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Isolation and Characterization of Crude Oil Degrading Bacteria in Association with Microalgae in Saver Pit from Egbaoma Flow Station, Niger Delta, Nigeria

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Abstract

The capability of indigenous bacteria and microalgae in crude oil effluents to grow in and utilize crude oil as their sole source of carbon and energy provides an environmentally friendly and economical process for dealing with crude oil pollution and its inherent hazards. In view of the toxicity of crude oil spillages to indwellers of the affected ecosystems and the entire affected environment, the isolation of pure bacterial and microalgae cultures from crude effluents is a step in the right direction, particularly for bio-augmentation or bioremediation purposes. The total heterotrophic bacteria count and hydrocarbon utilizing bacteria count, as well as the microalgae count, were determined with the pour plate technique. The physicochemical properties of the effluent samples were also analyzed. Identification of the hydrocarbon utilizing bacteria was performed with phenotypic techniques. The result shows a mean total heterotrophic bacterium count of 5.91 log CFU/ml and a mean microalga count of 4.77 log cells/ml. When crude oil and polycyclic aromatic hydrocarbon (PAH) were used as sole carbon sources, total hydrocarbon utilizing bacteria counts were respectively estimated at 3.89 and 2.89 log CFU/ml. Phenotypic identification of hydrocarbon utilizing bacteria in the crude oil effluents revealed the presence of two main bacterial genera: Streptococcus and Pseudomonas. Data obtained from this study confirmed the biodegradative abilities of indigenous bacterial species, thus, ultimately resulting in the amelioration of the toxicity associated with the crude oil effluents.

1. Introduction

Petroleum hydrocarbons have undoubtedly emerged as significant environmental contaminants (**Eriyamremu *et al.*, 2007; Liu *et al.,* 2010**). Environmental pollution especially oil spills are major threats predominantly in the Niger Delta region of Nigeria, due to intensive exploration and other petroleum related legal and illegal activities resulting in several environmental hazards ranging from soil infertility, erosion of soil microbial diversity and total damage to both flora and fauna in the environment (**Nweke and Okpokwasili, 2004; Head *et al.,* 2006; Emtiazi *et al.,* 2009; Mittal and Singh, 2009**). Despite the immense economic benefit of crude oil, oil spills in soils have been traceable to various hazards in the environment and could cause several diseases like Kidney disease, probable destruction of the bone marrow and a risk factor in cancer due to the presence of harmful carcinogenic and mutagenic substances (**Lichtfouse *et al.*, 1997; Lloyd *et al.,* 2001; Mishra *et al.,* 2001; Sing and Lin, 2008**), and even death in lower animals (**Phyllis *et al.,* 1989; Eriyamremu *et al.*, 2007**). The high demand for petroleum products contributes to the increase in crude oil extraction, processing and large amounts of oily waste through various means. Crude oil is a complex mixture of chemicals varying widely in their composition of hydrocarbon and hydrocarbon-like (polycyclic aromatic hydrocarbons; PAH) chemicals and may persist in the environment for prolonged periods, posing a major threat to ecosystems. Biodegradation by intrinsic microbial populations is one of the reliable tools through which a great deal of xenobiotic contaminants, comprising crude oil spill and effluents are gradually degraded and subsequently eradicated from the environment (**Cappello *et al.,* 2007**). The catabolic abilities of microorganisms such as fungi, bacteria and algae to degrade petroleum hydrocarbons had been previously reported (**Wang *et al.*, 2011**); these microorganisms possess specific enzyme systems that enable them to degrade and utilize hydrocarbons as their carbon and energy sources. The most important means of aerobic PAH biodegradation is the primary oxidation of the aromatic benzene ring through which molecular oxygen is incorporated by the dioxygenase enzymes to form *cis*-dihydrodiols. The intermediates of dihydrodiol dehydrogenation are metabolized to carbon dioxide and water through the catechols by the actions of catechol dioxygenases and other enzymes (**Chikere *et al.*, 2011**). However, one of the major limitations of (PAHs)

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biodegradation is the low bioavailability of the pollutants to the degrading microorganisms. The efficient disposal of crude oil waste has thus become a source of concern and has resulted in environmental pollution in the areas where crude oil exploration and exportation is predominant. Bioremediation has been used as a biological approach that involves the use of microorganisms' metabolic potential to degrade pollutants into innocuous compounds in the environment (**Hara *et al.*, 2013; Singh and Chandra, 2014**). Most constituents of crude oil sludge are biodegradable, and the use of bioremediation techniques has proven to be economical, environmentally friendly and flexible (**Niti *et al.*, 2013**). The present study deals with the isolation and characterization of bacteria that is capable of growing in and utilizing crude oil as their sole source of carbon and energy during compost bioremediation of crude oil effluents.

2. Material and methods

2.1 Sample Size and Collection

The sample site for this research was the Egbaoma flow station (Formerly Asuokpu/Umutu) located in the Northern Niger Delta of Delta State with coordinates 50 SS ON and 614' OE. Oil effluent samples were collected from the saver pit (crude oil effluent collection basin) into sterile plastic screw-caped bottles by dipping the bottles into the pit after which the bottle was corked and swabbed with cotton wool saturated with 95% ethanol. The samples were transported to the Microbiology Laboratory, Igbinedion University Okada, Edo State for analysis.

2.2 Enumeration of Total Heterotrophic Bacteria

The total heterotrophic bacteria count (HPC) were conducted according to protocols described in standard methods for the examination of water and waste water (**Baird *et al.*, 2017**). The HPC was determined with the pour plate technique. One milliliter of the crude oil effluent sample was aseptically mixed with 9 ml of sterile distilled water and serially diluted up to 10^{-3} dilution. One milliliter of each of the dilutions was subsequently poured into sterile Petri dishes containing 20 ml sterile molten nutrient agar, swirled gently and allowed to solidify. The plates were incubated at room temperature for 72 hours and colonies on each plate enumerated.

2.3 Enumeration of Total Hydrocarbon Utilizing Bacteria

The total hydrocarbon utilizing bacteria was counted on minimal salts agar with the pour plate method according to the method of **Mills *et al.*, 1978** as modified by **Okpokwasili and Odokuma (1990)**. Ten-fold serial dilutions were performed on the crude oil effluent. To isolate the hydrocarbon utilizing bacteria in the crude oil effluent, 1 ml of each dilution was mixed with 20 ml of sterile molten oil-agar medium (sterile minimal salts agar plus either sterile crude oil or PAH carbon sources) in a Petri dish The Petri dish was incubated at 30 °C for 5 – 7 days. Colonies on the respective oil-agar plates were then counted and subsequently expressed as hydrocarbon utilizing bacteria per ml of the effluent. The bacterial colonies which developed on the plates were randomly picked and streaked onto sterile nutrient agar slants. The pure isolates in the slants were then kept in the refrigerator until characterization/identification of the isolates.

2.4 Characterization of Hydrocarbon Utilizing Bacteria

The isolates were identified using various morphological and biochemical tests as described by **Holt (1995)** Bergey's Manual of Determinative Bacteriology.

2.5 Enumeration of Total Heterotrophic Micro Algae

The crude oil effluent samples from the saver pit were filtered using a sterile muslin cloth so as to recover a concentrated amount of the micro algae. A surface sterilized spatula sterilized with 95 % ethanol was then used to transfer the algae from the cloth to 2.5 ml of sterile Allen medium and the mouth of the flask was plugged with sterile cotton wool **(Stein, 1980)**. The flask was shaken vigorously for 30 seconds to allow the dispersal of algae in the medium as well as to discourage algae from setting at the base of the flask. The flask was then incubated for 7-14 days under an illumination provided by two (2) 2 ft. fluorescent tubes at room temperature (**Ajao and Fagade, 1990**). The cell was counted using a haemocytometer under × 40 objectives, and calculated using the standard formula.

2.6 Purification of Micro Algae Culture

The algae culture was plated out on Allen medium using the pour plate method and incubated for 24 hours after which distinct colonies were picked using a sterile inoculating needle and transferred into Allen medium. Incubation was for 7-14 days under an illumination provided by two (2) 2 ft. fluorescent tubes at room temperature after which the cells were viewed under × 40 objectives to ascertain purity (**Omoni and Abu, 2014**).

2.7 Characterization of Micro Algae

Pure algal isolates sub-cultured from the heterotrophic algal onto broth medium were examined based on their morphology and possible extracellular structures. A wet mount preparation was carried out and view under × 40 objectives. Characterization was done by comparison with those documented in the identification guide of fresh-water and terrestrial algae (**John *et al.*, 2003**).

2.8 Physicochemical Properties of the Effluent Sample

The physicochemical parameters assessed for the effluent samples include temperature, pH, and electrical conductivity, dissolved oxygen, turbidity, Polycyclic Aromatic Hydrocarbons (PAHs)-assessment, salinity and alkalinity and were determined using standard methods (**Baird *et al.*, 2017**).

3. Results

The pH of the effluent was estimated at 7.2, while the pH of the PAH and crude oil carbon sources ranged from 7.3 to 7.5. Hence there was no significant difference ($P < 0.05$) in the pH of the effluent and the carbon sources examined in this study. While the nitrogen content of the effluent was significantly higher than the values reported in the PAH and crude oil carbon sources; the phosphorus content in the PAH and crude oil were much higher than the value reported in the crude oil effluent. PAH was the least turbid while the crude oil effluent was found to be most highly turbid fluid. The lead content of the crude oil effluent was significantly lower than those reported in the PAH and crude oil carbon sources. Other heavy metals such as zirconium and copper were found at lower levels in the effluent when compared to PAH and crude oil carbon sources.

