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Production and characterisation of exopolymer from *Rhodococcus opacus*

Magdalena Czemierska[a], Aleksandra Szcześ[b], Anna Pawlik[a], Adrian Wiater[c], Anna Jarosz-Wilkołazka[a,*]

[a] Department of Biochemistry, Maria Curie-Sklodowska University, Akademicka 19, 20-033 Lublin, Poland
[b] Department of Physical Chemistry—Interfacial Phenomena, Maria Curie-Sklodowska University, M. C. Sklodowska Sq 3, 20-031 Lublin, Poland
[c] Department of Industrial Microbiology, Maria Curie-Sklodowska University, Akademicka 19, 20-033 Lublin, Poland

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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 μg/mg, 117.6 μg/mg, and 9.23 μg/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.

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 activity, 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-

* Corresponding author.
E-mail addresses: magdalena.czemierska@poczta.umcs.lublin.pl (M. Czemierska), aszczes@poczta.umcs.lublin.pl (A. Szcześ), anna.pawlik@poczta.umcs.lublin.pl (A. Pawlik), adrianw2@poczta.umcs.lublin.pl (A. Wiater), anna.wilkolazka@poczta.umcs.lublin.pl (A. Jarosz-Wilkołazka).

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Table 1
Studied strains of Actinobacteria.

Microorganism	Abbreviation	Source[a]
Rhodococcus opacus	1069	DSMZ
Rhodococcus rhodochrous	202 DSM	DSMZ
Pseudonocardia halophobica	89 DSM	DSMZ
Rhodococcus rhodochrous	273	DSMZ
Pseudonocardia autotrophica	100	DSMZ
Rhodococcus sp.	1	DSMZ
Pseudonocardia autotrophica	88	DSMZ
Pseudonocardia autotrophica	99	DSMZ
Rhodococcus opacus	89 UMCS	UMCS
Rhodococcus erythropolis	202 UMCS	UMCS

[a] DSMZ—Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures; UMCS—Maria Curie-Sklodowska 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 bioflocculants, e.g. the alkaliphilic and salt-tolerant bioflocculant 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 bioflocculant studies [8–11]. Because of their differential area of occurrence, Actinobacteria strains are known from high adaptation abilities, which contribute to production of bioflocculants 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 Sklodowska-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 KH_2PO_4, 5 g K_2HPO_4, 0.5 g NH_4Cl, 0.1 g NaCl, 0.5 g $MgSO_4$, 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(fD1) (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.11.0 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 $CaCl_2$ 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 $CaCl_2$ 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},

respectively) using a spectrophotometer. The flocculating activity (FA) was calculated as follows [13]:

$$FA = (OD_{blank} - OD_{sample})/OD_{blank} \times 100$$

2.5. Physical and chemical analysis of the exopolymer

2.5.1. Molecular weight analysis

The molecular weight of the obtained water-soluble exopolymer (at a concentration of 6 mg/mL of MilliQ water) was estimated by gel permeation chromatography on a Sepharose CL-6B column (0.7 cm × 90 cm). The separation was done at room temperature using MilliQ water as the eluent with a flow rate of 0.3 mL/min. Fractions of 1 mL each were collected and tested for carbohydrates by the phenol-sulphuric acid assay [19]. For column calibration, a mixture of dextran standards (150 kDa, 670 kDa, and 2000 kDa) was used.

2.5.2. Chemical composition analysis

The total sugar content of the *R. opacus* exopolymer was tested by the phenol-sulphuric acid method using glucose as a standard [19]. The total protein content was determined by the Bradford method, using bovine serum albumin as a standard [20]. In order to determine the sugar composition, the exopolymer at a concentration of 1 mg/mL was hydrolysed in 4 M trifluoroacetic acid (TFA) (4 h, 100 °C) in Nanocolor Vario equipment (Macherey-Nagel, Germany). The hydrolysate was cooled, evaporated in a vacuum concentrator (Eppendorf AG, Germany), and re-dissolved in distilled water (1 mg/mL). The resulting sugars and amino acids were analysed by appropriated assays. The reducing sugars were determined by a modified Somogyi-Nelson method using glucose as a standard [21]. The uronic acids were measured by the carbazole-sulphuric acid method [22]. Amino sugars were determined using the Elson-Morgan method with glucosamine as a standard [23]. The *N*-acetylate amino sugars content were determined using *p*-dimethyloaminobensoesane aldehyde and tetraborane potassium [24]. The amino acids were analysed by ninhydrin reaction [25]. Samples of hydrolysates were also analysed by thin-layer chromatography (TLC) on silica gel $60F_{254}$ Merck plates with propanol – acetate – distilled water (4/0.5/0.5, v/v) as the mobile phase. Monosaccharides were detected by spraying with 10% sulphuric acid in 95% ethanol (1/28, v/v), dried, and heated at 100 °C for 15 min. The composition of hydrolysates was determined by counting the R_f value, which is subtraction of movement of the solvent and solute molecules. Glucose, mannose, fructose, galactose, xylose, and lactose were used as standards (1 mg/mL). Sugar standards were purchased from Sigma Aldrich, United Kingdom. Other reagents used in hydrolysis and thin-layer chromatography were obtained from Avantor, Poland.

