Isolation And Identification of Thermophilic Bacteria from Oil Reservoir in Basrah, Iraq

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Abstract: Consortium of four thermophilic bacteria isolates from water production of oil reservoir through filtering large amount of water by millipore filter papers and cultured in peptone sulfur medium was powered by trace elements, vitamin solution and supplied with N_2:CO_2 gases. The thermophilic bacteria were isolated under anaerobic condition at 70°C and identified depended on the amplification of 16S rRNA gene. The result of 16S rRNA identification shows that this bacteria belongs to *Massilia sp.*R-1, *Stenotrophomonas rhizophila* SBANHCu14, *Geobacillus galactosidasius* CF2 and *Geobacillus toebii* R-32639. These results indicate that this strains are obligate thermophilic and it grows at 70°C as optimum temperature.

Keywords: Thermophilic, petroleum reservoir, Phylogenetic analysis

I. INTRODUCTION

Petroleum reservoirs constitute a group of very special terrestrial sites, as they show an exceptional combination of extreme environmental conditions, including temperature, pressure and salinity. It is also known that petroleum composition varies widely between reservoirs, which may have an influence on the microbial diversity of such environments. Attention has lately been paid to the microbial ecology of petroleum reservoirs, where anaerobes have always been estimated to be the dominant micro-organisms. They include fermentative, sulfate reducing bacteria and methanogenic Archaea [1]. Among the fermentative anaerobes, heat loving prokaryotes have been studied the most, probably because most oil reservoirs occur at a depth where in situ temperatures more than 100°C and many of them belong to the Bacteria.

Thermophiles, microorganisms that grow at temperatures exceed 45°C, are among the best studied extremophiles. They have been isolated from hot springs, solfataras, geothermally heated soils, oil reservoirs, and from some mesobiotic environments like soils, composting vegetation, river-, lake-, and sea water [2,3]. The enzymes created by these prokaryotes are extremely thermostable and commonly resistant to chemical denaturants such as detergents, chemotropic agents, organic solvents and extremes of pH [4]. Performing biotechnological processes at high temperature has many advantages: the elevation of temperature is accompanied by a reduced risk of contamination, a decreased viscosity and an increased diffusion coefficient of organic compounds, hence the bioavailability, solubility of organic compounds and reaction rates are improved [5]. As a consequence, there is a continuous interest in isolating and characterizing thermophilic bacteria and their enzymes in order to increase the possibilities for their industrial application. There are a great potential could be promote the application of thermophilic in the petroleum industry, since the bioavailability of less soluble hydrophobic substrates such as polyaromatic and aliphatic hydrocarbons could also be amend dramatically at elevated temperatures . In that respect one process have attracted attention recently the use of microbes down oil wells in order to enhance oil production after primary and secondary recovery procedures (Microbial Enhanced Oil Recovery, MEOR) [6]. Although mesophilic microorganisms have been used in this process, the application of thermophiles will provide crucial advantages due to the increase in bioavailability and reaction rates, and because thermophiles could survive such a harsh environment of the oil reservoir [7]. Nowadays the extremophiles' research has resulted in the isolation of thermophilic bacteria and Archaea from a

great variety of terrestrial and hot aquatic environments. Specially, aquatic ecosystems possess a very large microbial biodiversity which can be explored for isolation and discovering new microorganisms and biocatalysts [8,9]. This work is the first study focusing on isolation and culturable thermophilic bacteria from production water in two oil reservoir located in Basrah southern of Iraq, and analysis of their potential application in enhance oil recovery in future.

II. MATERIALS AND METHODOLOGY

A- Sampling

A total of 30 samples of produced water were collected during the period from September 2016 to October 2017. The samples were obtained from the separator tanks of non water flooded oil field (Nahran Omer) which is located in the north of Basrah city and water flooded oil field (Northern Rumaila) Extending from the west of the city of Basra heading south. The samples were collected in a 3 litter sterile dark glass bottles which were filled completely to prevent any contact with air, then were transported to the laboratory at ambient temperature, flushed with 10% N_2 gas and kept at room temperature until use [10] (Miranda-tello *et al.,* 2003). The physical and chemical parameters of the sampling are show in table (1).

