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Identification and Characterization of an Indigenous Hydrocarbonoclastic Bacterial Strain *Stenotrophomonas maltophilia* newly Isolated from Seawater and Marine Sediments of Oran Harbor Northwestern Algeria

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Abstract

Microorganisms have an important role in the bioremediation of petroleum contaminants that cause great concern about their persistent toxicity, carcinogenicity and difficult elimination. In this study, crude oil-degrading bacterial strain SP54N was isolated from the contaminated marine sediments and seawater at the harbor of Oran, Northwestern Algeria, using Bushnell-Hass salt medium. This strain could support high concentrations of crude oil (up to 10% v/v), and was identified as *Stenotrophomonas maltophilia* by sequencing and analyzing the 16s rDNA with the BLAST program on the NCBI website. The effects of pH, temperature and salinity on rate growth of strain SP54N, in BHSM medium supplemented with 2% (v/v) of crude oil as sole carbon and energy source, were studied. The results show that maximum growth rate was obtained at pH 7, temperature 25°C and 3% (w/v) of salinity, at 140 rpm. Moreover, *Stenotrophomonas maltophilia* could effectively utilize crude oil as its sole carbon and energy source. Therefore, *Stenotrophomonas maltophilia* SP54N can be used as an excellent degrader to develop one eco-friendly and cost-effective method for the bioremediation of harbor of Oran, as indigenous bacteria, and marine environments polluted by oil and petroleum hydrocarbons, and could be useful for biotechnological applications.

Keywords*: *Bioremediation; Crude oil; 16s rDNA; Marine sediments

Abbreviations:* *PAHs: Polycyclic Aromatic Hydrocarbons; BHSM: Bushnell Hass Mineral Salt Medium; TSI: Triple-Sugar-Iron

Introduction

Aromatic hydrocarbons and oil are the most important environmental pollutants. Hydrocarbon contamination in seawater and sediments has been considered as an international threat for environmental reasons [1]. Hydrocarbon pollution in the harbor and marine environments emerged from the emission of waste product processing, the utilization production, drilling, transportation and storage of petroleum product and from the oil spill accidents. The discharge of petroleum products in the environment causes environmental damage affecting directly or indirectly ecological systems and human health [2,3]. Several

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restoration techniques have been tested to eliminate or reduce hydrocarbon contaminants in recent decades. Bioremediation is an important strategy for utilizing biological activities for the rapid removal of environmental pollutants [4]. It has been confirmed that the application to be more appropriate and practical for cleaning-up petroleum hydrocarbons in contaminated harbors and marine environments [1].

Biodegradation by natural microbial population is the best reliable mechanism to eliminate xenobiotic pollutants, like crude oil from the environment [5]. Hydrocarbon-degrading microorganisms living in marine environments would be more adapted for restoring the hydrocarbon contamination in the sea. Various hydrocarbons degrading bacteria were isolated from marine environments such as *Psychrobacter sp., Rodoccocus sp., Arthrobacter sp., Micrococcus,* and *Spingomonas*. Though, the number of the bacterial species capable of degrading petroleum hydrocarbons isolated from the sea is less than that isolated from sediments [6-8].

Polycyclic Aromatic Hydrocarbons (PAHs) is a group of major environmental xenobiotic pollutants which raise significant concern over there toxicity, teratogenicity and carcinogenicity [9,10]. Previous studies confirmed that enormous bacteria, archaea, and fungi have the petroleum-degrading abilities [11,12]. However, these microorganisms were mainly isolated from terrestrial soils and sediments as well as marine sediments [13].

Our aim in present work is to isolate indigenous hydrocarbonoclastic bacterial strains with the abilities to degrade crude oil from contaminated marine sediments and seawater of Oran harbor and characterize the strain SP54N with its high growth rate on crude oil.

Materials and Methods

Samples collection

Samples of marine sediments and seawater of few millimeters of the surface of the sediments (-40 m depth) were collected, in sterile flasks, at the harbor of Oran, Northwestern Algeria (35°42’ 44’’N; 0°38’28’’W) (**FIG. 1**), in October, 2013, and transported immediately to the laboratory.

