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Original research

Effect of green synthesized iron oxide nanoparticles on bacterial microbiome for clean up the crude oil

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Abstract:

Nano-bioremediation approach involving the synergistic interaction between indigenous microorganisms and nanoparticles offers an affordable, environmentally, and beneficial solution for wastewater treatment. Herein, environmentally benign, *Eichhornia crassipes*-mediated green synthesized iron oxide nanoparticles (GS-IONPs) were prepared. The as-prepared GS-IONPs were properly characterized using different spectral analyses. Moreover, a bacterial microbiome was systematically isolated from a wastewater treatment unit located in the natural gas facility, Port Said, Egypt, and grown on a nutrient agar medium. The growth-enhancing effect of GS-IONPs on the bacterial community at different interval periods was studied. Bioremediation activity of employed bacterial consortium towards crude oil was carried out. The outcome data experimentally symbolized that the bacterial consortium was remarkably stable under pH of 7.0 and temperature of 37.0°C (optimized conditions). Besides, the growth of bacterial consortium was directly proportional to the concentration of GS-IONPs up to the optimum dosage of 0.04 g. Compared to the control sample (non-treated GS-IONPs specimen), the removal % of COD, BOD, and TOC interestingly improved by 74.76, 77.17, and 85.44%, respectively (e.g., 0.04 g of GS-IONPs). Overall, the present study illustrates an ample perspective of nano-bioremediation feasibility as a futuristic rational platform for decontamination of crude oil wastewater using a hydrocarbon-degrading bacterial consortium.

Keywords: Nanoparticles; Green synthesis; Nano-bioremediation; Crude oil; Clean water.

1- Introduction

Persistent water contamination is a critical worldwide crisis that endangers the globe, further organisms, and the entire ecosystem (Din Dar et al., 2021; Elbeltagi et al., 2021) . The problem intensifies with inadequate access to non-toxic drinking water, the quick expansion of the global inhabitants, industrial development, and climate issues (Ali et al., 2022).

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Most water bodies are extremely polluted by several types of hazardous materials such as crude oil, n-alkane, cycloalkane, diesel, polyaromatic hydrocarbons (PAHs), etc. (Saeed et al., 2022). Oil spills in the aquatic ecosystem are considered one of the most crucial catastrophes that happened globally. Oil spill impacts on ecosystems can be devastating, where they can destroy biota, disrupt the water quality such as salinity/pH scales (Chakravarty and Deka, 2021), and infect the atmosphere/water. Hydrocarbon pollution can also raise water's total organic carbon (Chakravarty and Deka, 2021) Ehmedan et al., 2021). A variety of treatment techniques presently exists to remove the hydrocarbons from contaminated wastewater. Treatment like skimming, solvent extraction, passive/reactive walls, and chemical dispersants have been utilized to treat contaminated oily spots (Tran et al., 2021). Numerous of these remediation technologies, nevertheless, are also overpriced or do not consequence in comprehensive mitigation of pollution. Conversely, bioremediation is a technique that relies basically on normal biological processes, in which microorganisms can destroy and even degrade toxic materials with eco-friendly effects, expenses, and energy supplies (Kaewlaoyoong et al., 2020). Bioremediation has been proved as a suitable and operative substitute to combat hydrocarbon pollution- (Ehmedan et al., 2021; Zhen et al., 2021).

Bioremediation is believed to be feasible, safe, and reasonably economical technique for capturing persistent organic compounds (Kaewlaoyoong et al., 2020; Singh et al., 2017). The efficacy of the bioremediation development may be impacted by the low existing microorganisms in the aquatic environments. More so, the oxidizing microorganisms may not be present in polluted water in the amounts needed for successful bioremediation (Ahmed et al., 2022). The hydrophobicity of organic compounds like hydrocarbons is one of the key obstacles to their effective biodegradation. The hydrocarbons degradation by microorganisms is manipulated by the bioavailability of hydrocarbons in the water ecosystem (Calvo et al., 2009). Due to the challenges, an unconventional methodology to hasten the degradation development is required. Nanotechnology has concerned prodigious attention owing to its anticipated influence on zones of catalysis and/or water remediation (Lodhi et al., 2021). Nanomaterials have demonstrated operative as exceptional materials for biodegradation activities due to their specific surface area and superior reactivity (Dhillon et al., 2012). Nanoparticles (NPS) have been broadly employed as reducing agents and/or catalysts to improve numerous applications, attributing to their considerable high surface-to-volume ratio and other unique physicochemical properties (Boz et al., 2009; Islam et al., 2021). Moreover, NPS consequence on microorganisms has also been placed into significant consideration. NPS applications have been suggested as successful heightened oil retrieval techniques. NPS can enter the opening throat and alter the reservoir characteristics to rise oil removal (Agista et al., 2018). Although NPS can support microbe processes, some relevant studies have identified the NP's impact on the biological reaction rates (Shin and Cha, 2008). The developed bustle of NPS is usually denoted by their exclusive characteristics and great accessible active surface areas and functionality (Singh et al., 2010).

Nanoparticles have a great impact on increasing the reaction rates of microorganisms by positioning the cells to develop the action of microbes (Ehmedan et al., 2021). It has also been noticed that the combination of nanotechnology with the enzymatic routes in the biodegradation activity could take the lead to overwhelming activity (Dixit et al., 2021). The iron oxide nanoparticles (IONPs) have been regarded as an effective bacterial growth-enhancing agent used to clean up contaminated hydrocarbon environments (Oyewole et al., 2019). The previous studies have demonstrated the significance of hydrocarbon-degrading bacteria for hydrocarbon

bioremediation and overseeing the hydrocarbon contamination issues using a sustainable and green strategy.

The present study aims to the assess the crude oil biodegradation using a hydrocarbon-degrading bacterial consortium isolated from the wastewater treatment unit located in the natural gas facility, Port Said, Egypt, in the presence of GS-IONPs. Specifically, the GS-IONPs can be introduced as an efficacious bacterial growth-improving agent applied to remediate contaminated hydrocarbons.

2. Materials and methods

2.1. Green synthesis of iron oxide nanoparticles (GS-IONPs)

The extract of the common water hyacinth *Eichhornia crassipes* biogenic material has employed as reducing, capping, and stabilizing agents for the formation of several types of NPs, for instance, iron oxide, zinc oxide, and silver NPs (Agarwal et al., 2017; Palai et al., 2022). For the preparation of *E. crassipes* extract, 30 g of plant leaves were cleansed steadily with deionized water, dried up, ground by mortar, and dunked within 100.0 mL of deionized water with constant agitation (~ 100 rpm) for 20 min at 75 °C, to optimize the release of contained bioactive compounds. After letting the homogenized solution cool down naturally, the aqueous solution was gently screened through a Whatman filter paper (diam. 45 mm). The pure liquid (supernatant) solution was stored for further purposes. To define the tannin concentration in the supernatant, a calibration curve was drawn applying standard tannic acid at different concentrations by the formerly stated procedure (Tinkílíç, 2001). For experimental testing, 6.0 mL of the supernatant was mixed with 6.0 mL of mixture solution [isopropyl alcohol + water (65 mL:35 mL)] and kept under stirring for 30 min. The U-VIS Spectrophotometer (T70 + UV/VIS) was used to analyze the samples at a wavelength of 540 nm (Tinkílíç, 2001). The concentration of tannin in the extract supernatant was 450 mg L^{-1}. On behalf of the hydrothermal supported green synthesis of IONPs by *E. crassipes*, about 50.0 mL of $FeCl_2.4H_2O$ and $FeCl_3.6H_2O$ (1:2 M ratio) solution was prepared in deionized water and kept under stirring at 75.0 °C for 20 min. Then the iron mixture solution was mixed with 10.0 mL of the supernatant under ~ 100 rpm at 65.0 °C for 5.0 h (Bhuiyan et al., 2020). Then an alkaline solution of 1.0 M NaOH (30 mL) was added to the corresponding solution (e.g., 2 mL min^{-1}) to facilitate the NPs precipitation consistently during the constant rotating system, and buffering the pH to reach 11. The temperature was adjusted at 85 °C for 4 h during the entire period of the experiment. Afterward, the resulting GS-IONPs material was magnetically separated from the aqueous medium using neodymium magnet, and cleansed numerous times with deionized water, methanol, and ethanol, dehydrated for 3 h in an oven at 60 °C, and finally retained for nano-bioremediation application.