B- Enrichment and isolation of thermophilic bacteria

For the enrichment and isolation of thermophilic Bacteria, the following medium was used: 0.33g KCl, 0.33g NH_4Cl, 0.33g $KH_2\ PO_4$, 0.33g $MgCl_2.6H_2O$, 0.33g $CaCl_2.2H_2O$, 18g NaCl, 0.5g $Na_2S.9H_2O$, 5g peptone, 0.1g yeast extract, 10g elemental sulfur, 2mg resazurin sodium salt, 1ml Trace elements solution [11] and 1ml vitamins solution [12]. The pH of the medium was adjusted to 6.8 at room temperature. Medium was dispensed in 35ml screw cap tubes that was filled completely to prevent any contact with air and flushed with oxygen free mixture of N_2:CO_2 (80:20 v/v) to provide anaerobic condition. Tubes were inoculated with Millipore filter paper after the filtration of (100-300) ml of produced water and incubated at 70°C. Pure cultures of thermophilic bacteria were obtained by serial 10-fold dilutions [13].

Table 1. Characteristics of sampling sites in oil separator tanks

Sampling sites	***In situ* Tem. °C**	***Ex situ* Tem. °C**	**Salinity PPT**	**Pressure PSI**	**pH**
Separator tank in Nahran Omer oil field	95.5-81.1	55-45	22.400	2680	5.66
Separator tank in Northern Rumaila oil field	93.7-80	48-45	19.836	3690	4.98

C- Characterization of the isolates

Characterization of each thermopiles colony was performed by detection color, size, elevation, margin of its colony, Gram and spore staining. Catalase, oxidase and nitrate reduction were tested according to Prescott *et al.* [14].

D- Phylogenetic analysis

Phylogenetic analysis of the thermophilic isolates bacteria was carried out by 16S rRNA gene sequencing. Total genomic DNA was extracted from bacteria strains by using Wizard Genomic Isolation DNA kit (Promega, USA) according to the company protocol. Quality of the genomic DNA was detected by agarose gel electrophoresis after used as template for PCR. Bacterial universal primers 8F forward (5-'AGAG TTTGATCTACTGGCTC-3') and 1492R reverse (5-'CGGCTACCTTGTTACG ACT T-3') were used for amplification of 16S rRNA gene [15]. The PCR was carried out in a total volume of 50 µl containing 25 µl of Master mix 2X (Bioneer, Korea), 2 µl of purified DNA template (50 ng /µl), 2 µl of each primer (10 pmol) and 18 µl Nuclease free water. The PCR was performed for 30 cycles in Thermal cycler (Bioneer, Korea) under

conditions that used for PCR amplification were: Initial denaturation for 2 min. at 94 ºC, cyclic denaturation for 45s at 94 ºC, annealing for 30s at 55 ºC and extension of 1 min. at 72 ºC with a final extension of 10 min. at 72 ºC. amplification of 1500pb of PCR products were analysis by 1% agarose gel electrophoresis with 100 bp DNA ladder at 65V and 120mA for 40 min. using TBE buffer 1x. The product was then purified and was further subjected to sequencing in (Macrogen, Korea). The sequence data of 16S rDNA gene was analyzed using BLAST (Basic Local Alignment Search Tool) at national center for biotechnological information, USA (http://www.blast.ncbi.nlm.nih) [16]. The bacterial sequence was identified by matching it with a sequence with the highest identity score from the GenBank.

III. RESULTS AND DISCUSSION

A- Isolated of thermophilic bacteria

The Filtrating method was used for isolated of thermophilic bacteria from temperate oil reservoir water (separator tanks) samples under anaerobic conditions by use millipore filter paper and inoculated in peptone sulfur liquid medium, showed efficiency for isolated of bacteria from these samples depended of the physical and chemical characteristics. After 3 days of incubation at 70°C, the bacteria was abundant growth of irregular bacilli cells. Four cultures were purified by serial dilution in roll tube technique and spreading on the solid media as showed in figure (1). The strains (AS2,AS4,AS9 and AS10) were chosen for a morphology, biochemical and genetic studies.