Total heterotrophic bacteria and microalgae isolated from the saver pit in Egbaoma flow station in Delta State is presented in Table 2. The result shows a total mean heterotrophic bacterium count of 5.91 log CFU/ml and a mean microalga count of 4.77 log cells/ml.

Table 1. Some physicochemical parameters of the crude oil effluent, polycyclic aromatic hydrocarbon (PAH) and crude oil carbon sources

Parameter	Crude Oil Effluent	PAH	Crude Oil
pH	7.2±0.1	7.5±0.1	7.3±0.1
Moisture %	20.1±0.1	14.1±0.7	13.4±0.7
Organic matter %	2.19	1.95	2.25
N %	5.3±0.1	0.1±0.0	0.17±0.0
P %	0.01+0.00	1.24+0.03	1.16+0.0
Turbidity µS/cm	367.3	95.0	248.0
Pb (mg/L sample)	57.8	337.8	347.8
Zr (mg/L sample)	247	556	504
Cu (mg/L sample)	111.8	143.8	164.8

Legend: N- Nitrogen; P- Phosphorus; PAH-Polycyclic Aromatic Hydrocarbons; Pb-Lead; Zr-Zirconium; Cu-Copper; some values are presented as Mean ± Standard Deviation

Table 2. Total microbial counts of Heterotrophic bacteria and Microalgae from the saver pit in Egbaoma flow station Delta State

Sample	Heterotrophic bacteria count		Micro algae	
	Rep. Bacteria (log CFU/ml)	Mean count (log CFU/ml)	Rep. microalgae count (log cells/ml)	Mean count (log cells/ml)
1	5.90		4.76	
2	5.94	5.91	4.87	4.77
3	5.90		4.66	

Table 3. Hydrocarbon utilizing bacteria isolated from the saver pit using different hydrocarbon materials as sole carbon sources

Hydrocarbon used as sole carbon source	Hydrocarbon utilizing bacteria counts	Mean count
	Rep bacteria count (log CFU/ml)	(log CFU/ml)
Polycyclic aromatic hydrocarbon (PAH)	1. 2.90 2. 2.87 3. 2.91	2.89
Crude oil	1. 3.89 2. 3.92 3. 3.85	3.89

Table 4. Phenotypic characterization of hydrocarbon utilizing bacteria

Carbon Source	Isolates	Cultural Examinations	Gram Staining	Biochemical Examinations								Probable Organism
				Co	Ca	Ox	Ur	In	Mr	Vp	Ci	
Crude oil	1	Mucoid colony with entire margin	Gram positive cocci in chains	-	-	-	-	-	+	-	-	*Streptococcus* species
Crude oil	2	Greenish colony with entire margin	Gram negative rods	-	+	+	-	-	-	-	+	*Pseudomonas* species
PAH	3	Mucoid colony with entire margin	Gram positive cocci in chains	-	-	-	-	-	+	-	-	*Streptococcus* species

Legend: Co-coagulase test, Ca-catalase test, Ox-oxidase test, Ur-urease test, In-indole test, Mr-methyl red test, Vp-voges Proskauer test, Ci-citrate test

Table 3 represents the hydrocarbon utilizing bacteria fraction of the total heterotrophic bacteria isolated from the saver pit in Egbaoma flow station in Delta state. When crude oil and PAH were used as sole carbon sources, hydrocarbon utilizing bacteria counts were 3.89 and 2.89 log CFU/ml for crude oil and PAH respectively.

Table 4 represents the use of phenotypic methods to identify isolated hydrocarbon utilizing bacteria. Upon colonial examination, three bacterial isolates were examined. Two of these isolates were indicated as Gram positive cocci occurring in chains, while the other isolate was observed as Gram-negative rods. The three isolates were subjected to an array of biochemical tests. The results indicated that the two isolates belong to the *Streptococcus* genus, while the other isolate was identified as *Pseudomonas* species. In essence, two main hydrocarbon utilizing bacteria were isolated from the saver pit in Egbaoma flow station, Delta state.

4. Discussion

Hydrocarbon utilizing bacteria exhibit extreme diversity in nature and can readily adapt to survive in unsuitable environments (**Sohal and Srivastava, 1994**). Previous studies (**Survery *et al.*, 2004; Chaillan *et al.*, 2004; Li *et al.*, 2005; Sathishkumar *et al.*, 2008**) have implicated some microbes such as *Achromobacter*, *Bacillus*, *Corynebacterium*, *Escherichia*, *Micrococcus*, *Vibrio* and *Pseudomonas* species as hydrocarbon utilizing bacteria. In this research, indigenous hydrocarbon utilizing bacteria were isolated from crude oil effluents collected from the saver pit in Egbaoma flow station in Delta state. Mean total heterotrophic bacteria count was estimated at 5.91 log CFU/ml (Table 2). From the pool of the total heterotrophic bacteria count, mean hydrocarbon utilizing bacteria counts of 3.89 and 2.89 log CFU/ml were respectively reported for crude oil and PAH carbon sources (Table 3). The hydrocarbon utilizing

bacteria that were isolated belong to the *Streptococcus* and *Pseudomonas* genera (Table 4). *Pseudomonas* species have been frequently detected by several authors in various hydrocarbon-contaminated environments (**Li *et al*., 2005; Sathishkumar *et al*., 2008**). Mean microalgae count was estimated at 4.77 g cells/ml (Table 2). This result was in line with the findings of **Tan and Ji (2010)** that these classes of bacteria and microalgae possess the ability to use the nitrogen–sulphur–oxygen (NSO) fractions of crude oil effluents as their sources of nitrogen, carbon and energy. This result is comparable to the findings of some studies (**Hara *et al*. 2013; Molina *et al*. 2009; Mishra *et al*. 2014**) that confirmed some organism including the ones isolated in this study to have the abilities to use crude oil effluents for their carbon and energy requirements.

5. Conclusion

The data from this study supports past research findings that indigenous bacteria and microalgae grow in and utilize crude oil effluents as their carbon source for energy. Our findings have indicated that the toxicity of crude oil effluents to the environment could be ameliorated by the biodegradative activities of indigenous microbes in the crude oil effluents. Data obtained from this study specifically confirmed the biodegradative abilities of *Streptococcus* and *Pseudomonas* species, their ubiquity in hydrocarbon polluted environments and their potential for bioremediation of hydrocarbon-polluted sites. Further work to determine the optimum environmental conditions favorable for their application in bioremediation is hereby suggested.

Declaration of interest

The authors report no conflicts of interest.

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In Vitro Antimicrobial Screening of *Momordica charantia* extracts against Multidrug-Resistant Bacterial Strains

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Abstract

A comparative study for screening the antibiotic potential of *Momordica charantia* successive extracts *in vitro* against eleven multidrug-resistant bacterial strains. *Momordica charantia* fresh fruit was extracted successively in different solvents in the order of increasing polarity from hexane to aqueous followed by screening against eleven antibiotic-resistant bacterial strains including both Gram-positive and Gram-negative bacterial strains using Kirby-Bauer's disk diffusion and agar well diffusion method. A study on *Staphylococcus epidermidis* and *Corynebacterium xerosis* are reported for the first time. Significant inhibitory activity was noted against most of the resistant human pathogenic strains. Findings reported that ethyl acetate fraction shows the highest zone of inhibition while hexane, petroleum ether, chloroform and aqueous extract were almost resistant. The antibacterial efficacy of *Momordica charantia* is found to be significant. It's also concluded that the controversy on the antibacterial activity of *Momordica charantia* fruit extract in different solvents is based on several factors like the solvent used for extraction, plant part, concentration, method of extraction, *etc*. *M. charantia* extracts could be used as an alternative anti-microbial to replace antibiotics for treating a broad spectrum of multidrug-resistant bacterial diseases.

1. Introduction

In the 21st century, antimicrobial resistance is the greatest challenge to public health and threatens modern medicine where common infections could become more deadly. Antimicrobial resistance is the ability of a microbe to resist the effects of medication that once could successfully treat the microbe. The discovery of antibiotics was one of the most important developments in medicine but misuse and overuse of antibiotics to treat viral infections, use of broad-spectrum antibiotics and as growth promoters in animals leads to emergence of bacteria that have evolved resistance to multiple antibiotics and gave birth to superbugs and multidrug-resistant microbes in the environment (**Sharma V.K, 2016**). The persistence of these antibiotic-resistant microbes not only creates increased opportunities to transfer the resistance genes to associated susceptible bacteria but also eventually lead to entry into the human food chain (**Founou, *et al.,* 2016**).

Globally, new resistance mechanisms are continuously being emerging and spreading. This has triggered initiatives worldwide to develop novel and more effective strategies to counteract antimicrobial resistance. Today in the synthetic world where common infection could become a threat, returning towards herbal therapy is a safe and noninvasive method for the betterment of healthcare. Since ages, natural products that include medicinal plants have been used to prevent, cure and treat multiple diseases. Herbal products from medicinal plants provide unlimited opportunities for new drug leads either as pure compounds or as standardized extracts. They possess a huge diversity of bioactive compounds having safer and more efficient therapeutic potential. As natural compounds have relatively better safety profile they are gaining a lot of attention in drug discovery programs. World Health Organization (WHO) reported about 80 % of the world's population that utilizes traditional medicine as their first line of therapy (**WHO, 2002-2005**). But today people are more focused on modern medicine. Though both therapies have several pros and cons. So, to find solutions to the severest problem facing the health system by intelligently selecting, harmonization of traditional and modern medicine promote the best care for patients.

