



Production and characterisation of exopolymer from *Rhodococcus opacus*



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ABSTRACT

Screening of Actinobacteria producing exopolymers with flocculating activity was carried out. An extracellular polymer with the highest flocculating activity extracted by *Rhodococcus opacus* was selected to characterisation. The water-soluble fraction of this exopolymer with molecular weight of about 760 kDa was found to be 64.6% polysaccharide and 9.44% protein. Chemical analysis showed the presence of reducing sugars, uronic acids, and amino sugars at concentrations of 184.79 $\mu\text{g}/\text{mg}$, 117.6 $\mu\text{g}/\text{mg}$, and 9.23 $\mu\text{g}/\text{mg}$, respectively. Additionally, the constituent sugars of the exopolymer were glucose, mannose, and galactose. The isoelectric point was measured at 2.5, and thermogravimetric analysis indicated the degradation temperature for this fraction at 275 °C. SEM microphotography showed a fibrillar structure with a sheet-like texture of the studied exopolymer. Infrared spectrophotometry analysis revealed that the exopolymer contained carboxyl, hydroxyl, acetyl, and carboxylate groups, preferred for the flocculation process. Additionally, the presence of these groups may facilitate the heavy metals adsorption and may influence carbonate minerals formation.

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1. Introduction

Flocculation is currently a widely investigated physicochemical process, which is mainly based on the presence of flocculants, synthetic or natural, with a tendency towards aggregation of particles suspended in water. This phenomenon can be applied in many fields from environmental engineering as a wastewater treatment factor to food and fermentation industries for removing pollutants [1]. Although many inorganic and organic synthetic flocculants performed well during the flocculation process, the health and environmental problems could not be neglected [2]. It has been reported that the use of synthetic flocculants is efficient, but still not safe for the environment. Therefore, natural flocculants have been intensively studied for the last decades. Despite many advantages like biodegradability and non-toxicity as well as the variety of preferable biopolymers with flocculation activ-

ity, it is necessary to optimise the whole process of derivation thereof. The macromolecules with flocculating activity are produced by microorganisms as their metabolites, mainly composed of proteins, lipids, polysaccharides, and nucleic acids [3,4]. Most bioflocculant-producing microorganisms have been isolated from soil and wastewater, and the methods of bioflocculant production and purification depend on the type of microorganisms from which this molecule is isolated. Although many microorganisms have been investigated as bioflocculant producers, probably there is still a multitude of new organisms with a potential use for flocculant application. Many factors connected with growth conditions have a crucial role in deriving of bioflocculants, and it is very important to optimise the culture parameters and all steps of biopolymer extraction and purification. The optimisation of the production and the process of purification of natural flocculants from microorganism cultures may encounter two problematic issues limiting the applications of these compounds in industry. The diversity of natural bioflocculant sources in terms of their composition and properties of analysed substances can be noticed [5]. Depending on microorganisms, it is possible to obtain products with unique features, characteristic only for a particular type of bacteria, fungi, or algae. The study performed by Patil et al. [6] demonstrates that an *Azotobacter indicus* ATCC 9540 strain is able to produce exopolysac-

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Table 1
Studied strains of Actinobacteria.

Microorganism	Abbreviation	Source ^a
<i>Rhodococcus opacus</i>	1069	DSMZ
<i>Rhodococcus rhodochrous</i>	202 DSM	DSMZ
<i>Pseudonocardia halophobica</i>	89 DSM	DSMZ
<i>Rhodococcus rhodochrous</i>	273	DSMZ
<i>Pseudonocardia autotrophica</i>	100	DSMZ
<i>Rhodococcus</i> sp.	1	DSMZ
<i>Pseudonocardia autotrophica</i>	88	DSMZ
<i>Pseudonocardia autotrophica</i>	99	DSMZ
<i>Rhodococcus opacus</i>	89 UMCS	UMCS
<i>Rhodococcus erythropolis</i>	202 UMCS	UMCS

^a DSMZ—Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures; UMCS—Maria Curie-Skłodowska University, Lublin, Poland (Fungal Culture Collection of Lublin).

charides which can be used in wastewater treatment, for example to remove dairy, starch, woollen, and sugar industry impurity. *Paenibacillus elgii* B69, producing an exopolysaccharide composed of glucose, glucuronic acid, mannose, and xylose, was effective in removal of pollutants such as dyes and heavy metal ions [7]. Newly isolated biofloculants, e.g. the alkaliphilic and salt-tolerant biofloculant produced by a *Bacillus agaradhaerens* C9 strain, are also effective in water purification to harvest some microalgae [2].

While analysing some investigations that have been conducted through decades, we can observe increased interest in Actinobacteria biofloculant studies [8–11]. Because of their differential area of occurrence, Actinobacteria strains are known from high adaptation abilities, which contribute to production of biofloculants in response to environmental stress factors. *Rhodococcus opacus* is a representative of a non-pathogenic lineage of nocardioform Actinomycetales. These bacteria are gram-positive and chemoorganotrophic organisms with high hydrophobicity (contact angle $70 \pm 5^\circ$) and have polysaccharides, carboxylic acids, lipids, and mycolic acids in the cell wall, which are responsible for its amphoteric behaviour [9,12]. Moreover, this strain produces extracellular polymers that can interact with different ions and particles to obtain flocs in the process of flocculation. In this work, we present an extracellular polymer with flocculating activity produced by *R. opacus* and the main focus was to isolate and characterise the physico-chemical properties of the exopolymer obtained. Probably not all the constituents of the tested exopolymer have flocculating capability. Hence, not all the analysis are related to the flocculating characterisation and some of them were done to determine the exopolymer composition, its stability and surface properties. The results of these studies may indicate the possibility of using the exopolymer or its fractions as natural flocculant for the removal of heavy metals and waste water treatment.