2.5.3. Physico-chemical analysis and ultra-structure characterisation

The morphology of the lyophilised samples was determined using a scanning electron microscope (Quanta 3D FEG). The Fourier transformation infrared spectrum (FTIR) was recorded with a Hyperion 2000 (Brucker) spectrophotometer between 250 and 4000 cm^{-1}. The exopolymer obtained was also characterised by X-ray photoelectron spectroscopy (XPS) with a UHV Prevac spectrometer equipped with a monochromatized aluminium X-ray source (30 mA, 12 kV) and charge stabilization devices. The binding energy was set by fixing the component of the C1s peak at 284.8 eV. The spectra obtained were decomposed using CasaXPS software. Thermal analysis was used to study the thermal decomposition of calcium carbonate particles, as well as to determine the presence of lipids in particles and was carried out on a STA 449 Jupiter F1, (Netzsch, Germany) under the following operational conditions: heating rate of 10 °C/min, a dynamic atmosphere of synthetic air (50 mL/min), temperature range of 30–900 °C, a sample mass ~10 mg, the sensor thermocouple type S TG-DSC. As a reference empty Al_2O_3 crucible was used. The isoelectric point of the exopolymer was determined in the 0.001 M NaCl solution. The pH value and the zeta potential were determined after 24 h using a pH-meter (CX-731, Elmetron) and Zetasizer Nano (Malvern, UK) for the exopolymer concentration of 40 mg/L.

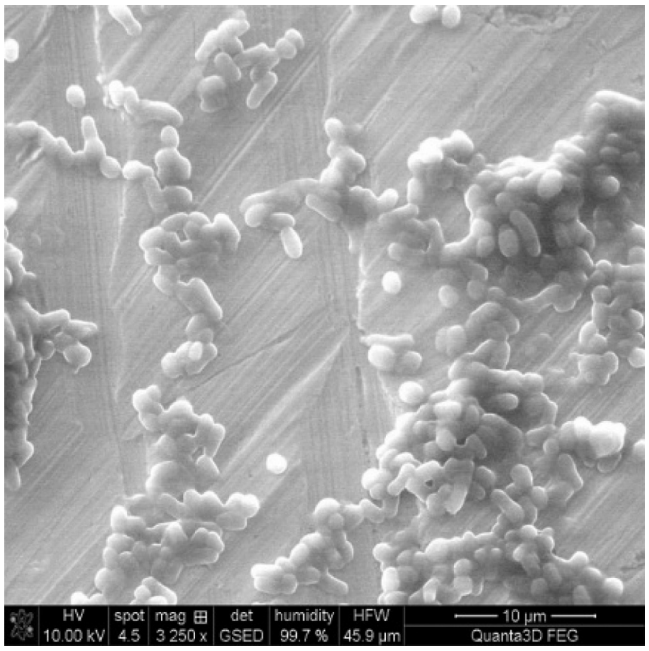

Fig. 1. Electron micrograph of *R. opacus* cells.

3. Results and discussion

Many bacterial members producing different kinds of polymers that exhibit flocculating activity are reported in the literature, namely *Arcuadendron* sp. [26], *Bacillus agaradhaerens* [2], *Azotobacter indicus* [6] and in Actinobacteria *Rhodococcus erythropolis* [13], *Nocardia amarae* [27], and *Streptomyces griseus* [28]. *R. opacus*, the bacterial strain tested in this work, is a unicellular gram-positive bacterium, which can interact with mineral surfaces due to presence of different types of compounds on the bacterial surface (Fig. 1). This microorganism has polysaccharides, carboxylic acids, lipid groups, and mycolic acids in the cell wall, which are responsible for the amphoteric behaviour on the cell surfaces [9]. The affinity of *R. opacus* cells for calcite and magnesite surfaces was studied in correlation to their application as a flotation collector [9]. On the other hand, this strain produces extracellular polymers that can interact with different ions and particles to obtain flocs in the process of flocculation. This exopolymer is released into the culture medium and is able to bind kaolin particles resulting in flocculation. In this work, the crude extracellular polymer with flocculating activity was obtained from the fermented broth of *R. opacus*.

3.1. Screening of flocculant-producing bacterial strains and their taxonomic analysis

The first stage of this work was the screening of cultures of Actinobacteria strains, stored in the collection of Maria Curie-Sklodowska University, Lublin, Poland (Fungal Culture Collection of Lublin, FCL), to test their ability to flocculate. Depending on the strain used in this study, the maximum of the flocculating activity