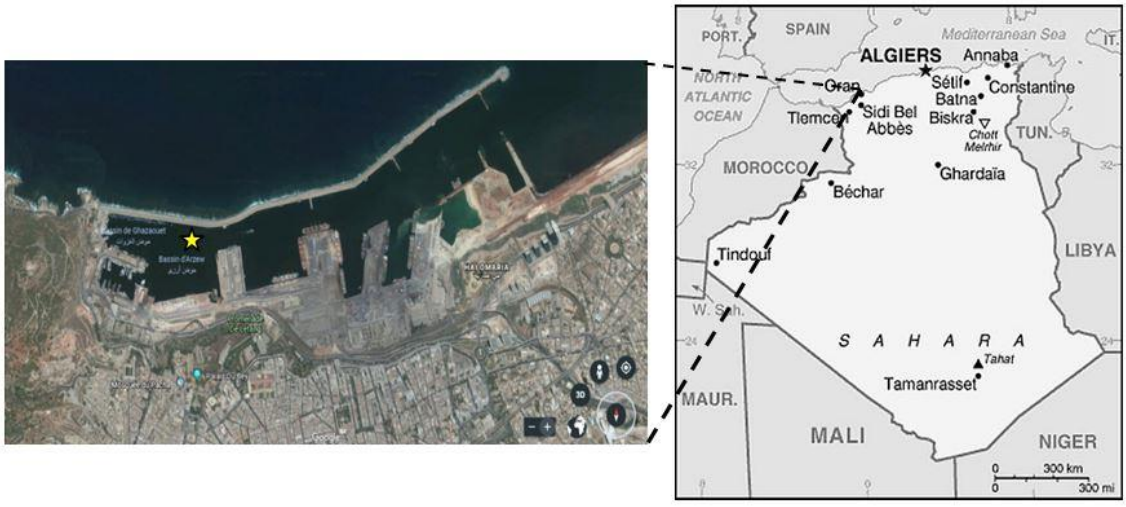

FIG. 1. **Localization of sampling site of the Oran harbor, Northwestern Algeria.**

Enrichment, isolation, and selection of hydrocarbon-degrading bacteria

A synthetic Bushnell Hass Mineral Salt Medium (BHSM) was used for isolation of hydrocarbon-degrading bacteria [14]. The pH was adjusted to 7.2 and then sterilized by autoclaving at 121°C for 20 min. After the BHSM was cooled, it was supplemented with 2% (v/v) of crude oil (Supplied by the Hassi-Messaoud oil Refinery, Algeria) as sole carbon and energy source. The crude oil was sterilized by 0.22 µm filter membrane.

From mixed sediments and seawater (of -40 m depth) 2 ml was taken after decantation, and added to 500 ml Erlenmeyer flask containing 100 ml BHSM, and incubated for 7 days at 25°C at 140 revolutions per minute (rpm) in a shaker incubator (Heidolph Inkubator 1000 coupled with Heidolph Unimax 1010, Germany). From the enrichment culture product, 1 ml aliquots were transferred to fresh BHSM medium for subsequent subculture. Then, 1 ml inocula from the flask were streaked on a series of four further subcultures on BHSM medium supplemented with 4%, 6%, 8% then 10% of crude oil (v/v) successively as sole carbon and energy source. Each subculture was incubated at 30°C and 140 rpm for 72 hours. Inoculums from the flask of 10% (v/v) of crude oil were streaked out into BHSM agar medium supplemented with 10% of crude oil (v/v) and incubated at 25°C for 3 to 7 days. Phenotypically different colonies were purified in nutrient agar medium (Sigma-Aldrich, Germany). Pure cultures were then inoculated another time in fresh BHSM medium with 10% (v/v) to be sure that it used crude oil as sole carbon and energy source, then stored at -20°C until characterization.

Morphological and biochemical characteristics

The typical biochemical and physiological characteristics of strain SP54N, including Gram staining, motility (Mannitol mobility medium), respiratory type (meat-liver agar), Triple-Sugar-Iron test (TSI test), Citrate utilization test (Simmons' Citrate agar), were systematically analyzed according to Bergey's Manual of determination for bacteriology [15]. All mediums were purchased from Sigma-Aldrich (Germany). The oxidase activity was determined by kit oxidase test (Sigma-Aldrich, Germany) and the catalase activity was determined by bubble formation in a 3% (w/v) hydrogen peroxide solution. All biochemical tests below were performed in triplicate.