Momordica charantia commonly called bitter gourd belongs to Cucurbitaceae family. It is widely distributed in tropical and subtropical regions of the world. *M. charantia* is frequently used in the traditional medicine due to its anti-bacterial, anti-viral, anti-tumor, anti-oxidant, anti-diabetes, antilipolytic, anti-inflammatory, anthelmintic, immunomodulatory and hepatoprotective properties (**Shuo Jia, *et al.,* 2017**). Although hundreds of plant species have been evaluated for phytochemical profile and screened for antimicrobial properties, the vast majority of the plants have not been

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adequately screened and evaluated. In the present study successive extraction of *Momordica charantia* was evaluated and each fraction was screened against several multidrug-resistant bacterial strains along with some novel findings.

2. Material and methods

All solvents used were of HPLC grade obtained from Sigma-Aldrich. Nutrient agar, Nutrient broth medium, and antimicrobial disks were purchased from Hi-media Labs, Mumbai. The bacterial cultures were kindly provided by Microbiology Lab, Department of Ilmul Advia and Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh.

2.1 Plant Material

Fresh *Momordica charantia* (MC) fruit was procured from the market, authenticated with the botanical literature available and identification made accordingly by pharmacognosy expert. The whole MC fruit comprising pericarp, pulp and seeds was washed thoroughly and subjected to grinding in a mixer to get a homogenized mixer.

2.2 Successive Extraction and Isolation of Chemical Constituents

The homogenized mixer of fresh MC fruit was subjected to successive extraction sequentially from hexane, petroleum ether, ethyl acetate, chloroform, acetone, n-butanol, ethanol and methanol to water (each 1:3 w/v ratio). Hexane and petroleum fractions were subjected to 24 hours maceration with continuous stirring while rest fractions were subjected to soxhlet hot extraction. Each fraction collected separately after filtration followed by drying.

2.3 Alcoholic and Aqueous Extraction

For alcoholic extraction the fresh MC fruit paste (250g) was subjected to extraction by heating to reflux with 95% ethanol (plant: solvent 1:2, m/v) in a soxhlet apparatus at 50°C for 5 hrs. Then filtered the ethanolic extract (HEE), collected the filtrate and reduce the concentration to a small volume by drying. Similarly, aqueous extraction (HAE) was done separately.

2.4 Antimicrobial Assay

Antibiotic susceptibility testing for multidrug-resistance: Multidrug-resistant testing for all Gram-positive and Gram-negative clinical strains was determined using Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI 2006) guidelines against certain antibiotics, namely, Cefixime (10µg), Amoxyclav (10µg), Cefotaxime (10µg) and Methicillin (10µg), and plates were incubated at 37°C for 24 hours. The next day, the diameters of the zone of inhibition around the discs were measured.
Drug susceptibility test: The susceptibility of different extracts of *M. charantia* against multidrug-resistant bacterial strains was determined using Kirby-Bauer's disk diffusion and agar well diffusion method according to CLSI (Clinical Laboratory Standard Institute) Guidelines as mentioned above. Seven Gram-positive (*Staphylococcus aureus, Streptococcus mutans, Streptococcus pyogenes, Streptococcus viridans, Staphylococcus epidermidis, Corynebacterium xerosis,* and *Bacillus cereus)* and four Gram-negative *(Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa,* and *Proteus vulgaris)* multidrug-resistant bacterial strains were used. About 50µl of the test sample was used against each strain swabbed on nutrient agar plates followed by incubation at 37°C for 24 hrs. Different extracting solvents were used as negative control while for positive control 10µg Streptomycin disk for Gram-positive and 10µg Norfloxacin disk for Gram-negative strains were used. Antimicrobial activity was assessed using the zone of inhibition (ZoI) measured after the incubation period against each tested micro-organisms and was compared with the standard used and analyzed.

2.5 Statistical Analysis

The experiment was performed in a triplet, compared with the standard used and analyzed statistically using graph-paid software by Tukey Kramer Comparison test, One way ANOVA.

3. Results

The findings show that all Gram-positive and Gram-negative clinical strains are resistant against antibiotics, namely, Cefixime (10µg), Amoxyclav (10µg), Cefotaxime (10µg) and Methicillin (10µg). The sequential successive extraction in ascending order of polarity from hexane to aqueous extract showed different zone of inhibitions against each strain (**Fig.1**) (**Table.1**). Hexane, petroleum ether, chloroform and aqueous extract are almost resistant to all eleven bacterial strains while ethyl acetate, acetone, n-butanol, the ethanolic and methanolic extract showed significant activity against all bacterial strains. On average, it's observed that as the polarity of the solvent increasing from ethyl acetate to methanol the antimicrobial activity of the extract decreases subsequently. Ethyl acetate crude extracts exhibited a considerably broader antimicrobial activity compared to other extracts. The maximum ZoI of 30mm was produced by ethyl acetate fraction against *Streptococcus pyogenes.* Besides, the ethanolic extract of successive extraction showed lesser antibacterial activity than extract obtained following non-successive extraction. Further, to the best of my knowledge antibacterial activity of *M. charantia* against *Staphylococcus epidermidis* and *Corynebacterium xerosis* is reported for the first time.

4. Discussion

Cefixime and cefotaxime are third generations broad-spectrum antibiotics while methicillin is narrow-spectrum β-lactam antibiotic. Amoxyclav is a combination of a salt of amoxicillin and clavulanic acid. Amoxicillin works as an antibiotic and interferes with the bactericidal effect by disrupting the cell wall of bacteria while clavulanic acid reduces resistance and acts as a β-lactamase inhibitor. Numerous extended-spectrum β-lactamases (ESBLs) cause resistance to the above antibiotics and other newer cephalosporin (**Bush, K, *et al.,* 1995**). Findings reported resistance to all the above antibiotics. This may be due to the high prevalence of extended-spectrum β -lactamases and metallo- β -lactamases amongst the bacteria. The possible reason for acquiring β -lactam resistance in bacteria includes the production of high β -lactamase, impermeability of outer membrane and active efflux mediated by RND-type efflux systems (**Poole K, 2011**).
To date, there is a controversy on the antibacterial activity of *Momordica charantia* fruit. Several studies reported antibacterial activity while others not. Earlier antibacterial activity of different plant parts has been reported against several Gram-positive and Gram-negative bacteria that are under the results gained (**M. Asan Ozusaglam *et al.,* 2013; P. Supraja *et al.,* 2013; Yang Lin Yeo, *et al.,* 2014 and Gulsum Yaldiza, *et al.,* 2015**). However, some of the researchers reported antibacterial activity of hexane, petroleum ether and aqueous extract (**YinYin Chia *et al.,* 2011; Abid Mahmood 2012; Yang Lin Yeo, *et al.,* 2014)** but in our study the results were negative.

Negative results were supported by the previous findings in which petroleum ether crude extracts of fruits showed no inhibitory activity against *Escherichia coli* and *Pseudomonas aeruginosa* (**K. D. Mwambete 2009**). The bitter melon extract was obtained from its interior, middle, and outside skin and mixed with sterile distilled water, the extract showed no inhibition against *S. aureus* and *E. coli* (**Debolina Ghosh, 2014**). Antibacterial activity of fresh juice of skin and pulp of *M. charantia* against different bacterial strains was reported earlier (**Sabahat Saeed and Perween Tariq, 2005**) with ZoI between 14-17mm. Beside, deseeded fresh fruit water and methanolic extract showed no inhibitory activity against Methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* while showed antibacterial activity against *Escherichia coli* and *Salmonella enteric* (**Yeh-Lin Lu *et al.,* 2011**). Also, several leave extracts of the plant were reported too. Aqueous, methanolic and ethanolic extracts of *Momordica charantia* leaves showed antibacterial activity (**G. Leelaprakash *et al.,* 2011; S. B. Mada *et al.,* 2013; Adegbola, *et al.,* 2016**). In the present study while going in deep and co-relating our results with the previous studies it's come to an end that the antibacterial activity of *Momordica charantia* is based on several factors.

Hexane and petroleum ether both have a polarity index of near-zero (P'=0.1). The extract of *M. charantia* in both solvents shows resistance, as might be antimicrobial compounds that are responsible for inhibitions are not extracted. However previous findings showed that *E. coli* was susceptible to hexane extract (**Yang Lin Yeo, *et al.,* 2014**). It might be possible that the antimicrobial compounds present in the hexane and petroleum ether with lower polarity index tend to be extracted at longer maceration time or by using a hot extraction method. Similarly, though the polarity index of chloroform is high (P'=4.1) but extraction time and method limit its activity. Ethyl-acetate with polarity index 4.4 is widely used as an extraction solvent. Extraction in ethyl acetate showed the highest antimicrobial activity against all bacterial strains with ZoI ranging from 20-30 mm. This indicates that the active ingredients of the plant are more readily dissolved and extracted in ethyl-acetate compared with other solvents used. Next, to ethyl acetate, acetone with polarity index 5.1 showed significant activity. Though the polarity index of acetone is higher than ethyl acetate during successive extraction most of the phytochemicals are already extracted in ethyl acetate. Preceding, solvents such as n- butanol (P'=3.9), ethanol (P'=4.3), methanol (P'=5.1) and distilled water (P'=10.2) extracted lower phytochemical profiles and thus show no or very little inhibition against different bacterial strains. Findings showed that intermediate-polar solvent systems used in extracting antimicrobial compounds from fruit of *Momordica charantia* L showed significant activity.

Further, when a comparative study on the ethanolic and aqueous extracts following successive extraction and non-successive extraction protocol was done, it's found that aqueous extracts of both the procedure do not show any antibacterial activity while the ethanolic extract of the non-successive extract showed more activity than successive extract. This might be because during successive extraction phytochemicals such as flavonols and phenols are extracted by solvents such as ethyl acetate and acetone so, the ethanolic extract shows less activity (**Table. 2**).