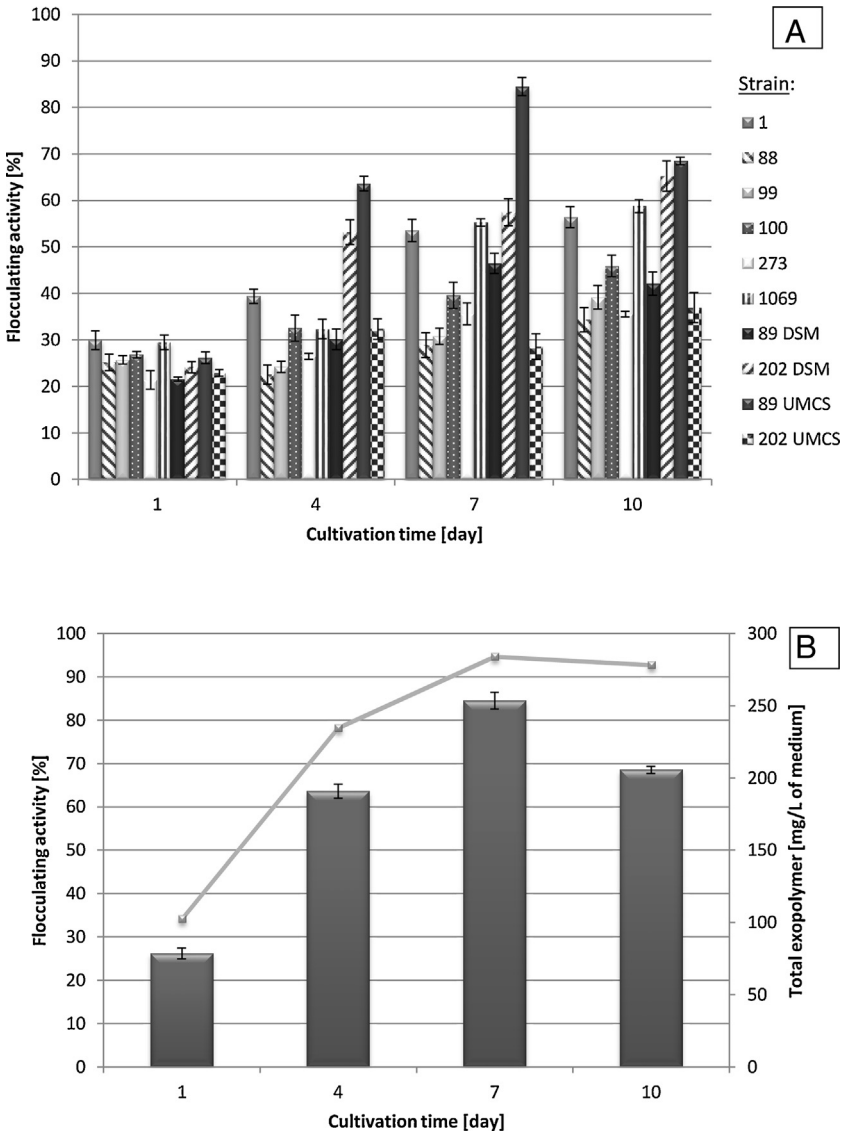

Fig. 2. (A) Flocculating activity of the studied strains of Actinobacteria and (B) the flocculating activity (bars) and exopolymer production (line) of the selected strain *Rhodococcus opacus* (89 UMCS).

was achieved from the 7th to the 10th day of cultivation on mineral liquid medium (Fig. 2A). Strains 1, 88, 99, 100, 1069, 202 DSM, and 202 UMCS reached the maximum activity on the 10th day of the growth and strains 273, 89 DSM, and 89 UMCS on the 7th day of cultivation. Between the ten tested strains, *R. opacus* (89 UMCS) was chosen as an exopolymer producer due to the presence of the highest flocculating activity during its growth on mineral liquid medium (Fig. 2B). Next, in order to maximise the amount of the exopolymer, cultures of the *R. opacus* strain were carried out in 3-L Erlenmayer flasks containing 1.5 L of LM medium. The cultures were performed at 26 °C on a rotary shaker (130 rpm). The studies performed on culture broth showed that *R. opacus* strain had the highest flocculating activity on the 7th day of the growth and that was the day of exopolymer extraction from the growing medium (Fig. 2B). During the cultivation time, the pH values were in the range from 6.7 to 7.0.

To determine the phylogenetic position of the tested bacterial strain, a 1428 bp fragment of the 16S rRNA gene was obtained from PCR with Eub27f(fD1) and Eub1525r(rD1) primers and followed by direct sequencing. The sequence of this product revealed over 99% identity of 89 UMCS to *R. opacus*, as shown in the NCBI-BLAST search system. The following GenBank accession number was assigned to the nucleotide sequence determined in this study: KM461686—*R. opacus* strain FCL89 16S ribosomal RNA gene, partial sequence. The NJ algorithm was employed to construct a phylogenetic tree for 89 UMCS and other strains tested in this work or described in databases (Fig. 3). *R. opacus* (89 UMCS) was clustered together with other closely related *R. opacus* species, which was further supported with a bootstrap value. The strains of *R. opacus* were clearly identified by forming a very well-defined cluster (98% bootstrap)

Two strains belonging to the same species—*R. opacus* (1069) and *R. opacus* (89 UMCS) showed high flocculating activity during their growth on the liquid mineral medium (Fig. 2A). This fact is unusual. Amjres et al. [29] found different efficiency, composition, and physical properties among the extracellular bioflocculants from different strains of the same species.

3.2. Chemical analyses of the water-soluble exopolymer and molecular mass determination

The growing medium of the 7-day-old culture of *R. opacus* was centrifuged and the supernatant was treated with cold ethanol. For standardisation of exopolymer yield, the polymer solution

