Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 24, No. (1) : 2022 : 7-12

ISSN-0972-3005
http://doi.org/10.53550/AJMBES.2022.v24i01.002

DEGRADATION OF ANTHRACENE BY *STREPTOMYCES* SPP. STRAIN AH4

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(Received 29 November, 2021; Accepted 1 December, 2021)

Key words: *Biodegradation, Streptomyces, Polycyclic Aromatic Hydrocarbons, Anthracene, Metabolites, Pollution.*

Abstract–Streptomyces strains AH4 performance degrading humic acid was isolated and selected from surface soils at Mitidja plain (North of Algeria) was used to biodegrade anthracene in cultured medium. *Streptomyces spp* strain AH4 was identified using 16S rDNA gene sequence analysis. Optimal conditions for the biodegradation of anthracene included: temperature of 30 °C, pH 7.2 and 50 ppm concentration of anthracene. Under these conditions and initial anthracene concentration of 50 mg. l^{-1}, more than 70% was removed within 5 days. The metabolites anthracene formations were examined by HPLC and FTIR. Appearance of new pic at tR= 3.75 min by HPLC. FTIR, appearance of new broad absorption bands at 2858 Cm^{-1}, 2927 Cm^{-1}, 2955 Cm^{-1} and another new strong absorption band at 1734 Cm^{-1} in metabolites demonstrates that carboxyl group produced during anthracene degradation.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) represent a wide and heterogeneous class of toxic organic compounds. They originate from the incomplete combustion of the organic matter, for this reason they are widely distributed, owing to both natural and anthropogenic sources (Amezcua-Allieri *et al*., 2012; Amir *et al*., 2005). PAHs are hydrophobic compounds and their persistence in the environment is chiefly due to their low water solubility. PAHs are even known as priority pollutants, such as anthracene exert a carcinogenic effect and may cause aberrance and mutations in biological tissues. However, they have been shown to be toxic to fish and algae (Amezcua-Allieri *et al*., 2012; Amir *et al*., 2005; Bamforth *et al*., 2005).

Environmental, anthracene contamination originates from a number of anthropogenic sources/ practices such as manufacturing of dyes, production of synthetic fibers, plastics and pesticides, petroleum spills as a result of pipeline rupture and tanker failure. Its natural sources are coal and tar, and can be released by incomplete combustion of fuels (such as coal, oil, gas) (Amir *et al*., 2005; Bamforth *et al*., 2005).

While traditional chemical/physical remediation techniques are currently critically acceptable from an environmental and economical point of view, there is an increasing interest in biodegradation, expected to be an economic and efficient alternative method to other remediation processes such as chemical or physical ones, has been developed as a soil and water clean-up technique. However, the success of PAHs biodegradation experiments mainly depends on microbial activities. The intensity of biodegradation is influenced by several factors, such as nutrients, oxygen, pH-value, composition, concentration and bioavailability of the contaminants (Ferradji *et al*., 2014; Margesin *et al*., 2012; Mollea *et al*., 2005).

A variety of non-actinomycete bacteria, such as *Pseudomonas* spp., *Burkholderia cepacia, Sphingomonas* spp., *Flavobacterium* spp., *Cycloclasticus* spp., and *Stenotrophomonas* spp., have been investigated and it has been proven that they can metabolize a large

number of PAHs, including naphthalene, phenanthrene, benz[a]anthracene, chrysene, fluoranthene, fluorine, anthracene, and pyrene in a soil environment (Zhang *et al.,* 2006). Actinomycetes, such as *Mycobacterium* spp., *Streptomyces* spp., and *Rhodococcus* spp., were isolated from various hydrocarbon-contaminated soils and each uses fluoranthene, pyrene, and chrysene as sole carbon and energy sources (Joanna *et al.,* 2001).

Degradation of anthracene, that is its conversion to both carbon dioxide and water (mineralization) or other organic substances (degradation products) which is not toxic is one of the inexpensive way of removing large concentrations of anthracene from soil and water (Bodour *et al*., 2003).