Molecular identification

DNA extraction: Genomic DNA of strain SP54N was extracted from grown cells using an EasyPure® Bacteria Genomic DNA Kit (TransBionovo Co., Ltd., China) by following the manufacturer's instructions. The DNA samples were stored at -20°C until use.

16s rDNA gene sequence analysis: The DNA sample were used as the template for PCR amplification of the 16s rDNA gene using the universal primers ben27F (5'-AGAGTTTGATCCTGGCTC-3') and ben1492R (5'-GGTTACCTTGTTACGCTT-3'), synthesized by Sigma (Germany). The 16s rDNA gene was amplified with the following mixture: 2 µL of DNA template (20 ng), 1µL of ben27F (25 µM), 1 µL of ben1492R (25 µM), 1.0 µL of dNTP (5 mM), 4.0 µL of $MgCL_2$ (25 mM), 5 µL of 10X buffer solution (20 mM), 0.5 µL of Taq DNA polymerase (5 μL^{-1}), and ddH_2O up to final volume of 50 µL. PCR conditions were described as below: 95°C for 5 minutes; 35 cycles, 94°C for 1 minute. PCR products were checked by running 1.5% agarose gel electrophoresis. This 16s rDNA was partially sequenced by Genewiz. The sequence similarity was searched by the National Centre for Biotechnology Information BLAST. Analysis of 16s rDNA

gene sequence data was performed using Clustal W and the software package MEGA (Molecular Evolutionary Genetics Analysis) version 7.0 [16,17] and the phylogenetic tree was inferred using neighbor-joining methods.

Effects different culture conditions on the growth of the hydrocarbon degrading isolate

The effects of pH, temperature and medium salinity on the growth rate of the strain SP54N were investigated using BHSM medium with 2% (v/v) of crude oil at 140 rpm shaking rate, in 250 ml sterile flasks with 50 ml BHSM medium.

In the effects of culture conditions tests, initial pH in the medium was adjusted to 6.0, 7.0, 8.0 and 9.0 with 1M HCl or 1M NaOH at 25°C. The incubation temperature was controlled at 20°C, 25°C, 30°C, 37°C, and 40°C at pH 7. The concentration of NaCl in the medium was adjusted to 0%, 3%, 6%, 9%, 12% and 15% (w/v) at 25°C and pH 7. The growth was assessed indirectly by measuring the turbidity (OD 600 nm) with the spectrophotometer (Perkin Elmer Lambda 35 UV/Vis Spectrometer, USA) after 48 hours of incubation.

Growth kinetics of the hydrocarbon degrading isolate

The strain SP54N was grown at an optimal culture conditions on BHSM supplemented by 3% (w/v) of NaCl and 2% of crude oil (v/v) as sole carbon and energy source at 25°C, pH 7 and 140 rpm on a orbital shaker (Heidolph Inkubator 1000 coupled with Heidolph Unimax 1010, Germany) for about 20 days. Cultures were performed in triplicate in 250 ml Erlenmeyer flasks containing 50 ml of medium, while the growth was assessed by measuring the optical density at 600 nm.

Nucleotide sequence submission

The 16s rDNA gene sequence of the crude oil-degrading bacteria strain SP54N was submitted in the NCBI-Gene Bank under the accession number MG738221.

Results

Isolation of hydrocarbon-degrading bacteria

24 crude oil degrading bacterial strains were isolated from sediments and seawater of Oran harbor, by enrichment culture and dilution subcultures with an increasing concentration of crude oil (2% to 10%, v/v) and purified on a plate. The degradation experiments demonstrated that these isolates exhibited their variable growth rate and degradability of crude oil in the BHSM medium with 2% to 10% (v/v) of crude oil as their sole carbon and energy source. Among, strain SP54N possessed a high growth rate on crude oil as a sole carbon and energy source, and supported a high concentration of crude oil (up to 10% v/v).

Identification of stain SP54N

To determine phylogenetic relationships between strain SP54N and other representatives strains of the genus Stenotrophomonas, about1500 pb fragments of the 16s rDNA gene were amplified by PCR (**FIG. 2**) using universal primers and only 932 pb were sequenced (GenBank ID: MG738221). This continuous stretch sequence was analyzed by the BLAST tool on the National Center for Biotechnology Information (NCBI) website using the Gen Bank database. The calculation of the sequence similarity showed that partial 16s rDNA gene sequence of strain SP54N has a high similarity score of 99% to those of the bacterial strains *Stenotrophomonas maltophilia*. Subsequently, a phylogenetic tree was constructed using similar 16s rDNA sequences (**FIG. 3**). The phylogenetic tree was performed using the Neighbor-Joining method [18]. The bootstrap

consensus tree inferred from 1000 replicates [19]. The analysis involved 8 nucleotide sequences, codon positions included were $1^{st}+2^{nd}+3^{rd}$ and all positions containing gaps and missing data were eliminated.