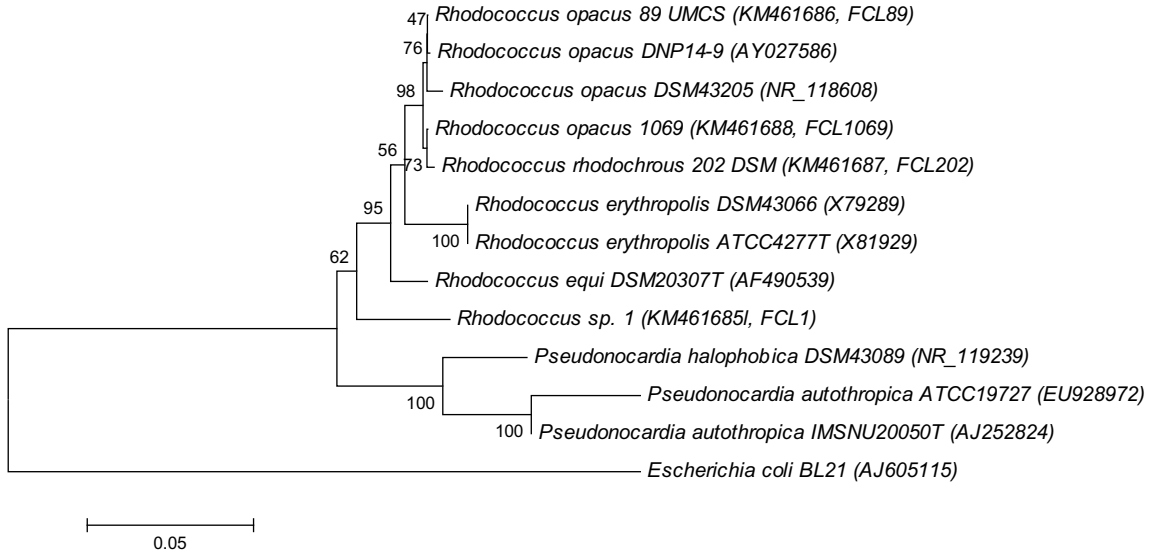

Fig. 3. Phylogenetic tree constructed with the NJ method based on 16S rDNA region sequences for the bioflocculant-producing *R. opacus* (89 UMCS) strain; the numbers in parentheses are accession numbers of 16S rDNA sequences; scale bar indicates base substitutions per 100 bases; bootstrap values at the nodes are percentages of 1000 replications.

was precipitated with different volumes of cold ethanol (95%). The double use of two volumes of cold ethanol showed better precipitation and recovery of the exopolymer. The precipitates recovered by centrifugation were dialysed overnight against distilled water at 4 °C. The dialysed material was freeze-dried, which yielded 266 ± 46 mg of the total exopolymer. The preparation of the exopolymer obtained showed flocculating activity in relation to the suspension of kaolin and $CaCl_2$ and was not totally solubilised in water after freeze-drying. The concentrations above 5 mg/mL formed a viscous solution with the precipitated material. This observation suggested a high molecular weight of the exopolymer, which was then analysed and validated by the gel permeation chromatography. Similar observation was described for an exopolysaccharide produced by *Gluconobacter diazotrophicus*, an endophytic nitrogen-fixing bacterium [30].

For further analysis, the extracellular polymer produced by *R. opacus* was divided into three fractions. The first fraction – the total exopolymer (*tP*) was solubilised in water at a concentration of 1 mg/mL during 24 h at 4 °C, centrifuged, and, after lyophilisation of the supernatant, the second fraction – the water-soluble exopolymer (*sP*) was obtained. The remaining sediment obtained after centrifugation was treated as the water-insoluble exopolymer (*inP*) – the third fraction.

In the case of the water-soluble exopolymer, the chemical analysis of its composition and the molecular mass were determined. This water-soluble exopolymer was dissolved in water (1 mg/mL) and analysed for polysaccharide and protein contents. The results showed that the *sP* fraction isolated from *R. opacus* and exhibiting flocculating activity was composed mostly of polysaccharides (646 ± 12.61 μg/mg of crude product). Additionally, the colorimetric Bradford reaction detected the content of protein (94.4 ± 6.56 μg/mg). The composition of this water-soluble exopolymer is similar to that of other reported products, which can mainly consist of polysaccharides and protein, e.g. a bioflocculant obtained from *Aspergillus parasiticus*—76.3% polysaccharides and 21.6% protein [31], a bioflocculant synthesised by *Micrococcus* sp. (28.4% polysaccharides and 2.6% protein) [32], or a bioflocculant obtained from *Nannocystis* sp. NU-2 composed of 40.3% proteins and 56.5% polysaccharides [33]. The dominant components of bacterial extracellular polymers are protein and carbohydrates (75–90%) but they also contain carbohydrates and protein derivatives such as lipopolysaccharides, glycoproteins, and lipoproteins [5], as in the case of bioflocculants that are mostly polysaccharides or their derivatives. *Bacillus* sp. I-471 [34] and *Alcaligenes cupidus* KT201 [35] produce bioflocculants that are polysaccharides. *Arathrobacter* sp. [36] and *Arcuadendron* sp. TS-4 [26] are able to synthesise glycoprotein bioflocculants. Kurane et al. [13] tested a bioflocculant with a glycolipidic structure produced by a *R. erythropolis* strain isolated from the soil. There are also bioflocculants that are only proteins such as the extracellular polymer produced by *N. amarae* YK1 [27] and by the fungus *Paecilomyces* sp. [37]. Table 2 compares data related to different flocculants about microbial origin.

Since polysaccharides are a mixture of many saccharides including neutral, uronic acid, and amino sugars, the water-soluble exopolymer (1 mg/mL) was hydrolysed with trifluoroacetic acid to determine the content of different sugars. The analysis showed that the polysaccharide fraction of the water-soluble fraction consisted of reducing sugars (184.8 ± 12.36 μg/mg), uronic acids (117.6 ± 1.85 μg/mg), amino sugars (9.23 ± 0.48 μg/mg), and *N*-acetylated amino sugars (4.17 ± 0.67 μg/mg). Additionally, the analysis showed the presence of amino acids from hydrolysed proteins (142.4 ± 3.71 μg/mg).

The sugar components of this exopolymer were identified by TLC analysis performed on silica gel plates using propanol: acetate: distilled water (4/0.5/0.5 v/v) as the mobile phase. The R_f values of three coloured spots of the *R. opacus* water-soluble bioflocculant corresponded with that of the standard sugars such as mannose, glucose, and galactose.

