Poll Res. VOL. 40 (May Supplement) : 2021
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ISSN 0257–8050

A NOVEL OPTIMISTIC OIL DEGRADING BACTERIA *KOCURIA FLAVA* BASRA AWS STRAIN

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(Received 1 January, 2021; Accepted 3 February, 2021)

ABSTRACT

The biodiversity and genetic identification of hydrocarbon-degrading microbes is a significant initiation to the bioremediation of contaminated soil. The aim of this study is screening the microbiome community with a highly adaptability of bacterial strains grown in extreme crude oil contaminated soil of the South of Basrah. The molecular identification of recovered bacterial isolates based on 16S rRNA sequencing indicated to isolation a novel *Kocuria flava* strain, identified as BasraAWS with accession number Ku715984 in the Gene Bank data. The main features of this isolate are Gram-positive, large cocci, arranged in different forms. The new strain exhibited a significant performance in crude destruction through consuming 70% of crude oil within 10 days of incubation. We can conclude register a novel bacterial strain with a notable capability in hydrocarbon remediation.

KEY WORDS : 16S rRNA gene, Actinobacteria, Bioremediation, *Kocuria flava*, Oil-contaminated

INTRODUCTION

Crude oil is the main source of energy and associated with many environmental risks due to oil pollution that leading to a serious environmental and health consequences (Xu *et al.*, 2018). The oil activities including transferring, production, and exploration, oil spill in addition to other anthropogenic activities are the major sources of contamination to the planet in both aquatic and landscape environments (Das and Chandran, 2011; Belvederesi *et al*., 2018). For this reason, many studies have been conducted for reducing and eliminating crude oil contamination. The main need is finding microorganisms exhibit the efficacy in converting toxic hydrocarbon to nontoxic particles or carbon dioxide and water either by genetic manipulation or via stimulation of indigenous microorganisms bioremediation activities (Albokari *et al*., 2015). Nowadays, studies shows the use of microorganisms, particularly bacterial isolates, is an optimistic tool for eliminating and controlling crude oil contamination due to their advantages such as low cost, effectiveness, and environmentally friendly (Guerra *et al*., 2018). Due to the capability of breaking down crude oil hydrocarbon molecules by some indigenous microorganisms, scientists have focused on their role in biodegradation and bioremediation for clearance oil pollutions. *Pseudomonas, Corynebacterium, Flavobacterium, Marinobacter, Micrococcus, Nocardia, Stenotrophomaonas,* and *Vibrio* are well known as hydrocarbon destructors (Varjani, 2017).

Actinobacteria are known wide distribution in natural habitat and involved in important processes, also in extreme soil condition, for example, the low level of moisture or high salinity, (extreme pH and temperatures), radiation and Drought (Zenova *et al*., 2011; Zhang *et al*., 2019). Olajuyigbe & Ehiosun (2016). The prevalence of actinobacteria within oil contaminated soils belongs to the ability of peroxidase enzymes production that significantly

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enhance crude oil degradation. *Kocuria* is genus of actinobacteria separated from *Micrococcus*, cocci cells characterized as Gram-positive and present in the form of single, pairs, groups and short chains (Stackebrandt *et al*., 1996).

Recently, identified based on 16S rRNA phylogenetic studies revealed that there were more than 18 species of *Kocuria* (Khalifa, 2017). Researchers reported the species of *Kocuria* to have ability to the utilization of hydrocarbons (Lalevic *et al*., 2014; Kumar *et al*., 2016). In Iraq, especially Basra Governorate, which is one of the oil-producing cities, has many oil wells as well as an oil refinery that refines crude oil, this caused an increase in environmental pollution resulting from petroleum hydrocarbons, the little studies on actinobacteria that degrade hydrocarbons, the current study aimed to the biodiversity of some species of *Kocuria*, genetic identification, and investigation their ability to degrade crude oil.