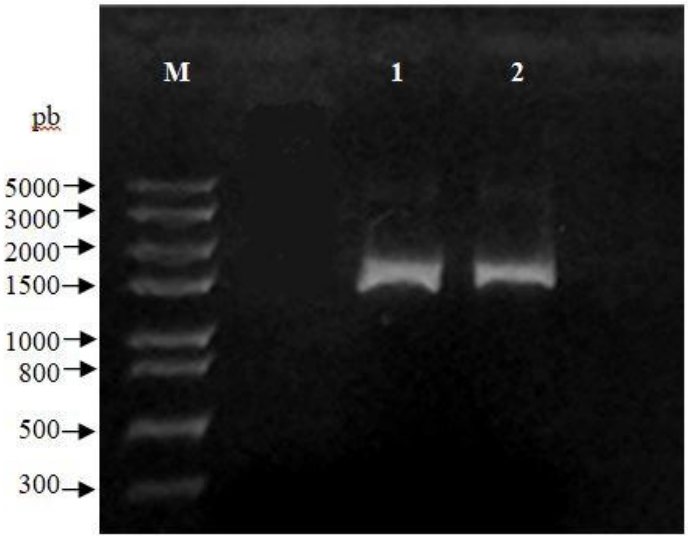

FIG. 2. **Electrophoresis of the 16s rDNA PCR product of the bacterial strain SP54N on 1% and 5% of agarose; M: Trans5K DNA Marker; lane 1 and 2: 16s rDNA.**

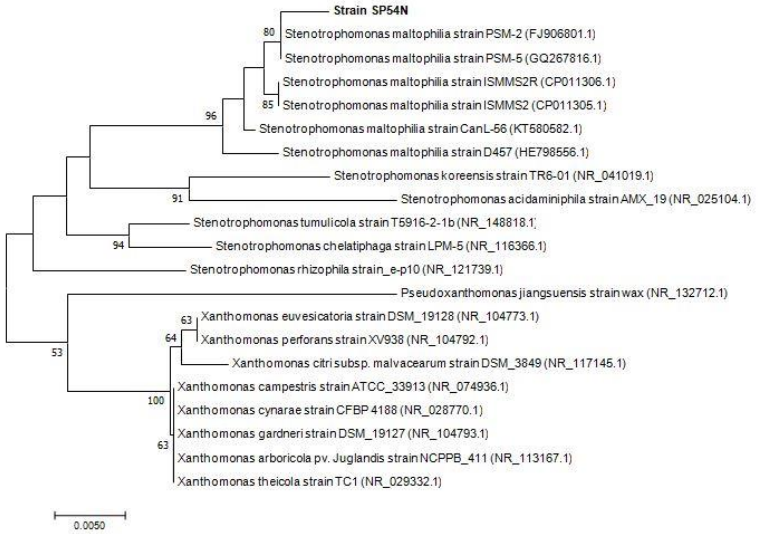

FIG. 3. **Phylogenetic tree of the partial 16s rDNA sequence of strain SP54N and the closest related species obtained from the GenBank database (NCBI); The calculation was performed according to the Neighbor-Joining method (bootstrap=1000); GenBank accession numbers are shown in parentheses; The scale bar presents 0.0050 sequence divergence.**

The phylogenetic tree indicated that the bacterial strain SP54N was related species *Stenotrophomonas maltophilia* (**FIG. 3**). Thus, strain SP54N was identified as *Stenotrophomonas maltophilia*. To highlight sequence similarity and differences between strain SP54N and the two closest strains PSM-5 (GQ267816.1) and PSM-2 (FJ906801.1) from GenBank matches, ClutalW multiple sequence alignment was used [15], and results are shown in **FIG. 4**. Results exposed in **FIG. 3 and 4** showed that strain SP54N has some differences with related strains so that it could be a new species of *Stenotrophomonas maltophilia*.