The chemical nature of bacterial flocculants is diverse and varies in terms of the concentration of carbohydrates, proteins, nucleic acid, and lipids and their form. There are examples of homopolymers such as the flocculant isolated from *Bacillus licheniformis*, which is a poly-glutamic acid polymer [3]. Most bioflocculants are heteropolymers such as the acidic bioflocculant DP-152 consisting of glucose, galactose, uronic acid, acetic acid, mannose, pyruvic acid, and fucose [38].

The monosaccharide composition is similar to that of other reported bioflocculants [10,30,39], which suggests that the exopolymer obtained has a straight structure and is similar to bioflocculants derived from bacteria. Moreover, analysis with the Fourier transformation infrared spectrum (FTIR) displayed

Table 2
The characteristics of bioflocculants about microbial origin with comparison to bioflocculant obtained from *Rhodococcus opacus* (this study).

Bioflocculant producing microorganism [reference]	Composition of bioflocculant	Molecular weight (Da) and flocculating activity (%)	Characteristic/properties
Rhodococcus opacus	64.6% polysaccharide 9.44% protein	7.6×10^5 Da	acidic isoelectric point correlated with the presence of anionic groups in the molecule
[this study]	carboxyl, hydroxyl, acetyl, and carboxylate groups	84.5%	
Aspergillus parasiticus [31]	76.3% polysaccharide 21.6% protein	3.2×10^5 Da	effective in the decolourisation of anionic dyes
	amine, amide and hydroxyl groups	98.1%	
Bacillus subtilis DYU1 [1]	14.9% polysaccharide 48.7% polyamides 4.4% protein	$3.16 - 3.2 \times 10^6$ Da	effective at pH 6–7
	amine, carbonyl and carboxyl groups	97%	
Corynebacterium glutamicum [8]	polygalacturonic acid	10^5 Da	high activity in the wide range of pH values, temperature and ionic strength
		80%	
Nannocystis sp. NU-2 [33]	56.5% polysaccharide	–	Fe^{3+} and Al^{3+} cations stimulate flocculating activity
	40.3% protein	90%	
Paenibacillus elgii B69 [7]	polysaccharide	3.5×10^6 Da	the bioflocculant removes turbidity and colour
	carboxyl groups	87%	
Bacillus sp. [50]	66.1% polysaccharide 29.3% protein	2.6×10^6 Da	high flocculating activity on real and synthetic wastewaters
	hydroxyl and carboxyl groups	98.1%	
Proteus mirabilis [51]	63.1% polysaccharide 30.9% protein	1.2×10^5 Da	alkaline condition promotes the flocculating efficiency
	carboxyl, hydroxyl, amino groups and hydrogen bonds	93.1%	
Micrococcus sp. Leo [32]	28.4% polysaccharide 2.6% protein 9.7% uronic acid	–	about 70% of flocculating activity was retained after heat treatment (100 °C)
	hydroxyl, carboxyl and amino groups	85.2%	

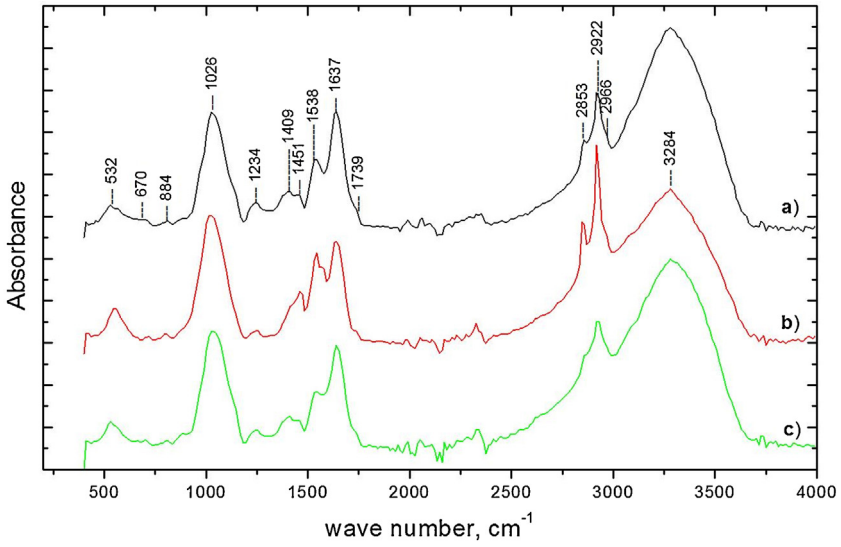

Fig. 4. FTIR spectra of the exopolymer from *R. opacus*: a) total exopolymer, b) water-insoluble fraction, c) water-soluble fraction.

absorption peaks characteristic for polysaccharides (1650 cm^{-1} and 1250 cm^{-1}) [40]. FTIR spectra of all the fractions (*tP*, *inP*, *sP*) of the *R. opacus* extracellular polymer are shown in Fig. 4. A broad intense peak at around 3289 cm^{-1} is characteristic for the hydroxyl group and the C—H bands at 2922 cm^{-1} and 2853 cm^{-1} are due to acyl chains [34]. It can be seen that the intensity of these two peaks in the fraction of the water-soluble exopolymer decreased (Fig. 4). The peak at ca. 1640 cm^{-1} may be due to the C=O stretching in the CONH group or NH_2 bending [31]. Peaks at 1739 and about 1226 cm^{-1} are attributed to acetyl groups [41]. Peaks at 1415 cm^{-1} and over 1700 cm^{-1} are due to carboxylate groups [41,42]. Bands in the region 1000–1100 cm^{-1} are characteristic for glucans [42]. Moreover, the band at ca. 890 cm^{-1} may indicate β configuration of the main glucan linkages [42]. The relatively strong peak at around 1650 cm^{-1} and the weak one around 1250 cm^{-1} indicate the characteristic IR absorption of polysaccharide [40].

