Isolation and Identification of Nitrate Reducing Bacteria from Produced Water of Oil Fields in Iraq

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Abstract: Nine Nitrate Reducing Bacteria NRB strains were isolated from produced water of Nahran Omer and Al Lahis oil fields in Iraq. 16S rRNA gene sequencing revealed that these reservoirs harbored many strains of NRB were belonged to *pseudomonas, Bacillus, Herbaspirillum* and *Shewanella*. These results show that the reservoirs have microbial population associated with oil recovery and have potential for microbial enhanced oil recovery.

Keywords: Nitrate reducing Bacteria, control souring, oil reservoirs

I. INTRODUCTION

Complex ecosystems comprising various types of microorganisms are present in petroleum reservoirs. Culture-dependent and -independent methodologies, in particular, 16S rRNA-based molecular identification methods, have revealed diverse microorganisms inhabiting petroleum reservoirs [1,2,3,4] Among them, hydrocarbon-degrading bacteria (HDB), nitrate reducing bacteria NRB, SRB, and methanogens are the important populations of reservoir ecosystems, and have critical roles in the microbial enhancing of the oil recovery process[5].

The presence of NRB in oil fields water has not been studied extensively. There are two major groups of bacteria that could be stimulated by the presence of nitrate in anaerobic environments condition; these are chemoorganotrophs (heterotrophs) that use organic compounds as electron donors and as their carbon source for growth. This group includes facultative anaerobes such as *Pseudomonas stutzeri*, *Citrobacter* and strict anaerobes such as some of *Desulfovibrio* the other group is chemolithotrophs (autotrophs) that typically use inorganic sulfur species as electron donors and CO_2 as their carbon source for growth. The latter group is also known as the colorless sulfur bacteria which include obligate chemolithotroph such as *Thiobacillus denitrificans*, *Thiomicrospira* and *Arcobacter* [6]. Recently the stimulation of NRB by the addition of nitrate, nitrite, or nitrate / molybdate mixtures has been used to inhibit SRB propagation and H_2S production by out-competing the growth of SRB [7, 8].

In this study 16S rRNA gene sequencing, were performed to investigate the microbial community of NRB in the reservoir of Nahran Omer and Al Lahis oil fields in Iraq.

II. MATERIAL AND METHOD

Sample Collection

A total of 40 samples were collected during the period of 6 months. The samples of produced water were obtained from the separator tanks of two oil fields, Nahran Omer which is located in the north of Basrah, the oil reservoirs are located at a depth of~ 2000-3000m below ground and the *in situ* temperature is~ 50-70 °C and Al-lahis oil fields located in the south of Basrah, the depth of the reservoirs were about~3700-3900m, and the temperature *in situ* was about~50-70°C. The samples were collected in sterile glass bottles which were filled completely to prevent contact with air, they were transported to the laboratory, flushed with %10 N_2 and kept at room temperature until used [9].

Isolation medium

Coleville Synthetic Brine (CSB) medium [10, 11] was used for enumeration of NRB after being modified by adding 10 g of NaCl. Prepare by dissolving 10 g Sodium chloride, 0.027 g Dipotassium hydrogen phosphate, 0.24 g hydrous calcium chloride, 0.68 g Hydrous magnesium sulfate, 0.02 g ammonium chloride, 0.68 g Sodium acetate, 0.1 g potassium nitrate in 1000 ml D.W. the pH of the medium was adjusted to between (7.2 - 7.5). After autoclaving the medium it was flushed with 90% N_2 and 10% CO_2 for 20 min., during the flushing add 30 ml/L of sodium bicarbonate solution [12], 2 ml/l sodium sulfide solution [13], 50 ml/l of trace element solution SL10 [14], 5 ml /l of mix vitamin solution and 1 ml of vitamin B_{12} solution [12]

Isolation of NRB

The mix cultures of NRB were obtained by inoculated 5-10 ml of sub-sample of produced water into a screw cup containing liquid CSB medium, the screw cup was completely filled up with medium and closed tightly, left in the incubator at 37°C for 30-35 days. Positive result was determined by the turbidity and phase contrast microscopy after staining.

Purification of NRB

The pure cultures of NRB were obtained by dilution plate technique; 1 ml of mix culture was used to prepare a serial dilution of buffer 10^{-1} - 10^{-7}. 0.1 ml of 10^{-7} was spread over the surface of solid CSB medium; the plates were placed in the anaerobic jar with gas generation kit and incubated at 37 °C for 10 days. The single colonies were picked and inoculated into liquid CSB medium, cultures positive for growth were checked for purity by gram staining under phase contrast microscopy [15].