FIG. 4. **Alignment sequence of partial 16s rDNA sequence gene of unknown stain SP54N and the two closest strains *Stenotrophomonas maltophilia* (code number in GenBank: in parentheses).**

The colonies of strain SP54N were light yellow, circular, smooth, convex, with entire margins and 1 mm to 2 mm in diameter after incubation at 25°C for 24 hours on a nutrient agar plate (**FIG. 5a**). The gram strain showed that strain SP54N belonged one of the Gram-negative rod-shaped bacteria (**FIG. 5c**). The **FIG. 5b** shows colonies of strain SP54N in BHSM agar medium with 10% of crude oil (v/v). The results of the biochemical characteristics of strain SP54N are summarized in **TABLE 1**.

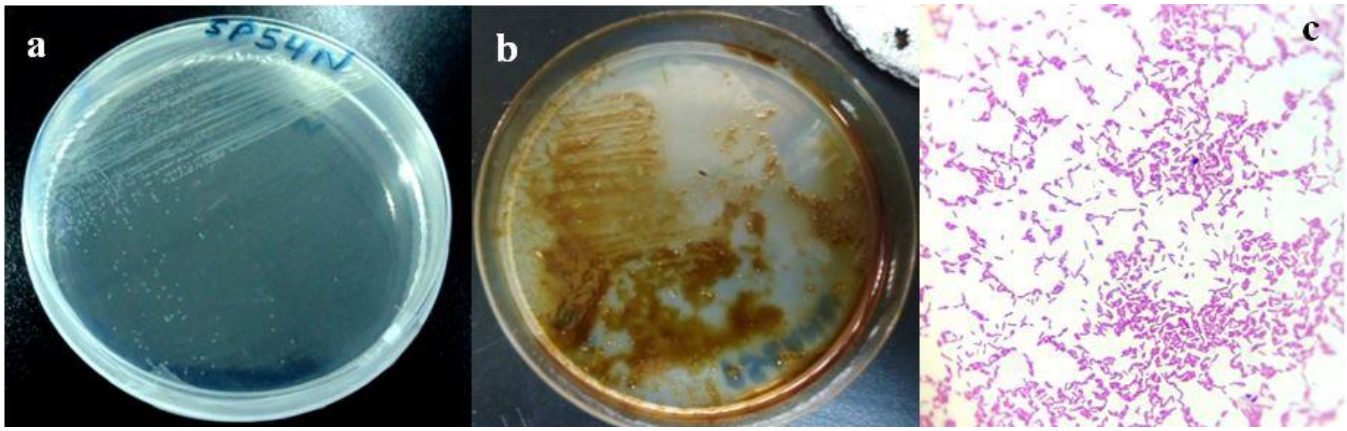

FIG. 5. **Morphological properties of the colonies and cells of strain SP54N; a: Colonies of strain SP54N on the nutrient agar plate; b: Colonies of strain SP54N on the BHMS medium agar plate with 10% (v/v) of crude oil; c: Gram-negative bacterium of strain SP54N (10 × 100).**

TABLE 1. **Morphological and biochemical characteristics of strain SP54N.**

Characteristics	Results
Gram staining	-
Shape	Rod-shaped
Oxidase test	-
Catalase test	+
Motility	+
Mannitol	-
Respiratory type	Strict aerobia
TSI test	K/K
H_2S production	-
Gas production	-
Citrate utilization	-
+: Positive reaction; -: Negative reaction	

Effects of different biodegradation conditions

In order to understand the crude oil degradation environmental characteristics by the isolate strain SP54N, the environmental factors affecting degradation were investigated, including initial pH, temperature, and NaCl concentration.

Results obtained from the effect of different pH on the growth rate of strain SP54N in crude oil as a sole carbon and energy source are represented in **FIG. 6a**. As shown in this curve, the strain could grow at pH 6-9 with the optimum growth rate at pH 7.