2.2. Characterization of GS-IONPs

The prepared GS-IONPs were characterized using FTIR analysis, X-ray diffraction analysis (XRD), Vibrating Sample Magnetometer (VSM), A Nano Zeta Sizer, and the transmission electron microscope (TEM). The detailed information about the utilized characterization techniques and equipment model are presented in the supplementary information (Section S1, see Supplementary Information).

2.3. Bacterial samples collection

A real sample of fresh bacterial inoculum was collected from natural gas wastewater treatment unit located in the industrial region, Port Said, Egypt. The fresh sample was obtained and stored through an icebox vessel. The trial was preserved in the fridge at 5 $^{\circ}$C to evade any deviations in the bacterial assemblages, and then transported to Environmental Science Department laboratory, Port Said University, Egypt for examination. Crude oil (real effluent) sample was assembled from a tailing pond of natural gas facility, Port Said, Egypt. The physicochemical parameters of the collected sample including biological oxygen demand (BOD), chemical oxygen demand (COD), total organic carbon (TOC) were monitored using the different standard analytical protocols (Section S2, see Supplementary Information).

2.4. Isolation and identification of bacterial microorganisms via 16S rRNA

Ten-fold in sequence diluted natural gas wastewater was spread onto a nutrient agar medium by means of appropriate dilution factors (10^7) (Osunla et al., 2021). Pure bacterial isolates were attained subsequently sub-culturing on a similar agar plate (Olaniyi et al., 2019). The obtained bacterial isolates were incubated at 30 $^{\circ}$C overnight. The 16S rRNA gene identification technique was completed intended for the eleven isolated bacterial species identification. The gene investigation was established by sigma company laboratory, Egypt (Salama et al., 2021). To obtain the maximum prolonged possible high-quality sequences, forward and reverse reads similar to each bacterial strain were gathered consuming Phrap (version 0.990329) with evasion parameters. Then, the collected structures were cut such that some basis with a valuable record of less than 30 (99.9% base call precision) was eliminated. The PCR result sequence study was accomplished by the software of Finch TV which completed the 16S rRNA genetic material of the nucleotide building. BLAST (Basic Local Alignment Search Tool) operational record that refers to NCBI (National Center for Biotechnology Information) was applied to describe the successes of the subject's sequences placed in the global nucleotide databases (Gen Bank), identifying the utmost identical with the query sequence (Kamil Jabbar Al Kaabi and Salman Al-Yassari, 2019).

2.5. Bioremediation experimental setup

2.5.1. Influence of some environmental factors on the microbial growth

To examine bacterial growth under different environmental conditions. one mL of the bacterial consortium (11 bacterial isolates) was inoculated inside 25 mL of nutrient broth medium and incubated at 37 $^{\circ}$C for 24 h (Oyewole et al., 2019). The Influence of three medium pH values (e.g., 6.0, 7.0, and 8.0), and three temperature degrees (e.g., 27, 37, 47 $^{\circ}$ C) were inspected. The solution pH was adjusted before sterilization step using HCl (0.5 M), and NaOH (0.5 M) to maintain pH values around 6.0, 7.0, and 8.0, respectively. The microbial growth was measured using Jenway model 6800 spectrophotometer at wavelength of 620 nm. Tests had been carried out in triplicate and the averages had been recorded.

2.5.2. Influence of different concentrations of GS-IONPs on the bacterial consortium growth enhancement and BOD, COD, and TOC reduction

To examine the influence of GS-IONPs on the microbial activity, different quantities of the prepared GS-IONPs (e.g., 10, 20, 30, 40, 50, and 60 mg) were dissolved in 50 mL of mineral salt medium containing [K_2HPO_4 (1.8 g), KH_2PO_4 (1.2 g), NH_4CL (4.0 g), $NaCl_2$ (0.1 g),

$FeSO_4.7H_2O$ (0.01 g) + 1.0 L distilled water] under optimized conditions of pH 7, and temperature of 37 °C. 10 mL of crude oil (real effluent) was introduced into flask (250 mL) containing 90 mL of mineral salt medium with the different GS-IONPs quantities, and autoclaved at 121 °C for 20 min. Afterwards, 1 mL of bacterium consortium was gently introduced into the flasks, incubated in a shaker incubator (150 rpm) at 37 °C for at different intervals times ranging from 3 to 21 days (Ehis-Eriakha et al., 2020; Oyewole et al., 2019). The bacterial growth was measured spectrophotometrically (Jenway's Model 6800) at a wavelength of 620 (Akinduti et al., 2019).

2.6. Statistical analysis

The whole experiments were performed in triplicate. Statistical Package for the Social Sciences (SPSS version 19. IBM Corporation) was employed to evaluate the obtained data.

3. Results and discussion

3.1. Characterization of produced GS-IONPs

As reported previously (Salama et al., 2022b), The FTIR spectra of GS-IONPs showed Fe_3O_4 identification peaks at 587 cm^{-1}, furthermore XRD pattern diffraction peaks of GS-IONPs planes obtained are aligned with standard JCPDS file no. 85-14366. The magnetic properties of GS-IONPs are displayed in the symmetric hysteresis loop, and their saturation magnetization abilities are determined to be 53.65 emu g^{-1}. TEM photomicrograph of the prepared GS-IONPs is given in Figure 1. The images showed that the particles get more or less spherical to elliptical shape with relatively uniform size. The images also showed that the average size was approximately 5 nm, while the results of Differential Light Scattering (DLS) showed that GS-IONPs are nanometer-size with an average value of 15.21 nm which is slightly higher than the observed in the TEM pictures. This result confirmed that selecting *E. crassipes* extract as a reducing and capping agent for GS-IONPs synthesis was successful. The Zeta potential value of the prepared Fe_3O_4 NPs was -26 mV confirming its negative surface charge (Section S3, see Supplementary Information).

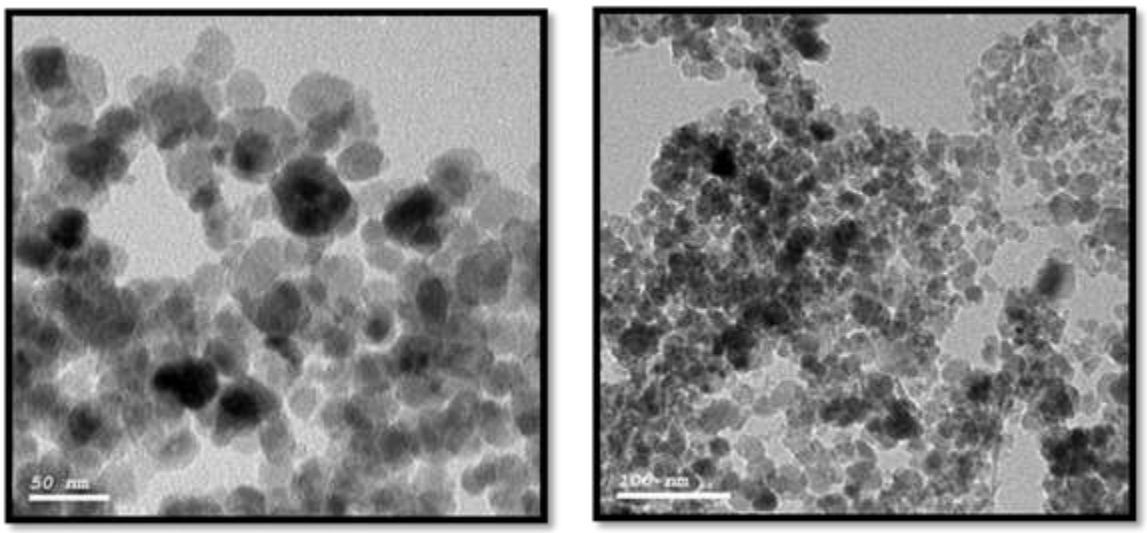


Figure 1. TEM photomicrograph of GS-IONPs capped with *E. crassipes* extract.