Figure 1: Isolation of thermophilic bacteria by used millipore filter papers method

The physical and chemical properties were measured for the samples of produced water collected from Nahar omer and Rumaila oil fields. During the production processes, this results in producing an oil-water mixture which is separated into produced oil and water in above ground facilities. Produced water from various oil fields has been used as sources of thermophilic bacteria [17,18]. The studied properties of petroleum reservoir, including temperature , salinity and pH are important role in the thermophilic bacterial growth and the range of their fittings to such extreme environment this is confirmed by Li *et al*. [19].Also Adelaja, *et al*. [20] indicated the probability of the growth and blooming of microorganism in oil field depends on the physical and chemical characteristics of an ecosystem such as temperature, salinity and pH are essential limited factor that can play important role for the growth of prokaryotes and activities in oil reservoir environments. Because the temperature is increase about 3°C for every 100m depth in the oil reservoir and reach more than 90°C, therefore the temperature has been considered the highest limited factor for growth of indigenous thermophilic prokaryote [1].

Nemati *et al*. [21] and Grigoryan *et al.* [17] were explained that the Produced water around the world was represented a source of thermophilic microorganism that used in petroleum industry and biotechnology. These properties were depended in the designing the media that used isolation of thermophilic bacteria anaerobically from the produced water that collected recently which represent the huge waste in petroleum industries [22].

The peptone sulfur liquid medium was used for isolating thermophilic bacteria from formation water in a temperature 70ºC under anaerobic conditions, the bacterial growth appear through (3-5) days. The peptone sulfur media was used for the isolating of thermophilic prokaryotes from oil reservoir many times, because it contains peptone and yeast extract as sole of carbon source, sodium chloride (1.8%), $Na_2S.9H_2O$ as oxygen reduced agent and resazurin sodium salt as indicator for the low redox potential [13].Because of a few number of thermophilic prokaryotes in the collected samples the filtration method were used to concentrate the number of thermophilic microorganism by filtration of (300) ml of produced water through 0.22µm sized millipore filter paper and that paper was moved in sterile conditions to inoculate a screw cup full of peptone sulfur liquid media after it has been flushed with 80% N2, 20% CO2 oxygen free gases this agreed with Mohammad *et al*.[23].

B- Morphology of thermophilic isolates

The bacterial growth on the solid media by using phytagel instead of agar to maintain its consistency in high temperature appear a spherical to irregular shaped and colonies are growing subsurface of medium partially to completely (Figure 2).Thermophilic bacteria isolates cells (AS2, AS4, AS9 and AS10) are irregular motile rod-shaped, 2.0–3.5µm, gram staining proved their variability between positive and negative (Figure 3).