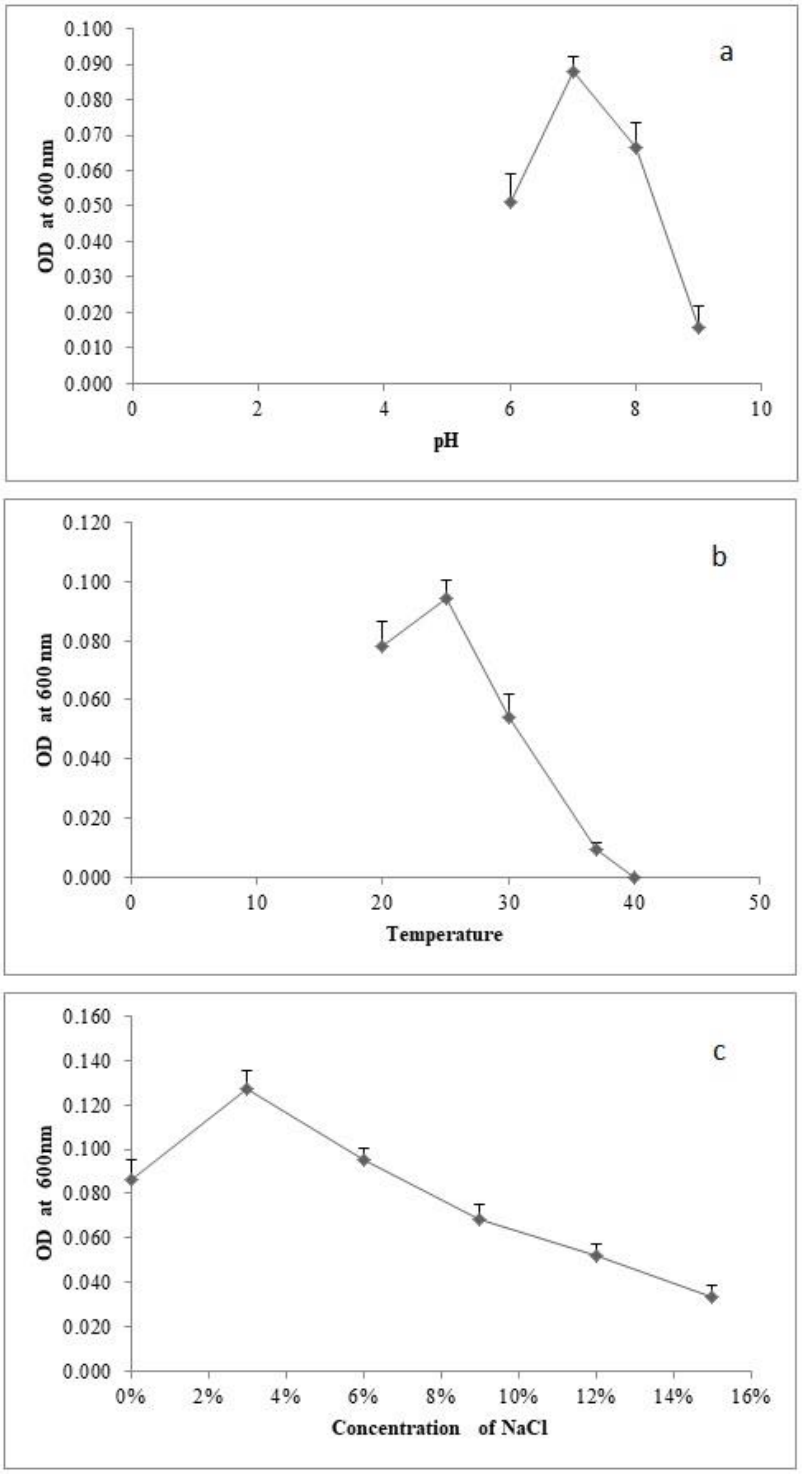

FIG. 6. **Effects of pH (a), temperature (b), and medium salinity (c) on the growth rate of strain SP54N on BHSM medium supplemented with 2% crude oil (v/v) as sole carbon and energy source for 48h at 140 rpm; Experiments were performed in triplicate.**

Effects of different temperature in the growth rate of strain SP54N on crude oil as a sole carbon and energy source are illustrated in **FIG. 6b**. The results demonstrated that this strain could utilize crude oil at temperature 20°C to 37°C, with a maximum growth rate at temperature 25°C. Strain SP54N can grow in temperatures less than 20°C.

Results obtained from the effect of different concentration of NaCl on rate growth of strain SP54N on crude oil as sole carbon and energy source are represented in **FIG. 6c**. From the curve, it can be seen that the optimum concentration of NaCl for the highest growth rate of strain SP54N was 3% (w/v). However, this bacterial strain can tolerate high salt concentrations (up to 15% w/v).