MATERIAL AND METHOD

Sampling and culturing of bacteria

Hydrocarbon-contaminated soil was collected from Basra Governorate (Basrah refineries company) in the south of Iraq, using a sterile spatula, the samples were collected from the ground level at depth 10-20 cm, placed in a sterile polythene bag, transferred to the laboratory for culturing. The bacteria were isolated by inoculated 0.1 mL of soil sample (10^{-4} dilution) on the plates contain actinomycetes isolation agar (AIA) supplied with 100 mg/L antibacterial (Nalidixicacid) and antifungal (Cyclohexamid). The plates remain under incubation for 7-14 days at 32°C until the colonies were appeared (Van Hop *et al*., 2011). The colonies selected according to morphology and color have been grown re-cultured by streaking to obtain pure culture and kept on glycerol asparagine agar base (ISP-5) medium slants. The bacteria cell's shaped and the arrangement was determined by Gram stain protocol.

Identification of strain

Extraction of DNA

The genomic DNA of strain isolate was extracted from cell pellets collected from a cultivated pure culture of strain in yeast malt broth medium for three days at 32°C in a rotary shaker by using a commercial kit protocol (Wizard Genomic DNA kit) provided by Promega company in the USA. Genomic DNA was isolated and purified according to the company's guidance, 25 mg/mL of lysozyme was ýadded to lysis the Gram-positive cell wall.

PCR program

The 16S rRNA, approximately 1500 bp, was amplified by universal forward primer 27F "(5ˆ-AGAGTTTGATCMTGGCTCAG-ý3ˆ)" and semi-specific reverse primer 1525R "(5ˆ CGGCTACCTTGTTACGACTT-3ˆ)". The reaction mixture 25 µL (Promega, USA) contained (DNA polymerase 2.5U, each dNTP 250 µM, tris-HCl (pH 9) 10mM, KCl 30mM, $MgCl_2$ 1.5 mM, 2 µL of purified DNA 1 µL (62.5 µmol/l) of both forward and reverse primer. A final amount of 50 µL was applied by diluted with free nuclease water. The initial denaturation was achieved in a thermal cycler at 94 ºC for 2 min., followed by 35 amplification cycles, denaturation at 94 ºC for 40s, primer annealing at 55 ºC for 30s, extension of primer setting for one minute at 72 ºC and finally extension ýat 72 ºC for 10min. The mixture after reaction was cooled to 4 ºC. The product was discrete on 1% agarose gel in TBE buffer 1x after stained with ethidium bromide visualized under a UV transilluminator (Burghal *et al*., 2015).

Sequencing of 16S-rDNA gene

The 16S rDNA of strain was purified and sequencing was completed at Applied Biosystems 3730XL Genetic Sequencing Analyzer in the USA, Sequences database were corrected and ýedited to compare by using BLAST with the sequences in ýthe gene bank at the "National Center for Biotechnology Information" (NCBI) http://www.ncbi.nlm.nih.gov/BankIt. The 16S rDNA was sequencing and determined by comparing to 16S rDNA sequences available in ýthe nucleotide databases of the GenBank to estimate the phylogenetic location and percent homology scores were collected. The tree was thus generated by the implemented neighbor-joining algorithm.

Biodegradation of crude oil assay

To evaluate the efficacy of *Kocuria flava* BasraAWS strain on crude oil degradation, the culture was activated on Nutrient Broth (NB) to obtain a sufficient amount of inoculum which was added at 3% (v/v) to a 100 mL conical flask containing 50 mL of sterile mineral salt (MS) medium composed of the

following salts (g/l): KH_2PO4 1g, Na_2HPO4 1g, NH_4NO_3 0.5 g, $(NH_4)_2SO_4$ 0.5g, $MgSO_4$ 0.2g, $CaCl_2$ 0.02g, $FeCl_3$ 0.002g, $MnSO_4$ 0.002, the pH was adjusted at 7±0.2 supplied with trace elements solution (0.5 ml/L) consisted of (g/l) $ZnSO_4$, 0.29; CaCl2, 0.24; CuSO4, 0.25; MnSO4, 0.17 and 1% (w/v) light crude oil as a single source of energy was added, control sample flasks were prepared without bacterial inoculum. All flasks were incubated in an orbital shaker at 120 rpm for 10 days at 32 °C (Shahriari Moghadam *et al*., 2014).