This study is a contribution to assess the ability of hydrocarbon biodegradation by *Streptomyces* spp. Strain AH4 isolated from Algerian soil samples. This isolate is expected to provide new valuable strains for the production of active and stable enzymes (peroxidase) in highly acid conditions. The extracellular peroxidase from *Streptomyces* spp. strain AH4 is highly attractive for the biodegradations of humic acids (Badis *et al*., 2009; Fodil *et al*., 2012). Therefore, the aims of the present work were (1) to find out the potential of *Streptomyces* isolated locally for the ability of degradation of anthracene (2) to study the structural changes of the anthracene used as carbon source by this strain and metabolites formation.

MATERIALS AND METHODS

Streptomyces strain and culture media

Isolation was made at 30 °C from soil 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) and supplemented with actidione (antifungal) (500 $mg.l^{-1}$). After one week of incubation, colonies were numbered, coded and transferred onto the same medium devoid of actidione to test purity. These isolate was stored on the yeast-malt extract–glucose-agar-slants (in l: yeast extract: 4 g; malt extract: 10 g; glucose: 4 g; agar: 12 g) Badis *et al*. (2009).

Badis *et al.* (2009) demonstrated our results indicate that Streptomyces strain have the capability to degrade humic acids and play a part role in lignin degradation and humus turnover in natural waters and production the extracellular peroxidase (Badis *et al*., 2009; Fodil *et al*., 2012).

Screening for biosurfactant producing isolates

The medium used for preculture of the Streptomyces contained Mineral Medium (MM), 0.1$g.l^{-1}$ glucose (Sigma) and 100 $mg.l^{-1}$ anthracene (Panreac). MM (per liter): $(NH_4)_2SO_4$ (Merck): 2,84g, KH_2PO_4 (Fluka): 2.38g, K_2HPO_4 $3H_2O$ (Prolabo): 5.65g, $MgSO_4$ $7H_2O$ (Fluka): 1g, and 1 ml of solution containing trace elements prepared in 1L ($CuSO_4$ $5H_2O$ (Prolabo): 0.64g, $FeSO_4$7H2O (Merck): 0.11g, $MnCl_2$4H_2O (Fluka) : 0.79 g, $ZnSO_4$$7H_2O$ (Prolabo) : 0.15 g).

The pH of the medium was adjusted to 7.2 medium was sterilized by autoclaving at 120 °C for 20min.

The broth cultures were incubated at 30 ± 1 °C on a reciprocal shaker at 150 rpm for 7 days.

The culture broth was then tested for the production of extracellular biosurfactants with: Emulsification measurement was measured according to the method of Bodour *et al*. (2003). One volume of culture supernatant, and one volume of gazoil were added and vortexed (IKA Vortex Genuis) for 2 min. The mixture was allowed to stand for 24 hours prior to measurement. The emulsification activity is defined as the height of the emulsion **(he)** layer divided by the total height **(ht)** and expressed as percentage (E_{24}= (he/ht)*100) (Mnif *et al*., 2011).

Biodegradation experiments

Two factors were examined for their influence on the biodegradation of anthracene. These included temperature and different anthracene concentration: incubation temperatures of 20, 30 and 40 %C, and anthracene at concentrations of 22.5, 50, 500, 1000 and 2000 ppm were used for this experiment. The cultures were incubated in a shaking incubator at 150 rpm (Stuart, orbital incubator), and pH of the medium was adjusted to 7.2 medium.

Anthracene stock solutions were prepared by dissolving anthracene in hexane (Sigma) with a final concentration of 1 $g.l^{-1}$, and kept in a brown bottle at 4 °C .

The anthracene concentration after degraded in various samples was determined using HPLC at various time intervals, pH values of the culture media were determined by pH meter (PHS-3C, Shanghai, China).