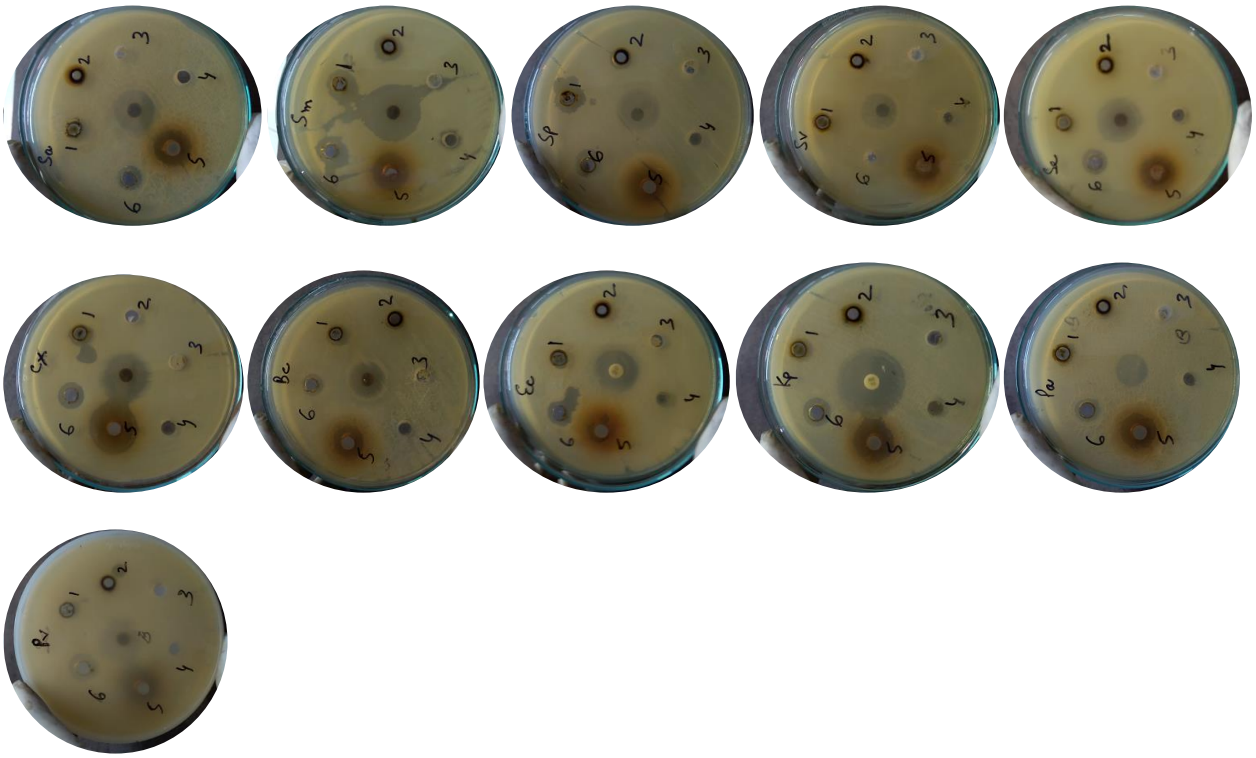

Figure 1. *In vitro* antibacterial activity of *M. charantia* extracts

Table 1. Drug susceptibility readings against various multidrug-resistant microbial strains

Name of Microbial Strains		Zone of Inhibition (in mm) [Mean±SEM (SD)]								
		HE	**PE**	**EAE**	**CE**	**AcE**	**BE**	**EE**	**ME**	**AE**
Gram Positive Bacteria	*Staphylococcus aureus*	6.6±0.3 (0.57)	6.3±0.3 (0.57)	27.6±0.3 (0.57)	6.6±0.3 (0.57)	19.6±0.3 (0.57)	10.6±0.3 (0.57)	6.6±0.3 (0.57)	6.6±0.3 (0.57)	6.3±0.3 (0.57)
	Streptococcus mutans	6.3±0.3 (0.57)	6.6±0.3 (0.57)	28.6±0.3 (0.57)	6.6±0.3 (0.57)	14.6±0.3 (0.57)	10.6±0.3 (0.57)	11.6±0.3 (0.57)	11.6±0.3 (0.57)	6.6±0.3 (0.57)
	Streptococcus pyogenes	6.3±0.3 (0.57)	6.3±0.3 (0.57)	30.6±0.3 (0.57)	6.6±0.3 (0.57)	22.6±0.3 (0.57)	10.6±0.3 (0.57)	11.6±0.3 (0.57)	10.6±0.3 (0.57)	6.6±0.3 (0.57)
	Streptococcus viridans	6.6±0.3 (0.57)	6.3±0.3 (0.57)	26.6±0.3 (0.57)	6.6±0.3 (0.57)	21.6±0.3 (0.57)	11.6±0.3 (0.57)	7.6±0.3 (0.57)	6.6±0.3 (0.57)	6.6±0.3 (0.57)
	Staphylococcus epidermidis	6.6±0.3 (0.57)	6.6±0.3 (0.57)	25.6±0.3 (0.57)	6.6±0.3 (0.57)	15.6±0.3 (0.57)	12.6±0.3 (0.57)	12.6±0.3 (0.57)	12.6±0.3 (0.57)	6.6±0.3 (0.57)
	Corynebacterium xerosis	6.3±0.3 (0.57)	6.6±0.3 (0.57)	27.6±0.3 (0.57)	6.6±0.3 (0.57)	16.6±0.3 (0.57)	6.6±0.3 (0.57)	8.6±0.3 (0.57)	8.3±1.3 (2.3)	6.6±0.3 (0.57)
	Bacillus cereus	6.6±0.3 (0.57)	6.6±0.3 (0.57)	27.6±0.3 (0.57)	6.6±0.3 (0.57)	14.6±0.3 (0.57)	11.6±0.3 (0.57)	8.6±0.3 (0.57)	8±1.0 (1.7)	6.6±0.3 (0.57)
Gram Negative	*Escherichia coli*	6.3±0.3 (0.57)	6.3±0.3 (0.57)	20.6±0.3 (0.57)	6.3±0.3 (0.57)	13.6±0.3 (0.57)	11.6±0.3 (0.57)	7.6±0.3 (0.57)	8.3±1.3 (2.3)	6.3±0.3 (0.57)
	Klebsiella pneumonia	6.3±0.3 (0.57)	6.6±0.3 (0.57)	27.6±0.3 (0.57)	7.3±0.3 (0.57)	15±1.0 (1.7)	11.6±0.3 (0.57)	10.6±0.3 (0.57)	11.3±0.8 (1.5)	6.3±0.3 3 (0.57)
	Pseudomonas aeruginosa	6.6±0.3 (0.57)	6.3±0.3 (0.57)	26.6±0.3 (0.57)	6.3±0.3 (0.57)	17.6±0.3 (0.57)	10.6±0.3 (0.57)	7.6±0.3 (0.57)	11.6±0.3 (0.57)	6.3±0.3 (0.57)
	Proteus vulgaris	6.6±0.3 (0.57)	6.3±0.3 (0.57)	24.6±0.3 (0.57)	7.3±0.3 (0.57)	17.6±0.3 (0.57)	11.6±0.3 (0.57)	10.6±0.3 (0.57)	13.6±0.3 (0.57)	6.6±0.3 (0.57)

Legend: Successive extraction= HE: Hexane extract, PE: Petroleum ether extract, EAE: Ethyl acetate extract, CE: Chloroform extract, AcE: Acetone extract, BE: n-butanol extract, EE: Ethanol extract, ME: Methanol extract, AE: Aqueous extract

Table 2. A comparative study on successive and non-successive ethanolic and aqueous extracts against various multidrug-resistant microbial strains.

	Name of Microbial Strains	Zone of Inhibition (in mm) [Mean±SEM (SD)]					
		Ethanolic (SE)	**Ethanolic (NSE)**	**Aqueous (SE)**	**Aqueous (NSE)**	**Positive control**	**Negative control**
Gram Positive Bacteria	*Staphylococcus aureus*	6.6±0.3 (0.57)	10.3±0.8 (0.57)	6.3±0.3 (0.57)	6.6±0.3 (0.57)	18.3±0.3 (0.57)	6.6±0.3 (0.57)
	Streptococcus mutans	11.6±0.3 (0.57)	12.3±0.3 (0.57)	6.6±0.3 (0.57)	6.6±0.3 (0.57)	27.3±0.3 (0.57)	6.3±0.3 (0.57)
	Streptococcus pyogenes	11.6±0.3 (0.57)	19. 3±0.3 (0.57)	6.6±0.3 (0.57)	6.6±0.3 (0.57)	19.3±0.8 (0.57)	6.3±0.3 (0.57)
	Streptococcus viridans	7.6±0.3 (0.57)	19.3±0.8 (0.57)	6.6±0.3 (0.57)	6.3±0.3 (0.57)	12.3±0.3 (0.57)	6.3±0.3 (0.57)
	Staphylococcus epidermidis	12.6±0.3 (0.57)	14.3±0.6 (0.57)	6.6±0.3 (0.57)	6.3±0.3 (0.57)	12.3±0.3 (0.57)	6.3±0.3 (0.57)
	Corynebacterium xerosis	8.6±0.3 (0.57)	19.3±0.6 (0.57)	6.6±0.3 (0.57)	6.3±0.3 (0.57)	15.3±0.6 (0.57)	6.3±0.3 (0.57)
	Bacillus cereus	8.6±0.3 (0.57)	12.3±0.6 (0.57)	6.6±0.3 (0.57)	6.6±0.3 (0.57)	13.3±0.6 (1.15)	6.3±0.3 (0.57)
Gram Negative Bacteria	*Escherichia coli*	7.6±0.3 (0.57)	14.3±0.3 (0.57)	6.3±0.3 (0.57)	6.6±0.3 (0.57)	12.3±0.3 (0.57)	6.6±0.3 (0.57)
	Klebsiella pneumonia	10.6±0.3 (0.57)	15.3±0.6 (0.57)	6.3±0.33 (0.57)	6.6±0.3 (0.57)	22.3±0.3 (0.57)	6.3±0.3 (0.57)
	Pseudomonas aeruginosa	7.6±0.3 (0.57)	15.3±0.6 (0.57)	6.3±0.3 (0.57)	6.6±0.3 (0.57)	9.3±0.6 (0.57)	6.3±0.3 (0.57)
	Proteus vulgaris	10.6±0.3 (0.57)	15.3±0.6 (0.57)	6.6±0.3 (0.57)	6.6±0.3 (0.57)	16.3±0.3 (0.57)	6.3±0.3 (0.57)

Legend: SE: Successive extract; NSE: Non-Successive extract

5. Conclusion

It's concluded that the antibacterial activity of *M. charantia* is based on several factors like the solvent used for extraction, plant part, concentration, method of extraction, etc. However, the isolation of the principal compound and structural elucidation would have yet to be achieved.