3.2. Isolation and identification of bacterial isolates

After identification via morphology and microscopy properties of bacterial isolates, the phylogenetic analysis of eleven different bacterial strains was successfully performed. The results of nucleotides sets were checked and confirmed by using (NCBI) Basic Local Alignment Search Tool (BLAST analysis) nucleotide blast Search a nucleotide database using a nucleotide query online as displayed in Table 1 and Figure 2. Sequences alignment was done by using references 16srRNA gene of different world bacterial species sequences databases information recorded in Gene Bank to find identity and similarity score degrees of 16srRNA gene and compared with our bacterial isolates. The results showed great identity, highly query cover, max score, and a total score while zero for E-value with other world bacterial species (Kamil Jabbar Al Kaabi and Salman Al-Yassari, 2019). Referring to the literature, the isolated bacterial species in the present study have been demonstrated to exhibit a beneficial contribution in the remediation process of collected crude oil sample as illustrated in Section 3.3.

Table 1. Bacterial isolates 16S sequence information.

Sequence association description	Sequence association No.	Genome size (bp)	Identity	Associated NCBI tax ID
Staphylococcus hominis **strain DM 122 16S ribosomal RNA, partial sequence**	NR_ 036956.1	1544	99.85%	1290
Bacillus subtilis **strain SBMP4 16S ribosomal RNA, partial sequence**	NR_ 118383.1	1463	85.14%	1423
Bacillus safensis **strain NBRC 100820 16S ribosomal RNA, partial sequence**	NR_113945.1	1474	99.79%	561879
Staphylococcus xylosus **strain KL 162 16S ribosomal RNA, partial sequence**	NR_036907.1	1477	99.57%	1288
Staphylococcus epidermidis **strain NBRC 100911 16S ribosomal RNA, partial sequence**	NR_ 113957.1	1476	99.54%	1282
Staphylococcus haemolyticus **strain JCM 2416 16S ribosomal RNA, partial sequence**	NR_113345.1	1433	99.88%	1283
Bacillus infantis **strain SMC 4352-1 16S ribosomal RNA, partial sequence**	NR_ 043567.1	1367	97.33%	324767
Shewanella fodinae **strain NBRC 105216 16S ribosomal RNA, partial sequence**	NR_ 114281.1	1468	99.56%	552357
Mangrovibacter phragmitis **strain MP23 16S ribosomal RNA, partial sequence**	NR_ 156930.1	1368	96.94%	1691903
Mangrovibacter plantisponsor **strain MSSRF40 16S ribosomal RNA, partial sequence**	NR_ 116079.1	1368	99.97%	451513
Agrobacterium fabrum **strain C58 16S ribosomal RNA, partial sequence**	NR_ 074266.1	1480	92.60%	1176649

Figure 2. (a) The phylogenetic tree of *Staphylococcus hominis* strain DM 122 16S ribosomal RNA (NR_036956.1) (Strain1) (b) The phylogenetic tree of *Bacillus subtilis* strain SBMP4 16S ribosomal RNA (NR_118383.1) (Strain2) (c) The phylogenetic tree of *Bacillus safensis* strain NBRC 100820 16S ribosomal RNA (NR_113945.0) (Strain3 (d) The phylogenetic tree of *Staphylococcus xylosus* strain KL 162 16S ribosomal RNA (NR_036907.1) (Strain4) (e) The phylogenetic tree of *Staphylococcus epidermidis* strain NBRC 100911 16S ribosomal RNA (NR_113957.1) (Strain5) (f) The phylogenetic tree of *Staphylococcus haemolyticus* strain JCM 2416 16S ribosomal RNA (NR_113345.1) (Strain6) (g) The phylogenetic tree of *Bacillus infantis* strain SMC 4352-1 16S ribosomal RNA (NR_043267.1) (Strain7) (h) The phylogenetic tree of *Shewanella fodinae* strain NBRC 105216 16S ribosomal RNA (NR_114281.1) (Strain8) (i) The phylogenetic tree of *Mangrovibacter phragmitis* strain MP 23 16S ribosomal RNA (NR_156930.1) (Strain9) (j) The phylogenetic tree of *Mangrovibacter plantisponsor* strain MSSRF40 16S ribosomal RNA (NR_116079.1) (Strain10) and (k) The phylogenetic tree of *Agrobacterium fabrum* strain C58 16S ribosomal RNA (NR_074266.1) (Strain11).

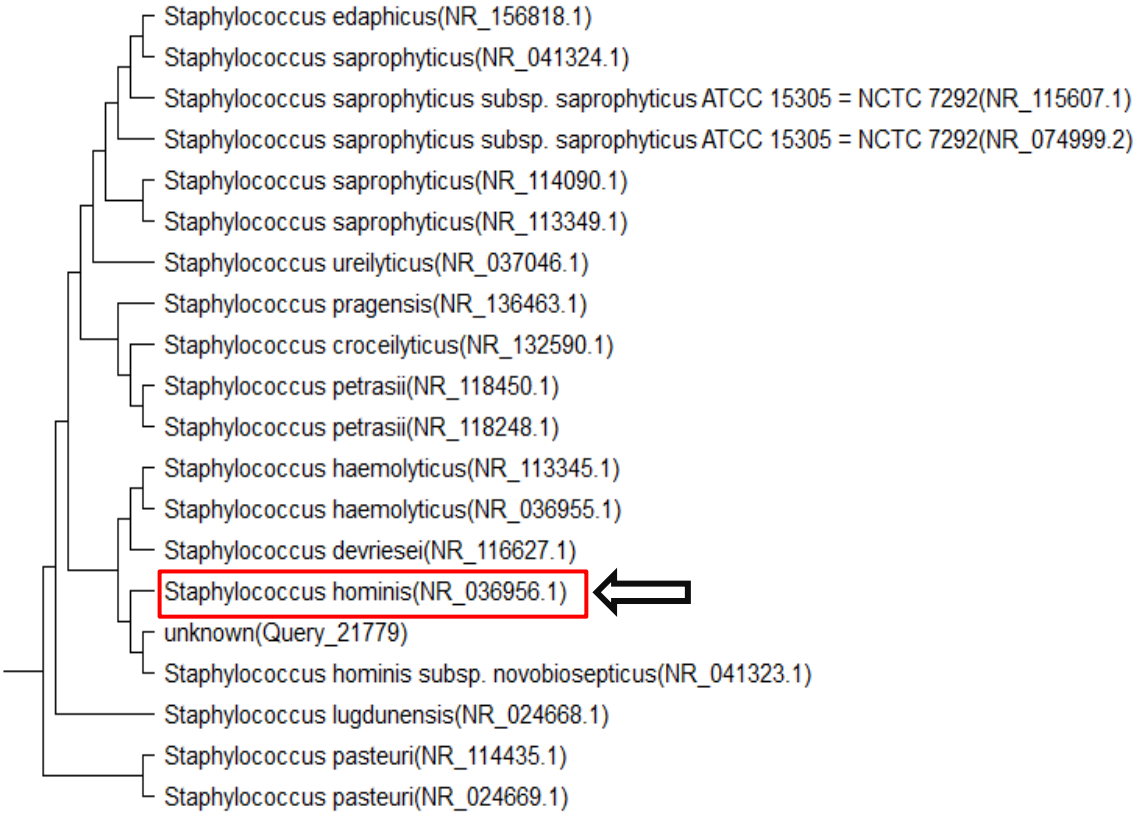
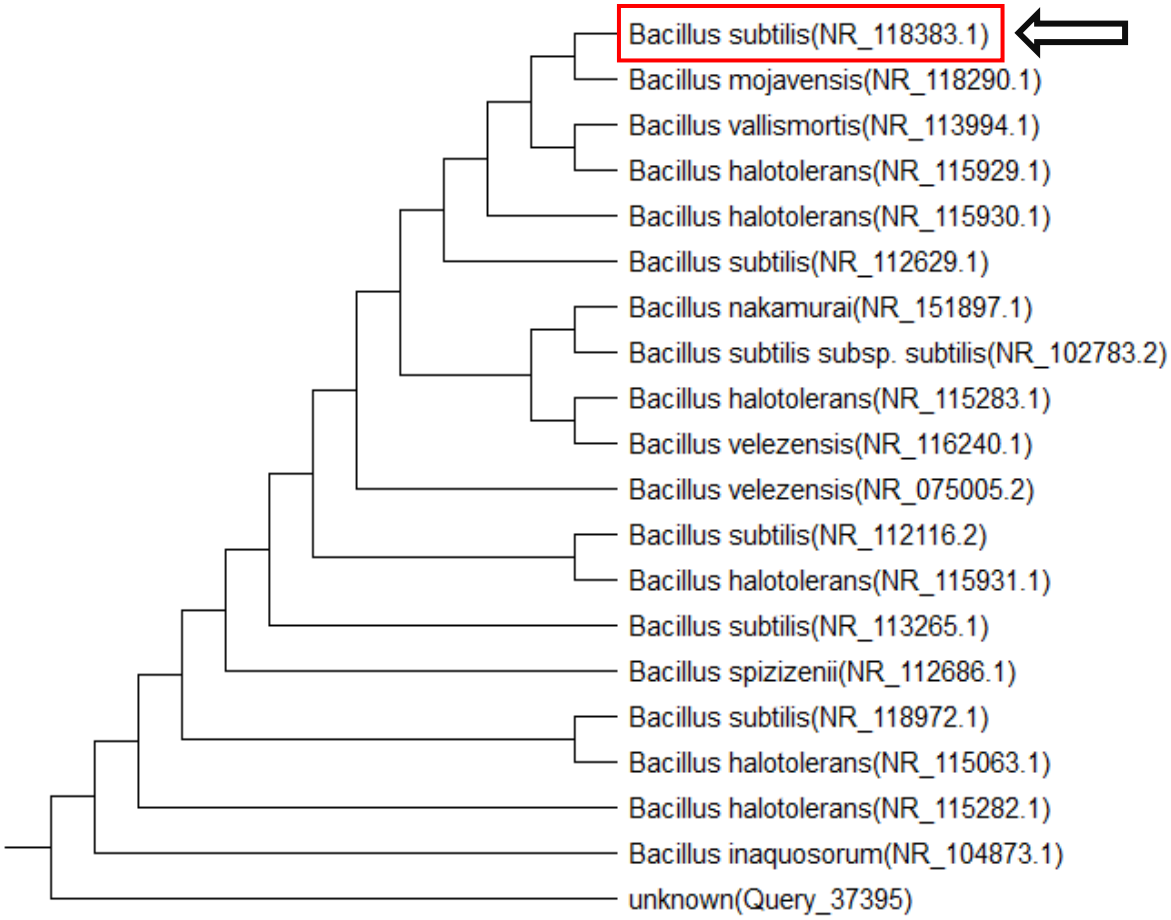


