BIODIVERSITAS
Volume 23, Number 7, July 2022
Pages: 3462-3470

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230720

Screening for extracellular synthesis of silver nanoparticles by bacteria isolated from Al-Halfaya oil field reservoirs in Missan province, Iraq

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Manuscript received: 8 June 2022. Revision accepted: 20 June 2022.

Abstract. *Alshami HGA, Al-Tamimi WH, Hateet RR. 2022. Screening for extracellular synthesis of silver nanoparticles by bacteria isolated from Al-Halfaya oil field reservoirs in Missan province, Iraq. Biodiversitas 23: 3462-3470.* Microorganisms that live in deep environments are thought to be adapted to the conditions of these environments and possess enzymatic systems that can be useful in scientific and commercial applications. The study aimed to isolate, identify, and screening of silver nanoparticles (AgNPs)-producing bacteria from five samples of produced water of the Al-Halfaya oil field reservoirs in Missan governorate, Iraq. The results revealed a total of 22 bacterial isolates were isolated and identified by morphological features and amplification of the 16S rDNA gene. The isolates are belonging to nine species, including *Bacillus cereus* strain DBA1.1, *Bacillus thuringiensi*s strain MSP51, *Neobacillus drentensis* strain ROA042, *Enterococcus faecalis* strain 2674, *Exiguobacterium mexicanum* strain AB201, *Klebsiella quasipneumoniae* strain KP18-31, *Klebsiella quasipneumoniae* subsp. *similipneumoniae* strain 2437, *Klebsiella pneumoniae* strain IOB-L, and *Acinetobacter lwoffii* strain K34. The Gram-positive bacteria were the most dominant (55.56%), and the most frequent isolate was *Klebsiella quasipneumoniae* strain KP18-31 (63.64%). Six species out of nine species were recognized as AgNPs producing bacteria and *B. cereus* strain DBA1 was the best and most potent isolate in the synthesis of AgNPs. The phylogenetic tree was constructed depending on 16S rDNA gene sequences to determine the evolutionary relationship among the isolated bacteria.

Keywords: 16S rDNA gene, nanoparticles producing bacteria, nitrate reductase enzyme, oil reservoirs, silver nanoparticles

INTRODUCTION

The petroleum systems or oil field reservoirs are regarded as difficult habitats for various microbes to survive due to their harmful nature, poor water activity, extreme heat, salt content, and pressure (Pannekens et al. 2019). On the other hand, oil field reservoirs offer extensive habitats for bacteria and archaea, especially sulfate, nitrate, and iron reducers, as well as microorganisms such as acetogenins, fermenters, and methanogens (Cai et al. 2015). Oil reservoirs contain diverse stage, such as crude oil, formation water, and solid surfaces formed from rock and organic materials, where microorganisms can thrive. The oil-water transition zone (OWTZ), located between the oil and water legs, is a center for microbial growth and oil degradation in oil reservoirs (Pannekens et al. 2019).

The majority of research on oil microbes divides samples of oil field reservoirs into two types: water phase and oil phase samples. Formation or produced water, which is normally found inside porous rock, forms a major part of the water phase. A number of bacteria may adhere directly to the oil phase, while others may stay in the water phase. *Nitrospira*, *Actinobacteria*, *Acidobacteria*, *Thermodesulfobacteria*, and *Fusobacteria* were identified in the oil phase samples, while *Thermotogae*, *Betaproteobacteria*, *Atribacteria*, *Bacteriodetes*, *Synergistetes*, and *Alphaproteobacteria* were obtained from the water phase samples (Li et al. 2017). A number of studies have employed the polymerase chain reaction (PCR) technique to amplify the 16S rDNA gene for the identification and determination bacterial diversity in oil reservoir samples (Frank et al. 2016).

The prevalence of bacteria in deep habitats is well known, and include a diverse range of mesophilic bacteria, thermophilic bacteria and archaea, many of which have the ability to metabolize organic and inorganic substances. Many researches are currently concentrated on deep environments, as bacteria that live in these places are believed to be adapted to extreme environments and therefore contain enzymatic systems that might be significant in scientific and commercial applications (Silva et al. 2013).

AgNPs have gained a lot of attention in scientific studies due to their usability in a variety of fields like medicine, the environment, food technology, and other new applications (Dawadi et al. 2021). Depending on where the nanoparticles are manufactured, nanofabrication can be done extracellularly (Shanshoury et al. 2020) or intracellularly (Gloria Martin and Vergara Padilla 2020).