For further characterisation of all fractions of the exopolymer from *R. opacus* (*tP*, *sP*, *isP*), the XPS spectra were obtained and complete atomic compositions are presented in Table 3 . The significant changes in the atomic percentage of atoms appeared only for carbon. Surprisingly, the water-insoluble fraction contains the smallest amount of this element; however, it also contains more phosphorus and silica.

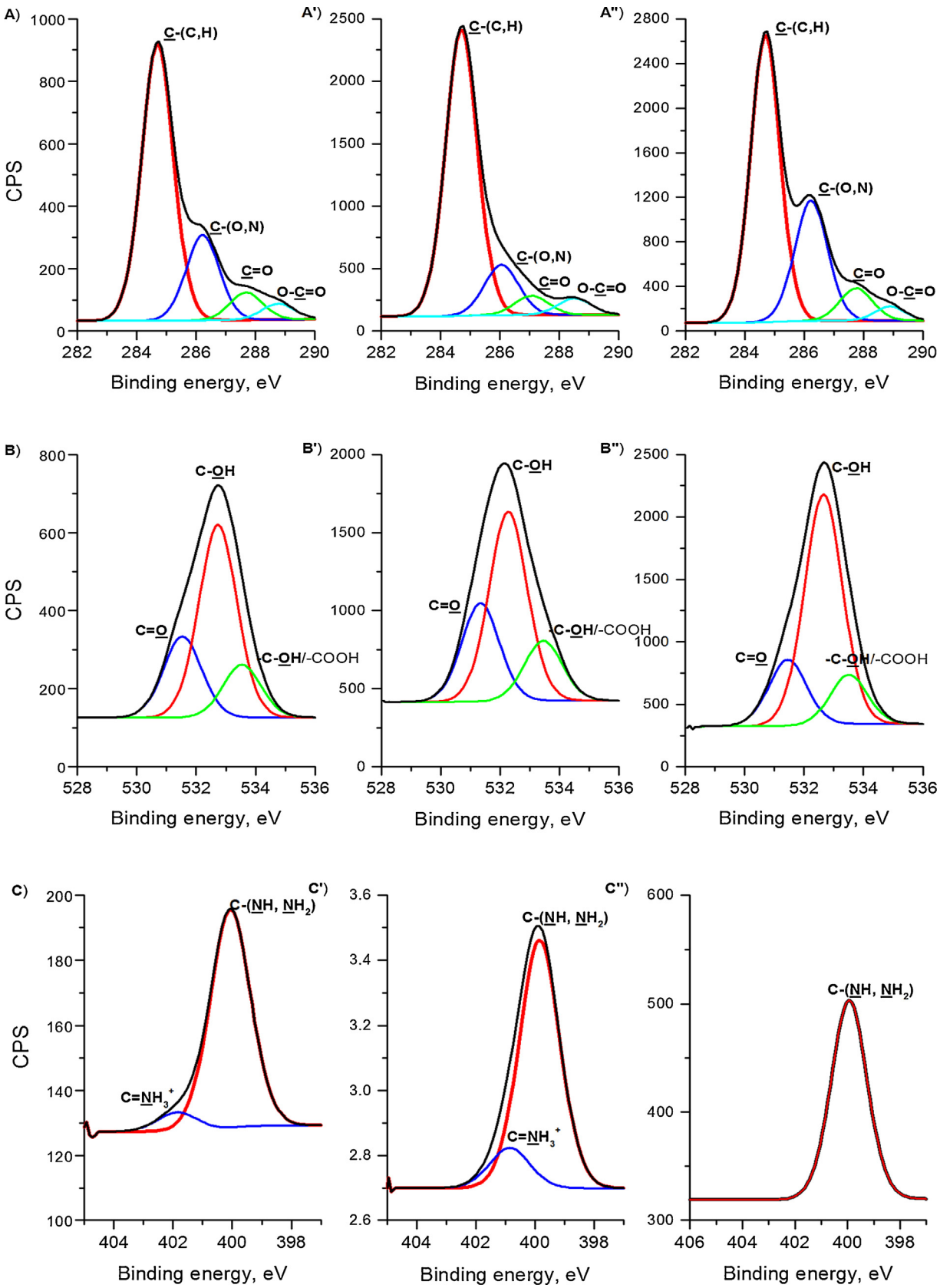

Fig. 5. XPS peaks (P1s, C1s, N1s, and O1s) recorded for: A–C—total exopolymer, A'–C'—water-insoluble exopolymer, A"–C"—water-solubl e exopolymer.

The C1s, O1s, and N1s spectra for the three fractions of the exopolymer (total, water-soluble, water-insoluble) are shown in Fig. 5. The C1s peak for all the exopolymer fractions was decomposed into four components (Fig. 5A-A") at the binding energies: 284.7, 286.2, 287.7, and 288.8 eV corresponding to the C in the C-(C,H), C-(O,N), C=O, and O—C=O groups, respectively [43,44]. The

Table 3
Complete elemental composition (atom percentage) of *R. opacus* exopolymer analysed by XPS.