Figure 2(a)



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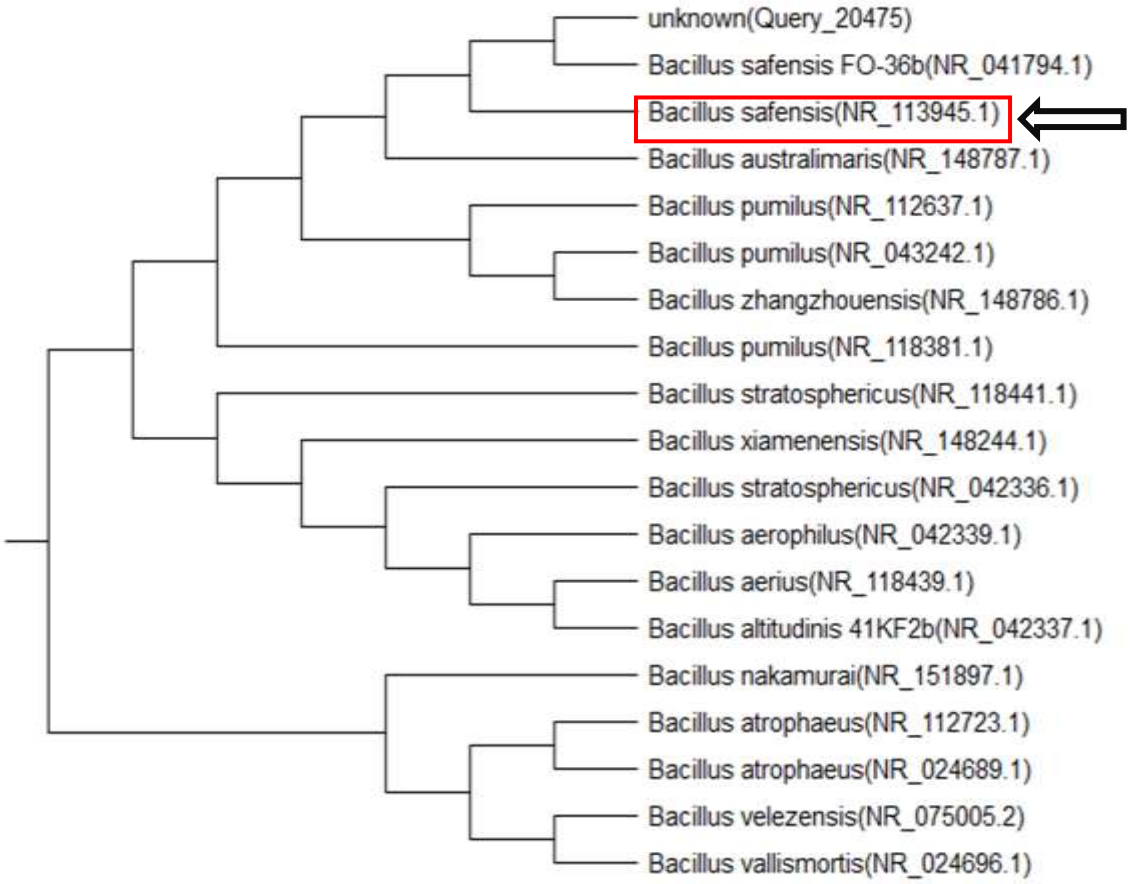

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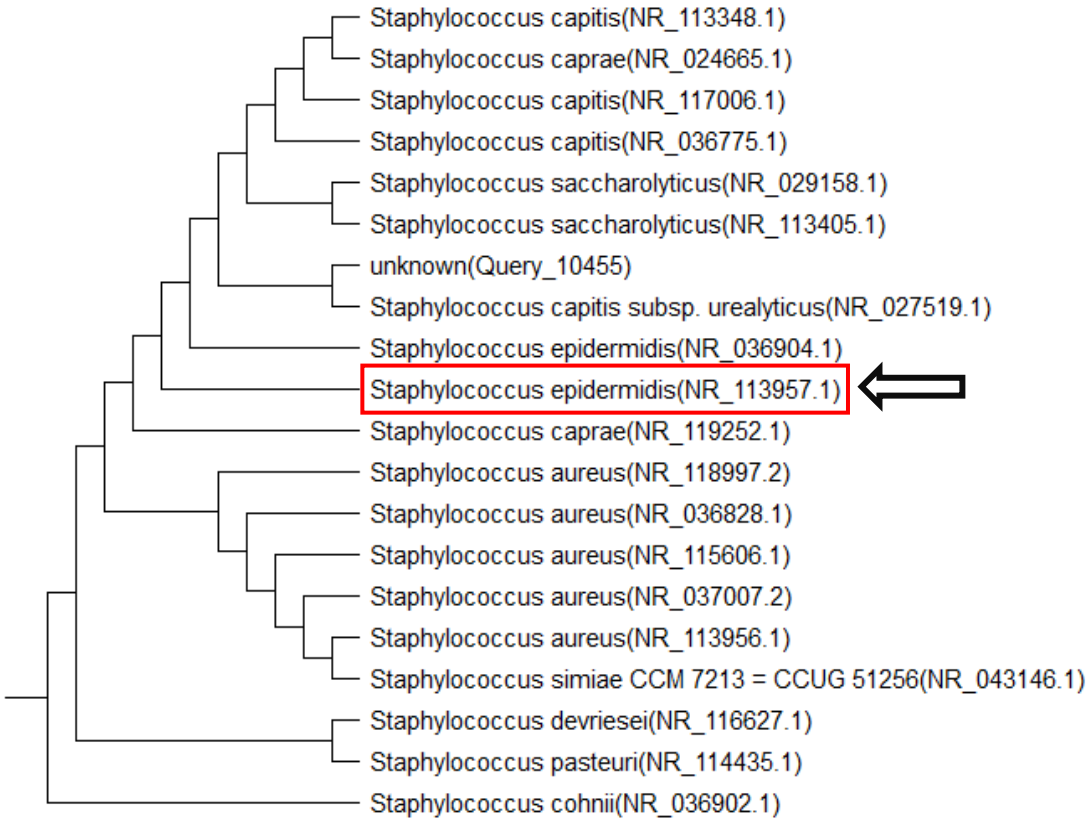
Figure 2(d) (continued)