Mechanisms involved in the synthesis of nanoparticles (NPs) in microbes include enzymatic action, proteins, peptides, and efflux pump systems. Enzymes are thought to be the most important agents in the reduction and capping of metals in microorganisms, via redox processes that take place in either the intracellular or extracellular space (Ghosh et al. 2021). In bacteria, NADH-dependent enzymes like nitrate reductase (NR) have been found to be involved in the reduction process and help in the production of NPs from metal ions. Such a mechanism was

shown in *Pseudomonas aeruginosa* ATCC 27853 and AgNPs were produced using this bacterium when the supernatant was exposed to silver ions(Ag^+), leading to reduce metal ion extracellularly and hence synthesis of AgNPs (Peiris et al. 2017). During the reduction process, the NR enzyme converts nitrate to nitrite, and the electron is delivered to the silver ion, reducing it to silver (Ag^+ to Ag^0) (Tashi et al. 2016).

The bacteria of the Al-Halfaya oil field have not been investigated, and no information is available. Hence, the present study aimed to isolate, identify the bacteria and screening of AgNPs-producing bacteria from the Al-Halfaya oil field reservoirs in Missan governorate, Iraq.

MATERIALS AND METHODS

Samples collection

Following *crude* oil extraction from oil wells, the produced water is separated through the separator tank and then treated in various ways before being re-injected, while the remainder of the produced water is drained into the ponds (Siagian et al. 2018). In January 2021, five Samples of produced water were collected from different sources of Al-Halfaya oil field, south of Missan Governorate in Iraq (31°42′N 47°19′E). Three samples were obtained from the ponds (each sample from a pond), and two samples from the separator tank water. The samples were collected using 3 liter sterilized glass containers, and directly transferred to the laboratory.

Bacterial isolation

To isolate the bacterial strains, 5 mL of produced water samples were inoculated into a 250 mL glass Erlenmeyer flask containing 95 mL of sterile nutrient broth. The flask was placed in a shaker incubator at 37°C for 48 h with 120 rpm. After the incubation, 1 mL of the suspension was diluted serially in glass tubes containing 9 mL of distilled water (the serial dilution was achieved up to 10^{-6}). The volume of 0.1 mL from each dilution tube was added by pipette to the center of nutrient agar and MacConkey agar, then dispersed via spreading plate technique and incubated at 37°C for 24 h. To obtain pure isolates, the various colonies that have grown recultured using the streaking plate method on nutrient agar plates (Sirisha et al. 2017). All isolates were subjected to Gram staining protocol and preserved on nutrient agar slants for further experiments (Dash and Payyappilli 2016).

Molecular Identification

Extraction of bacterial genomic DNA

The genomic DNA was extracted from all bacterial isolates grown on nutrient agar plates by using Presto™ Mini g DNA bacteria kit (Geneaid, Taiwan) according to the manufacturer's instructions. The extracted DNA was preserved at -20°C.

Amplification of bacterial 16S rDNA gene

All the isolated bacteria were identified by 16S rDNA gene (Approximately 1500 bp). Amplification of 16S rDNA using thermocycler (TECHNE prime, USA) was done by universal 27F forward primer (5´-AGAGTTTGATCCTGGCTCAG -3´) and 1492R reverse primer (5´-GGTTACCTTGTTACGACTT -3´) adopted from a previous study (Alyousif et al. 2020). The reaction mixture was carried out in a volume 50 µL, where 7 µL of template DNA, 2.5 µL of each forward and reverse primers (10 pmol), and 38 µL of distilled water were added into each tubes of Maxime™ PCR PreMix Kit (i-Taq) (Dried and aliquoted master mix) (iNtRON, Korea). The steps of the thermal cycle were as follows: 3 min initial denaturation at 96ºC, followed by 27 cycles of 30 s denaturation at 96ºC, 25 s primer annealing at 52ºC, 15 s extension at 72ºC and a final 10 min extension at 72ºC (Alyousif et al. 2020). The amplified DNA fragments were separated using an electrophoresis apparatus (Bioneer, Korea) on a 1% (w/v) agarose gel stained with 2 µl of ethidium bromide solution (Promega, USA). The running was carried out for 45 minutes at 120 mA 80V. Finally, the results of gel electrophoresis were visualized by gel documentation system (Biometra, Germany) (Lee et al. 2012).

Sequencing of the 16S rDNA gene

Each sample was labeled and sent to Macrogen biotechnology company (South Korea). Purification of the PCR product and analysis the sequence of forward and reverse 16S rDNA primers were done by the same company. The sequences results were then compared with the ready gene sequences to National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) website by using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov) and analyzed for detecting the closest match for the bacterial isolates. The phylogenetic tree was constructed using the neighbor-joining tree (NJ) method with 1000 bootstraps using the MEGA X (Molecular Evolutionary Genetic Analysis) program.

Bacterial screening for the ability to synthesize AgNPs

Nitrate reductase assay

To demonstrate the existence of the enzyme in different bacteria, the nitrate reductase test was performed according to Bhusal and Muriana (2021).