Extraction of crude oil

A liquids culture media were extracted three times with equal volume of organic solvent (chloroform/methanol 80:20% v/v) in a separator funnel mixture were shake vigorously for a few minutes and left for 30 min. for separated the mixture into two-layer, chloroform layer was collected and dried with anhydrous sodium sulfate, the solvent and any volatile components are evaporated, the residue was weighed for determining the percentage of crude oil intake by bacteria according to (El Mahdi *et al*., 2015).

RESULTS AND DISCUSSION

According to hydrocarbon analysis, collected soil samples shown a highly contamination with crude oil, hydrocarbon contain 0.5 gm/Kg. The results showed some isolates of actinomycetes that isolated from contaminated soil samples appeared as small yellowish colonies, round, mucoid, convex, grown within 5-7 days at 35 °C on AIA medium. The cells under the microscope were cocci, large, single or pairs, short-chain, cluster, and Gram-positive (Table 1 and Figure 1). Generally, Kocuria species tend to be Gm+, nonencapsulated, coccoid, lacking for spore

Table 1. Phenotypic and biochemical features of *Kocuria flava* Basra AWS strain.

No.	Features	Consequence ý
1	Colony shape	Small, Smooth, Regular edges
2	Colony color	Yellow
3	Gram's stain	Gm +ve
4	Cells ýmorphology	Miscellaneous (single, pair, chains, aggregates)
5	Pigment	Yellowish
6	Oil degradation	Positive
7	Biosurfactant	Producer
8	Catalase	Positive

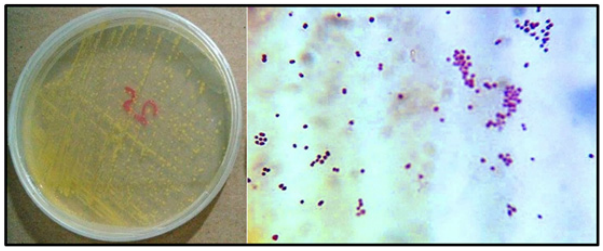

Fig. 1. Colony and Gram's stain showing the *Kocuria flava* yellowish colony, the cells appeared as cocci, pairs, short chains, tetrads, groups, single and clusters big cocci.

forming, yellow colonies in colour (Zhou *et al*., 2008).

The result of molecular identification using 16S rRNA gene sequencing of recovered bacterial strain showed revealed a 99 % similarity with *K. flava*. Similar studies reported isolation of *Kocuria* from crude oil contaminated soil (Khalifa, 2017). The distribution of the biodegradation strain was shown in the phylogenetic tree (Figure 2). The tree was designed to determine their related ýrelationship and evolution between different strains.ý For decades, 16rRNA gene sequence analysis has been the mainstay of diagnosing bacterial isolates. In the experiments conducted by Johnson *et al*., (2019) to study species based on the 16rRNA gene sequence, confirm that gene sequencing with few variants makes discovery of ýmicrobial strains more realistic.

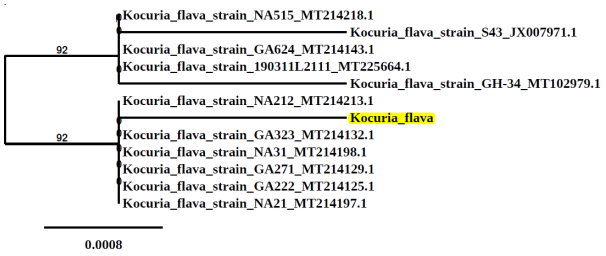

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences revealing evolutionary biodegradation isolate associations accessible in the NCBI GenBank database with close relatives.