Residual anthracene in milieu culture was

extracted from spent medium with and equal volume of hexane. The mixture was vortexed for 5min in order to dissolve the anthracene in the organic phase. The mixture was then transferred to a core true and centrifuged at 6000 rpm for 30 min in order to separate the organic and aqueous phases. A 20µl aliquot was taken from the organic phase and anthracene was quantified using HPLC (type SHIMADZU). HPLC equipped with a UV–Visible at 254nm detector. Chromatographic analyses were conducted using a 125×4.6 mm RP-C18 column with an isocratic mobile phase of acetonitrile: deionized water 65:35 (v/v) at a flow rate of 0.9 ml min^{-1} (Moody *et al*., 2001).

Extraction of metabolites

Streptomyces AH4 was grown in MM supplemented with anthracene (50 ppm) as a sole source of carbon and energy at 30 °C and 150rpm. After incubation for 7 days the cultures were centrifuged at 6000rpm for 30 min at room temperature three times.

Supernatant was acidified to pH 2.3 with 6 mol. l^{-1} hydrochloric acid (Fluka) and extracted with ethyl acetate (Sigma. Alarich). The combined organic phase was extracted three times with aqueous sodium hydroxide (30 ml, 10mmol.l^{-1}, Fluka). The remaining organic phase was dried over anhydrous sodium sulphate (Fluka) and concentrated to 5 ml of ethyl acetate (neutral fraction: NF). The aqueous phase was acidified to pH 2.3 and extracted with ethyl acetate (3×30 ml, acidic fraction: AF). Control experiments were performed with autoclaved cells according to the same procedures (Almlof *et al*., 1996).

Metabolites extracted by acetate ethyl were analysed by FTIR spectrophotometer (Shimadzu FTIR 9800 spectrophotometer over the 4000–400 Cm^{-1} range at a rate of 16 $nm.s^{-1}$) after packed with potassium bromide at room temperature. Spectra FTIR were treated by IRsolution.

RESULTS AND DISCUSSION

***Screening for biosurfactant-producing isolates**

The emulsification activities (*E24*) of cell-free culture broth during the growth on anthracene AH4 isolate were variety between 01 to 85% after 7 day of incubation. These results indicated these *Streptomyces* strain is able to produce biosurfactants (Fig. 1).

Fig. 2 show the effect of temperature on the rate

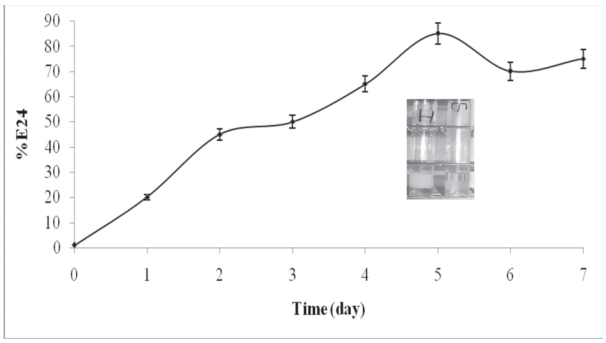

Fig. 1. Biosurfactant activity of *Streptomyces* spp. AH4. Emulsion activity (E24) using gazoil as substrate.

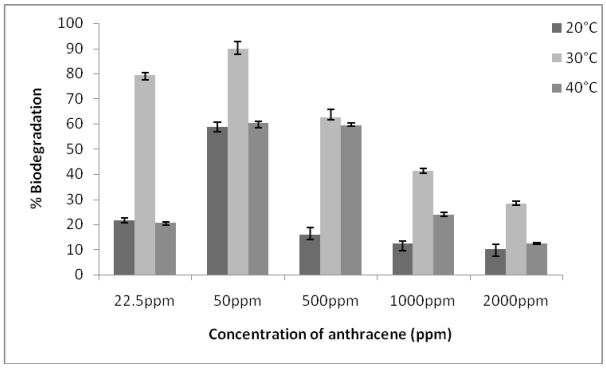

Fig. 2. The effect of temperature on the rate of anthracene biodegradation by the *Streptomyces* spp AH4.

of anthracene biodegradation by the *Streptomyces* spp AH4. It was observed that AH4 caused the highest level of anthracene degradation at 30 °C and 50ppm concentration of anthracene. Streptomyces spp AH4 degraded 89.8% of anthracene at 30 °C as compared to 58.73 and 60.31 % at 20°C and 40 °C respectively in 5 days. AH4 degraded 79.57% of 22.5 ppm anthracene at 20 °C and 40°C as compared to 22% degradation of the anthracene in 5 days. The lowest level of anthracene degradation by *Streptomyces* AH4 occurred at 1000 ppm (12.5, 41.25 and 23.73 % of rate degradation at 20, 30 and 40 °C respectively), while the lowest level of anthracene degradation by *Streptomyces* AH4 occurred at 2000 ppm (10.22, 28.4 and 12.5 % of rate degradation at 20, 30 and 40 °C respectively).

