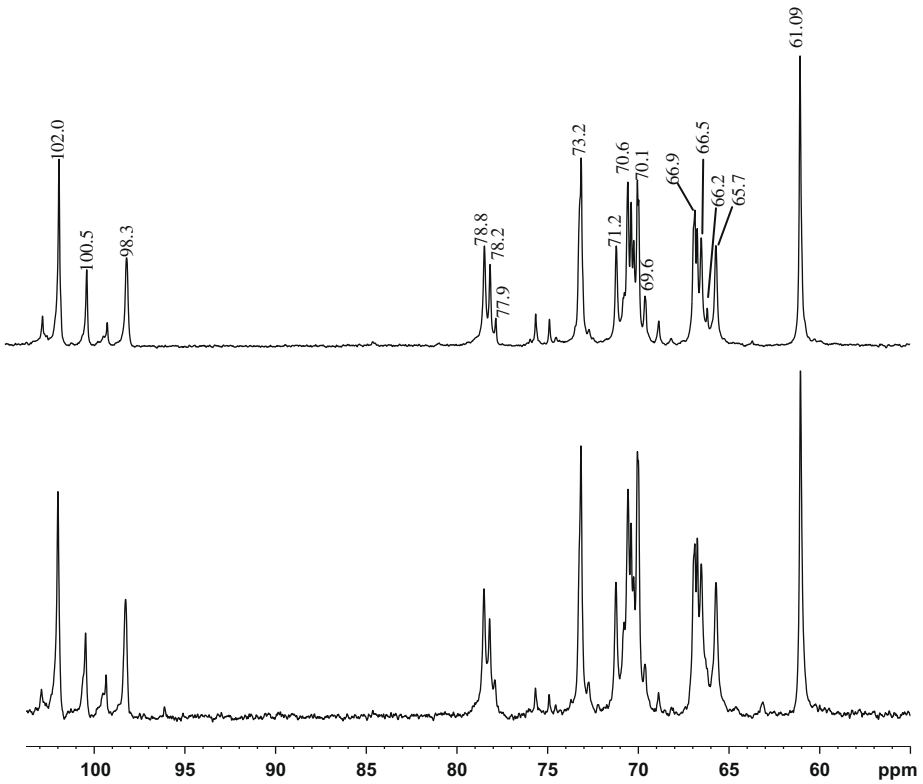

Fig. 3. ^{13}C NMR spectra of the yeast extract mannan from Vetec (A) and Usb (B) in D_2O at 70 °C (chemical shift are expressed in ppm).

When the basidiomycete *Lyophyllum decastes* was cultivated in submerged culture using different concentrations of yeast extract, it was observed that an increase of the yeast extract concentration was accompanied by an increase of EPS yield (Pokhrel & Ohga, 2007). Comparing this previous result to ours, we can suggest that the increase of EPS quantity is related to the presence of mannans on the yeast extract used in the culture medium.

For these reasons, if the intention is to cultivate organisms in submerged cultures aiming the study of EPS, it is necessary to be cautious when selecting the organic nitrogen and carbon sources, in order to prevent doubtful judgment regarding the polymers produced by the organisms.

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