The changes in the color of bacterial culture supernatants

This approach was performed according to Singh et al. (2015) with some slight modifications. The isolated bacteria were grown in flasks filled with 50 mL of sterile nutrient broth (NB). After 24 hours of incubation at 37 °C in an orbital shaker (150 rpm), the cultures were centrifuged at 6000 rpm for 10 min to get the supernatant. The supernatant of each isolate was mixed with 50 mL of 1 mM silver nitrate (AgNO3) solution in a 250 mL flasks, the flasks were then wrapped with foil and incubated at 37 °C for 3 days at 150 rpm. A mixture of medium and AgNO3 solution (1mM) was used as a control. The biosynthesis of AgNPs was visually checked for any changes in the color

of the supernatant. In the case of the biosynthesis of AgNPs, the color changes from pale yellow to brown or dark brown.

Absorbance measurement of bacterial culture supernatants

After the incubation period, to confirm the presence of AgNPs in the supernatant of different strains, 2 mL of each reaction mixture that changed color to brown or dark brown were taken and poured into quartz cuvettes. The absorbance (in the range of 200-800 nm) was measured using the Dual Beam UV-1800 Spectrophotometer instrument (Shimadzu, Japan) (Valentina 2022).

Product weight of synthesized AgNPs

Following the measurement of absorbance, samples were centrifuged at 10000 rpm for 10 minutes using Table Top Centrifuge (Gemmy, Taiwan), and the supernatants were discarded. The precipitates (AgNPs) were then dispersed using deionized water. This process was carried out three times. The purified precipitates were air-dried, collected in powder form, and then weighed (Singh et al. 2018).

RESULTS AND DISCUSSION

Physiochemical properties of produced water samples

The physiochemical properties of produced water were obtained from the daily production reports of the oil field laboratories as shown in tables (1) and (2). The age of oil wells ranges from 1 to 9 years, and their depths range from 1903 to 4433m. The water physiochemical properties of the separator tank sampled in the current study (high temperature, slightly acidic pH, high salinity) were similar to previously reported data in other oil reservoirs (Kobayashi et al. 2012; Lenchi et al. 2013), while contradict the research results of Tang et al. (2012) (less salinity, alkaline PH, lower temperature). The differences in physiochemical characteristics are due to the geographic location and environmental characteristics of the various oil reservoirs, as reviewed by Li et al. (2017). Gao et al. (2016) discovered that temperature, salinity, and ion concentrations varied significantly even among reservoirs in the same oilfield. For example, the temperature rises by about 2-3°C for every 100 m of depth, implying that the impacts of depth and temperature are closely related (Lenchi et al. 2013).

Bacterial isolation

For ponds water samples, the ideal dilution to get single colonies was 10^{-5}, whereas, for separator tank samples, it was 10^{-4}, indicating that the bacterial load in separator tank water was lower. Water injection into wells is used to produce a large portion of the oil in the world (Folarin et al. 2013). Produced water (used as injection water) in the separator tanks of Al-Halfaya oil field reservoirs is frequently chlorinated and treated with a biocide before being injected back into wells. The chemical treatment has a significant impact on bacterial populations by stopping bacterial growth and activity. This may explain why the bacterial load in separator tank water samples is relatively lower compared to pond water samples. Upon reviewing relevant publications on oil reservoirs, the best dilution for obtaining separate colonies from separator tank water was not stated, with the exception of Hamzah et al. (2020), who revealed that 10^{-2} was the ideal dilution. Regarding the pond water samples, there are no studies that have investigated their microbiology; they were sampled for the first time in the current study. A total of 22 pure bacterial isolates were obtained in the current study, namely P1 to P11 for ponds water samples and T1 to T11 for separator tank water samples. The size of the colonies was the most noticeable difference, with P11,T8, and T10 being the smallest, P1-P8, T2-T7, T9, and T11 being medium, and P9, P10, and T1 being the largest. Gram staining revealed that 55.56% of the isolates were Gram-positive and 44% were Gram-negative. In the current study, it was noticed that Gram-positive bacteria were more widespread than Gram-negative bacteria. Ozyurek and Bilkay (2017) who used an oil field as a microbial source, showed the same results, in their study, different Gram-negative and positive bacteria were isolated from different sources of oil fields. Many other studies have found that the majority of bacteria found in produced water samples were Gram-positive bacteria (Elumalai et al. 2019; Hamzah et al. 2020). The reason for Gram-positive dominance is that Gram-positive bacteria have strong cell wall, which allow them to adapt more easily to extreme conditions such as high temperature and osmotic pressur (Prakash et al. 2014). Other reason is that many Gram-positive bacteria have the ability to form endospores (Elumalai et al. 2019).