Many published studies have found that biodegrading efficiency depends on environmental factors because they vary from site to site. Such factors include pH, temperature, nutrient availability and bioavailability of the contaminant. These can influence the biodegrading process by inhibiting growth of the pollutant-degrading (Lin *et al*., 2010; Sihag *et al*., 2014; Swaathy *et al*., 2014 a, b). The main environmental factors that could

determine biodegrading suitability were examined in this section to understand the biodegradation of anthracene. Temperature at 30 and 40 °C increased further the remaining concentration of anthracene. This explains the increased solubility of anthracene at higher temperatures, there by causing a noticeable improvement in the bioavailability of anthracene molecules (Amellal *et al*., 2001; Das *et al*., 2008). Further increase in temperature caused a reduction in metabolic activity of aerobic microorganisms due to the decline in oxygen solubility (Bamforth *et al*., 2005). Bioavailability, however, was lower when the temperature rose above 40 °C, indicating that the adverse effect of high temperature on the cell is more important than any increase in substrate availability (Sihag *et al*., 2014).

The removed amount of anthracene using strain AH4 however increased as the initial concentration of anthracene, which is due to the initial concentration that provides an important driving force to overcome all mass transfer resistances of the anthracene between the aqueous and solid phases. Consequently a higher initial concentration of anthracene may enhance the process (Kumar *et al*., 2010; Jacques *et al*., 2005). This suggests that strain AH4 could survive and rapidly degrade a high concentration of antharcene at 50 ppm. So at despite the substrate concentrations being high enough to support growth, this may cause toxic effects, and toxic metabolites may accumulate in the growth medium (Jacques *et al*., 2005).

Kinetic degradation of anthracene

Streptomyces strains AH4 can utilize anthracene as a sole source of carbon and energy. Degradation rate of anthracene rapidly increased after 1 day incubation (Fig. 3). Anthracene (50ppm) was degraded completely after 5 days of incubation. The pH was around 7.0 and it even decreased to 6.7 (Fig. 3). The results indicated some acidic materials produced during biodegradation process. According to Swaathy *et al*. (2014 a, b), observed an decrease in pH (6.5± 0.2) degradation of anthracene at 500, 750 and 1000 ppm in aqueous phase by marine *Bacillus licheniformis* MTCC 5514.

The pH was monitored thought the experiment. It was found to be decreasing from 7 to the acidic range during the biodegradation. The pH was measured every 24 hr. The pH change of the experimental media may signify metabolic activity leading to production of acidic or alkaline metabolites during breakdown of anthracene. Kim *et al*. (2005) observed that acidic pH conditions promote uptake of PAHs for degradation in *Mycobacterium vanbaalenii*. It can therefore be suggested that monitoring pH of media may be used to check the progress of PAHs degradation (Lin and Cai, 2008). This corroborates previous research where the dynamics of pH changes in cultures were consistent with that of PAH concentration change (Lin *et al*., 2010).

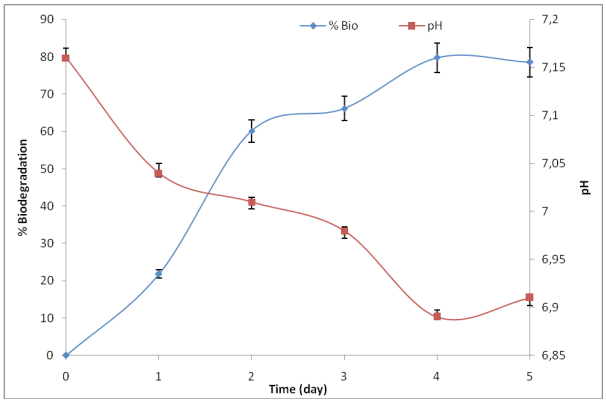

Fig. 3. Variation in pH and time course of the degradation of anthracene by *Streptomyces spp* AH4.