Morphological characterization

Selected colonies of NRB were characterized on solid CSB media by recognizing of shape, color, and size [15].

Nitrate reduction test

The ability to reduce nitrate by NRB was determined by the presence of nitrite in the culture. The following solutions were mixed together by using a Pasteur pipette: 3 drops of 1% sulfanilamide, 1 drop of 0.02% naphthylethylenediamine. 50 µl of the culture medium was added to the above solution. In the presence of nitrite in the culture medium, the solution would change color from colorless to bright pink /purple. If no color had developed, a few grains of zinc powder were added, the development of a purple color around the zinc particles indicated the presence of nitrate in the culture medium [16].

Genetic identification of NRB

1-Genomic DNA extraction

DNA extraction from 1.5 ml of cultures of NRB was performed using a DNA extraction kit (Promega, USA) according to the manufactures protocol.

2-Detection of genomic DNA

According to Sambrook and Rusell [17], 1% of agarose was dissolved in tris- borate- EDTA buffer (1X), heated, touched by very small amount of ethidium bromide stain and poured into casting tray with the insertion of the comb for making wells and let till it was solidified. After that agarose was get away of the cast with comb carefully out and put down the gel gently in the electrophoresis tank soaked by diluted TBE. Then 6 µl of extracted DNA was mixed with 3 µl of bromophenol blue and loaded in wells of agarose by micropipette and electrophoresed by ban electric current from a power supply adjusted at 60 V and 120 mA for 35 min. UV light transmitter was used to recognize the migrated bands.

3-PCR amplification of 16S rRNA gene

The 16S rRNA genes from NRB were amplified by PCR using the universal bacterial specific primer 1492R and 27F [18] listed in table (1). The reaction mixture for PCR amplification consist of green master mix 25 µl mixed with 2 µl of each forward and reverse primers (10 pmol/ µl) and 5 µl of template DNA and equal volume to 50 µl by added nuclease free water, The thermal cycling conditions were as follows: initial denaturation at 94°C for 2 min, 35 cycles of 40 s of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of elongation at 72°C. Cycling was completed by a final elongation step at 72 °C for 10 min.

Table 1: primers used in PCR amplified

Primer	Primer sequence
1492R	5'-CGGCTACCTTGTTACGACTT-3'
27F	5-'AGAGTTTGATCCTGGCTCAG-3'

4-Analysis of the PCR products

After the amplification, the PCR products for NRB were electrophoresed with 100bp ladder marker in 1.5% agarose gel for 35 min. using TBE buffer. The gel were stained with ethidium bromide, visualized under the UV light and documented using a Bio imaging system [19].

5-Sequence of PCR products

The~ 1500 bp 16S rDNA of each isolates were purified and sequencing at NICEM company laboratories /USA, the bacterial 16S rDNA obtained sequencing were then aligned with known 16S rDNA sequences Gen bank using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI) and percent homology scores were generated to identify bacteria.

III. RESULTS

Isolation of NRB

For enrichment cultures was used modified CSB medium under anaerobic condition which showed a growth of NRB present in the community of produced water. The growth of NRB was indicated by turbidity of the medium and phase microscopy after 30 - 35 days of incubation as shown in (figure 1).

Figure 1: Cultures of NRB.

Purification of NRB

The Isolated colonies of NRB, obtained by dilution plate technique appeared after 10 days of incubation under anaerobic conditions, 30 isolates were selected for morphological and molecular identification.

Colonies characteristics

The NRB isolates grew on CSB medium, some of the colonies grow below the surface, were fine, small, milky white pellicle and round, shape about 0.5 - 1 mm in diameter as shown in (figure 2).

Figure 2: Colonies of NRB on solid CSB medium.

Nitrate reduction test

The nitrate reduction test showed the isolates with vigorous growth had strong nitrate reduction ability where isolates of NRB reduced nitrate to N_2 in *Pseudomonas sp*., *Bacillus licheniformis* and *Shewanella hafniensis.* They gave no change in color when a sulfanilamide / naphthylethylenediamine solution and zinc powder was added. The other isolates reduced nitrate to nitrite in *Herbaspirillum sp.* giving change to pink color as shown in (figure 3).