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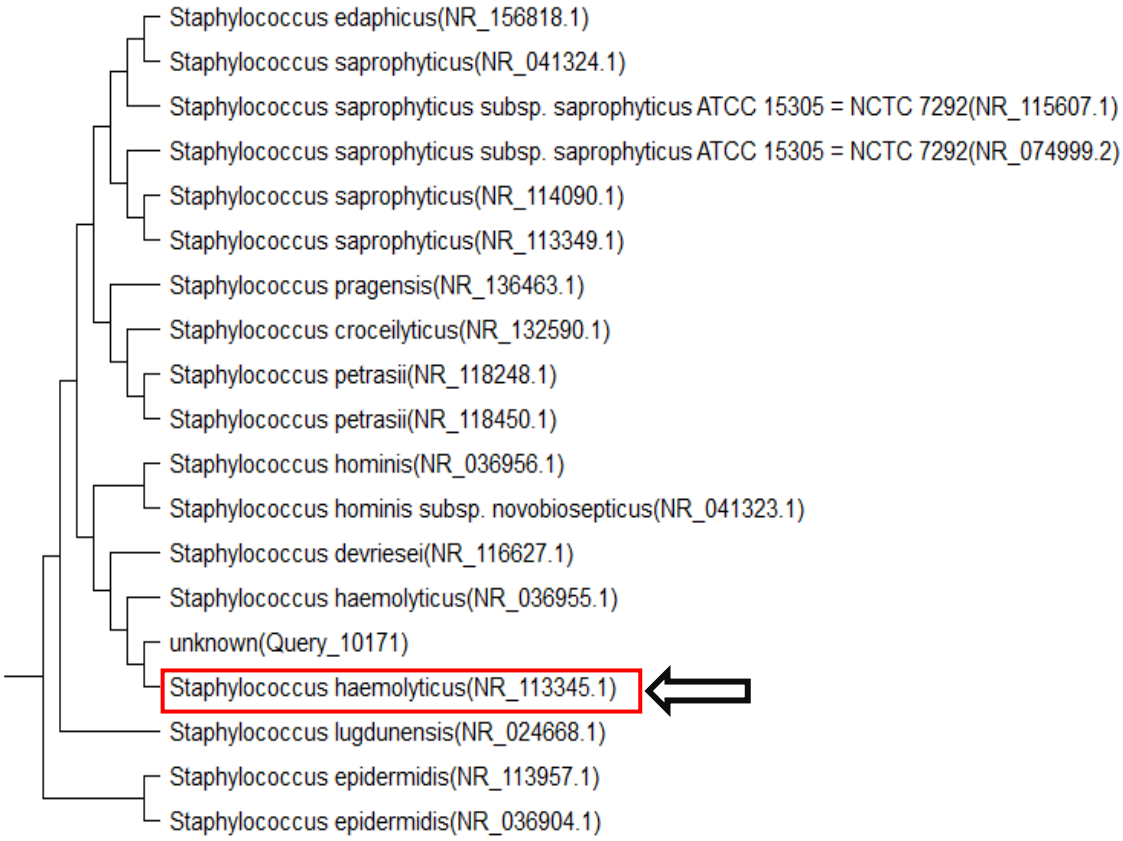

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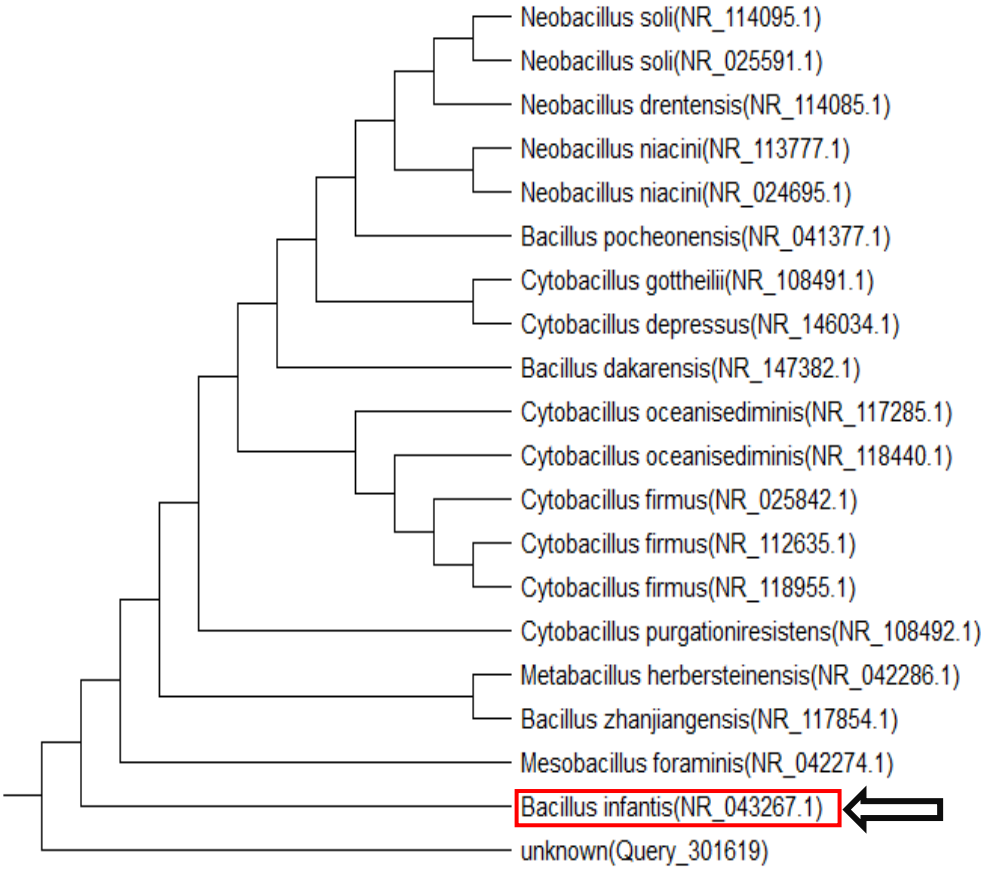

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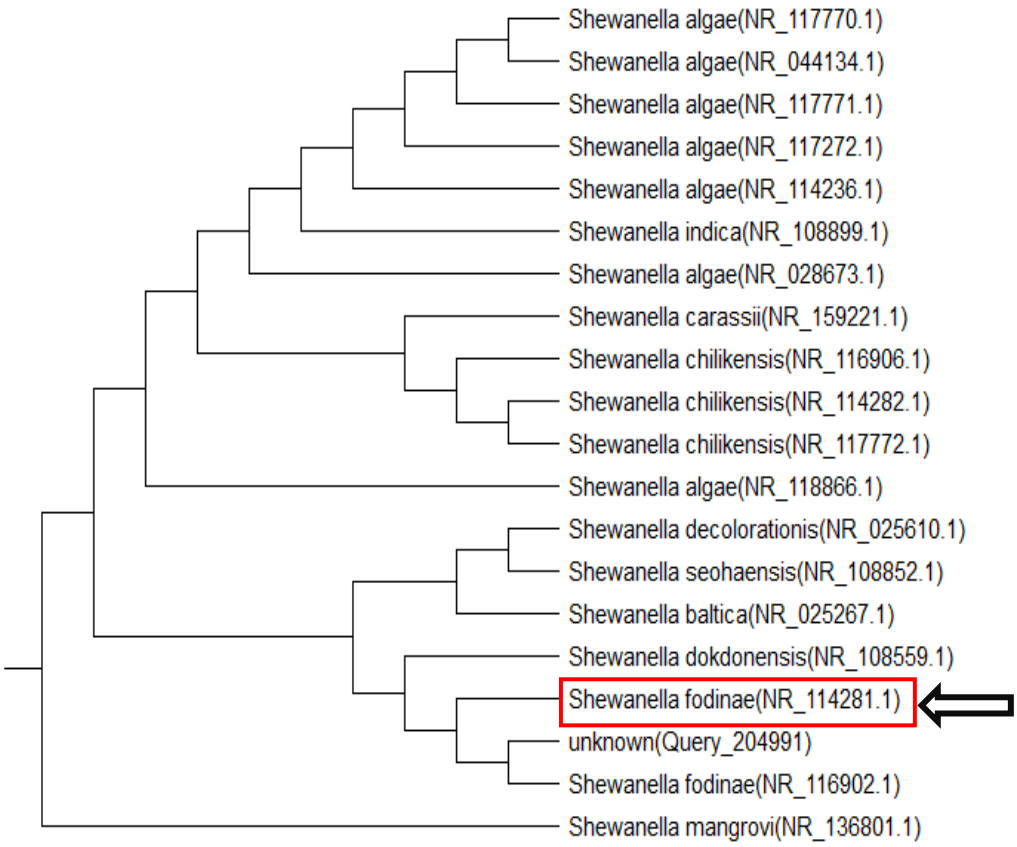

