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Review

Characterization and biodegradation of soil humic acids and preliminary identification of decolorizing actinomycetes at Mitidja plain soils (Algeria)

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There are abundance of lignite resources in Algeria, particularly in Mitidja plain soils which is known by its fertility and a rapid disappearance of natural organic matters (NOM). The scanning electron microscopy (SEM) morphologies and optical parameters observed for soil humic acids (SHAs) made them different to 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 SHAs 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. AB1, *Streptomyces* sp. AM2 and *Streptomyces* sp. AH4, respectively). As compared with initial and final structures of SHAs after incubation (28 days), the structural changes in FTIR spectrum and metabolite products analysed by HPLC indicate the capability of the selected *Streptomyces* sp. strains to degrade SHAs and to play a part role in lignin degradation and humus turnover in local soils.

Key words: Soil humic acids, *Streptomyces,* actinomycetes, decolorization, biodegradation.

INTRODUCTION

Humic substances (HS) represent the main carbon reservoir in the biosphere, estimated at 1600 x 10^{15} g C. Due to their crucial role in reductive and oxidative reactions, sorption, complexation and transport of pollutants, minerals and trace elements, sustaining plant growth, soil structure and formation, and control of the biogeochemistry of organic carbon in the global ecosystem, HS are then extremely important to environmental processes (Grinhut et al, 2007).

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 (Chilom et al, 2009).

In recent years, more and more research for producing several kinds of fuel and industrial materials by biodegradation have been taken into account (Polman et al., 1995; Yong et al., 1995; Thygesen et al., 2009). It has been established that treatment of low-rank coals with aerobic coal-solubilizing microorganisms results in the production of highly polar, heterogeneous materials with a relatively high oxygen content (Davison et al., 1990).

Microbial treatment has been considered as an economically effective and environmentally safe way of pro-

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Table 1: Some criteria of three sites used for HAs extraction and actinomycetes isolation.

Sites	Depth of soil sampling (cm)	Humidity at the time of sampling (%)	Organic matter (%)	Total limestone (%)	Texture	Soil classification
Meftah	10	22-05	3-06	3-33	silty	Vertisols
Boufarik	10	21-50	1-65	4-86	clay	Humid
Hadjout	10	17-25	2-37	6-08	clay	red mediterranean

cessing coal via degradation of the macromolecular network into simpler, low molecular mass products (Zheng, 1991; Fakoussa and Hofrichter, 1991; Gupta and Birenda, 2000).

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 (Kontchou and Blondeau, 1992; Dari et al, 1995; Yanagi et al., 2002). There has been great progress in the analytical methods which can be used to characterize humic substances (Blondeau, 1989; De Nobili and Chen, 1999; Käcker et al., 2002; Campitelli et al., 2006; Peña-Méndez et al., 2007). By applying these methods during biodegradation, a better understanding of the mechanisms governing this process can be achieved.

Soil organic matter (SOM) is a key factor in ecosystem dynamics. A better understanding of the global relationship between environmental characteristics, ecosystems and SOM chemistry is vital in order to assess its specific influence on carbon cycles (Vancampenhout et al., 2009). In addition, the HAs 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 (Moriguchi, 2005). HAs can form complex with heavy metals and hydrophobic polychlorinated organics (Bratskaya, 2004), influencing their fate and transport (Qi et al, 2004).

The Mitidja plain a North location of Algeria is known by its fertility and a rapid disappearance of NOM (Senoussi, 1992). The location sites for HAs extraction and actinomycetes isolation are: Hadjout: Alti 90m, long 2°. 24°12''E. Lat 36°. 30'. 39 N, Nord-ouest ; Boufarik: Alti 70m, long 0,65 grandes SE lat.40.61 grandes SE. Nord-est and Meftah : Alti 45 m, long 03°. 12'.54'' E. Lat 36°. 38'. 9''N. Criteria for these three sites are presented in Table 1 and citrus trees are the most types of corps cultivated in this plain.

Until now, this is the first report on the decolorizing actinomycetes being isolated and identified from local surface soils and we suggest that they may play a significant role in the turnover of HS in soils. Moreover it seems that these actinomycetes are the major element causing rapid disappearance of NOM in this plan and few studies have described the degradation of HAs by actionmycetes especially by *Streptomyces*, is why we concentrated only on this genus as first step. It should be noted that further work on the degradation by fungi strains and bacteria isolated from the same sites are underway as second step. Therefore, the aim of the present work was (1) to find out the potential of actinomycetes isolated locally for the degradation of SHAs under static and shaking conditions at laboratory scale and (2) to study the structural changes of these macromolecules used as carbon and nitrogen sources by the same strain isolated from the same soil sample.