Bacterial identification by 16S rDNA

The polymerase chain reaction (PCR) technique was conducted to determine the identities of bacterial isolates through the amplification of the 16S rDNA gene. Figures (1) and (2) show agarose gels (1% w/v) indicating PCR-amplified fragments of the 16S rDNA gene using genomic DNA extracted from bacteria obtained from pond and separator tank water samples, respectively.

The BLAST program was used to analyze and match the DNA sequencing results of isolates with their reference strains in the GenBank. DNA sequencing of bacterial populations from produced water samples revealed that bacterial isolates are belonging at the genus level to six different bacterial genera and at the species level to nine species were identified and recognized as shown in Table 3. The bacterial genera include *Bacillus*, *Neobacillus*, *Enterococcus*, and *Exiguobacterium*, all of which belong to the class *Bacilli* and are Gram-positive bacteria.

Table 1. physiochemical properties of the separator tank water samples obtained from Al-Halfaya oil field

Water chloride (mg/L)	Water salinity (mg/L)	PH	Temp. (°C)
90043-122302	148570-201798	5.48-6.28	58-65

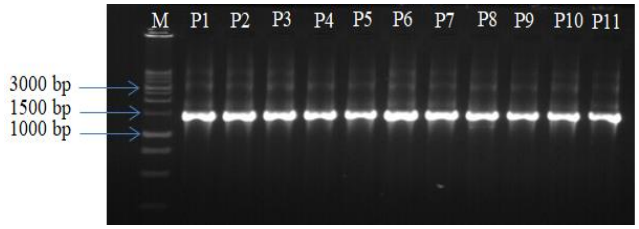

Figure 1. The 16S rDNA gene electrophoresis exhibit amplified genomic DNA extracted from bacterial species obtained from pond water samples. Lane M: 1kb DNA Ladder; Lane P1-P11: PCR products of the 16S rRNA gene of bacterial species

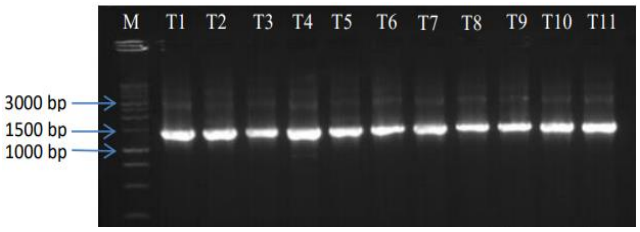

Figure 2. The 16S rDNA gene electrophoresis exhibit amplified genomic DNA extracted from bacterial species obtained from separator tank water samples. Lane M: 1 kb DNA Ladder; Lane T1-T11: PCR products of the 16S rRNA gene of bacterial species

Table 2. physiochemical properties of the ponds water samples obtained from Al-Halfaya oil field, Iraq

Samples	Turbidity (NTU)	Oil content (ppm)	Slinity (ppm)	PH	Temperature (°C)	Concentration of trace elements (mg/L) ++Fe
Pond 1	18.70	4.30	91200	6.58	25	0.63
Pond 2	20.00	1.80	90950	6.57	25	0.82
Pond 3	19.00	3.90	90600	6.57	25	0.48

Table 3. Bacterial populations in the total produced water samples that were identified using the 16S rDNA gene

Isolation source		Code of isolate	Closet bacteria	Accession no. of closet bacteria	Match ratio with reference strain
Pond water	Pond 1	P1	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		P2	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		P3	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		P4	*Klebsiella pneumoniae* strain IOB-L	MN555336.1	100%
	Pond 2	P5	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		P6	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		P7	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	99%
		P8	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	99%
	Pond 3	P9	*Bacillus thuringiensis* strain MSP51	MG984081.1	98%
		P10	*Bacillus cereus* strain DBA1.1	MT332156.1	100%
		P11	*Enterococcus faecalis* strain 2674	MT611693.1	99%
Separator tank water		T1	*Neobacillus drentensis* strain ROA042	MT525288.1	99%
		T2	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		T3	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		T4	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		T5	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		T6	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		T7	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		T8	*Exiguobacterium mexicanum* strain AB201	MT436082.1	99%
		T9	*Klebsiella quasipneumoniae* strain KP18-31	CP045641.1	100%
		T10	*Acinetobacter lwoffii* strain K34	MK548539.1	99%
		T11	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* strain 2437	MT604862.1	99%

Other genera are *Klebsiella* and *Acinetobacter* which belong to the class *Gammaproteobacteria* and are Gram-negative bacteria. The species that fell under these genera included *Bacillus cereus* strain DBA1.1, *Bacillus thuringiensis* strain MSP51, *Neobacillus drentensis* strain ROA042, *Enterococcus faecalis* strain 2674, *Exiguobacterium mexicanum* strain AB201, *Klebsiella quasipneumoniae* strain KP18-31, *Klebsiella quasipneumoniae subsp. Similipneumoniae* strain 2437, *Klebsiella pneumoniae* strain IOB-L, and *Acinetobacter lwoffii* strain K34. The most frequent isolate as shown in figure (3), was *K. quasipneumoniae* strain KP18-31, which constituted 14/22 (63.64%) of all isolates identified in produced water samples.