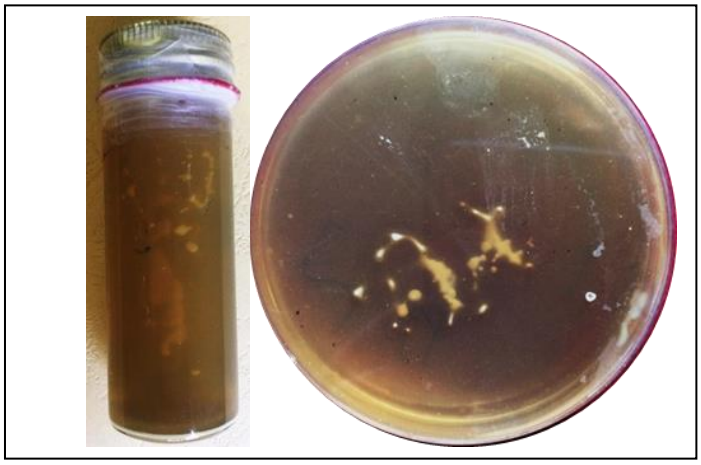

Figure 2: Morphology of thermophilic bacteria colonies in solid medium

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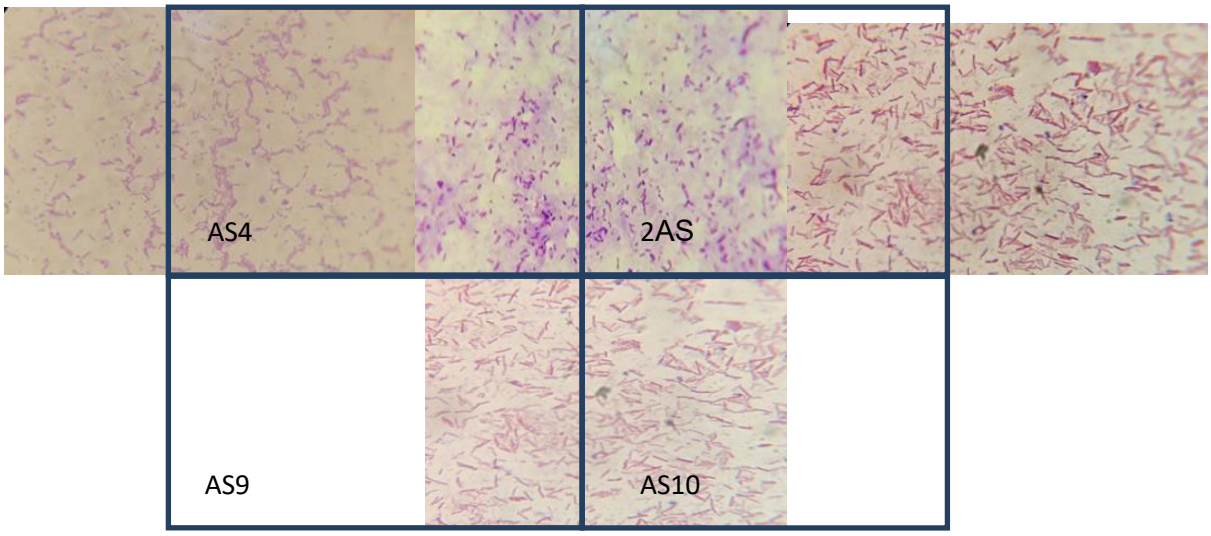

Figure 3: Shaped of thermophilic bacteria cells under light microscope (100X)

The thermophilic bacteria appear in a round or irregular shape partially or completely subsurface with a white color on the solid peptone sulfur media using phytagel instead of agar because its more stable at high temperature during the microscopic examination the thermophilic isolate approved to be variable for Gram staining and some of them are nitrate reducer, this has been published by Nazina *et al*. [24].

C- Physiology of growth

All of the isolates (AS2,AS4,AS9 and AS10) were thermophilic grow in the temperature range from 40° to 80°C (optimal growth up to 70 °C), no growth below 37°C or above 90°C. The pH range of growth was from 5.8 to 9.0 with the optimum at (7.0-6.8). All of the isolates were catalase, oxidase and nitrate reduction positive as showed in (Table 2). The isolation of *Geobacillus* from oil reservoir as well as most of the isolated thermophilic bacteria give a positive reaction for catalase and oxidase this result that are compatible with a study done by Annarita *et al*. [25] when they isolate a new subspecies belong to *Geobacillus* which is positive for catalase, oxidase and a nitrate reducer.

Table 2: Morphological and biochemical test of isolates strains.

Isolates	Cells shape	Gram stain	Spore stain	Oxidase test	Catalase test	Nitrate reduction
AS2	Bacilli	-	-	-	-	-
AS4	Bacilli	+	+	+	+	+
AS9	Bacilli	+	+	+	+	+
AS10	Bacilli	+	+	+	+	+

D- Genetic identification of thermophilic strains

On quality evaluation of DNA extracted from bacterial isolates, A representative image of an agarose gel electrophoresis is shown in figure (4) which display bacterial DNA bands observed under UV trans-illuminator. Related to amplification of 16S rRNA gene for bacterial isolates the result are shown in figure (5). The 16S rRNA gene sequence of the bacterial strains was submitted to GenBank through aligned with BLAST. The of 16S rRNA gene analysis are shown in Table (3). The result of phylogenetic relationship of these bacteria with other reference species was determined using phylogeny tree figure (5).