The 16S rRNA gene sequence obtained for the strain was contained replacement mutation (Figure 3). As a result of the presence of the genetic mutation the isolation was registered as a new strain *Kocuria flava* BasraAWS and deposited in GenBank with the accession number Ku715984.

The distinctive microbiome is usually diverse, exists in extreme ecosystems such as hot, salty, polluted, etc., and includes several unique taxa with a large competitive spectrum of relative abundance.

Ahmed *et al*., (2010) isolate two *Kocuria* species from crude oil contaminated beaches. Al-Buhamrah & Hamid (2020) reported the isolation of *Kocuria rosea* from soils contaminated with petroleum hydrocarbons. The alignment of the 16S rRNA gene sequences of strain with sequences collected from a Blast quest El Mahdi *et al*., (2015) showed a 96% resemblance to the *Kocuria palustris* isolated from Refinery water samples which contaminated with oil.

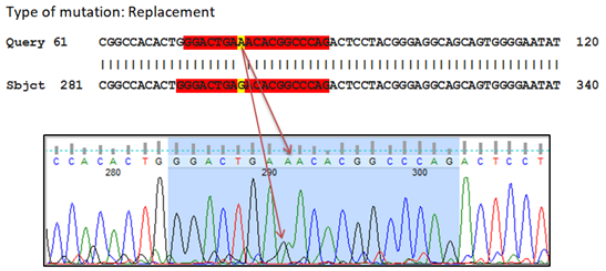

Fig. 3. Replacement mutation in 16SrRNA gene of *Kocuria flava* BasraAWS strain.

The selection of the *Kocuria flava* Basra AWS was depended on its high ability to degrade crude oil, due to emulsification the crude oil with water in the inoculated culture, it became turbid gradually, whereas not observed in control which remained clear, on the water surface the dispersed crude oil was as same as the beginning. The turbidity of culture and disappearance of oil film refer to the degradation of hydrocarbons has occurred. The results showed that the amount of oil extracted from *Kocuria flava* BasraAWS culture was less than that extracted from the control sample. The concentration of the oil in MSM was decreased from (100%) to (30%) by strain after 10 days of incubation. This result has shown that *Kocuria flava* BasraAWS has the potential for used hydrocarbons in metabolites activity as energy source, this results was explained by Tumaikina *et al.* (2008); Ahmed *et al*. (2010).

Khalifa (2017) reported a newly isolated *Kocuria sediminis* DDK6 which have the ability to consume a variety of biochemical compounds, this provides clarification about using of variety range metabolic by *Kocuria sp.* in diversified ecological niches especially, in ecosystems contaminated with oil, so in particular areas, it's important to the isolated new bacterial strain that adapted to a local condition in that area for the efficiency cleanup of hydrocarbon contaminant.

Tseng *et al*. (2007) showed that the rapid enzyme secretion enables actinobacteria for bioremediation of complex substrates such as xenobiotics, hydrocarbons, plastics, toxic pesticides and rubber. Standard methods for chemo-physical remediation are either incompetent or costly and can induce secondary contamination. Soil composting technologies with the support of available nutrients and active microbes to degrade pollutants for soil remediation. It is extremely eco-friendly and effective (Ren *et al*., 2018). In 2019, Wang and his group illustrated the capability of *K. flava* grow in significantly crude oil contaminated soil.

CONCLUSION

Based on the results, it can be concluded that *K. flava* Basra AWS has a pivotal role in bioremediation of hydrocarbon due the ability of growing in extreme crude oil contaminated soil. The degradation efficiencies exhibited 70% after 10 days by this strain, so it can be used for bioremediation of hydrocarbon. This new indigenous actinobacteria strain was detected in soil contaminated oil recorded as novel strain by genetic identification according to 16SrDNA gene.

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