When the 16S rDNA gene sequences of bacterial isolates were compared with sequences in GenBank, it was observed that isolates P7, P8, P11, T1, T8, T10, and T11 were similar to *K. quasipneumoniae* strain KP18-31, *K.quasipneumoniae* strain KP18-31, *E. faecalis* strain 2674, *N. drentensis* strain ROA042, *E. mexicanum* strain AB201, *A. lwoffii* strain K34, and *K. quasipneumoniae subsp. similipneumoniae* strain 2437 respectively, with similarity of 99%, while isolate P9 demonstrated 98% identity to *B. thuringiensis* strain MSP51.

These isolates were recorded as new strains in the GenBank database. The names and accession numbers of these strains are listed in Table 4).

The Neighbor-Joining method was used to construct the phylogenetic tree based on the 16S rDNA gene sequences of all nine identified species. The original tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed, and it was constructed using the Jukes-Cantor model (Rastogi et al. 2019). As shown in figure (4), this tree depicts the distribution and evolutionary relationships among the nine species were selected and their type strains downloaded from NCBI database.

The bacterial populations in the current study were varied greatly from those found in Bohai S-Oilfield, China, where the diversity of the bacteria found in produced water samples was related to genera *Sphingomonas*, *Hydrogenophilus*, *Achromobacter*, *Brevundimonas*, *Methylobacterium*, *Pseudomonas*, *Bosea Caulobacte*r, and other genera, according to their data (Zhou et al. 2020). Furthermore, the bacterial diversity obtained from produced water samples of the deep oil reservoir in the study of Orphan et al. (2003) was also different from the bacterial diversity in our study and the study of Zhou et al. (2020). The reason for this difference in the bacterial diversity among different types of oil reservoirs as described above, is that microbial communities are identified by the geology of oil reservoirs. Temperature, pH, and salinity, which influence the structure and metabolic activity of the indigenous microbiota, are all determined by the geology of an oil reservoir (Vigneron et al. 2017).

In current study, members of the *Bacilli* class were more abundant than *Gammaproteobacteria* class members and the *Bacillus* species had a wide spread in total produced water samples. These species are *B. cereus*, *B. thuringiensis*, and *Neobacillus drentensis* (formerly *Bacillus drentensis* by Patel and Gupta (2020)). These findings are consistent with those published by Hamzah et al. (2020) and Al-Tamimi et al. (2019). According to their findings, *Bacillus* species were the most frequent isolates detected in the produced water samples. Similarly, Nazina et al. (2001) demonstrated the same results.

Bacillus is a big and diversified genus of rod-shaped, Gram-positive, endospore-forming bacteria that are aerobic and facultatively anaerobic, and they are a genus of the Bacillaceae family that belongs to the *Firmicutes* phylum (Errington and van der Aa 2020). In fact, it appears that the physiological aspects of *Bacillus* genus members have enabled them to inhabit almost all natural environment, such as water, air, soil, lake sediments, and fodder, and also harsh environments including thermal acid water, salt marshes, and hot springs (Gopal et al. 2015; Alyousif 2022). The survival of *Bacillus* genus members for a long time in various extreme conditions is due to the formation of protective endospores that withstand unfavorable environmental conditions (Zammuto et al. 2020).

In present work, three *Klebsiella* species were found, including *K. quasipneumoniae* strain KP18-31, *K. quasipneumoniae subsp. similipneumoniae* strain 2437, and *K. pneumoniae* strain IOB-L. Through reviewing previous studies, these bacteria have never been isolated from the produced water of oil reservoirs so far, and they were identified for the first time in current study. These bacteria (exogenous bacteria) may have arrived from external sources such as lakes or rivers water used as injection water in Halfaya oil field reservoirs.