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Declaration of interest

The authors have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Hydrocarbon-Degrading Bacterial Strain *Pseudomonas mendocina* Newly Isolated from Marine Sediments and Seawater of Oran Harbor (Algerian Coast)

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Abstract

Contamination by petroleum hydrocarbons causes serious dangers to human health and the environment, whether by accidental or chronic contamination. Due to the large flow of ships, the commercial harbor of Oran is subject to pollution particularly by polycyclic aromatic hydrocarbons. For that, bioremediation by indigenous microorganisms is the most important method to eliminate or decrease this contamination. In the present paper, hydrocarbon-degrading bacterium strain SP57N has been studied, newly isolated from contaminated marine sediments and sea water from the harbor of Oran (Northwestern-Algeria), using of Bushnell-Hass salt medium (BHSM). The strain SP57N was Gram-negative, oxidase negative, catalase negative, motile, Rod-shaped bacteria, identified molecularly as *Pseudomonas mendocina* based on partial 16S rDNA gene sequence analysis, using the BLAST program on National Centre for Biotechnology Information (NCBI) and the EzBioCloud 16S rDNA databases. This isolate could growth on high concentrations of crude oil (up to 10 %, v/v). The effects of some culture conditions such as temperature, NaCl concentration and pH on growth rate of strain SP57N on crude oil as the sole carbon and energy source were studied. In addition, growth kinetic of this isolate on crude oil during 20 days of culture at 140 rpm, under optimal culture conditions was considered. The results showed maximum growth rate at temperature 25°C, 3% (w/v) of NaCl concentration and pH 7. Results of growth kinetic on crude oil as sole carbon and energy source showed that the stationary phase was attained at day 12. Thus, *Pseudomonas mendocina* SP57N had effectively hydrocarbon-degrading potential, and could be used as an efficacy degrader to initiate a biological eco-friendly method for the bioremediation of the hydrocarbon pollution on the port of Oran, and marine environment.

1. Introduction

Marine environments harbour a constant microbial seed that can be shaped by changes in environmental conditions including contamination by petroleum components. Oil spills are a major source of oil pollution in the marine environments, even though they are spilled in small but continuous discharges of hydrocarbons from transportation and recreational activities. Therefore, prokaryotic communities are well pre-adapted to oil pollution, and several microorganisms exposed to this xénobiotiques have developed an active degradation response **(Acosta-González and Marqués 2016**). The rate of oil-contamination in sediments is significantly reduced by natural processes over a long duration. Therefore, various methods have been studied to achieve rapid and complete removal of oil from sediments **(Agarwal and Liu 2015).** Also
In-situ bioremediation is particularly considered as one of the most effective and sustainable means to clean up oil-contaminated sediments. However, they can be exploited more efficiently if their multifactorial environmental and pollution parameters are known, even their metabolism, physiology and ecology **(Mapelli *et al.* 2017)**.
Amongst petroleum hydrocarbons, the polycyclic aromatic hydrocarbons (PAHs) are the major common xenobiotic pollutants presenting serious risks to human health and all living organisms, become a most important concern, due to their bioaccumulation, ubiquitous in nature, having mutagenic, toxic and carcinogenic properties **(mnif *et al.* 2017; Patel *et al.* 2018)**.
Different remediation technologies are available to remove PAHs from polluted environments. Conventional physical and chemical treatment methods are incapable to provide sustainable technology, because of their diverse disadvantages. The recently developed bioremediation approach is an alternative technology to effectively remediate the polluted by PAHs in environment **(Patel *et al.* 2018)**.
Bioremediation become an interested eco-friendly, economical and cost-effective substitution for removing petroleum hydrocarbons from contaminated environment **(Logeshwaran *et al.* 2018; Tiralerdpanich *et al.* 2018)**. Various microorganisms such as fungi, archaea and bacteria have

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capacity to degrade oil and petroleum hydrocarbons-degrading **(Atlas and Hazen 2011)**. In addition, indigenous hydrocarbonocalstic bacteria living in contaminated environment, such as marine sediments, are more suitable and better adapted for restoration of the hydrocarbon contamination in sea **(Capello *et al.* 2007; Acosta-González and Marqués 2016).**

The commercial port of Oran (northwestern Algeria), an important port economically, is actually polluted essentially by HAPs due to a large flow of ships, hence the need to carry out an eco-friendly and cost effective technique for it decontamination and remediation.

The aim of the present work is to isolate indigenous hydrocarbonoclastic bacterial strains from contaminated marine sediments and seawater at the port of Oran, having to capability grown on and utilize crude oil, and to study the newly isolated hydrocarbons-degrading bacterial strain SP57N, with its interesting potential growth on crude oil as carbon and energy source.

2. Material and methods

2.1 Localization of sampling site and samples collection

Samples of mixed seawater and marine sediment were collected, a few millimeters above the surface of the sediments, at - 40 m depth, from the port of Oran (Algerian coast) (latitude: 35°42'44''N; longitude: 0°38'28''W), in October 2013, and were immediately transported to the Aquaculture and Bioremediation laboratory (AquaBior).

2.2 Enrichment, isolation and selection of hydrocarbon-based bacteria

From samples of mixed seawater-marine sediment, the isolation of hydrocarbon-degrading bacteria was isolated out using a Bushnell Hass synthetic mineral (BHSM) medium and crude oil (from the Hassi-Messaoud refinery, Algeria) as the sole source of carbon and energy, at 4%, 6%, 8% then 10% of crude oil (v/v) subsequently according to the modified protocol of **Mehdi and Giti (2008)**. Each subculture was incubated for 72 hours at 25°C and 140 rpm Heidolph Inkubator 1000 coupled with Heidolph Unimax 1010, Germany). From the product of the last subculture, containing 10% (v/v) crude oil, inocula were seeded in BHSM agar medium, supplemented with 10 % (v/v) of crude oil, and incubated at 25°C during 7 days. The purification of the different colonies was carried out by successive subcultures on nutrient agar medium. Purity of cultures is analyzed morphologically, macroscopically and microscopically (Gram stain). Pure cultures with highest and outstanding visible growth rate and crude oil degradation on BHSM medium supplemented with 10% (v/v) of crude oil were selected, and stored at -20°C until use.

2.3 Study of physiological and biochemical characteristics the isolated bacterial strain SP57N

Physiological and chemical characteristics of the SP57N strain (carried out in triplicate) was determined, including Gram staining, the oxidase activity [kit oxidase test from (Fluka)], the catalase activity, motility, respiratory type, triple-sugar-iron test (TSI test), Citrate utilization test, and the ability to growth, BCPL medium, Chapman agar medium, King A agar medium, King B agar medium, and SS agar medium were systematically analyzed according to Bergey's Manual of determination for bacteriology **(Holt *et al.* 1998).**

2.4 Partial 16S rRNA gene sequencing and phylogenetic analyses

Genomic DNA extraction of strain SP57N was carried out using an *EasyPure*® Bacteria Genomic DNA Kit (TransBionovo Co., Ltd., China), according to the manufacturer's instructions. DNA template sample was used for PCR amplification of the 16S rRNA gene, using universal primers, forward primer ben27F: 5'-AGAGTTTGATCCTGGCTC-3'; and reverse primer ben1492R: 5'-GGTTACCTTGTTACGCTT-3', synthesized by Sigma (Germany). In total volume of 50 µl, mixture of the PCR reaction contained: 2 µL of DNA template (20 ng), 1µL of ben27F (25 µM), 1 µL of ben1492R (25 µM), 2.0 µL of dNTP (2,5mM), 2.0 µL of $MgCL_2$ (50 mM), 5 µL of 10X buffer solution (20 mM), 0.5 µL of taq DNA polymerase (5 UL^{-1}), and ddH2O. The PCR programmer was: 95°C for 5 min; 35 cycles, 94°C for 1 min denaturation, 55°C for 1min annealing and 72°C for 2 min extension; and final extension at 72°C for 10 min. For analysis of the PCR products electrophoresis was carried out on 1.5% agarose gel, and then partially sequenced by Sanger sequencing services laboratory (GENEWIZ, Inc., South Plainfield, NJ, USA). Sequence similarity research was required using the alignment method on the EzBioCloud 16S rRNA database **(Yoon *et al.* 2017),** using reference sequences, and the National Biotechnology Information Center NCBI database, using published 16S rDNA sequences. Subsequentlly, phylogenetic trees was carried out using data analysis of 16S rDNA gene sequences by MEGA software package Version 7.0 **(Kumar *et al.* 2016)**, using neighbor-joining methods.