2. Materials and methods

2.1. Microorganisms and culture conditions

Ten strains of gram-positive bacteria belonging to Actinobacteria class were compared in relation to flocculating activity (Table 1). The studied strains were stored in the collection of the Department of Biochemistry, Maria Skłodowska-Curie University, Lublin, Poland (Fungal Culture Collection of Lublin—FCL), at 4 °C in agar medium consisting of (g/L) yeast extract 0.04, malt extract 0.1, glucose 0.04, and agar 0.2. The cultivations of the bacteria were carried out in liquid medium (LM) for 10 days on a rotary shaker (130 rpm, 26 °C). The medium used in this study consisted of 20 g glucose, 2 g KH₂PO₄, 5 g K₂HPO₄, 0.5 g NH₄Cl, 0.1 g NaCl, 0.5 g MgSO₄, and 0.5 g yeast extract dissolved in 1 L of distilled water [13]. The yeast extract was purchased from Difco Laboratories, USA, whereas other components of the medium were purchased from Avantor, Poland.

2.2. PCR amplification and sequencing of the bacterial 16S rDNA region

The total genomic DNA of the bacterial strain was isolated according to the method of Sharma and Singh [14]. The purity and quantity of the DNA samples were evaluated using an ND-1000 spectrophotometer (Thermo Scientific, Palm Beach, FL, USA). PCRs were performed using Thermo Scientific DreamTaq Green PCR Master Mix in a MyCycler Personal thermal cycler (Bio-Rad, USA). To confirm the genetic identity of the bacteria, the 16S rRNA gene was amplified using the universal prokaryotic primers Eub27f(rD1) (AGA GTT TGA TCC TGG CTC AG) and Eub1525r(rD1) (AAG GAG GTG ATC CAG CCG CA) as described previously [15]. The amplified region was analysed by direct sequencing of the PCR products. Automatic sequencing was performed using a BigDye™ Terminator Cycle Sequencing Kit and ABI PRISM 310/3730 XL sequencers (Applied Biosystem). Data from 16S rDNA sequencing was analysed with ChromasPro v.1.5 (Technelysium Pty Ltd, Australia) and Lasergene v.1.1.0 software (DNASTAR, Inc). Database searches were performed with the BLAST program at the National Centre for Biotechnology Information (Bethesda, MD, USA) [16]. The multiple DNA sequence alignments were performed with the Clustal-W algorithm [17]. The neighbour-joining (NJ) algorithm was employed to construct a phylogenetic tree as implemented in MEGA v.6.0 software [18]. The topology of the tree was evaluated by bootstrap analysis of the sequence data based on 1000 random resamplings.

2.3. Exopolymer production and purification

Production of *R. opacus* was performed in 3-L Erlenmeyer flasks containing 1.5 L of LM medium. 3-day-old *inocula* were added to the medium (10% v/v) and, after the incubation on a rotary shaker during 7 days (26 °C, 130 rpm), the culture solution was centrifuged twice at 9200 rpm for 30 min to remove bacterial cells. One volume of distilled water was added to the supernatant and the solution was concentrated about five times using a reverse osmosis process. The concentrated solution was centrifuged twice at 9200 rpm for 30 min and the supernatant was filtered using a Durapore membrane (0.45 µm diameter of pore; Millipore). Two volumes of cold ethanol (95%) were added to the filtrated solution, and then the mixed solution was left to stand at 4 °C for 72 h. After this time, the solution was centrifuged at 9200 rpm for 30 min and *precipitate I* was dissolved in distilled water and left at 4 °C overnight. The supernatant was again precipitated by adding one volume of cold ethanol (95%) and incubated for the next 72 h at 4 °C. After centrifugation (9200 rpm, 30 min), *precipitate II* was resuspended in distilled water and combined with *precipitate I*. The combined precipitates were dialysed for 3 days at 4 °C to remove ethanol and after dialysis freeze-dried using a lyophilisator (Labconco, USA) and powder of the *total exopolymer (tP)* was obtained. Next, the total exopolymer was solubilised in water at a concentration of 1 mg/mL during 24 h at 4 °C, centrifuged (9200 rpm, 30 min) after this time, and the supernatant was lyophilised and a *water-soluble exopolymer (sP)* was obtained. The solid, remaining after solubilisation and centrifugation, was treated as a *water-insoluble exopolymer (inP)*.

2.4. Assay of the flocculating activity

Kaolin suspension with CaCl₂ was used to measure the flocculating activity of the exopolymer obtained at a concentration of 1 mg/mL. 4.5 g of kaolin and 11 g of CaCl₂ were suspended in 1 L of distilled water. 0.1 mL of the exopolymer was added to 9 mL of this solution, stirred during 30 s using Vortex, and left to stand for 5 min. The absorbance of the upper phase and blank control without the exopolymer was measured at 550 nm (as OD_{sample} and OD_{blank}).

ID	Title	Pages
2717	Production and characterisation of exopolymer from Rhodococcus opacus	10

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