HPLC chromatogram indicated one fraction was received (tR= 3.75 min) (Fig.4). The fraction reveals the nature of the degraded products.

FTIR spectroscopy

Infar Red spectra (Fig. 5) of initial structure of anthracene (a) and structure after incubation of anthracene degraded by *Streptomyces spp* AH4 (b).

Results the change of functional groups between initial and after incubation of anthracene by

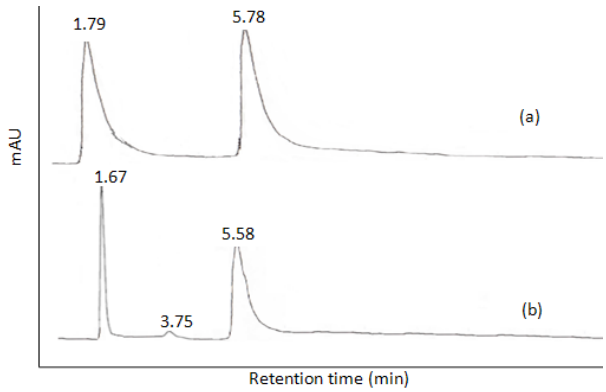

Fig. 4. HPLC analysis of anthracene biodegradation; a: anthracene control, b: After degradation by *Streptomyces spp* AH4

Streptomyces spp AH4 were shown in Fig. 5.

The bands for anthracene (Fig.5a) between 3500-2800 Cm^{-1} are due to these proton (H) stretching bands. The skeleton vibration peak of benzene ring appears between 1600-1500 Cm^{-1}.

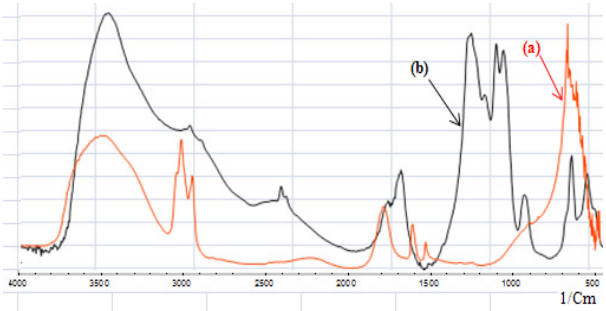

Fig. 5. FTIR spectra of (a) anthracene, (b) structure after incubation degraded by *Streptomyces* spp. AH4.

The spectra of metabolites are shown in Fig. 5b, the band between 1450 Cm^{-1} and 1640 Cm^{-1} is the skeleton vibration peak of benzene ring. The new strong absorption band appears between 1500 and 900 Cm^{-1} of anthracene degraded by Streptomyces spp AH4.

The appearance of those new bands demonstrates that carboxyl group. In addition to this, the new width weak absorption band appearing between 2500 and 2000 Cm^{-1} relates to stretching vibration peak of hydroxyl (OH).

Above results indicated that for comparison with anthracene, the metabolites of anthracene by *Streptomyces spp* AH4 make a difference during degradation, and carboxylic acids with aromatic ring, phenolic compounds, arone, salicylic acid and catechol in metabolites were identified by FTIR (Chandrasekhar and Karigar, 2010; Smith *et al*., 1999; Wu *et al*., 2010).

CONCLUSION

From the data presented in this study, it can be concluded that the investigated strain *Streptomyces* spp. AH4 could be considered to have good prospects for its application in remediation of anthracene contaminated environment and improvement of anthracene removing treatment of industrial wastewater.

ACKNOWLEDGEMENTS

The authors duly acknowledge instrument support from the department of chemical industrial laboratoire department and laboratory of natural products chemistry and biomolecules Blida, university, ALGERIA

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