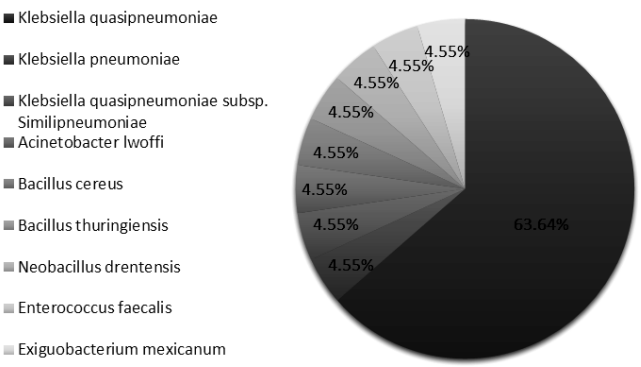

Figure 3. The frequency of bacterial isolates identified in the total produced water samples

Table 4. The new bacterial strains that were deposited in NCBI GenBank database

Isolates code	Name of the new strain in GenBank	Accession no.
P7	*Klebsiella quasipneumoniae* strain AMHWRB1	MZ379583.1
P8	*Klebsiella quasipneumoniae* strain AMHWRB2	MZ379584.1
P9	*Bacillus thuringiensis* strain AMHWRB3	MZ672038.1
P11	*Enterococcus faecalis* strain AMHWRB4	MZ672033.1
T1	*Neobacillus drentensis* strain AMHWRB5	MZ667879.1
T8	*Exiguobacterium mexicanum* strain AMHWRT1	MZ947162.1
T10	*Acinetobacter lwoffii* strain AMHWRT2	MZ947165.1
T11	*Klebsiella quasipneumoniae subsp. Similipneumoniae* strain AMHWRT3	MZ947166.1

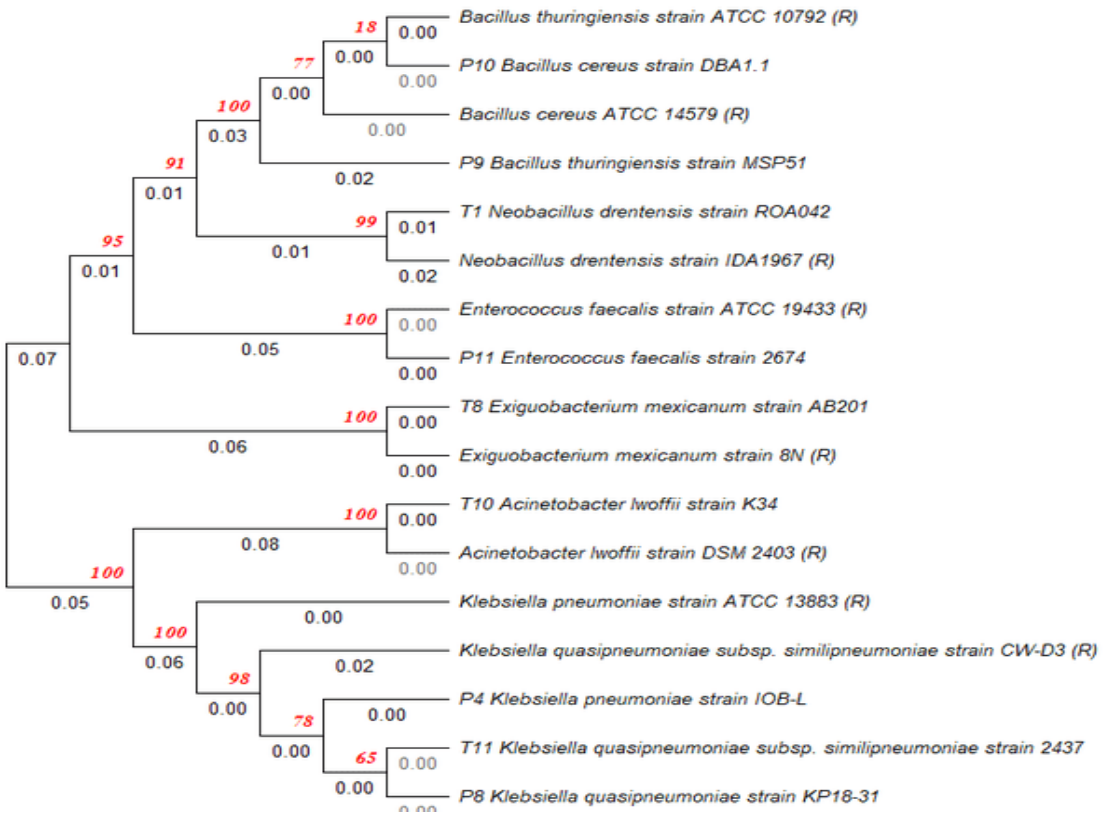

Figure 4. The Neighbor-joining method of phylogenetic tree construction shows the evolutionary relationships between of 9 different strains isolated from produced water samples (separator tank and ponds water samples) in the current study and their 8 type strains (the letter R) downloaded from the NCBI database

Bacterial screening for the ability to synthesize AgNPs

All nine identified species, including *K. quasipneumoniae* strain KP18-31, *K. pneumoniae* strain IOB-L, *K. quasipneumoniae* subsp. similipneumoniae strain 2437, *B. cereus* strain DBA1, *B. thuringiensis* strain MSP51, *N. drentensis* strain ROA042, *E. faecalis* strain 2674, *E. mexicanum* strain AB201, and *A. lwoffii* strain K34 were selected to test their ability to synthesis AgNPs. The screening results for all nine species are shown in Table 5).