MATERIALS AND METHODS

Extraction and characterization of soil humic acids

Soils were collected on March, 2007 from the top 0 - 20 cm layer of cultivated soils from three sites on the Mitidja plain. 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 HS were carried out according to Lopez et al. (2006). One gram fresh sample was treated with 20 ml pyrophosphate-NaOH solution (0.1 mol l^{-1} Na_4 P_2O_7.10 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 pyrophos-phate-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. (2000).

Characterization of SHAs was performed using (i) scanning electron microscopy (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. (2008); (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-

Figure 1. SHAs samples dissolved with NaOH 2 N.

dried NHAs pressed under vacuum).

Humic acids preparation and purification

Commercial humic acids (CHAs) were purchased from Aldrich and soil humic acids were obtained after extraction (as above). A solution containing 5% HAs (SHAs 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 (SHAs 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. A photo at Figure 1 shows HAs samples dissolved with NaOH 2 N.

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) (Shirling and Gottlieb, 1966) 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).

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 as reported by Dari et al. (1995). 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.

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. A 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. (2008) using the following equation:

$$\text{Decolorization extent} = [(OD1 - OD_t)/OD1] \times 100 \quad (1)$$

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

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 (1966) and Nonomura (1989). The morphological and cultural characteristics of the organisms were determined by macromorphologic examination of 7-days-old cultures grown on various International *Streptomyces* Project (ISP) media (Shirling and Gottlieb, 1966): yeast extract–malt extract agar (ISP2), oatmeal agar (ISP3) and inorganic salts-starch agar (ISP4) (Nonomura Nonomura, 1989). 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. (2006).

Chemotaxonomic analysis : Biomass for chemotaxonomic analysis was obtained from a culture grown in shake ISP2 medium (Shirling and Gottlieb, 1966)) 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 (1974) using Thin Layer Chromatography (TLC) on plastic sheets cellulose (Merck) with methanol / water / HCl 6N / pyridine (80 / 26 / 4 / 10, v / v) as solvent system. Sugar pattern was analyzed according to the method of Lechevalier and Lechevalier (1970).

Kinetic of SHAs removal and biodegradation during incubation

In this step kinetic of SHAs removal was performed using the performance strains and the main aim of this experience was to test

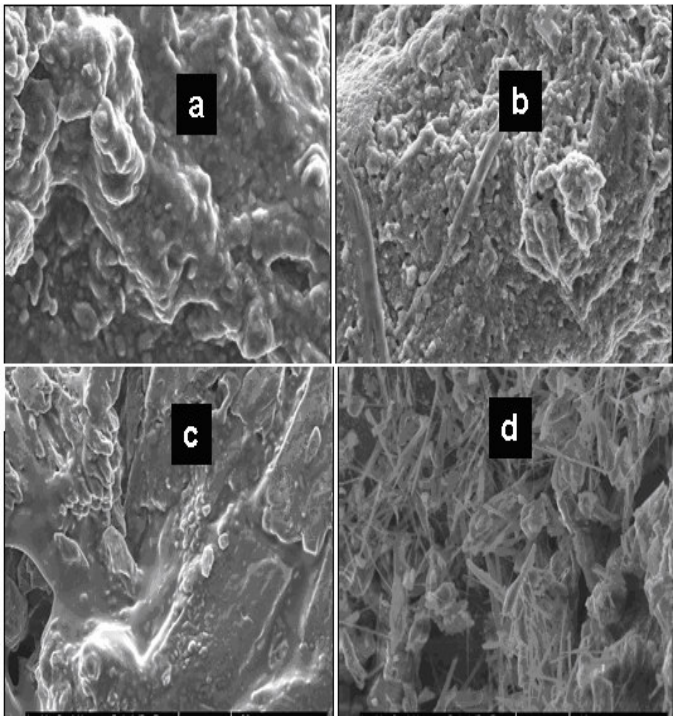

Figure 2. Morphology of SHAs extracted from local soils (a, SHAs from Boufarik; b, SHAs from Hadjout and c, SHAs from Meftah) and synthetic humic acid (d). Magnification (2000X).

the utilization of these macromolecules as carbon and nitrogen sources in PLM by the strain isolated from the same soil sample. Culture conditions were prepared as described in *Section "Screening assays and selection of decolorizing strains"*. Strains were inoculated in test tubes containing 10 ml of PLM containing glucose (0.1 g L^{-1}) and traces of $(NH_4)_2SO_4$ (0.084 g L^{-1}) as easily carbon and nitrogen sources, respectively to starting growth of microorganisms. This medium was supplemented with SHAs (each strain was incubated with its correspondent SHAs at a final concentration of 0.5 g L^{-1}) under static or shaking conditions. After incubation, centrifugation, filtration and dilution (as before), *A*350 of the solution was measured. A time course experiment of HAs removal was conducted at 7-days intervals from time zero to day 28. The mean and standard error of the data were calculated using Excel (Microsoft Office Excel 2003, USA).