Sample	C1s (%)	O1s (%)	N1s (%)	P2p (%)	Si2p(%)	Ca2p (%)	Mg2p (%)
total exopolymer	77.8	18.0	2.4	0.7	0.3	0.8	0.0
water-insoluble fraction	65.9	20.0	2.4	3.1	4.4	0.0	4.2
water-soluble fraction	72.4	20.4	2.5	1.2	1.7	0.0	1.7

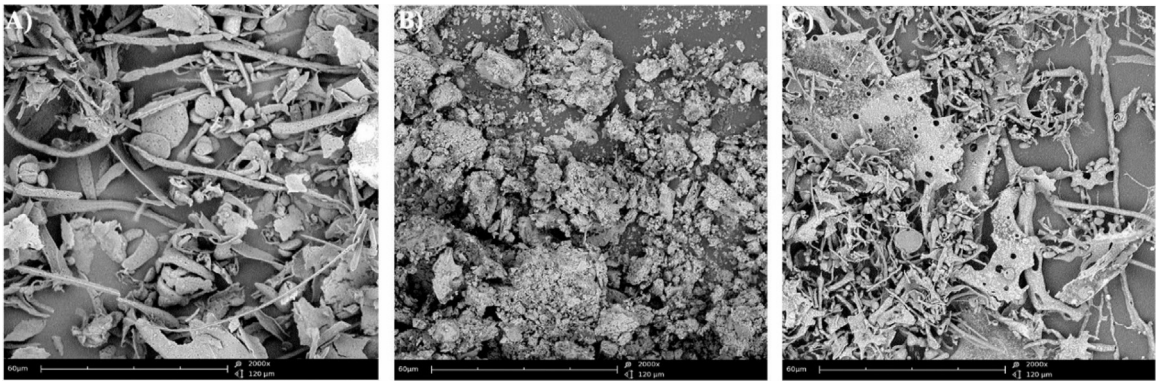

Fig. 6. SEM images of the biopolymer of *R. opacus*: a) total exopolymer, b) water-insoluble exopolymer, c) water-soluble exopolymer.

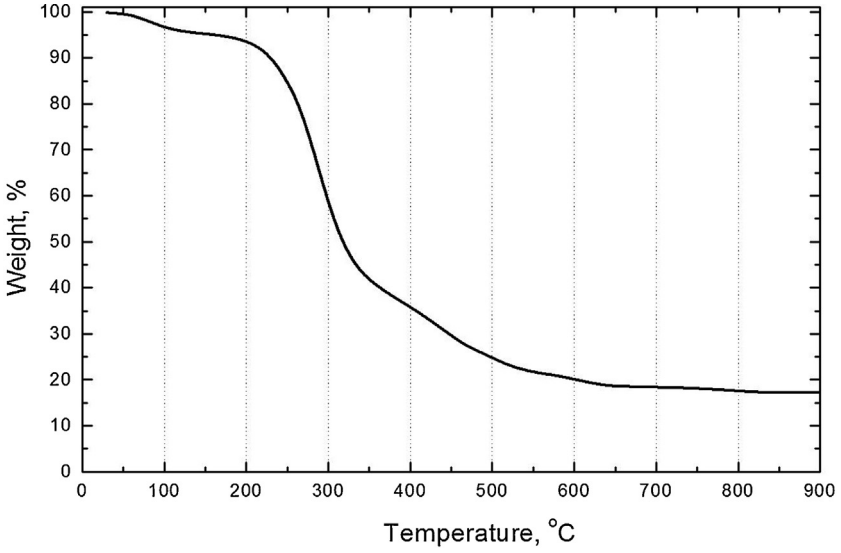

Fig. 7. TGA thermogram of the total exopolymer from *R. opacus*.

peak at 287.6 eV may contain contribution of acetal or hemiacetal functions present in carbohydrates [44]. The highest intensity of this peak appears in the water-soluble exopolymer. For the water-insoluble exopolymer, the peak intensity corresponding to hydrocarbons is the highest in comparison to the two other C peaks. The O1s spectrum (Fig. 5. B-B") can be resolved into three peaks corresponding to C═O/COOH (532.1 eV), C—OH in alcohol or (hemi)acetal (532.7 eV), and C—OH in carboxylic acid and ester (533.5 eV) [44,45]. Also in this case, the intensity of the $O_{532.7}$ peak in comparison to the two other O1s peaks is the highest in the case of the water-soluble exopolymer. The N1s spectrum (Fig. 5C—C") was decomposed in the C—NH/C—NH_2 group (399.8 eV) and nitrogen atoms C—NH_3^+ (401.6 eV) was protonated in the case of the total exopolymer as well as in the case of the water-insoluble fraction. In the case of the water-soluble exopolymer, only a peak contributing to the amide or peptidic link function appeared at 399.8 eV [44,45]. These results are correlated with the FTIR analysis and prove the presence of hydroxyl, carboxylic, amide, and amine groups in the exopolymer.

To establish the average molecular mass of the water-soluble exopolymer from *R. opacus*, the purified preparation was dissolved in MilliQ water. After gel permeation chromatography, it exhibited a single symmetrical peak, indicating homogeneity. The preparation of the exopolymer from *R. opacus* was eluted close to a void volume of the column giving a molecular mass of about 760 kDa. Among other parameters, during the flocculating process, the efficiency of the bridging mechanism in flocculation is related to the molecular weight [46]. The flocculation with a high-molecular-weight bioflocculant involves more adsorption points, stronger bridging ability, and higher flocculating activity [46].