The current study revealed that six out of nine species (66.67%) tested were positive for NR enzyme as shown in figure (5). These six species can reduce nitrate to nitrite. This reduction is facilitated by the NR enzyme produced by these bacteria. The NR enzyme is a NADH-dependent enzyme that accepts two electrons freed via NADH oxidation to reduce nitrate to nitrite. Silver ions (Ag^+) can also be reduced to AgNPs by liberated electrons (Bharti et al. 2020).

The extracellular formation of AgNPs was initially validated by observing the color change in the supernatant of bacteria treated with 1mM AgNO3 solution. Following the incubation period, the color of the supernatants changed from pale yellow to brown or dark brown due to surface plasmon resonance (SPR), while no color change was noticed in the control flask as shown in figure (6). This result is similar to those observed by Syed et al. (2019). The color change occurred only in the strains that were positive for the NR enzyme. This indicates that there is a close relationship between the presence of this enzyme and the biosynthesis of AgNPs (Khodashenas 2019). *A. Acinetobacter lwoffii* strain K34, *E. faecalis* strain 2674, and *E. mexicanum* strain AB201, when incubated at the same conditions, exhibited no color change. This could be explained by the lack of these species to NR enzyme or redox mediators such as ubiquinol and NADH, which act as electron carriers during NPs biosynthesis (Gahlawat and Choudhury 2019; Van Tan et al. 2021).

The UV-vis spectrum of the reaction mixtures was used to monitor the biosynthesis of AgNPs only by six strains whose supernatants changed from pale yellow to brown or dark brown. In the UV-vis spectrum, peaks at 407 nm, 424 nm, 432 nm, 435 nm, 440 nm, and 448 nm were observed for NPs synthesized using the culture supernatant of *K. quasipneumoniae* strain KP18-31, *B. thuringiensis* strain MSP51, *B. cereus* strain DBA1, *K. pneumoniae* strain IOB-L, *N. drentensis* strain ROA042, and *K. quasipneumoniae* subsp. *similipneumoniae* strain 2437, respectively (Figure 7), This is owing to the SPR band of the AgNPs.

According to studies, the peak in the range of 400-475 nm corresponds to the SPR of AgNPs (Alsamhary 2020; Jahan et al. 2021). As a result, the reaction mixtures

showed the biosynthesis of AgNPs using all six strains. As shown in Figure 7, the peaks for supernatant of *B. cereus* strain DBA1, *K. quasipneumoniae* strain KP18-31, and *K. pneumoniae* strain IOB-L are sharp and high, indicating the formation of monodisperse AgNPs, whereas the peaks for supernatant of *B. thuringiensis* strain MSP51, *K. quasipneumoniae* subsp. *similipneumoniae* strain 2437, and *N. drentensis* strain ROA042 are broad and less absorbent. Broad peaks suggest aggregation or polydispersion of NPs (Alahmad et al. 2021).

In current study, *B. cereus* strain DBA1 was considered the best and most potent in the synthesis of AgNPs due to NR enzyme secretion, the development of a dark brown color in solution, and higher absorbance (0.65 at 432 nm). Furthermore, the weight of AgNPs powder (product) obtained from the supernatant of this strain is heavier (7.2 mg) than other bacteria screened.

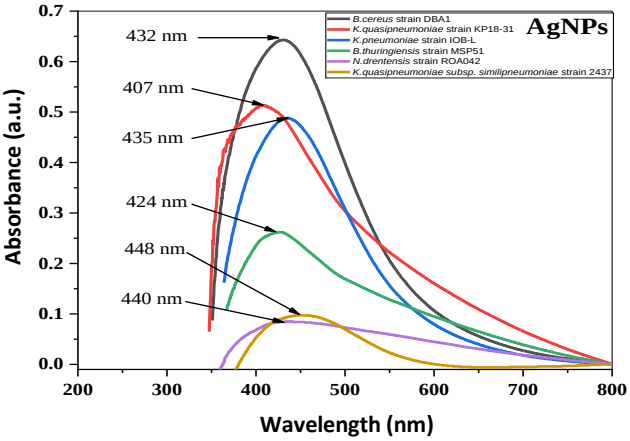

Figure 7. UV Vis-Spectra of AgNPs synthesized by the supernatant of six bacterial strains subjected to the same condition