The change in the structure (degradation) of HAs molecule during incubation was monitored by Fourier transform infrared spectroscopy (FTIR) using selection samples from the shake cultures supplemented with SHAs. After complete decolorization (28 days incubation at 30°C) culture broth was centrifuged at 5000 rpm for 15 min. FTIR spectra were recorded from KBr pellets with a SHIMADZU FTIR 9800 spectrophotometer over the 4000 - 400 cm^{-1} range at a rate of 16 nm s^{-1} (1 µl of supernatant containing rest of HAs was spread on pellets and then quickly dried).

CHAs were chosen as a common carbon source for all strains tostudy metabolites products after degradation analyzed by HPLC. Similar cultures containing CHAs were prepared as before in shaking conditions (28 days incubation at 30°C). HPLC equipped with a UV-Visible at 254 nm detector (type SHIMADZU UV-VIS DETECTOR). Chromatographic analyses were conducted using a 125 × 4.6 mm RP and C18 column with an isocratic mobile phase of acetonitrile: deionized water 0.3:0.7 (v/v) at a flow rate of 0.6 ml min^{-1}.

RESULTS AND DISCUSSION

Humic acids characterization

SEM morphologies observed for SHAs and CHAs samples are represented in Figure 2. SHAs are characterized by globular and filamentous aggregates while CHAs are characterized by filamentous morphology. The E_4/E_6 ratios are 2.8, 3.2, 2.9 and 3.9 for SHAs from Boufarik, Hadjout, Meftah and CHAs respectively. Functional groups detected by FTIR are represented in Table 2.

On the basis of the SEM morphologies, our SHAs show an abundance of the aromatic structure rather than aliphatic. These characteristics confirm their non-complexity structure comparatively with commercial humic acids and those of literature (Eyheraguibel, 2004; Eyheraguibel et al., 2008). Evaluation of HAs molecular weight by calculation of E_4/E_6 ratios (< 5) and presence of typical functional groups have confirmed the efficiency of the technique employed to extract SHAs from local soils.

Isolation and selection of decolorizing actinomycetes

Nineteen strains, isolated on the selective medium supplemented with actidione (antifungal), were preserved after several subcultures. The number of isolates was important in Hadjout (8 isolates), followed by strains isolated from Boufarik (6 isolates) and by strains isolated from Meftah (5 isolates). These isolates were examined further to detect bleaching of liquid medium containing CHAs (bleaching has been considered as an indicator of degradation rate, with a dark colour measured at 350 nm). Decolorization of CHAs inoculated with 19 strains ranged from 0 - 46% (Table 3). Among the 19 strains, three were selected for further studies according to the extent, stability and competence of their activity on SHAs degradation.

The selection procedure indicated that the number of strains that were capable of decolorizing the HAs-containing medium was important in the soils of Mitidja plain. These degrader microorganisms seems to be one cause behind the rapid disappearance of organic matter known at this plain.

Preliminary identification of decolorizing actinomycetes

AB1, AM2 and AH4 strains were grown on various media at 30°C for one week and have formed colonies with approximatively 10 mm in diameter, circular and smooth. Good growth was observed on ISP2 and moderate on ISP3 and ISP4 media. They have developed aerial mycelium on sporulation media. The aerial mycelium (AM) is gray and the substrate mycelium (SM) is brown and not fragmented. The AM2 produces chain spores

Table 2. Assignments of the infrared spectral peaks of soil humic acids.

Wave numbers (cm^{-1})	Assignments	HAs from Boufarik	HAs from Hadjout	HAs fromMeftah
3300 - 3500	O–H vibrations of the hydroxyl groups of phenols, alcohols and carboxyl functions and N–H vibrations from amides and amines.	3421.42	3452.34	3456.20 2933.88
2960 - 2920	Symmetric CH stretching in $–CH_3$ and $–CH_2–$ of aliphatic chains.	2927.27	2974.03	
2855 - 2840	Asymmetric CH stretching in $–CH_3$ and $–CH_2–$ of aliphatic chains.	2850.45		
2573 - 2000	C=N stretching in secondary amides	2368.42	2488.00	2488.80
1720 - 1712	C=O stretching in quinones and/or in ketonic acids and primary amides.	1712.67	1720.00	1716.50
1650 - 1600		1635.52	1635.52	1643.24
1225 - 1200	O–H deformation, C=O stretching of phenols, anti-symmetric COO^- stretching and aliphatic C–H deformation.	1203.5	1215.07	1215.07
1130 - 1000	C–OH stretching of aromatic groups and C–O–C stretching of aryl ethers and phenols.	1053.06 1006.77	1056.92 1006.77	1006.77
890 - 700	C–O–C stretching of carbohydrates	879.48	879.48	
	Out-of-plane bending of aromatic C–H and O–H phenols.	590.177	547.7	875.622

Table 3. Decolorization of CHAs by the nineteen actinomycetes isolated from three soils.