Growth kinetics of the hydrocarbon degrading isolate

The bacterial stain SP54N was cultured under optimal culture conditions obtained in this study, pH 7, 3% (w/v) of NaCl at 25°C, on BHSM medium with 2% (v/v) of crude oil as sole carbon and energy source over 20 days. The optical density at 600 nm was measured to assess bacterial growth, and the results are shown in **FIG. 7**. The strain started the logarithmic growth phase from the 1st day to 13th day, and then the stationary phase was attained at day 14.

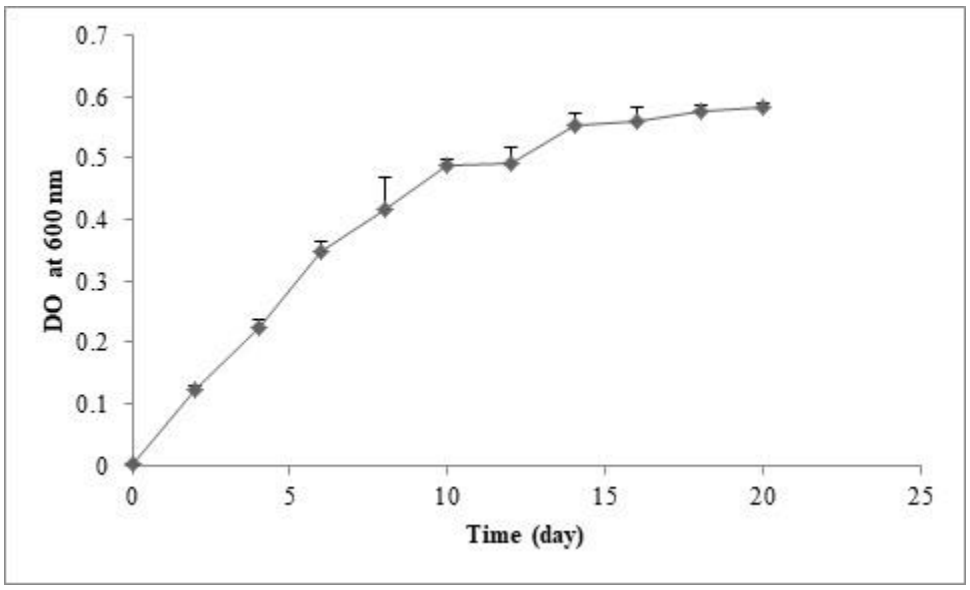

FIG. 7. **Growth kinetics of the SP54N strain on crude oil (v/v) as a sole source of carbon and energy over 20 days; Bars represent standard deviation.**

Discussion

Strain SP54N was one of the hydrocarbon-degrading bacteria belonging to *Stenotrophomonas maltophilia*, which was firstly isolated from seawater and sediment of the harbor of Oran-Algeria, and had potential to develop a method to remediate marine environments polluted by petroleum hydrocarbons.

The molecular identification of the SP54N strain was considered. Therefore, the partial sequence of 16s rDNA gene (932 bp) confirmed the identification as *Stenotrophomonas maltophilia* species with 99% of similarity (**FIG. 3 and 4**). Furthermore,

the morphological characteristics of strain SP54N were very similar to those of the reported strains *Stenotrophomonas maltophilia*. The result of the biochemical characteristics of strain SP54N is summarized in **TABLE 1**.

The study of the effect of culture conditions on petroleum hydrocarbons degradation is very important because it can provide information about the microorganism and their growth requirements, before any contribution for bioremediation. To reveal the crude-oil-degrading conditions of strain SP54N, pH from 6 to 9, temperature from 20°C to 40°C and NaCl concentration from 0% to 15% (w/v) were investigated, as shown in **FIG. 6** respectively.

Maximum growth was obtained at pH 7.0 (**FIG. 6a**). This result is in agreement with those obtained by Neethu et al., [20], Deng et al., [1] and by Kumari et al., [21], who showed that the maximum growth rate of *Stenotrophomonas maltophilia*, isolated from contaminated soil refinery was at pH 7.5. According to Vandecasteele, extreme pH inhibited the biodegradation of the oil [22]. However, in the marine environment as fresh water, the favorable range of pH was between 7 and 8.

