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Removal of natural humic acids by decolorizing actinomycetes isolated from different soils (Algeria) for application in water purification

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ABSTRACT

Humic acids are considered problematically in drinking water because it can react readily with chlorine to form carcinogen compounds and its biological removal is much recommended. The scanning electron microscopy morphologies and optical parameters observed for natural humic acids (NHAs) extracted from different soils at Mitidja plain (Algeria) made them different from the commercial ones. Three of the most active strains of 19 actinomycetes were isolated and selected from surface soils at this plain. These strains were identified based on cultural characteristics and chemotaxonomic analysis and classified in the genus *Streptomyces*. Growth of these strains was assured on a poor liquid medium containing NHAs as carbon and nitrogen sources and degradation occur only in the presence of glucose. A maximal decolorization extent was obtained for 28 days at 30 °C under shake culture (67%, 66% and 57% for *Streptomyces* sp. strain AB1, *Streptomyces* sp. strain AM2 and *Streptomyces* sp. strain AH4, respectively). As compared with initial and final structures of NHAs after incubation (28 days), the structural changes in FTIR spectrum and metabolite products analyzed by HPLC indicate the capability of the selected *Streptomyces* sp. strains to degrade HAs and to play a part role in humus turnover in natural waters.

1. Introduction

Humic substances (HS) can be operationally divided into three fractions based on their solubility in aqueous solutions as a function of pH. Humic acid (HAs) is the fraction soluble in an alkaline solution, fulvic acid (FA) is the fraction soluble in an aqueous solution regardless of pH, and humin is the fraction insoluble at any pH value. The characteristic that remains associated with each humic fraction after their separation from a natural organic matter (NOM) sample is the high degree of their heterogeneity [1].

It is now known that several microorganisms, including fungi, actinomycetes and bacteria, can decolorize and even completely mineralize HAs under certain environmental conditions. The changes in the chemical properties of a limited range of HAs degraded (and therefore decolorized) by actinomycetes have been investigated [2–4]. There has been great progress in the analytical methods which can be used to characterize humic substances [5–9]. By applying these methods during biodegradation, a better understanding of the mechanisms governing this process can be achieved.

In addition, the HA fraction of NOM is considered problematically in drinking water because it can react readily with chlorine to form carcinogen compounds. Therefore, there exist two reports on endemic diseases that are harmful to those who used to drink well water near peat bogs: Kaschin–Beck disease, a chronic osteoarthritic disorder with necrosis of condrocytes prevailing in China [10]. HAs can form complexes with heavy metals and hydrophobic polychlorinated organics [11], influencing their fate and transport [12].

The Mitidja plain a North location of Algeria is known for its fertility and a rapid disappearance of natural humic acids causing saturation of surface waters by FA-like metabolite products (Ghrib and Keddara dam's waters, north of Algeria) [13]. Until now, this has been the first report on the decolorizing actinomycetes being isolated and identified from surface soils and we suggest that they may play a significant role in the turnover of HS in local soils and surface waters. Moreover it seems that these actinomycetes are the major element causing rapid disappearance of natural humic acids in this plan. Therefore, the aim of the present work was (1) to find out the potential of actinomycetes isolated locally for the degradation of NHAs under static and shaking conditions at laboratory scale and (2) to study the structural changes of these macromolecules used as

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carbon and nitrogen sources by the same strain isolated from the same soil sample.

2. Materials and methods

2.1. Extraction and characterization of natural humic acids

Soils were collected on March 2007 from the top 0–20 cm layer of cultivated soils from various sites on the Mitidja plain (red Mediterranean soil at Hadjout, Vertisols at Meftah and humid soil at Boufarik). The soil was air-dried and sieved through a 40-mesh screen to remove coarse plant debris, and stored for less than 1 week at 4 °C before use. The extraction and fractionation of humic substances were carried out according to Lopez et al. [14] method. One gram of the fresh sample was treated with 20 ml pyrophosphate-NaOH solution (0.1 mol L^{-1} $Na_4P_2O_7 \cdot 10\,H_2O$ + 0.1 mol L^{-1} NaOH, pH 13 measured by a pH meter type Cyber Scan pH 510) in sealed bottles by shaking at 200 rpm for 30 min, and then the samples were conserved for 12 h at 4 °C. The pyrophosphate–NaOH extract was acidified to pH 2 with H_2SO_4 solution (6 N). The insoluble fraction that contained SHAs was separated from the fulvic acid solution by filtration and then re-dissolved in 0.1 N NaOH. SHAs solutions obtained were incubated at 60 °C until dried. For characterization, they were converted to the acid form HAs following the method of Fukushima et al. [15].

Characterization of NHAs was performed using (i) scanning electron microscopy (SEM) (ESEM XC30FEG) for morphology observation; (ii) UV–visible spectrophotometer (Technicomp 8500) with a 10 mm quartz cell was used for absorbance measurements taking 0.3 mg HAs which dissolved in 10 ml $NaHCO_3$ (0.05 M) at pH 8.3 ± 0.2 and E_4/E_6 ratios (the absorbance at 465 nm divided by that at 665 nm) were calculated according to Eyheraguibel et al. [16]; and (iii) Fourier transform infrared spectroscopy (FTIR) (Shimadzu 9800) to detect the presence of typical functional groups: FTIR spectra were recorded from KBr pellets over the 4000–400 cm^{-1} range at a rate of 16 nm s^{-1} (250 mg dried KBr and 2 mg freeze-dried NHAs pressed under vacuum).

