Bioresource Technology 114 (2012) 603–609

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effects of extraction methods on the composition and molar mass distributions of exopolymeric substances of the bacterium *Sinorhizobium meliloti*

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ARTICLE INFO

Article history:
Received 23 January 2012
Received in revised form 22 March 2012
Accepted 24 March 2012
Available online 29 March 2012

Keywords:
Biopolymers
Extracellular polymeric substances
Bacteria
Characterization
Extraction methods

ABSTRACT

The influence of the extraction methods on the composition, size diversity, molar mass and size distributions of exopolymeric substances (EPS) from the bacterium *Sinorhizobium meliloti* wild type (WT) and by the exoY strain deficient in exopolysaccharide production was investigated. EPS obtained by centrifugation, EDTA and formaldehyde/NaOH were compared. It was found that the extraction method influenced TOC, TN and total protein content in EPS from both strains. However, no difference between EDTA and formaldehyde/NaOH methods was observed for the exopolysaccharide components. Similar functional groups and fluorescence pattern were found in the EPS obtained by different methods; however their relative abundance was method dependent. The extraction method also affected the molar mass and size distribution, HP SEC diversity among different treatment and bacterial strains.

1. Introduction

Extracellular polymeric (or exopolymeric) substances (EPS) are composed of a wide range of organic polymers such as polysaccharides, proteins, nucleic acids and phospholipids, excreted by eukaryotic and prokaryotic organisms (Wingender et al., 1999). EPS are considered to play a key role in bacterial flocs and biofilms (Flemming and Wingender, 2010), to be central in toxic metal bioremediation (Pal and Paul, 2008) as well as to greatly influence the performance in waste water treatment systems (Sheng et al., 2010; Subramanian et al., 2010). Exopolymeric substances can be divided to: (i) soluble EPS in the extracellular environment, not covalently linked to the cell surface and (ii) bound EPS tightly linked via a covalent or non-covalent association to the cell wall (Wingender et al., 1999). Usually the soluble and bound EPS are separated by centrifugation and different procedures have been developed to extract bound EPS based on physical (e.g. centrifugation, ultrasonication, blending and heat), chemical treatments (e.g. extraction with ethylenediamine tetraacetic acid (EDTA), NaOH, NaCl or formaldehyde) or their combination (Donot et al., 2012; Pal and Paul, 2008; Sheng et al., 2010). The available methods were evaluated and compared with respect to their extraction efficiency, the chemical composition and fluorescence properties of the extracts (Comte et al., 2006a; Domínguez et al., 2010a; Donot et al., 2012; Ni et al., 2009; Sheng et al., 2010). It was shown that different extraction procedures influence the quantity and the composition of the extracted EPS (Comte et al., 2006a; Domínguez et al., 2010a; Donot et al., 2012; Ni et al., 2009; Sheng et al., 2010), the quantity and the composition of the mineral fraction present in the EPS extracts (Bourven et al., 2011), as well as EPS binding properties to protons and different metals (Comte et al., 2006b; d'Abzac et al., 2010; Kenney and Fein, 2011). EPS are also broadly distributed in size and molar mass, thus the extraction procedure could be expected to affect their molar mass and size distribution and thus their environmental reactivity. However, very few and often contradictory studies are available about the physicochemical characterization of EPS, their molar mass and size distributions and the effects of extraction treatments. Several studies employed high pressure size exclusion chromatography (HP-SEC) to elucidate the influence of the EPS extraction on their HP-SEC fingerprints and

Abbreviations: EPS, extracellular polymeric substances; WT1, soluble EPS isolated from wild type *Sinorhizobium meliloti*; WT2, EPS of wild type bacteria extracted by EDTA; WT3, EPS of wild type bacteria extracted by formaldehyde/NaOH; EXOy1, soluble EPS isolated from exoY mutant *S. meliloti*; EXOy2, EPS of exoY mutant extracted by EDTA; EXOy3, EPS of exoY mutant extracted by formaldehyde/NaOH; FT-IR, fourier transform-infrared spectroscopy; EEM, excitation emission matrix; AFlFFF-UV-DRI-MALS, asymmetrical flow field-flow fractionation hyphenated with UV, differential refractive index and multiangle laser light scattering detection; HP-SEC, high pressure size exclusion chromatography; FPLC, fast protein liquid chromatography; TN, total nitrogen; TOC, total organic carbon.