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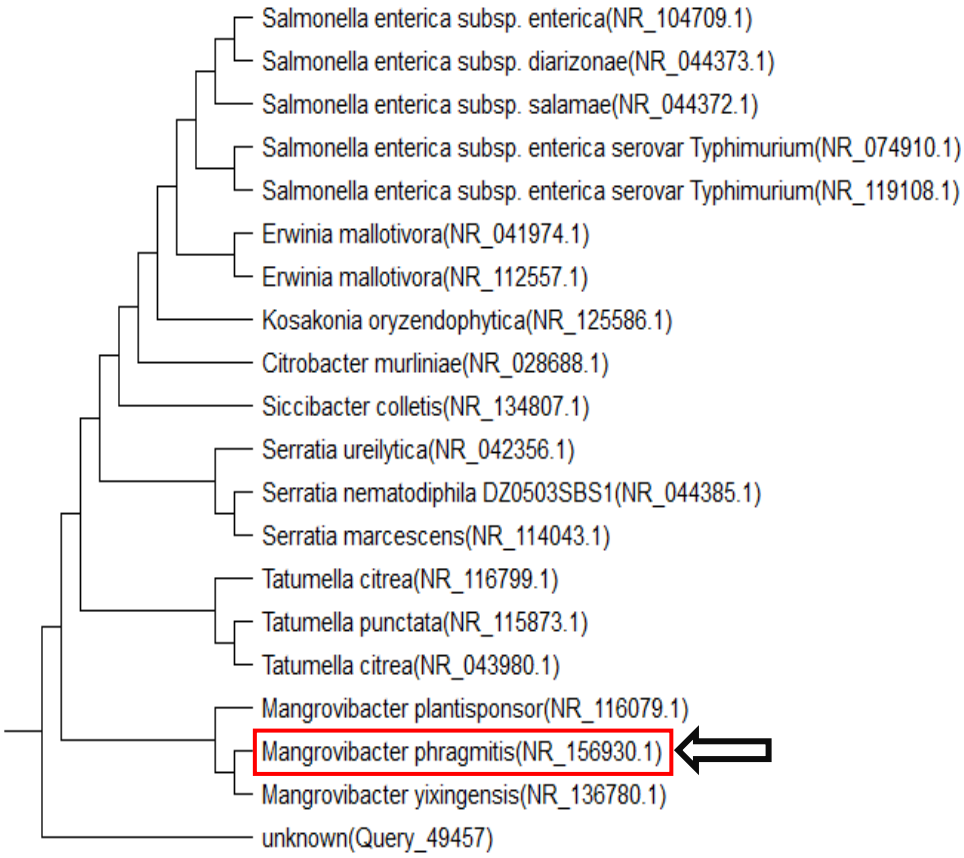
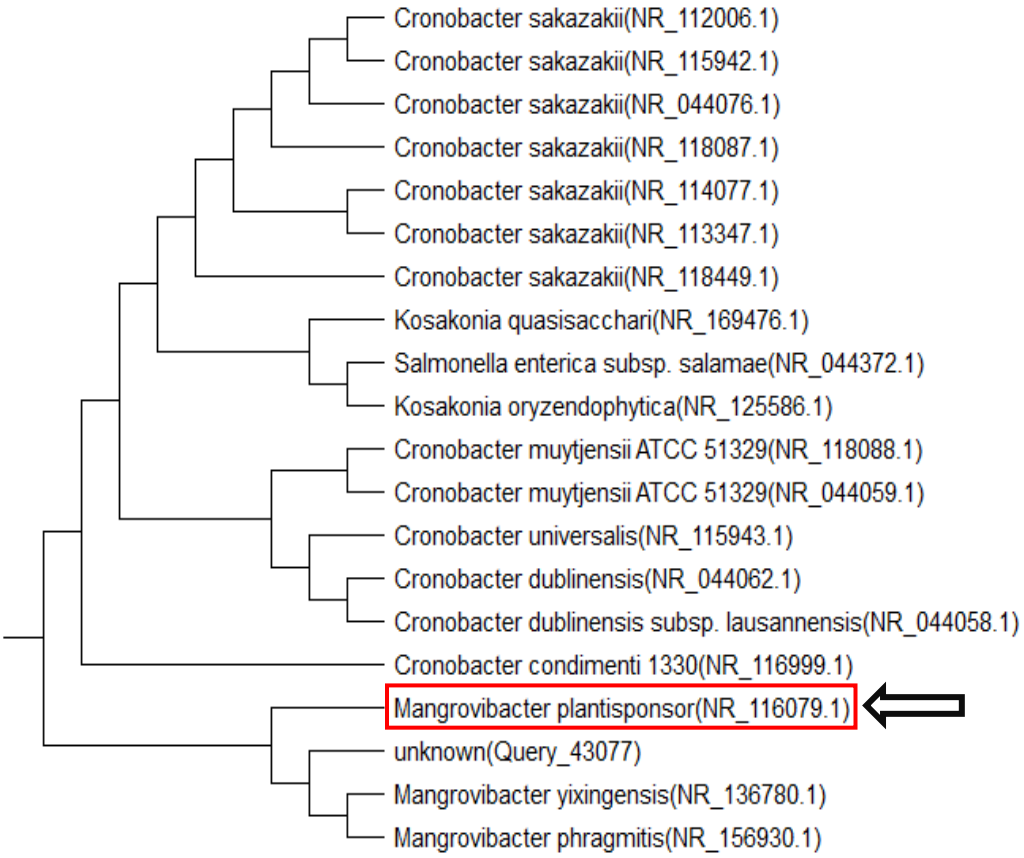


Figure 2(i) (continued)



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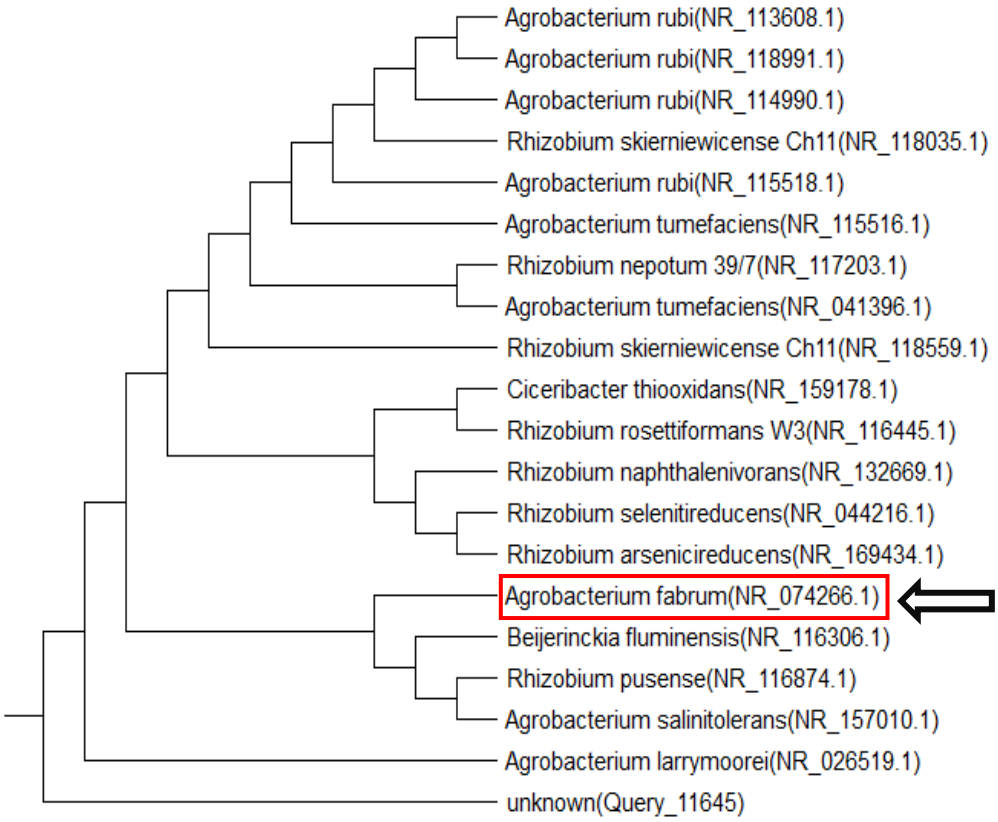