Decolorization (%) after 21 days measured at 350 nm		
Strains from Boufarik	**Strains from Meftah**	**Strains from Hadjout**
AB1 30.48 ± 0.85	AM1 31.50 ± 0.89	AH1 11.58 ± 0.89
AB2 4.79 ± 2.36	AM2 41.20 ± 1.45	AH2 23.28 ± 1.23
AB3 4.69 ± 2.97	AM3 8.37 ± 0.73	AH3 1.67 ± 0.23
AB4 13.1 ± 1.63	AM4 2.34 ± 0.22	AH4 45.79 ±1.45
AB5 1.17 ± 0.45	AM5 4.69 ± 0.43	AH5 7.58 ± 0.65
AB6 5.53 ± 1.36		AH6 0.167 ± 0.004
		AH7 24.28 ± 1.24
		AH8 17.08 ± 0.98

with *Rectus Flexible* type and non-mobiles. Soluble pigment was produced on all media used.

The chemotaxonomic study showed the presence of LL-diaminopimelic acid isomer in the cell-wall in addition to glycine, and not characteristic sugar in the whole-cell hydrolysates. This corresponds to *Chimiotype IC* according to Lechevalier and Lechevalier (1970). On the basis of its morphological and chemical properties, these three strains AB1, AM2 and AH4 were classified in the genus *Streptomyces*.

We have applied the methods recommended by the International *Streptomyces* Project (Shirling and Gottlieb, 1966), which is the basis of the classification of the genus *Streptomyces* in Bergey's Manual of Systematic Bacteriology (Williams, 1989). The phenotypic characters were not sufficient for identification to species levels. In this regard, genotypic tests should be necessary to confirm the phenotypic results. Detailed analysis is underway in our laboratory to make clarify the identification of these strains by 16s RNA sequencing.

Kinetic of soil humic acids biodegradation

Firstly, bleaching increased in the course of the experiment, showing that SHAs was removed. Indeed, the decolorization started after an initial lag-period of approximatively 4 days for AM2 and continued through the 28 days of the experiment. Secondly, SHAs removal started when easily carbon and nitrogen sources were added and, in the best case, maximal decolorization (>60%) was obtained with strains AB1 and AM2 under

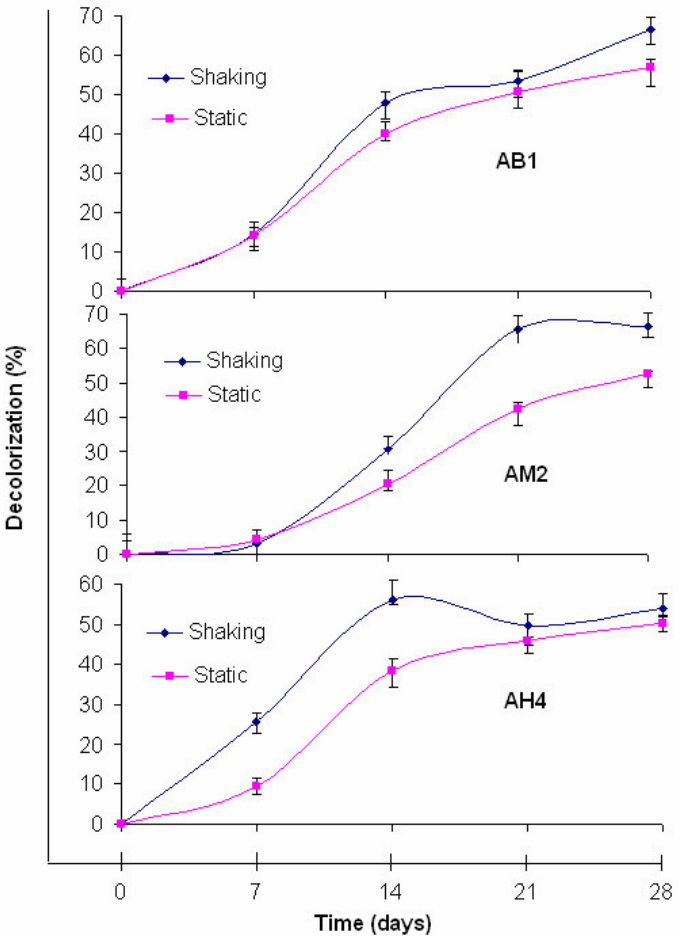

Figure 3. Time course of SHAs removal by *Streptomyces* sp. strains under shaking or static conditions. The data values have been given as mean ±standard error and shown as Y-error bars.