From the curve of **FIG. 6b**, we note that our isolate strain grows in a wide range of temperature, from 20°C to 37°C (and less than 20°C). These results are according to those obtained with Rouf et al., [23]. Also, Rodriguez-Blanco and Antoine reported that the degradation of crude oil might as well be done at 4°C, 10°C and 25°C with controlled conditions in Mediterranean water [24]. Temperature is the most important factor influencing the biodegradation rate by affecting directly bacterial metabolism [25].

Concerning salinity, it may have a positive impact on hydrocarbon degrading aptitude. There is a correlation between the mineralization rate of several hydrocarbons and salinity [26]. However, contamination by both salt and oil posed a problem for cleaning up using bioremediation, because externally added bacteria do not support high salinity [27].

In this study, results obtained for optimum salinity test showed that strain SP54N supported a high concentration of NaCl and grown in a large range of salinity (0% to 15%, w/v), with a maximum growth rate at 3% (w/v) of NaCl (**FIG. 6c**). These results are consistent with those obtained by Roder et al., who showed that *Stenotrophomonas maltophilia* decreased growth rate when exposed to more than 3% (w/v) of NaCl [28].

For results obtained from the kinetics of growth on crude oil by the isolated bacterial strain (**FIG. 7**), under the optimal conditions, the stationary phase was attained at day 14. These results indicate that this bacterial strain had effectively the ability to degrade crude oil.

Several strains of *Stenotrophomonas sp*. were previously reported to own their hydrocarbon-degrading capacity [29,30]. Other *Stenotrophomonas sp*. strains have demonstrated the ability to degrade organic pollutants, such as pesticides, insecticides, chitin, steroid hormones, and Keratin [31-35]. Also, this species possessed the ability to remediate the hydrocarbon contaminated environments [36,37].

Strains of *Stenotrophomonas maltophilia* were capable of utilizing crude oil as a sole carbon and energy source [38], and could polycyclic aromatic hydrocarbons (HAPs), such as naphthalene, fluorene, and anthracene [39], Phenanthrene [39,40],

benzo[a]pyrene [41]. In addition, Urszula, et al., showed that *Stenotrophomonas maltophilia* (strain KB2) was capable to degrade high concentrations of aromatic compounds, and might utilize phenol as a sole carbon and energy source of, and different kind of aromatic compounds such as protocatechuic acid (3,4-DBH), 4-hydroxybenzoic acid (4-HB), benzoic acid, vanillic acid and catechol [42].

Mahjoubi et al., showed that *Stenotrophomonas maltophilia*, isolated from petroleum contaminated sediments and seawater at the refinery harbor of the Bizerte coast, in the north of Tunisia, were the most active hydrocarbonoclatic isolated strain with significant production of biosurfactants and high emulsification activity of crude oil (up to 46%), and suggest by their results that *Stenotrophomonas maltophilia* may constitute a potential candidate for bioremediation, and could be useful for biotechnological applications [7]. It was demonstrated that this species had the ability to utilize a wide range of aromatic substrate as a sole carbon source, and could degrade 80% of phenanthrene in 48 hours comparing to Pseudomonas that degraded 70% of phenanthrene in 40 days [43,44]. Moreover, *Stenotrophomonas maltophilia* is a very powerful and useful means in the biotreatment of sole and wastewater decontamination [7].

The isolated strain from contaminated marine sediments and seawater in our study tolerate significant concentrations of NaCl (up to 15% w/v) and crude oil (up to 10% v/v) because of the polluted area and mechanisms of induction of developed enzymes of interest and this tolerance may reflect the evolutionary adaptation. Thus, strain SP54N may be a new species of *Stenotrophomonas maltophilia*. This adaptation translates in high stability which gives bacteria the ability to answer more quickly to a new source of hydrocarbons then bacteria without spoilage [45-48].

Conclusion

In the present study, a firstly isolated marine petroleum hydrocarbon-degrading bacterium strain SP54N, from mixture seawater and marine sediments of the Oran harbor-Algeria, was identified as *Stenotrophomonas maltophilia* according to its phenotypic and phylogenetic characteristics, possessed an outstanding growth capacity in crude oil, and excellent adaptability in pH and salinity, and could be used as a good degrader, to establish one eco-friendly, and cost-effective method for the removal of hydrocarbon contaminants in diverse marine environments polluted by petroleum hydrocarbons and crude oil, particularly in harbor of Oran.

Conflicts of Interest

The authors declare no conflicts of interest.

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