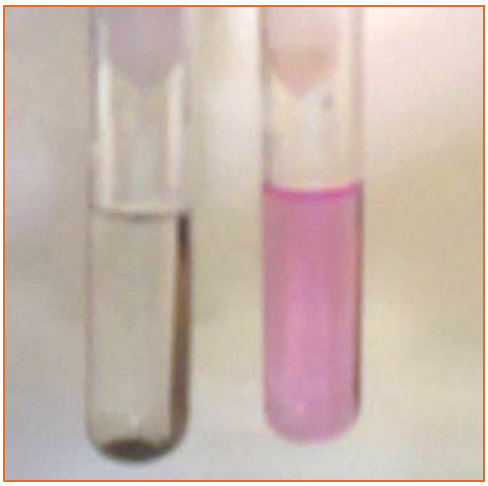

Figure 3: nitrate reduction test

Genomic DNA extraction and detection

The results of electrophoresis technique for genomic DNA extraction was showed clear isolated DNA for all isolates of NRB.

Amplifying of the 16S rDNA gene by PCR technique

The results revealed obtained the required bands of 16S rDNA gene for each isolates along with electrophoresd ladder in the region of 1500 bp as in the (figure 4).

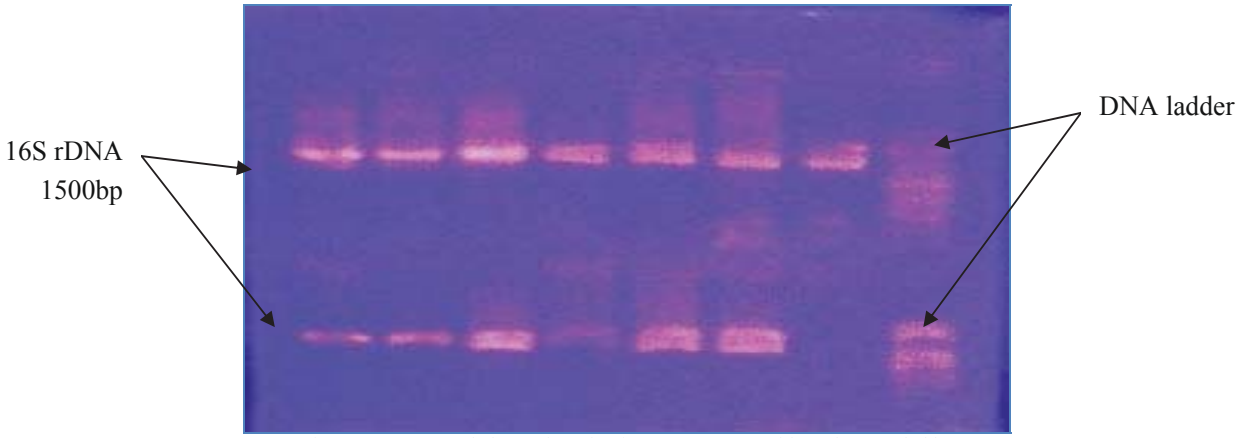

Figure 4: Agarose gel electrophoresis of 16S rDNA gene with 100bpDNA ladder

The isolates were identified to the level of strain, depending on 16S rRNA gene sequences which indicate that NRB isolates were belonged to the species of *Pseudomonas stutzeri, Pseudomonas putida, Herbaspirillum huttiense*, *Shewanella hafniensis* and *Bacillus licheniformis* as shown in (table 2).

Table 2: The BLAST results of the16S rDNA gene sequences of the NRB isolates

NRB strain	Identity	Accession number	Length bp
Isolate 12N *Pseudomonas stutzeri* strain LH-Ka9	99%	KJ1071780.1	737
Isolate 18N *Pseudomonas stutzeri* strain ACM2-32	99%	KP019212.1	734
Isolate 16N *Pseudomonas stutzeri* strain 16	99%	HG780372.1	981
Isolate 17N *Pseudomonas stutzeri strain* NRC25	99%	KP29744.71	734
Isolate 5N *Pseudomonas stutzeri* strain ZH-1	99%	KM278988.1	906
Isolate 19N *Pseudomonas putida* strain NBAllCK-8c	99%	HM439953.1	712
Isolate 20N *Bacillus licheniformis* strain MSEB	99%	KP261081.1	717
Isolate 35 N *Herbaspirillum huttiense* strain LAMA	99%	KM272770.1	923
Isolate 37S *Shewanella hafniensis* strain P010	99%	NR041296.1	941

IV. DISCUSSION

The isolation and enumeration of NRB under anaerobic conditions was performed in a selective modified CSB medium containing sodium acetate as a sole carbon source with reducing and enrichment agents such as trace elements solution, mix vitamins solution gives the growth of this bacteria after 30 – 35 days of incubation, the growth of NRB on this medium was indicated by turbidity and phase contrast microscopy, also the presence of bubbles resulting from reduction of potassium nitrate to nitrogen gas can be indicative of the growth of these bacteria as shown in (figure 1).