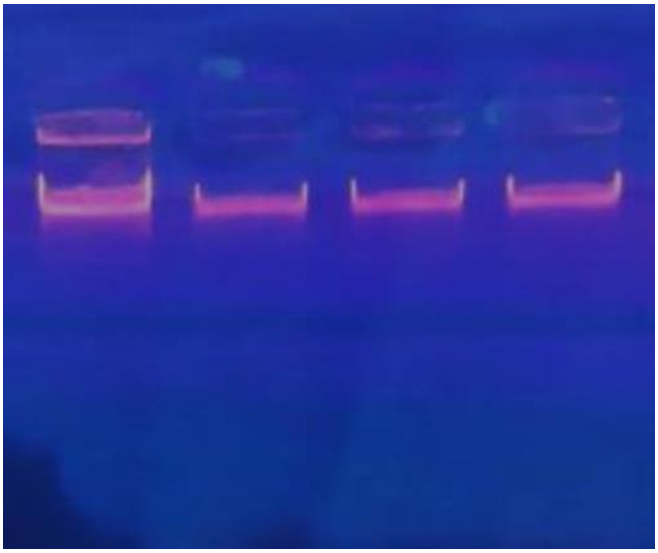

Figure 4: Agarose gel electrophoresis of bacterial DNA using a 0.8 % agarose gel containing ethidium bromide.

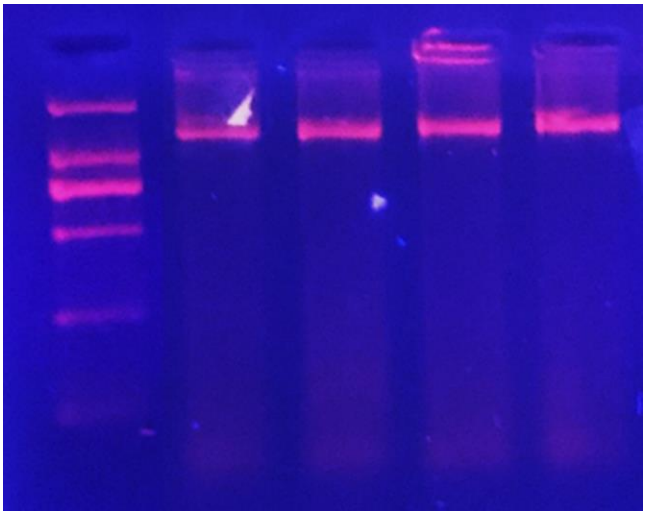

Figure 4: Amplification of 16S rRNA gene form bacterial isolates on agarose gel containing ethidium bromide.

Table 3: Analysis of 16S rRNA gene for identification of bacterial isolates

Isolate	**Strain**	**Accession No.**	**Identity**
AS2	*Massilia sp.*R-1	HF548445.1	**%95**
AS4	*Stenotrophomonas rhizophila* SBANHCu14	KR259223.1	**%90**
AS9	*Geobacillus galactosidasius* CF2	FR689752.1	**%90**
AS10	*Geobacillus toebii* R-32639	FN538992.2	**93%**