2.2. Humic acids preparation and purification

Commercial humic acids (CHAs) were purchased from Aldrich and natural humic acids were obtained after extraction (as above). A solution containing 5% HAs (NHAs or CHAs) is dissolved in 0.1 N NaOH and then agitated under nitrogen atmosphere for 20 minutes. The pH was adjusted at 1.0 ± 0.2 by HCl (1 N). The obtained aggregate was dissolved with NaOH 0.1 N and then treated again (agitation, flocculation, re-solubilization). Both types of HAs, employed throughout this study, were dissolved as follows: 1 g of dried HAs (NHAs or CHAs) was dissolved in 62.5 ml of NaOH (2 N), and then completed to a liter with distilled water. This solution was stirred for 48 h and stored at 4 °C in the dark.

2.3. Isolation of actinomycetes

Isolations were made at 30 °C from three soil samples of various origins that were freshly collected from the same location as described above. The samples were collected from the first 10 cm below the surface, aseptically transferred to sterile vials and stored at 4 °C until used. After mechanical stirring of samples in sterile water serial 10-fold dilution were made in NaCl 0.15 mol L^{-1} and spread on yeast extract–malt extract agar (ISP2) [17] and supplemented with actidione (antifungal) (500 mg L^{-1}). Actidione was filter-sterilized (final pH 7.2 ± 0.01). After one week of incubation, colonies were numbered, coded and transferred onto the same medium devoid of actidione to test purity. These isolates were stored on the yeast-malt extract–glucose-agar-slants (in L^{-1}: yeast extract: 4 g; malt extract: 10 g; glucose: 4 g; agar: 12 g).

2.4. Screening assays and selection of decolorizing strains

All 19 isolates were examined in the screening tests using CHAs. Decolorization was monitored on poor liquid medium (PLM) containing traces quantity of carbon and nitrogen sources. The medium containing: (1) mineral salts (in L^{-1}: KH_2PO_4: 2.38 g; $K_2HPO_43H_2O$: 5.65 g; $MgSO_4.7H_2O$: 1 g; (2) 1 ml of solution containing trace elements prepared in L^{-1} ($CuSO_4$ $5H_2O$: 0.64 g; $FeSO_4.7H_2O$: 0.11 g; $MnCl_2.4H_2O$: 0.79 g; $ZnSO_4.7H_2O$: 0.15 g), (3) glucose (0.1 g L^{-1}), (4) $(NH_4)_2SO_4$ (0.084 g L−1) (5) CHAs (0.5 g L^{-1}). The growth medium and dissolved CHAs were adjusted to pH 7.2 ± 0.01 and sterilized by membrane filtration (pore size 0.22 µm). Ten milliliters of the medium were added to three test tubes (16 cm high and with 2 cm in diameter) for each combination of HAs and microbial strains. Spore culture incubated for 2–4 weeks in ISP2 solid medium were used as inoculation culture. One colony (0.5 cm in diameter) was added to each test tube and they were kept in the dark under shake (150 rpm) during 21 days at 30 °C.

2.5. Humic acids quantification in culture medium

Degradation (and therefore decolorization) of HAs was measured as the rate of decrease of absorbance compared with non-inoculated cultures. After incubation, the tubes were centrifuged by EBA 20 centrifuge (5000 rpm for 15 min) and the supernatant fraction was filtered using Microfilter syringe system (Thomapor®-Membranfilter, 5FP 025/1). The supernatant fraction (2 ml) was diluted five-fold with 0.5 M NaOH solution and the absorbance measured at 350 nm (*A*350) at pH 4.5 ± 0.01. Standard curves were drawn by measuring the absorbance of known concentrations of HAs. All studies were performed in triplicates and decolorization extent was calculated as reported by Wang et al. [18] using the following equation:

$$\text{Decolorization extent}\,(\%) = \frac{OD_1 - OD_t}{OD_1} \times 100 \quad (1)$$

where OD_1 refers to the initial absorbance, OD_t refers to the absorbance after incubation at time t (days).

2.6. Preliminary identification of decolorizing actinomycetes

Preliminary bacterial identification to genus level was carried out by morphological and chemical studies as follows:

- Morphological and cultural characteristics
  Taxonomic studies of three of the most active strains of 19 actinomycetes isolates (AB1 originated from Boufarik, AM2 from Meftah and AH4 from Hadjout soils) were performed based on morphological and chemical analyses using the methods described by Shirling and Gottlieb [17] and Nonomura [19]. The morphological and cultural characteristics of the organisms were determined by macromorphologic examination of 7-day-old cultures grown on various International *Streptomyces* Project (ISP) media [17]: yeast extract–malt extract agar (ISP2), oatmeal agar (ISP3) and inorganic salts–starch agar (ISP4) [19]. The micromorphology and sporulation were observed by light microscopy (Carl Zeiss, Germany). Colors of aerial and substrate mycelia were determined with the ISCC-NBS centroïd color charts (US National Bureau of Standard, 1976) which were used by Boudjella et al. [20].
- Chemotaxonomic analysis
  Biomass for chemotaxonomic analysis was obtained from a culture grown in shake ISP2 medium [17] and incubated at 30 °C for 14 days. Analysis of cell-wall components (diaminopimelic acid isomers) was done according to the method of Staneck and Roberts [21] using Thin Layer Chromatography (TLC) on plastic sheets cellulose (Merck) with methanol/water/HCl 6 N/pyridine (80/26/4/