The microbial composition of oil field fluids is required to precise a success of nitrate and nitrite addition for oil field souring control. This result might be achieved by using a multipronged approach for microbial community characterization including cultivation of microorganisms and polymerase chain reaction PCR analysis of 16S rRNA genes using universal primers [1]. In the past, culture-based methods were used as the primary means of bacterial identification and enumeration in oil fields, cultivation of bacteria from extreme environment was difficult and may lead to errors in identifying the microbial community members and incorrect conclusions regarding the diversity and metabolic activity of the microbial consortium and thus, to improper design of control strategies [20]. Therefore molecular techniques and nucleic acid based analyses of bacterial community have been used to provide data from bacterial communities in inhospitable environment [21]. An important objective of this study is to find the most dominant bacteria, which are the major contributors to the microbial process of these systems.

The results of sequences which were analyzed by use BLAST program in NCBI showed that most of the NRB strains identified were well known components of the oil field consortia. The major hNRB populations were found in the produced water of oil fields sharing sequences identity with genus *Pseudomonas* sp. within the Alfa subclass of the proteobacteria (5N, 12N, 16N, 17N and 18N) isolates with 99% similarity to *Pseudomonas stutzeri* and (19N) with 99% similarity to *Pseudomonas putida*. The results of this study were in agreement with Grigoryan *et al.* [22]. Who found that 16S rRNA gene sequences for five colonies purified from produced water of Neuquen Basin oil field in Argentina belonged to the genus *Pseudomonas* with 100% identity to *P. putida* and *p. stutzeri*. Pseudomonadas can reduce nitrate to nitrogen and metabolize a wide spectrum of oil organics [23]. The tow species of *pseudomonas* are hNRB and able to utilize a range of fatty acid as the sole carbon and energy source during anaerobic growth by denitrification which was energetically more favorable than nitrate reduction to nitrite or ammonium. If the inhibitory mechanism of SRB in a system is dominated by denitrifies the nitrite accumulation will probably be transient due to additional reduction to nitric oxide and nitrous oxide and finally nitrogen gas, all these compounds are considered to be potential inhibitory agents of SRB, in addition to increasing redox potential [24]. The isolates (35N) NRB belonged to the genus *Herbaspirillum sp.* with 99% identity to *H. huttiense* strain LAMA. This genus was previously classified as

Pseudomonas huttiensis, based on analysis of nucleotide sequences of the 16S rRNA transfer to other taxa of *Herbaspirillum* [25]. Its gram negative, growing under microaerobic condition and grows well with nitrogen as the sole nitrogen source. Some species assimilate or dissimilate nitrate to nitrite under oxygen limitation [26].

Isolate of (20N) was belong the genus of *Bacillus sp*. with (99%) identity to *B. licheniformis* strain MSEB, this bacteria is a facultative anaerobe capable of reducing nitrate to nitrogen gas [27]. Its play a role in production of surfactant, acid, gas and etc. therefore these bacteria can be used in enhanced oil exploitation from reservoirs also are useful for removing asphaltenes sediments around wells [28]. Within the microbial community of produced water there was isolate of (37S) which is Iron Reducing Bacteria (IRB), identified belong to the genus of *Shewanella* with identity of 99% to *Shewanella hafniensis* strain P010, this genus is facultative anaerobic, heterotrophic, [29]. All *Shewanella* strains can use nitrate, nitrite and iron Fe^{+3} as an electron acceptor for growth [30], also can grow anaerobically by reduction of various sulfur compounds to H_2S [31]. The results of this study were compatible with previous studies which isolated of *Shewanella spp.* from oil fluids of petroleum reservoirs [32,33].

V. CONCLUSION

In the present study, Nitrate Reducing Bacteria isolated from oil fields in Iraq. Identification of these isolates by 16S rRNA gene show the presence of different strains of heterotrophic NRB.

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