shake culture. Thirdly, in shake culture, the SHAs decolorization was more efficient than in static culture for strain AM2 after 7 days and for AH4 before 21 days and a slight difference was observed for AB1 through the 28 days followed by AH4 from 21 days to 28 days. Finally, under shaking conditions the decolorizing activity was particularly significant for AB1 followed by AH4 and AM2 between 7 days and 14 days and between 21 days and 28 days of incubation. It was particularly significant for AM2 followed by AB1 and AH4 between 14 days and 21 days. Under static conditions, decolorization increased fortify during the first 7 days for AM2 followed by AH4 and then AB1 and it increased fortify between 7 days and 14 days for AM2 followed by AB1 and then AH4. From 14 days to 21 days and under shake culture, decolorization is practically unchanged for strain AB1 but increase for AM2 and decrease for AH4. While the strain AM2 showed the lowest rate between 7 days and 14 days under both static and shaking conditions, this strain marked the lowest percent decolorization after 21 days under shake culture. A maximal decolorization extent was observed for 28 days for the three strains under shake culture (67, 66 and 57% for AB1, AM2 and AH4, respectively) (kinetic of soil HAs biodegradation is graphically presented in Figure 3).

The lag-period observed on HAs degradation was related to the slowing of microbial growth which started by adding trace quantities of easily carbon and nitrogen sources untypical for usual soil conditions as reported by Panikov et al. (1982). In shake culture oxygen was favorable to the growth of the bacterium and also favorable to the yield process of the degradation related to enzyme (Dari et al., 1995). However, in absence of glucose, the three *Streptomyces* isolates were unable to grow in the medium supplemented with HAs as the sole carbon source. Some authors reported the possible use of HAs as sole carbon source by *Streptomyces* as reported by Dari et al. (1995) and Hayakawa and Nonomura (1987a; 1987b), but these results were obtained with humic fractions of undefined purity. Therefore, the carbon utilized by the bacteria in such studies might have been organic fractions not fully humified and not wholly resistant to biodegradation. The presence of an additional assimilable carbon source was also essential for the expression of the decolorizing activity on melanoidins from *Streptomyces* isolates of Murata et al. (1992).

Two phenomena accompanying HAs removal were observed, the first one is related to the adsorption of HAs on actinomycetes mycelia but with negligible rate comparatively with adsorption of HAs on fungi mycelia (photo at Figure 4, data not shown). This low adsorption rate suggests that catabolic activity of these *Streptomyces* could be in part surface-bound and in contrast with the extracellular decolorizing system from the white-rot fungi (Grinhut et al., 2007). Adsorption was also signaled by Vukovic et al. (2008) on fungal pellets of *Aspergillus Niger* 405, *Aspergillus ustus* 326, and *Stachybotrys* sp. 1103 which were used for the removal of humic substances from aqueous solutions. The second one concerns percent bleaching below zero which refers to an increase in absorbance (350 nm) relative to the control; which might be the result of a secretion of melanoidins pigments known for these actinomycetes (Sabaou et al., 1992). These results provided obvious evidence of biodegradation of HAs by *Streptomyces* in the degradation process, and also supported the earlier conclusion that decolorization by bacteria is due to biodegradation, rather than active surface adsorption (An et al., 2002; Rezacova et al., 2006; Asad et al., 2007; Fan et al., 2009).

The elimination rate of SHAs, quantified by decolorization or degradation varies between 57 and 67% with three strains of actinomycetes. This activity lies within the range published in literature concerning fungi strains (2.6 - 90%). Decolorization or bleaching of the medium of a dark color, representing a higher concentration of HAs is considered as an indicator of degradation (Grinhut et al., 2007). However, few works have been published on HAs degradation by actinomycètes