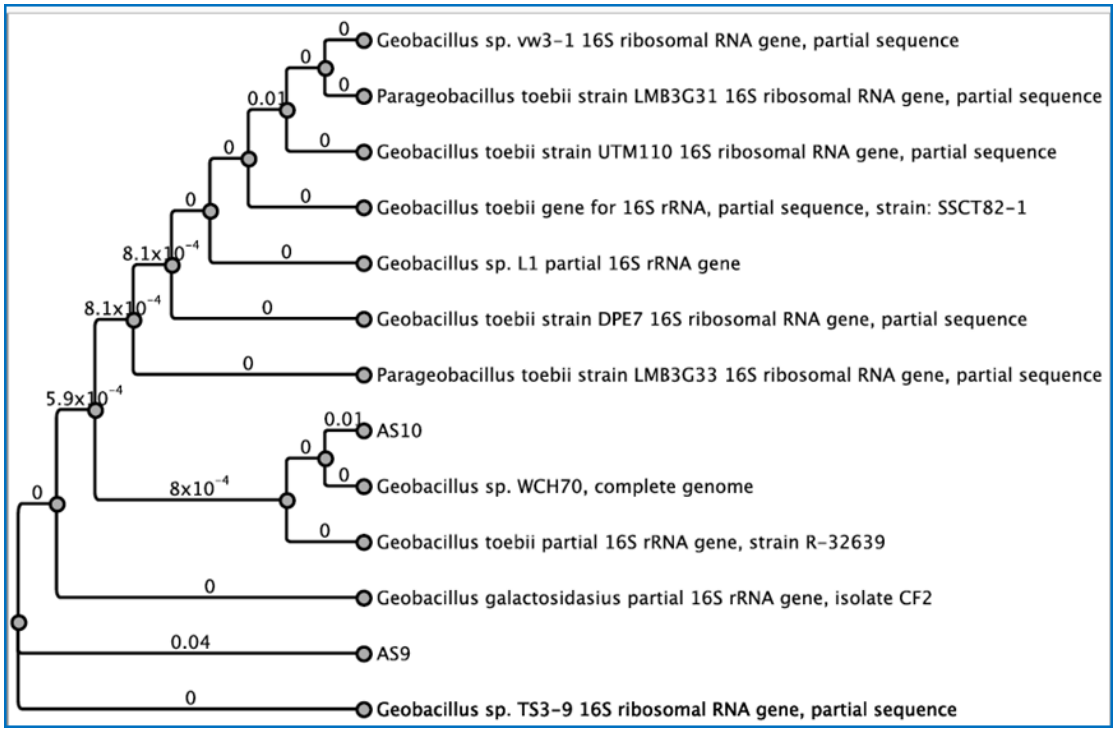

Figure 5: Phylogenetic tree of *Geobacillus galactosidasius* CF2 (FR689752.1) and *Geobacillus toebii* R-32639 (FN538992.2) with other related bacteria. Software available at https://mafft.cbrc.jp

The genetic identification based on the PCR for the 16S rRNA gene using the universal primers (8F and 1492R). This results might be achieved by using a multipronged approach for microbial community characterization including cultivation of microorganisms and PCR analysis of 16S rRNA genes [25]. Previously, culture-based methods were used as the first way for bacterial identification and enumeration in oil fields. Cultivation of bacteria from extreme environment was complicated and may lead to defect in identifying the microbial community members [26]. Therefore Kjellerup *et al.*[27] ware refer to molecular methods and nucleic acid are based analyses of bacterial community have been used to provide data from bacterial communities in such harsh environment. The results of gene sequences which were analyzed by use BLAST program in NCBI showed that most of the thermophilic strains identified were well known components of the oil fields society and most of them are nitrate reducer chemo-organotroph [28,29].

Two common thermophilic nitrate reducer genera that seems to be especially abundant in oil reservoirs are *Geobacillus* and *Deferribacter*. The genus *Geobacillus* belongs to the order Bacillales within the Firmicutes, and oil reservoir-derived *Geobacillus* isolates are thermophilic microaerophiles that degrade single of hydrocarbon and some could reduce nitrate anaerobically isolated from oil reservoir in Russia, china and Kazakhstan [24].

The genus *Massilia* belong to the family *Oxalobacteraceae* isolated from different environment. It's a Gram negative non spore forming bacilli isolated for the first time in a pure culture from the polluted soil with Polycyclic aromatic hydrocarbons (PAHs) in china. the study evidence the ability of *Massilia* sp. WG5 to degrade PAHs in a good efficiency which make a suitable choice for the bioremediation of oil polluted soil [30].While the genus *Stenotrophomonas* one of its strains *S. maltophilia* IPN-TD isolated from the polluted soil with diesel in Mexico that strain showed a high efficiency to degrade Methyl tert-butyl ether [31] (Guadalupe *et al.*, 2016).

VI. CONCLUSION

Use thermal environments in oil fields (Nahran Omer and Northern Rumaila) of Basrah, Iraq for isolation of extremophilic bacteria are a good source for these Bacteria. Four isolates of thermophilic were isolated under thermophilic conditions by using filtration method with peptone sulfur medium that incubated anaerobically.

These strains were identified depended on analysis of 16S rRNA genes which are considered a based analyses of bacterial community have been used to provide data from bacterial communities in such harsh environment.

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