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http://dx.doi.org/10.1016/j.biortech.2012.03.071

molar mass distributions showing a variable size diversity and fingerprints (Bourven et al., 2011; Comte et al., 2007; Domínguez et al., 2010b; Ras et al., 2011). It was found that different physical extraction methods give rise to different EPS HP-SEC fingerprints and molar mass distributions, while the chemical extraction procedures neither affected the HP-SEC fingerprints, nor molar mass distributions (Domínguez et al., 2010b). These findings disagree with the distinct HP-SEC fingerprints of the activated sludge EPS extracted by chemical methods (Comte et al., 2007). However, SEC with fluorescence detection showed similar number of fingerprints of activated sludge EPS extracted with EDTA as compared to those isolated by centrifugation (Bourven et al., 2011).

In the present study we characterized the soluble and bound EPS fractions obtained from pure bacterial cultures of *Sinorhizobium meliloti* to evaluate the influence of extracting methods on the EPS composition, molar mass and size distributions. We compared the EPS obtained by chemical extraction using EDTA and formaldehyde/NaOH methods, shown to be highly efficient (Donot et al., 2012; Sheng et al., 2010). We focused on the EPS of the gram negative bacterium *S. meliloti*, which is known to be able to produce measurable quantities of proteins, siderophores and low and high molar mass exopolysaccharides (LMM and HMM) (Alasonati et al., 2010; Alasonati and Slaveykova, 2011). The EPS extracted from the wild type (WT) and the exoY strain deficient in the production of the LMM (Reinhold et al., 1994) were compared to get a deeper insight on the relationship between the chemical composition of EPS and their molar-mass distributions. Asymmetrical flow field-flow fractionation hyphenated with UV, DRI detectors and multiangle laser light scattering, (AFlFFF-UV-DRI-MALS) have been employed for this purpose. The AFlFFF-UV-DRI-MALS is one of the best suited techniques that find increasing application for the separation and characterization of biopolymeric samples as recently reviewed (Yohannes et al., 2011), including bacterial EPS (Alasonati et al., 2010; Alasonati and Slaveykova, 2011).

2. Methods

2.1. ESP production and extraction procedure

The *S. meliloti* wild type strain *Rm1021* and the mutant exoY were cultivated on Luria broth (LB) agar plates for 7 days at 30 °C, then inoculated in 40 mL of 20 g L^{-1} LB at 30 °C and a rotary shaking of 160 rpm (Infor). Bacterial growth was followed by the measurements of the optical density at 660 nm. Once bacteria reached the exponential growth phase, they were isolated from the growth medium by gentle centrifugation (10 min, 2500*g*, 15 °C) then washed twice and transferred to a "minimal growth medium" (dilution 1:10). The minimal medium contained 2.05 g L^{-1} K_2HPO_4, 1.45 g L^{-1} KH_2PO_4, 0.15 g L^{-1} NaCl, 0.5 g L^{-1} NH_4NO_3, 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.01 g L^{-1} $CaCl_2{\cdot}2H_2O$, 6 g L^{-1} D-glucose and vitamins (0.4 ml L^{-1} of the Gamborg 1000 × stock solution). Next bacteria were grown at 30 °C, 160 rpm. When they reached the stationary growth phase (ca. 42 h) they were centrifuged 15 min at 2500*g* and 4 °C. The supernatant was collected, re-centrifuged in order to eliminate residual bacteria, and then filtered on 0.22 μm polyvinylidene fluoride (PVDF) filters (Millipore) under sterile conditions. The obtained fractions contained soluble EPS, denoted as WT1 and EXOy1 when isolated from the wild type strain and from the exoY mutant, respectively. To extract bound EPS bacterial pellets were washed twice with physiologic solution (9 g L^{-1} NaCl) then re-suspended in 10 mL milliQ water and divided in two portions. To the first bacterial suspension 2% EDTA was added and incubated for 3 h at 4 °C, while the second suspension was supplemented with 36.5% formaldehyde and incubated for 1 h at 4 °C, then 1 N NaOH was added and incubated for additional 3 h at 4 °C (Liu and Fang, 2002). Thereafter, the mixtures of bacterial suspensions and extracting agents were centrifuged for 15 min at 4 °C and 2500*g* to remove the remaining cells. The supernatants were filtered on 0.22 μm PVDF filters under sterile conditions in a laminar flow hood. The EPS extracted with EDTA were labeled as WT2 and EXOy2, while those obtained by formaldehyde/NaOH as WT3 and EXOy3. To remove the residual salts from the growth medium and the extraction agent, each EPS fraction was dialyzed against milliQ water for 96 h at the temperature of 4 °C. The regenerated cellulose tubular membrane of 3500 Da cut-off was employed (CelluSep, Membrane Filtration Products, Inc., USA). Dialyzed EPS were stored at 4 °C until analysis for a maximum of four weeks.