Figure 2(k) (continued)

3.3. Role of isolated bacterial species in the crude oil bioremediation process

To assess the inclusive performance advantage of the isolated bacterial microbiome species, a comprehensive survey was particularly researched to reveal the contribution of various species in the crude oil bioremediation studies. Satisfactory findings have been reported through biodegradation of diesel oil in contaminated soil. Two main bacterial species of; *Staphylococcus hominis and Kocuria palustris* were isolated, identified, and introduced to speed up the bioremediation of diesel oil. The results revealed that the bioremediation technique improved the treatment step of the polluted soil via biostimulation and bioaugmentation, and the treatment with nutritional enrichment had the greatest outcomes. Regarding the respirometry data, the isolated species have doubled the biodegradation efficiency of total petroleum hydrocarbons content up to 45.5 % within 55 days of treatment (Mariano et al., 2007). Similarly, Ehis-Eriakha et al. (2020) announced that *Staphylococcus hominis* could serve as a potential hydrocarbon bioremediation agent (Ehis-Eriakha et al., 2020). Wang et al. (2019) stated that a hydrocarbon-degrading bacterium of *Bacillus subtilis* BL-27 was separated from hydrocarbons polluted areas. Under the best experimental conditions (e.g., pH = 7, NaCl concentration = 10 g/L, and temperature = 45 $^{\circ}$C), the isolated strain effectively degraded 65 % of crude oil within 5 days with preferentially destroying of long-chain alkanes. *The Bacillus subtilis* BL-27 can be regarded as a promising bio remediating material to be employed through the petrochemical industry (Wang et al., 2019). Hanano et al. (2017) illustrated the biosynthesis process of efficacious biosurfactant from petroleum crude oil dwelling microorganism (*Bacillus safensis PHA3*). Their findings clarified that *Bacillus safensis PHA3* was a suitable producer of biosurfactant (e.g., 9.8 ± 0.5 mg mL^{-1}). Moreover, 2.5 folds improvement in the degradation of petroleum crude oil was successfully achieved with more adding of purified glycolipid (e.g., 0.15%). The optimized biosurfactant emulsifying activity of biosurfactant towards crude oil was reported at medium pH = 8, temperature range = 30 – 60 °C, pH 8, and a high saline environment, whilst the lowest activity was found against naphthalene. Attributing to the bacterial species' characteristics, *Bacillus safensis* is viewed as an outstanding model for upgrading the microbial oil recovery (Hanano et al., 2017).

Ziagova et al. (2010) isolated two bacterial species of; *Pseudomonas sp. and Staphylococcus xylosus and* compared their degradation adequacy towards three aromatic compounds (e.g., 1, 2-dichlorobenzene, 2, 4-dichlorophenol, and 4-Cl-m-cresol). They presented that 1, 2-dichlorobenzene exhibited a better effective concentration (EC50), of 0.84 and 1.04 mM for *S. xylosus* and *Pseudomonas sp*, respectively. Contrarily, the m-cresol displayed a more persistent behavior for bacterial biodegradation. Furthermore, the assessment of the half-saturation constants of 0.26 and 0.36 mM characterized to *S. xylosus* and *Pseudomonas sp*, respectively revealed that 2, 4-DCP was less assimilate than 1, 2-DCB, though bacterial specificity was greater (Ziagova and Liakopoulou-Kyriakides, 2010). Lauprasert et al. (2017) evaluated the bioactivity of the immobilized lipase enzyme derived from selected *Bacillus subtilis, Staphylococcus epidermidis, and Pseudomonas aeruginosa* species on the degradation of fats, oils and grease and wastewater remediation. The experiment's findings were gathered after 5 days of bacterial culture in the wastewater. Results indicated that lipase enzyme activity in oily wastewater utilizing continuous pathway shown more effectiveness than batch strategy. The lipase enzyme activities were 559.95-, 579.95-, and 819.92 unit ml^{-1} for *S. epidermidis*, *B. subtilis*, and *P. aeruginosa*, respectively. Moreover, the BOD treatment efficacy was found to be

60.72 %, 63.58 %, and 64.51 %, for *P. aeruginosa*, *S. epidermidis*, and *B. subtilis*, respectively (Jenkins and Bean, 2020; Lauprasert et al., 2017).

Tanzadeh et al. (2020) (Tanzadeh et al., 2020) isolated 115 bacterial strains across the shorelines of the Caspian Sea. Among them, three bacterial species of *Bacillus cereus, Staphylococcus haemolyticus, and Pseudomonas aeruginosa* displayed the best competence for removing crude oil. They announced that the *Bacillus cereus* was more able to break down short and medium n-alkanes chains rather than long ones in crude oil residues. The depression in the total petroleum hydrocarbons content were 88.8 mg g^{-1} (60 %), 113.2 mg g^{-1} (50 %), and 123.6 mg g^{-1} (55 %) for *B. cereus*, *P. aeruginosa*m and *S. haemolyticus*, respectively. The total petroleum hydrocarbons content decreased within consortia inoculating with the three species by 18.3 (mg g^{-1}), 83 % of crude oil residues (1%, v/v) during 22 days of treatment (Tanzadeh et al., 2020).

Dealtry et al. (2018) scanned the bacterial community of petroleum hydrocarbon degrading consortium inoculated on mangrove tree *Avicennia schaueriana* for decontamination of artificial petroleum medium. They considered the bacterial 16S rRNA gene partial sequence and analyzed for the determination of incompatible plasmids as well as naphthalene dioxygenase genes via dot blot hybridization. They expounded the prevalence of *Bacillus infantis* after 10 days on incubation, with other bacterial species assigned to *Pseudomonas sp.*, *Ochrobactrum sp*, and etc (Dealtry et al., 2018). Joe et al. (2019) (Joe et al., 2019) isolated, identified a rhamnolipid-producing bacterial species (e.g., *Shewanella sp. BS4*) from the contaminated soil, and trialed it for hydrocarbon decontamination approach through a new developed treatment system (pilot scale) simultaneously containing rhamnolipid, along with *Shewanella sp. BS4*. They revealed that the designed system increased the hydrocarbon-degrading bacteria population, boosted their microbial activity, promoted the soil respiration activity, and upgraded the treatment of contaminated soil. The proposed system resulted in 75.8 % hydrocarbon removal efficacy, which was greater than the non-treated soil (Joe et al., 2019).

3.4. Optimized conditions for the growth of isolated bacterial consortium

The growth of isolated bacterial consortium under different environmental factors of medium pH (e.g., 6, 7, and 8), and temperature (e.g., 27, 37, 47 ^{o}C) was regularly evaluated. A considerable (optimized) microbial consortium growth was achieved at pH of 7.0 and running temperature of 37 ^{0}C. The absorbance pattern values of microbial growth media at wavelength 620 nm among different environmental factors were illustrated in Tables S1& S2 (see Supplementary Information). Our experimental outcomes are in accordance with other studies from the literature, indicating that pH range from 5 to 8 and a temperature range from 30 to 40 °C for bacterial bioremediation are the key factors that present the best experimental conditions for the greatest hydrocarbon bioremediation by enhancing the bacterial activities and degradative enzymes. In addition, the formation of biofilm in hydrocarbon-polluted environments may increase the microbial adaptation to the low bioavailability of hydrophobic compounds, and genes that encode for hydrocarbon degradative enzymes, which are paramount for microbes to bioremediate the environment contaminated with hydrocarbon pollutants.