3.3. Physico-chemical analysis and ultra-structure characterisation

All the preparations of *R. opacus* exopolymers obtained (total exopolymer, water-soluble exopolymer, and water-insoluble exopolymer) were analysed for their physico-chemical characterisation. At the beginning, the morphology of the lyophilised

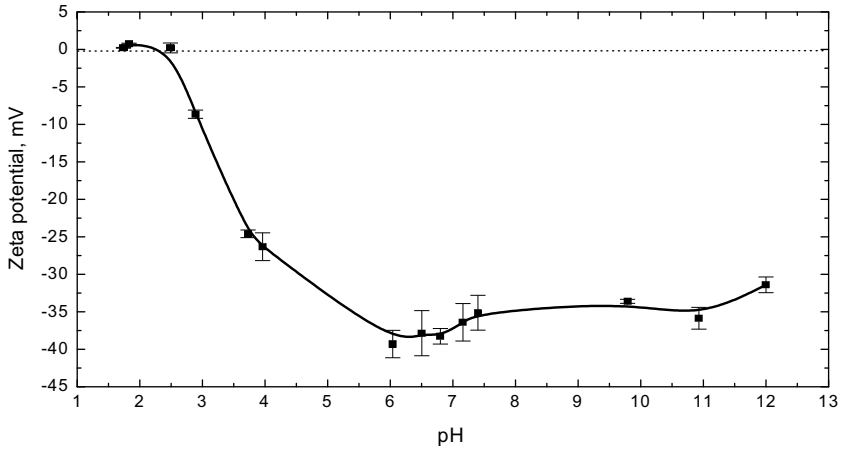

Fig. 8. Zeta potential changes versus the pH values of the total exopolymer from *R. opacus*.

samples was determined using a scanning electron microscope. Fig. 6 presents SEM photography of three fractions of exopolymer isolated from bacterial growing medium. The total exopolymer and the fraction of the water-soluble exopolymer show a fibrillar structure with a sheet-like texture. This indicates the thin web structure of these preparations with higher capillary forces to hold water molecules. In the fraction of the water-insoluble exopolymer, no fibrillary structure is present and typical powder is seen (Fig. 6).

In the case of the fraction of the total exopolymer from *R. opacus*, a thermogravimetric analysis (TGA) of thermal decomposition patterns and an analysis of the isoelectric point were performed. The TGA analysis was carried out dynamically (weight loss versus temperature) and the results are presented in Fig. 7. An initial weight loss due to the moisture content appeared between 50 and 150 °C. The initial moisture content in the total exopolymer is due to the increased level of carboxyl groups in the polysaccharide, which is correlated with greater affinity for the interaction with water molecules [34]. The decline in the weight above this temperature is attributed to the degradation of the sample. The degradation temperature (T_d) for this exopolymer is 288 °C. The onset of decomposition occurred at 220 °C and the recorded mass loss was 60%. The weight of the total fraction of exopolymer was dramatically lost around 275 °C and gradually decreased (Fig. 7).

The zeta potential curve (Fig. 8) shows the behaviour of the *R. opacus* fraction as a charged particle in the aquatic medium. As can be seen in Fig. 8, the pH of the isoelectric point (IEP) for the exopolymer obtained is equal to 2.5. In a neutral and alkaline solution, the electrokinetic potential is negative and fluctuates around −37.0 mV.

The acidic IEP value could be due to the presence of anionic groups in the molecule of the exopolymer. Botero et al. [9] described that the IEP value of the cell wall of *R. opacus* cells was also acidic (around 3.2), which was correlated with the domination of anionic groups. The presence of polysaccharides and amino groups on the cell wall or in the molecule of the exopolymer gives a net charge on the surface that depends on the pH value [47]. A majority of bacterial cells have isoelectric points below pH 4 [48] due to the presence of glucuronic acids or other polysaccharide-associated carboxyl groups. An acidic IEP may reflect mixed contributions of polysaccharide- or protein-associated $COOH^-$, NH_3^+, and phosphate groups. The bioflocculant produced by *S. griseus* tends to act under acidic conditions and achieve the highest flocculating activity at pH 4 [28]. *Enterobacter aerogenes* W-23 [49] was isolated from soil and identified as an acidic polysaccharide containing uronic acid (13.2%), pyruvic acid (7.4%), and acetic acid (1.6%). Bioflocculant MBF3-3 extracted by *Bacillus* sp. BF3-3 is composed of an acidic polysaccharide (66.1%) and protein (29.3%) and such ions as Al^{3+}, Mg^{2+}, Ca^{2+}, K^+, and Na^+ stimulate the flocculating activity of this exopolymer [50]. *Proteus mirabilis*, a microorganism that produces flocculant TJ-F1 from mixed activated sludge, was investigated by Zhang et al. [51], who showed that alkaline condition improved its flocculating activity.

4. Conclusions

This study shows that *R. opacus* is a bioflocculant producing strain and this extracellular polymer is not totally solubilised in water. This is very important because this strain was earlier described as a biocollector for flotation of different minerals. Probably, this is due not only to the amphoteric behaviour of the surface of the bacterial cell wall but also to the production of extracellular polymers. This exopolymer can form flocs in the presence of kaolin and different ions. The extracellular polymer produced by *R. opacus* is an acidic molecule due to the mixed contributions of polysaccharide- or protein-associated $COOH^-$, NH_3^+, and phosphate groups. The exopolymer produced by *R. opacus* possesses all the characteristics favouring the flocculation process (the molecular weight, the presence of functional group, the acidic behaviour). This macromolecule can interact with positively charged functional groups of particles suspended in water causing their aggregation, as well as binding metal cations, e.g. Ca^{2+}, Fe^{2+}, Mg^{2+}. Stabilising properties of layers of long polymer chains may also play role in the flocculation process. This characteristic indicates that the obtained polymer may be not only the good flocculating agent but also have the ability to bind heavy metal cations and modulate mineralisation of calcium carbonate.

Acknowledgments

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