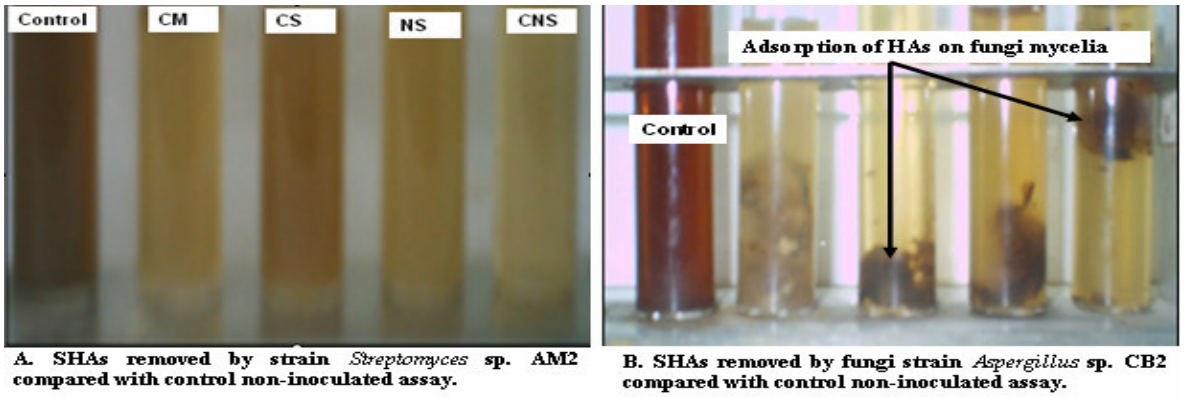

Figure 4. Adsorption of HAs on actinomycetes mycelia but with negligible rate comparatively with adsorption of HAs on fungi mycelia. CM; completed medium contain sufficient carbon and nitogen source plus SHAs, CS; SHAs as the alone carbon source, NS; SHAs as the alone nitrogen source, CNS; SHAs as the alone carbone and nitrogen sources

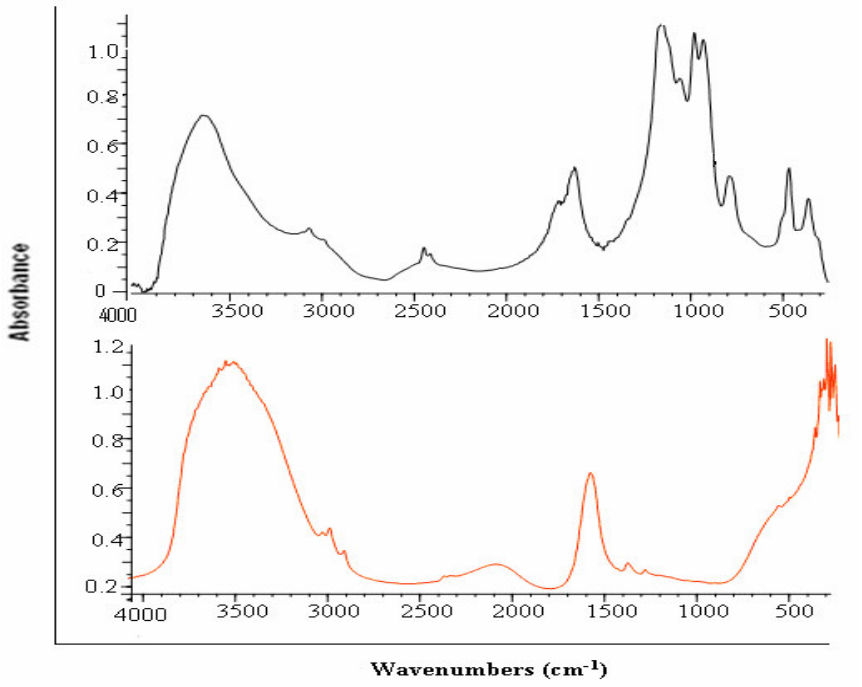

Figure 5a. Fourier transform infrared spectra of the HAs extracted from Boufarik soil incubated with *Streptomyces* sp. AB1 for 28 days at 30°C. Initial structure (black) and structure after incubation (red).

(Kontchou and Blondeau, 1992; Dari et al., 1995; Yanagi et al., 2002).

Structural changes in soil humic acids during incubation

As compared with initial and final humic acids structures under incubation for 28 days at 30°C, the FTIR spectrum (Figure 5a, b and c) shows two substantial changes after incubation as follows: (1) new absorption peaks appeared in the regions at 350 - 700 cm^{-1}, 1200 - 1500 cm^{-1} for HAs extracted from Boufarik (Figure 5a) and in the regions at 600-700 cm^{-1}, 1300 - 1450 cm^{-1}, 1600 - 2100 cm^{-1} for HAs extracted from Meftah (Figure 5b) and in the regions at 350 - 400 cm^{-1}, 500 - 600 cm^{-1}, 1500 - 1600 cm^{-1} and 2300 - 2350 cm^{-1} for HAs extracted from Hadjout (Figure 5c) ; (2) some absorption peaks were totally disappeared in the regions at 700 - 1200 cm^{-1}, 1600 - 1800 cm^{-1}, 2000-3200 cm^{-1} for HAs extracted from Boufarik (Figure 5a)