Table 5. Results of the bacterial screening for the biosynthesis of AgNPs

Code of isolate	Name of bacteria	NR enzyme test	Color change/ color	Absorbance (a.u.)	Wavelength (nm)	weight of AgNps powder (mg)
P3	*Klebsiella quasipneumoniae* strain KP18-31	Positive	Yes/dark brown	0.51	407	4.8
P4	*Klebsiella pneumoniae* strain IOB-L	Positive	Yes/ dark brown	0.49	435	0.4
P9	*Bacillus thuringiensis* strain MSP51	Positive	Yes/ brown	0.26	424	0.3
P10	*Bacillus cereus* strain DBA1	Positive	Yes/ dark brown	0.65	432	7.2
P11	*Enterococcus faecalis* strain 2674	Negative	No			
T1	*Neobacillus drentensis* strain ROA042	Positive	Yes/brown	0.085	440	0.2
T8	*Exiguobacterium mexicanum* strain AB201	Negative	No			
T10	*Acinetobacter lwoffii* strain K34	Negative	No			
T11	*Klebsiella quasipneumoniae subsp. similipneumoniae* strain 2437	Positive	Yes/brown	0.11	448	0.2

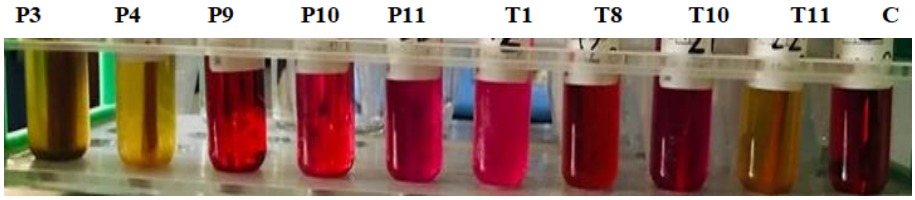

Figure 5. The test results for the nitrate reductase enzyme. P3: *Klebsiella quasipneumoniae* strain KP18-31, P4: *K. pneumoniae* strain IOB-L, P9: *Bacillus thuringiensis* strain MSP51, P10: *Bacillus cereus* strain DBA1, P11: *Enterococcus faecalis* strain 2674, T1: *Neobacillus drentensis* strain ROA042, T8: *Exiguobacterium mexicanum* strain AB201, T10: *Acinetobacter lwoffii* strain K34, T11: *Klebsiella quasipneumoniae subsp. similipneumoniae* strain 2437, C: Control (nitrate broth only)

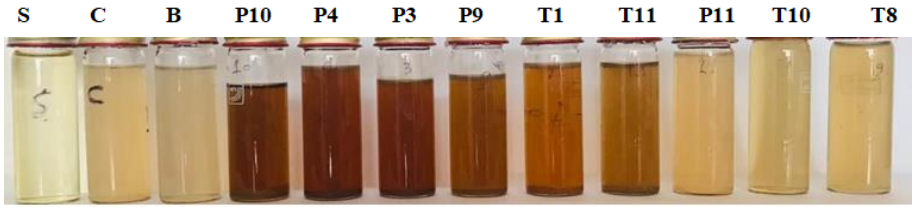

Figure 6. The color change of the culture supernatant of the nine strains treated with (1 mM) AgNO3 solution. S: Supernatant only, C: Control (medium with only AgNO3 solution), B: Supernatant treated with AgNO3 solution before incubation, P10: *Bacillus cereus* strain DBA1, P4: *Klebsiella pneumoniae* strain IOB-L, P3: *Klebsiella quasipneumoniae* strain KP18-31, P9: *Bacillus thuringiensis* strain MSP51, T1: *Neobacillus drentensis* strain ROA042, T11: *Klebsiella quasipneumoniae* subsp. similipneumoniae strain 2437, P11: *Enterococcus faecalis* strain 2674, T10: *Acinetobacter lwoffii* strain K34, T8: *Exiguobacterium mexicanum* strain AB201

Several studies have reported the use of *B. cereus* for the fabrication of AgNPs. Silambarasan and Abraham (2012) reported the synthesis of AgNPs using supernatant of *B. cereus* isolated from soil samples. Similarly, the extracellular biosynthesis of AgNPs by *B. cereus* isolated from wastewater-contaminated soil was described by Ahmed et al. (2020).

In conclusion, regardless of the harsh environmental conditions in oil reservoirs, there is a significant diversity of bacterial communities in these habitats, impacted by several factors. The Gram-positive bacteria were more prevalent than Gram-negative bacteria, and *Bacillus* species were widely distributed. The majority of bacteria (66.67%) in produced water samples were able to synthesize AgNPs, and there was a close relationship between the presence of the NR enzyme and AgNPs synthesis. *Bacillus cereus* strain DBA1 was the most potent and successful in the synthesis of AgNPs.

ACKNOWLEDGEMENTS

The authors are grateful to Missan Oil Company (MOC) for assisting with samples collection and to the Department of Biology, Faculty of Science, University of Misan, for providing laboratory equipment needed to complete this study.

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