2.5 Nucleotide sequence submission

The partial 16S rDNA gene sequence of the hydrocarbon-degrading bacteria strain SP57N was submitted to the NCBI-Gene Bank under the accession number MK825733

2.6 Investigation of culture parameters effects on growth rate of the bacterial isolate on crude oil

The optimum growth rates of the strain SP57N were investigated using different culture parameters such as temperature, NaCl of concentration and pH, using 50 ml BHSM medium, in 250 ml sterile flasks, with 2% (v/v) of crude oil, at 140 rpm shaking rate (Heidolph Inkubator 1000 coupled with Heidolph Unimax 1010, Germany), for about 48 h. All tests were performed in triplicate, and the analysis of variance (ANOVA) is conducted to analyze the data with significance levels less than 0.05 using the statistical analysis tool STATISTICA (Version 10).

2.7 Effect of temperature

The bacterial strain SP57N was inoculated aerobically into BH medium at different temperatures (20°C, 25°C, 30°C, 37°C and 40°C) at pH 7. After incubation, the growth rate was assessed by measuring optical density (OD 600 nm) with spectrophotometer (Perkin Elmer Lambda 35 UV/Vis Spectrometer, USA).

2.8 Effect of salt concentration

For the effect of the NaCl concentration test on the growth rate of strain SP57N, 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, and the growth was investigated after 48 h of incubation indirectly by measuring the turbidity (OD 600 nm) with spectrophotometer (Perkin Elmer Lambda 35 UV/Vis Spectrometer, USA).

2.9 Effect of pH

The isolate bacterial strain SP57N was inoculated with different pH of the medium, (6.0, 7.0, 8.0 and 9.0), at 25°C. The pH was adjusted with 1 M NaOH or 1 M HCl. After incubation, the growth rate was measuring by the optical density (OD 600 nm) with spectrophotometer (Perkin Elmer Lambda 35 UV/Vis Spectrometer, USA).

2.10 Turbidimetric analysis of growth kinetic of the bacterial isolate on crude oil at optimal culture conditions

For the study of the growth kinetic on crude oil the bacterial strain SP57N, it was grown under optimal cultivation conditions in BHSM medium with 2% of crude oil (v/v) as sole source of carbon and energy, based on the results of these experiments, such as 3% (w/v) of NaCl, pH 7 and temperature 25°C on a orbital incubator shaker at 140 rpm (Heidolph Inkubator 1000 coupled with Heidolph Unimax 1010, Germany), for about 20 days. Cultures were performed in 50 ml of BHSM medium, on 250 ml flasks, in triplicate. Estimation of the growth rate of the isolate strain was carried out indirectly using turbidimetric analysis by measuring the optical density with spectrophotometer (Perkin Elmer Lambda 35 UV/Vis Spectrometer, USA), at 600 nm.

3. Results

3.1 Isolation and selection of hydrocarbonoclastic bacteria

Hydrocarbon-degrading bacteria were enriched then isolated from polluted marine sediments and seawater collected at the harbor of Oran (Algeria), using enrichment cultures and successive subcultures, with increasing crude oil concentrations (2-10 %, v/v). These isolates showed variable growth rates on the BHSM medium, supplemented by crude oil as sole source of carbon and energy (data not shown). Among these strains, the SP57N strain had remarkable and outstanding growth rate on crude oil as it sole carbon and energy source, at concentrations up to 10 % (v/v).

3.2 Identification of stain SP57N

The phylogenetic relationship of the hydrocarbon-bacterial bacterial strain SP57N, and the other representative strains of the genus *staphylococcus*, was determined. For that, after amplification of approximately 1,500 bp fragments of 16S rDNA **(Figure 1.)**, this amplified rDNA was partially sequenced. Analysis of the continuous sequence thus determined of 928 bp (GenBank ID: MK825733) by the BLASTn alignment program, on the NCBI database, and on the EzBiocloud database, reveals the belonging of the strain SP57N to the species *Pseudomonas mendocina*. Indeed, the calculation of the similarity of the sequences, using the BLASTn program on the basis of NCBI data, showed that the partial sequence of the 16S rDNA gene of the strain SP57N had a similarity score of 98.92 % with the strain *Pseudomonas mendocina* NK-01 (CP002620.1), and 98.60 % with the strain *Pseudomonas mendocina* strain IITR46 (MF321766), and the strain *Pseudomonas mendocina* NCIB 10541 (NR_043421.1). While by analysis on the EzBiocloud database, we recorded a similarity rate of 97.90% with the strain *Pseudomonas mendocina* NBRC 14162 (BBQC01000018). Subsequently, two phylogenetic trees are constructed using similar 16S rDNA sequences from the two databases **(Figure 2a,b).** The results of the phylogenetic analysis show that the bacterial strain SP57N was related to the species of *Pseudomonas mendocina* **(Figure 2)**. The phylogenetic trees indicated that the bacterial strain SP57N was related to *Pseudomonas mendocina* species **(Figure 2a,b)**. For that, the strain SP57N was identified as *Pseudomonas mendocina.*

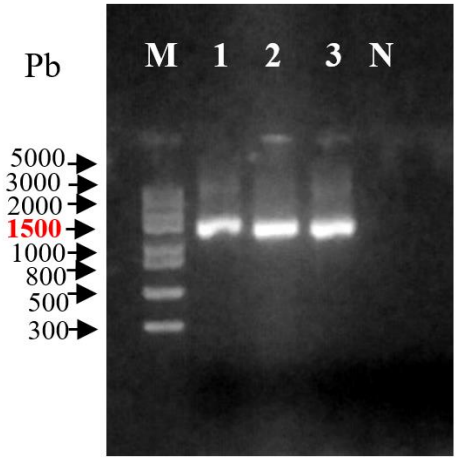

Figure 1. Electrophoresis of the PCR product of the bacterial strain SP57N 16S rDNA gene on 1.5% agarose. Lanes 1, 2 and 3: Amplified 16S rDNA; M: Trans5K DNA marker, N: negative control.

The isolate strain SP57N was found to be Gram negative, strict aerobic, motile, Rod-shaped bacteria **(Figure 3c)**. The colonies on nutrient agar plate were translucent greenish-beige, Smooth, brilliant, convex, with irregular margin and 1-2 mm in diameter after incubation for 24 h at 25°C **(Figure 3a,f)**, and approximately 3-4 mm in diameter after incubation for 48 h at 25°C **(Figure 3b)**. The results of the biochemical characteristics of the SP57N strain are shown in **Table 1**.

3.3 Effects of culture conditions on the growth rate of the hydrocarbon degrading bacterial isolate on crude oil

To understand the environmental characteristics effects on the growth rate of the isolate strain SP57N on crude oil as sole source and energy source, factors such as temperature, NaCl concentration, and pH were studied.

Effects of different temperature in growth rate of strain SP57N on crude oil as sole carbon and energy source are illustrated in **Figure 4a**. The results in the curve indicate that this strain could utilize crude oil at temperature 20°-37°C, and it maximum growth rate was at temperature 25°C.

Results of effect of different concentration of NaCl on growth rate of the isolate strain SP57N on crude oil as sole carbon and energy source are showed in **Figure 4b**. As it can be seen from the curve, the optimum NaCl concentration for maximum growth rate of strain SP57N was 3% (w/v). However, this bacterial strain could tolerate high salt concentrations (up to 15%, w/v).

Results obtained by effect of different pH of the medium on growth rate of strain SP57N in crude oil as sole carbon and energy source are represented in **Figure 4c**. This curve demonstrated that the strain SP57N had optimum growth rate at pH 7, and might grow at pH 6-9.