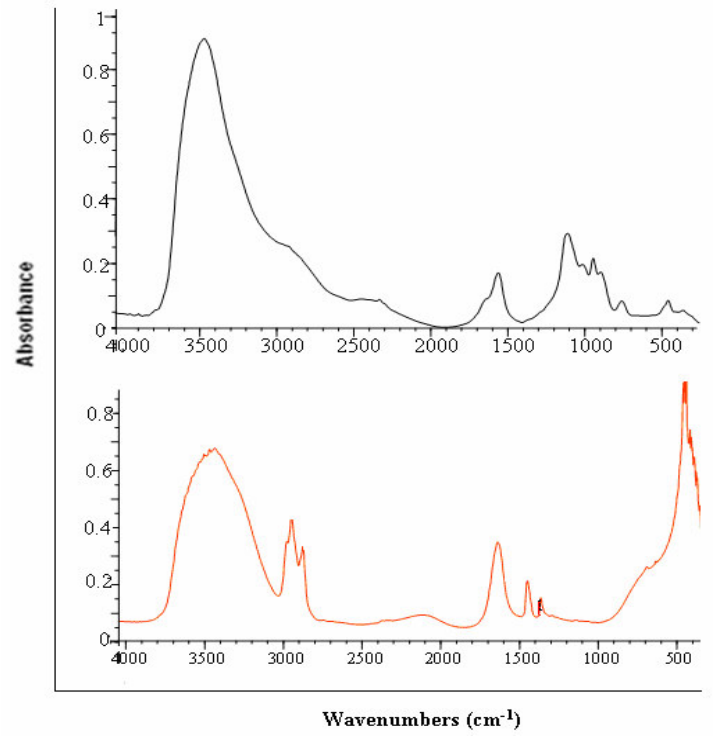

Figure 5b. Fourier transform infrared spectra of the HAs extracted from Meftah soil incubated with *Streptomyces* sp. AM2 for 28 days at 30°C. Black: initial structure and red: structure after incubation.

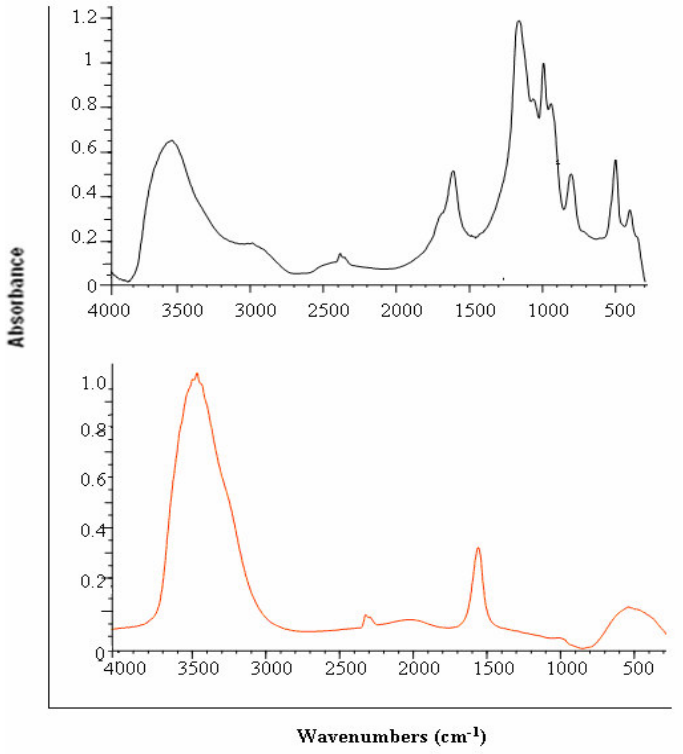

Figure 5c. Fourier transform infrared spectra of the HAs extracted from Hadjout soil incubated with *Streptomyces* sp. AH4 for 28 days at 30 °C. Black: initial structure and red: structure after incubation.

and in the regions 700 - 800 cm^{-1}, 900 - 1000 cm^{-1} and 1500 - 1600 cm^{-1} for HAs extracted from Meftah (Figure 5b) and in the regions 700 - 1000 cm^{-1} and 1500 - 1600 cm^{-1} for HAs extracted from Hadjout (Figure 5c). The shoulders at 2980 cm^{-1} and 2924 cm^{-1} (Figure 5a) indicated the appearance of organic matter containing aliphatic methylene. The peaks appearing in the region from 1200 - 1500 cm^{-1} (Figure 5a) suggest the presence of aliphatic C–H and O–H deformation, C = O stretching of phenols in the humic acids after degradation. The disappearing bands at 700 - 1200 cm^{-1},1600 - 1800 cm^{-1}, 2000 - 3200 cm^{-1} (Figure 5a) implied the complete removal or transformation of organic components containing phenolic O–H or N–H stretches, C = O stretching in carboxyl, ketonic, aldehyde and COO^{-}, respectively (Gerasimowisz and Byler, 1985; Hernández et al., 1990; Ricca and Severini, 1993; González-Vila, 1999; Amir et al., 2005).