2.2. Characterization of the chemical composition of the EPS

The EPS were partially characterized by measuring the content of organic carbon, nitrogen, proteins and glucose, and then fractionated and characterized by AFlFFF-UV-DRI-MALS and Fast protein liquid chromatography (FPLC). Fourier transform-infrared spectroscopy (FT-IR) was used to probe the influence of the extraction procedure on different functional groups. Three dimensional excitation emission matrices (EEMs) analysis allowed further characterization of the fluorescent components of the extracted EPS. Total organic carbon and total nitrogen were measured using a Shimadzu TOC 500/5000A and calibration was performed with potassium hydrogen phthalate or potassium nitrate. Carbon and nitrogen values were then normalized to the bacteria dry weight. Total protein content was determined according to the Bradford method (Bio-Rad Protein Assay), using bovine serum albumin (BSA) as standard. Protein content was expressed in mg or mg L^{-1} of BSA equivalents, normalized to the bacteria dry weight. Total saccharide content was determined by measuring the glucose content, using the Amplex Red Reagent Assay Kit (Molecular Probes, Invitrogen AG) after hydrolysis by adding 1 N HCl and heating for 20 h at 80 °C (Myklestad et al., 1997). The solution was neutralized with 1 M NaOH. The absorbance was measured at 570 nm with a Microplate reader (MRX, Dynatech laboratories). D-glucose was used as standard and the exopolysaccharide content was expressed in mg L^{-1} of glucose equivalent, normalized to the bacteria dry weight. EEMs were determined using a luminescence spectrometer (LS-55, Perkin-Elmer Co., USA). The spectra were collected varying the emission spectra from 270 to 450 nm at 1 nm increment and the excitation wavelengths from 200 to 320 nm at 10 nm increments. The scanning speed was set at 500 nm min^{-1}. FT-IR was used to determine the chemical groups in the EPS obtained by the different extraction procedures. IR spectra were collected from 3900 to 800 cm^{-1}. Spectra were baseline corrected in Origin 8G data analysis and graphing workspace (OriginLab Corporation).

2.3. AFlFFF-UV-DRI-MALS

The instrumental set-up used in this study consisted in an asymmetrical flow field-flow fractionation system (AF2000 Focus, Postnova Analytics) coupled to UV–VIS (PN3241 UV/VIS, Postnova Analytics), differential refractive index (DRI, Shodex RI 101) detectors and seven angles laser light scattering (Postnova Analyitics). System control as well as data reduction treatment were performed using the AF2000 Control software (Postnova Analytics). The focusing and elution conditions were optimized in a previous study (Alasonati and Slaveykova, 2011) and were as follows: (i) focusing conditions: inlet flow rate V_{in} = 0.09 mL min^{-1}, focus flow rate 5.00 mL min^{-1} and crossflow rate 4.09 mL min^{-1}; (ii) elution conditions: inlet flow rate 1.1 mL min^{-1}, crossflow rate 0.1 mL min^{-1} and outlet flow rate 1.00 mL min^{-1}. A regenerated cellulose membrane with 10 kDa molecular weight cut-off (Post-