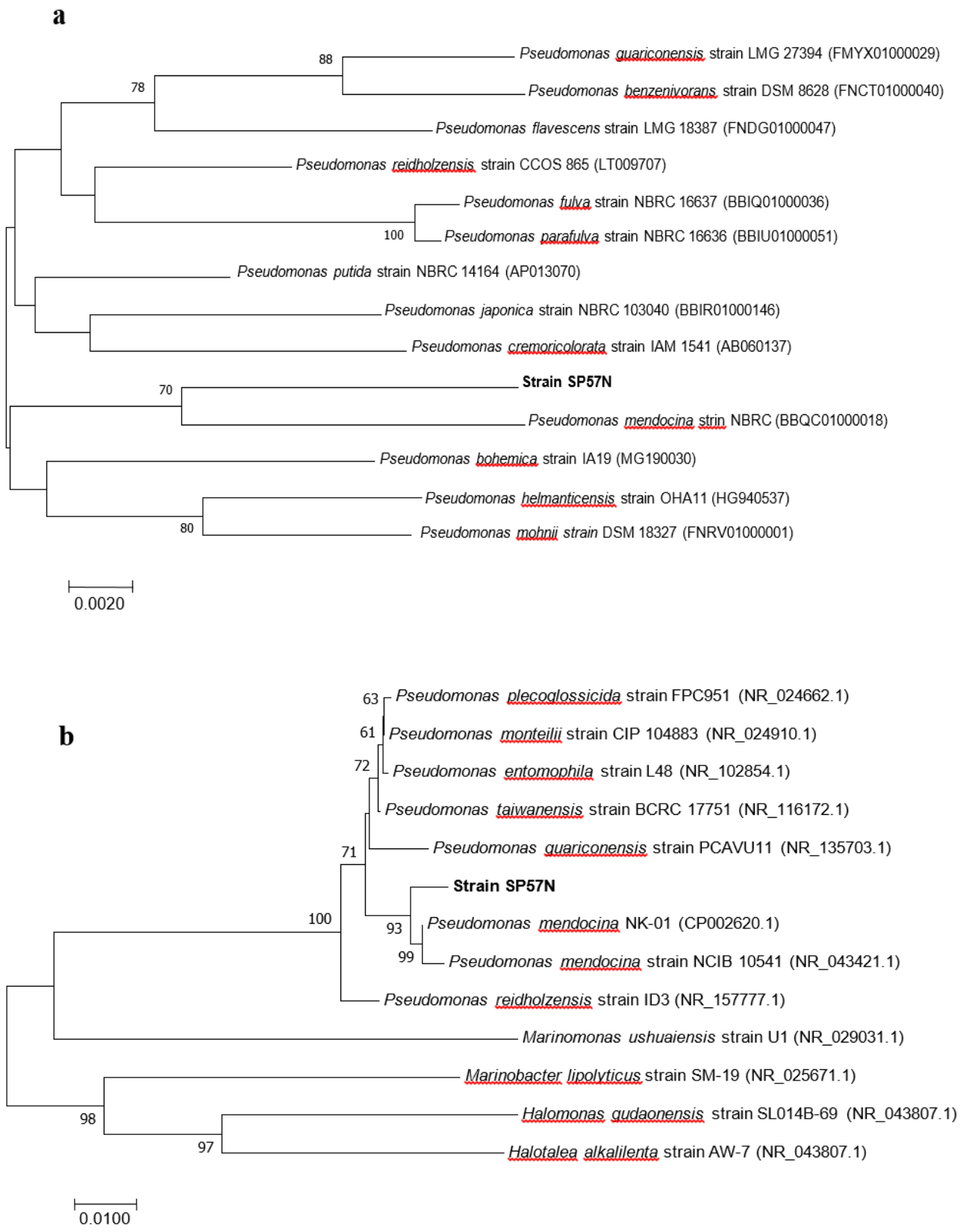

Figure 2. Phylogenetic trees of the16S rDNA sequence of the SP57N strain and the clostest-related species carried out using the software MEGA 7.0; (a) From the NCBI database, (b): From EzBioCloud database, using the neighbor Neighbor-Joining method (bootstrap = 1000); The scale bar represents the sequence divergence. GenBank accession numbers are indicated in parenthesis.

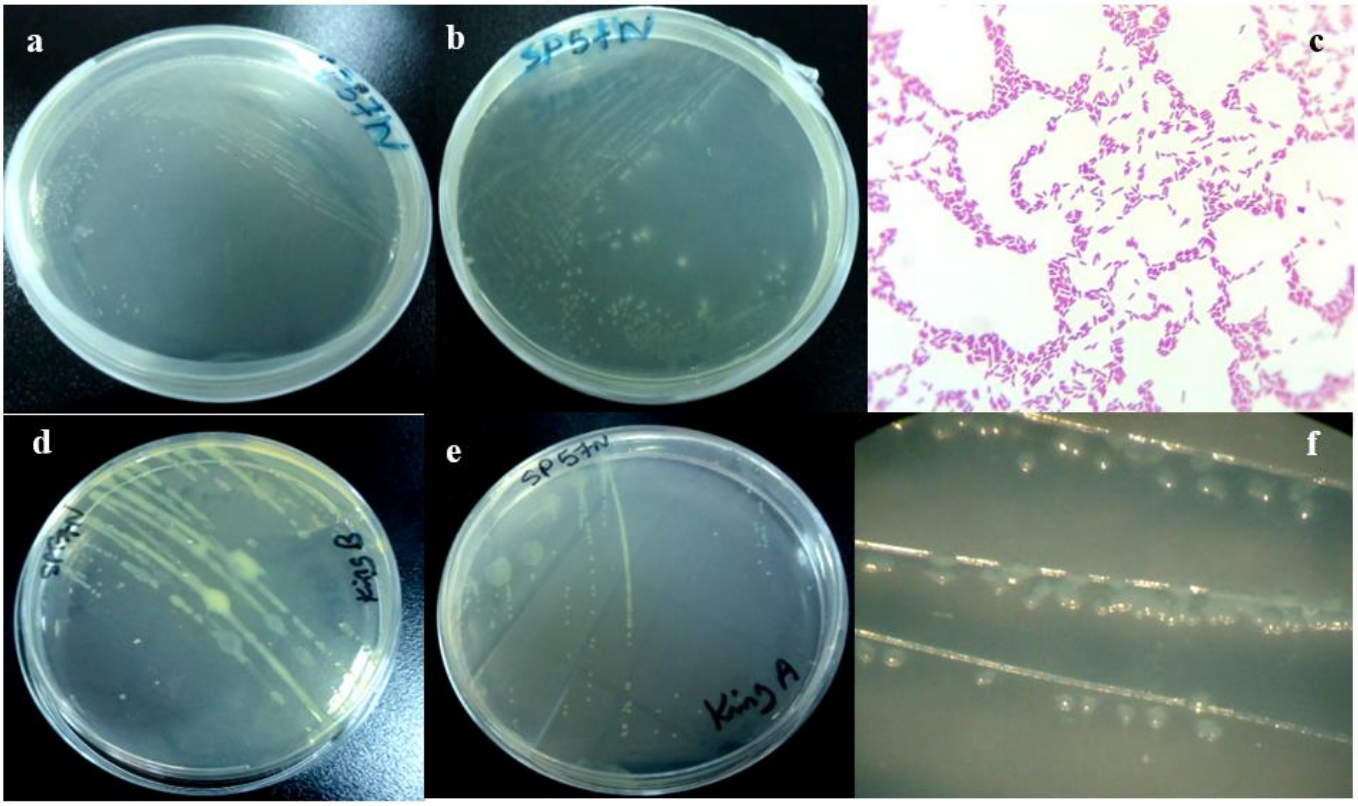

Figure 3. Colonies growth and cells morphological aspects of the bacterial strain SP57N on different culture medium; (a): Colonies of the strain SP57N on the nutrient agar plate after 24h of incubation; (b): colonies of strain SP57N on the nutrient agar plate after 48h of incubation; (c): Gram negative bacterium of strain SP57N (10 x 100); (d): Colonies of the strain SP57N on the King B medium agar plate after 24h of incubation; (e): Colonies of the strain SP57N on the King A medium agar plate after 24h of incubation; (f): Colonies of strain SP57N on the nutrient agar plate after 24h of incubation observed with Binocular magnifier (x 20).

Table 1. Morphological and biochemical characteristics of the isolated bacterial strain SP57N.

Characteristics	Results
Gram straining	- (Fig. 3c)
Shape	Rod-shaped (Fig. 3c)
Oxidase test	-
Catalase test	-
Motility	+
Mannitol	-
Respiratory type	Strict aerobia
TSI test	K/K
Lactose	-
Saccharide	-
Glucose	-
Indole	-
H_2S production	-
Gas production	-
Citrate utilization	+
Chapman	-
SS	+
King A	+ (Fig. 3f)
King B	+ (Fig. 3d)
BCPL	-

3.4 Growths kinetic of growth the hydrocarbonoclastic isolate SP57N under optimal culture conditions on crude oil

The bacterial stain SP57N was grown on 2% (v/v) of crude oil as sole carbon and energy source in BHSM medium, for 20 days, on their optimal culture conditions (Temperature 25°C; pH 7; and 3% (w/v) of NaCl concentration). The growth rate of this bacterial isolate was estimated using turbidimetric method by measuring at the optical density 600 nm, and the results are shown in **Figure 5**. As shown in the curve, strain SP57N started the logarithmic growth phase from the first to 12th day, and then the stationary phase was attained at day 12.

4. Discussion

The SP57N strain was part of the hydrocarbon-degrading bacterium belonging to *Pseudomonas mendocia*, which was firstly isolated from marine sediments and seawater at the port of Oran (Algeria), having potential to develop a method of bioremediation to clean up petroleum hydrocarbon contamination in marine environments.

Partial sequence analysis of the 16S rDNA gene (928 pb) of isoled strain SP57N confirmed the identification as *Pseudomonas mendocina* specie, with a similarity to *Pseudomonas mendocina* specie of 98.92 % using BLASTn alignment on NCBI database, and 97.90% of similarity using BLASTn alignment on EzBioCloud database. In addition, the morphological characteristics of the strain SP57N were very similar to those of the *Pseudomonas mendocina* reported previously.