Decreasing absorbance at the two regions 1500 - 3000 cm^{-1} and 3200 - 3800 cm^{-1} for HAs extracted from Boufarik (Figure 5a), for HAs extracted from Meftah (Figure 3b) and for HAs extracted from Hadjout (Figure 5c) often indicates the degradation level. However, in the remaining SHAs the carbon content increased, indicating that aliphatic structural units were preferentially utilized, while more condensed aromatic structures were rather resistant to biodegradation. The appearance of strong IR-bands at 1599 cm^{-1}, attributed to quinones seems to confirrm this suggestion; similar results were reported by

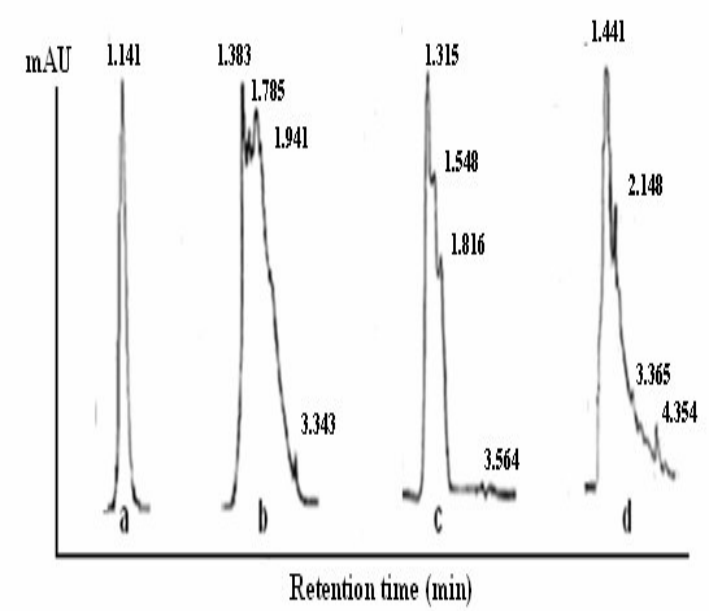

Figure 6. Metabolites from CHAs degradation detected by HPLC analysis. The retention time correspond to the flowing metabolites: a. Initial concentration of CHAs as control, b. Metabolites obtained after incubation of CHAs with AB1, c. Metabolites obtained after incubation of CHAs with AH4, d. Metabolites obtained after incubation of CHAs with AM2.

Filip and Tesarova (2004). Results show the microbial degradation of SHAs used in our study and the structural changes differed among HAs and *Streptomyces* strains. Indeed, structural changes during incubation prove clearly degradation of SHAs tested in our study as as they showed Liang et al. (2008) in their study about decomposition and mineralization of aquatic humic substances (AHS) in treating landfill leachate using the Anammox process using FTIR analysis.

The HPLC analysis (Figure 6) shows different metabolites produced at the end of the incubation compared with the initial peak of SHAs (t_R = 1.141 min). Four metabolites produced in the case of SHAs incubated with strain AB1 (t_{R1} = 1.383; t_{R2} = 1.785; t_{R3} = 1.941; t_{R4} = 3.344), four in the case of SHAs incubated with AH4 (t_{R1} = 1.315; t_{R2} = 1.548; t_{R3} = 1.816; t_{R4} = 3.564) and four in the case of SHAs incubated with AM2 (t_{R1} = 1.441; t_{R2} = 2.148; t_{R3} = 3.563; t_{R4} = 4.354). However, there were difficulties in the determination of metabolites which are related to standards lacks.

According to their morphologies, SHAs have a dominant globular structure indicating high aromatic rate. Therefore, this structure is not more difficult to be degraded by *Streptomyces* strains isolated from local soils. In contrast, Yanagi et al. (2008) reported that HAs with higher aromaticity have greater resistance to decolorization by a particular fungi (*Coriolus consors*).

However, there is significantly less information regarding chemical and physical changes undergone by HAs acids during biodegradation. Their structural complexity makes the analytical detection of changes extremely difficult. Not only is it hard to characterize HAs, the secretion of enzymes and other compounds and the possible sorption of HAs to the mycelium are liable to cause difficulties in interpretation. Different size-exclusion chromatography methods are commonly used to show that bleaching of HAs is associated with degradation (Blondeau 1989; Hofrichter et al. 1998; Ziegenhagen and Hofrichter, 1999; Gramss et al. 1999). Nevertheless, the accuracy of HAs molecular weight and size determinations have been criticized due to changes that occur with different methods and conditions (De Nobili and Chen, 1999). HAs mineralization has been demonstrated by using ^{14}C-labeled SHAs (Steffen et al., 2002). Unfortunately, this HAs is far from being representative of natural ones.

In this study decolorizing actinomycetes strains was investigated. These strains could use HAs as carbon and nitrogen sources showing that the percentage of degradation differs among the strain and the origin of SHAs. Our results indicate that *Streptomyces* strains have the capability to degrade SHAs and play a part role in lignin degradation and humus turnover in local soils. Moreover, high decolorization extent and facile culture conditions of these strains make them potential candidates for applications in biological processes for fossil energy using HAs and specially to remove these macromolecules present in drinking water. Detailed analysis is underway

in our laboratory to identify up to species level by 16s RNA sequencing and highlighting the mechanism responsible of HAs degradation.

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