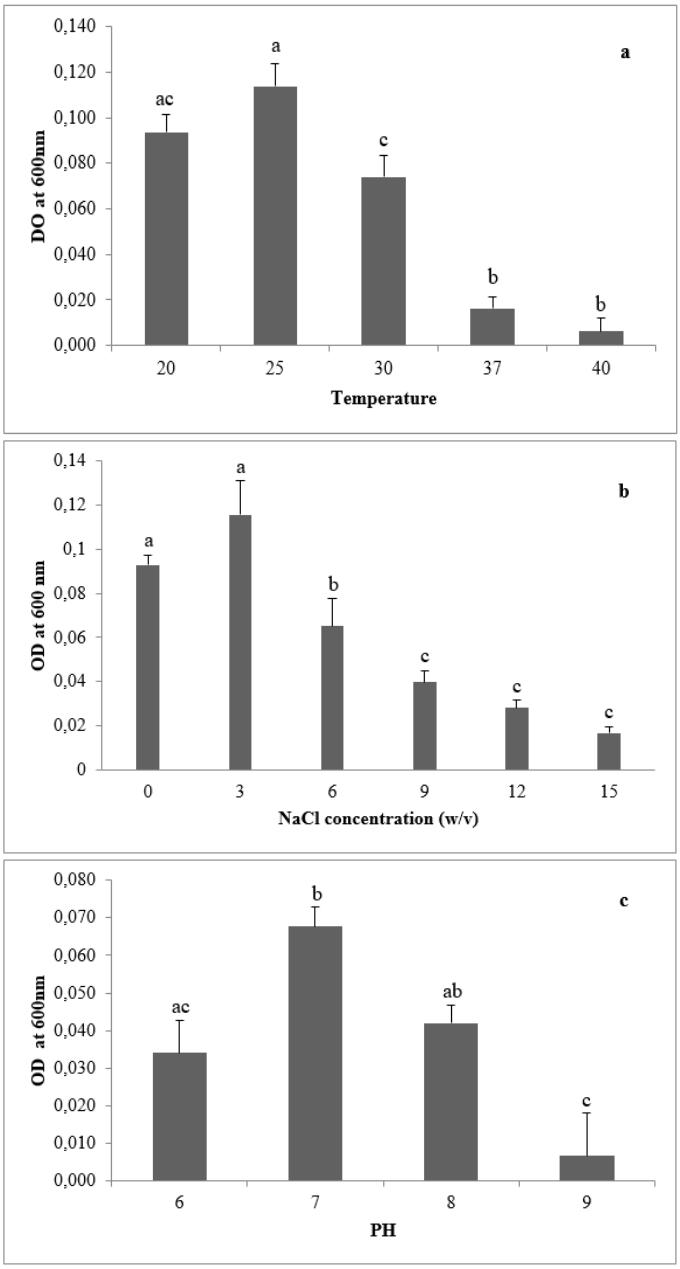

Figure 4. Effects of culture conditions on growth rate of strain SP57N in BHSM medium supplemented with 2% of crude oil (v/v) as sole source of carbon and energy, after incubation time of 48 h at 140 rpm. The histograms with different indices are significantly different ($p < 0.05$). (a): Effect of temperature; (b): Effect of medium salinity (NaCl concentration); (c): Effect of pH.

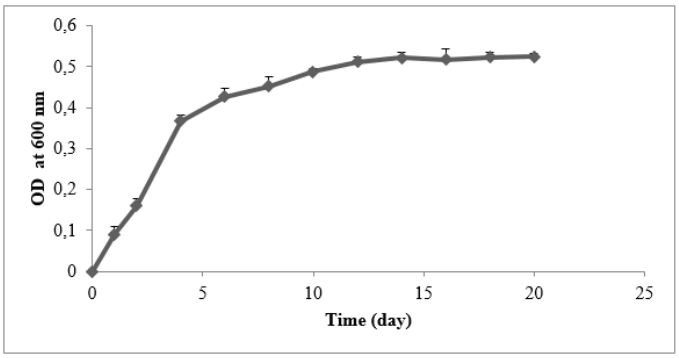

Figure 5. Growth kinetic of the bacterial strain SP57N on 2% of crude oil (v/v) as sole source of carbon and energy at optimal culture conditions and 140 rpm over 20 days of incubation. Bars represent standard deviation and experiments were performed in triplicate.

Before contribution to bioremediation, it is very important to study effects of culture conditions on petroleum hydrocarbons degradation, since it furnishes information about the bacteria and their growth requirements.

Several bacteria have the capacity to biodegrade hydrocarbons. These microorganisms are available in sites polluted by hydrocarbons. The number and diversity of these hydrocarbonoclast bacteria are influenced by various environmental, physical and chemical factors, such as temperature, pH, salinity, and the source of carbon. Seasonal and climate change also plays a role in the number and diversity of bacteria in general and hydrocarbonoclast bacteria in particular **(Acosta-González and Marqués, 2016; Kumar et *al.* 2019).**

Given the complexity of petroleum products and environmental conditions, the study of the effect of environmental conditions on oil degradation is essential. Indeed, before contribution to bioremediation, it is very important to study effects of culture conditions on petroleum hydrocarbons degradation, since it furnishes information about the bacteria and their growth requirements.

To disclose the crude-oil-degrading conditions of strain SP57N, temperature from 20° to 40°C, NaCl concentration from 0 to 15% (w/v) and pH from 6 to 9 were investigated, as shown in **(Figure 4a,b,c).**

From the curve of Fig. 3a, we note that our isolate had capability to growth in a wide temperature range, from 20°C to 37°C, with maximum growth rate at 25°C. These results are according with those obtained by **Chang et *al.* (2011).**

Concerning temperature, it is responsible for controlling the nature and quantity of microbial metabolism in hydrocarbons, and diffusion rates, solubility and bioavailability. It is the main factor that influences the rate of biodegradation, by directly effecting bacterial metabolism and growth rate. It affects also the chemical and physical properties of oil **(Wong et *al.* 2012).**

Results of effect of NaCl concentration test **(Figure 4b)** showed that strain SP57N had maximum growth rate at 3% (w/v) of NaCl, corresponding to the salinity of seawater which is 37–39.2 ‰ in the Mediterranean Sea **(Effrosynidis et *al.* 2018)**. Also, this isolate could support high concentrations of NaCl and grown in wide range of salinity (0-15%, w/v). These results are consistent with those obtained by **Abou-Elela et *al.* (2010)**. According to **Shiaris (1989)**, salinity could have a positive impact on the capability of degrading hydrocarbon. There is correlation between degree of salinity and the rate of several hydrocarbons mineralization.

Maximum growth was obtained at pH 7.0 **(Figure 4c)**. These results consist with those obtained by **Chang et *al.* (2011)**. According to **Deng *et al.* (2014)**, extreme pH inhibited the biodegradation of the oil. However, in the marine environment, as in fresh water, the favorable range of pH was between 7 and 8 **(Varjani and Upasani 2017)**.

For results obtained from kinetic of biodegradation of crude oil by the isolated bacterial strain in the optimal culture conditions **(Figure 5)**, the stationary phase was attained at day 12. These results indicate that this bacterial strain SP57N had effectively the ability to utilize crude oil as the sole source of carbon and energy, which reflects the potential for degradation of hydrocarbons.

Formerly, several studies have shown the capacity of *Pseudomonas* sp. to degrade hydrocarbons **(Nwinyi et *al.* 2016; Chebbi et *al.* 2017; Nogales et *al.* 2017; Ramadass et *al.* 2018)**. Also, the genus *Pseudomonas* is among the species that show a high efficiency in degrading polycyclic aromatic hydrocarbons **(Lamichhane et *al.* 2017; Sun et *al.* 2019)**.

These microorganisms have the property of colonizing environments contaminated by hydrocarbons **(Liu et *al.* 2019)**. In addition, many strains of *Pseudomonas mendocina* had the capacity to degrade various environmental pollutants **(Zhang et *al.* 2017; Mudhoo et *al.* 2018)**. In effect, *Pseudomonas mendocina* NSYSU was able to mineralize a high concentration of pentachlorophenol (PCP) (150 mg / l) according to **Kao et *al.* (2005)**, and the *Pseudomonas mendocina* strain ZAM1 degraded more than 64.5% of endosulfan after twelve days of incubation according to **Mir et *al.* (2017)**.

Pseudomonas mendocina could grow in crude oil as the sole carbon and energy source **(Kumari et *al.* 2018)**. In addition, this bacterial specie had effectively the ability to degrade polycyclic aromatic hydrocarbons (PAHs) **(Nogales et *al.* 2017)**. Indeed, according to **Barman et *al.* (2017)**, almost 98.8% and 98.6% of the degradation of acenaphthene and naphthalene respectively were recorded by *Pseudomonas mendocina* under optimal growth conditions. Degradation of naphthalene by *Pseudomonas mendocina* isolated from seawater has also been reported by **Mangwani et al. (2014)**. This species has been reported to have the ability to degrade phenanthrene **(Tian et *al.* 2002)**. *Pseudomonas mendocina* was reported to have the gene encoding the enzyme catechol dioxygenase, the key enzyme for the breakdown of PAHs **(Heinaru et *al.* 2000; Kahlon 2016; Nogales et *al.* 2017)**.

Also, other studies have demonstrated the production of biosurfactants by the *Pseudomonas mendocina* strain, which has potential for application for the bioremediation of hydrocarbons **(Kumari et *al.* 2018; Tripathi et *al.* 2019)**.

Consequently, the species *Pseudomonas mendocina* is a powerful and interesting means for the bioremediation of the environment contaminated by PAHs, and for biotechnological applications.

In our study, the bacterial strain SP57N isolated from contaminated marine sediments and seawater tolerated high concentrations of NaCl (up to 15%, w/v) and crude oil (up to 10%, v/v), because of the polluted environment, that induce development of enzymes of interest. This tolerance may reflect evolutionary adaptation that results in high stability in presence of hydrocarbon sources **(Al-Dahash and Mahmoud 2013; Acosta-González and Marqués 2016; Joy et *al.* 2017)**.

Therefore, as an indigenous microorganism, *Pseudomonas mendocina* SP57N is a very useful biological means, as an efficient degrader, for the decontamination and bioremediation of marine sediments polluted by hydrocarbons such as the harbor of Oran.

5. Conclusion

In the present study, indigenous hydrocarbon-degrading bacterium strain SP57N was newly isolated from mixed seawater and marine sediment at the port of Oran (Algeria), and identified as *Pseudomonas mendocina*, based on their phenotypic phylogenetic characteristics, which possessed high growth capacity in crude oil as sole carbon and energy source, and excellent adaptability to salinity. Therefore, having capability to grown in the divers environmental conditions, the isolate strain SP57N could be used as a suitable degrader, to initiate an cost effective eco-friendly method for the removal of hydrocarbon contaminations in different marine environments polluted by hydrocarbons, in particularly, the harbor of Oran.

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Declaration of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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