3.5. Influence of GS-IONPs on bacterial culture growth

Numerous studies demonstrated that NPs might simultaneously have synergism/antagonism effects on the microbial growth. The bacterial cell walls may be physically destroyed by the

effect of nanoparticles, or by oxidative stress, in which the generation of reactive oxygen species can be simulated, and thence provides the NPs with their antibacterial features. In contrast, NPs can inhibit the development of bacteria, possibly because of their high specific surface area and ability to release electrons. The generated electrons by the effect of NPs may improve the enzymatic activity of proteins in the external membrane, in which the electron transport chain may be accelerated, and aid in the bacterial cell metabolism (Liu et al., 2013).

The effect of GS-IONPs concentrations ranging from 10 to 60 mg on the growth of bacterial consortium is presented Figure 3. Compared to the control sample, the enhancing-growth effect of GS-IONPs on bacterial consortium was directly proportional to the minuscule concentrations of GS-IONPs up to 40 mg over different intervals times (e.g., from 3 to 21 days). The detectable growth in bacterial cell density with lower concentrations (e.g., < 40 mg) of GS-IONPs may be consistent with the microbial adaptation with the presence of GS-IONPs. However, greater concentrations of GS-IONPs (e.g., > 40 mg) negatively inhibited the growth of bacterial consortium may particularly be due to the GS-IONPs stress (overloading) on them, with definite agglomeration, promoting the toxic effects on the bacterial community (Tables S3 & S4, see Supplementary Information). Moreover, the bacterial growth inhibition may assume to be caused by reactive oxygen species (e.g., O^{2-}, OH^{-}, and $_1O^2$) produced by the IONPs (Sies, 1997). The generation of reactive oxygen species has been demonstrated in a wide variety of NPs, which may cause oxidative stress, inflammation, and subsequent damage to proteins, membranes, and DNA, regarding as one of major causes of nanotoxicity (Chatterjee et al., 2011).

Bhatia et al. (2013) (Bhatia et al., 2013) suggested biochemically that the progress in the microbial population in the presence of NPS might be attributed to shortening in the lag phase and lengthening the exponential and stationary phases. Kiran et al. (2014) reported a remarkable promotion of *Nocardiopsis MSA13A* growth, associated with noticeable improvement in the microbial activity in the presence of iron NPS (e.g., 10 mg L^{-1}), which may be referred to the microbe's capacity to get energy necessary for development via the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) (Kiran et al., 2014). Besides, the produced biosurfactants by the action of microbes may increase the bioavailability of hydrophobic compounds to microorganisms via lowering their surface tension, and consequently improve the bioremediation process (Cameotra and Makkar, 2010). Moreover, iron atom is commonly regarded as one of the essential metal ions for producing biosurfactants in a variety of bacteria (Haferburg and Kothe, 2007). Due to its low toxicity and intrinsic biocompatibility, microbes often use it in various essential metabolic processes (Liu et al., 2013). Meanwhile, iron nanoparticles are recognized as enzyme activators, such as *isocitrate lyase*, which are employed by microbial cells to grow on hydrophobic substrates, incorporate acetyl-CoA into C4 compounds, and/or produce biosurfactants (Hommel, 1993; Ramesh et al., 2021).

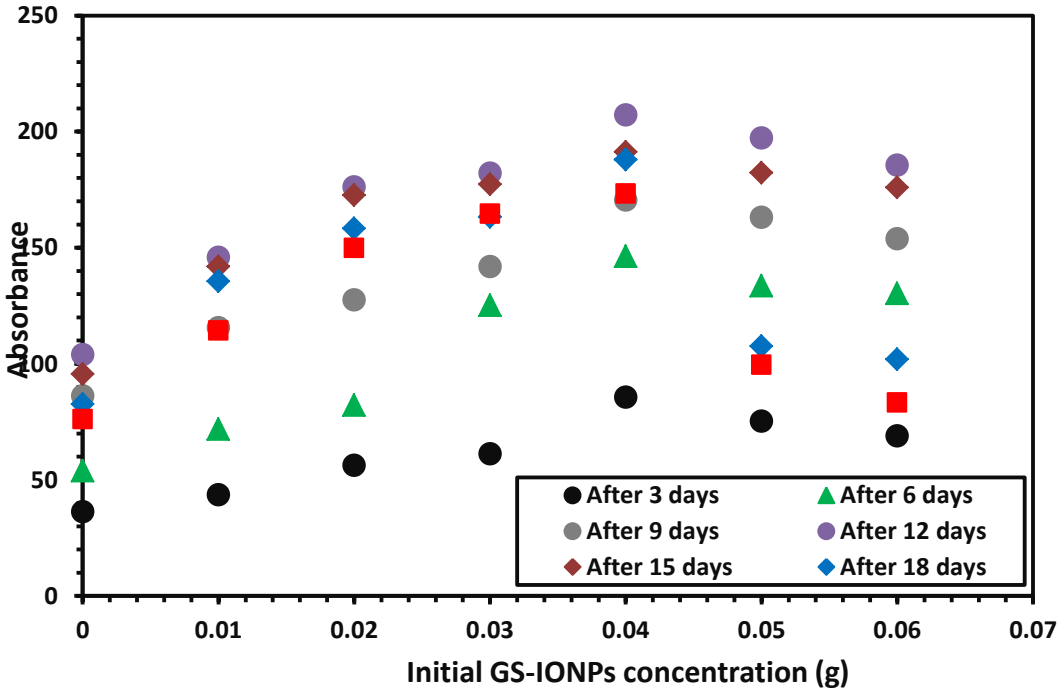

Figure 3. Bacterial growth media absorbance at wavelength 620.0 nm using different concentrations of GS-IONPs (mg) at different time intervals.

3.5. Influence of GS-IONPs on BOD, COD, and TOC mitigation (nano-bioremediation)

The bioremediation properties of isolated bacterial consortium were inspected to visualize the relative decline in the BOD, COD, and TOC content. The bacterial role dynamics on the bioremediation of crude oil was studied under various concentrations of GS-IONPs (e.g., from 10 to 60 mg). As displayed in Figure 4, an apparent decrease in the BOD concentration (mg L^{-1}) with an enhancement in the GS-IONPs concentration from 10 to 40 mg was noticed. Compared with the reported remediation efficiency of GS-IONPs-free sample, the BOD reduction efficiency improved by 77.17 %, which is closely correlated to the supreme bacterial growth, accomplished using 40 mg of GS-IONPs at the temperature of 37 °C, and pH 7, after 12 days of incubation. This may be expounded by an increment in the total freely available GS-IONPs, which in turn increased the interaction between GS-IONPs and microbial system and improved the wastewater reclamation process (Elgarahy et al., 2021; Gabrielyan et al., 2019). On the contrary, the higher concentration of GS-IONPs (e.g., 50 and 60 mg) showed lower bioremediation efficiency. This may be assigned to the inadequate interface between GS-IONPs and bacterial system, in addition to substantial inhibition in the biosurfactant production (Salama et al., 2022a).

Moreover, the microbial enzymes may play a decisive role in the bio-degradation of organic pollutants attributing to their biodegradability (Teng et al., 2019). In the case of oxidation processes, the enzymes can obtain electrons from the substrate and distributes them to the electron acceptors. As a result, once the process is accomplished, the enzymes are regenerated and intended for the upcoming catalytic cycle. Substantially, the microbial enzymatic can be conceived as an environmentally beneficial technology for wastewater treatment.

The presence of [Fe-S] and [Fe-Fe] at nitrogenase and hydrogenase receptor sites, respectively, can improve their enzymatic performance via conjunction of Fe^{2+}/ Fe^{3+} within bacterial oxidation/reduction processes. Furthermore, it can accelerate electron transfer rates, hence promoting wastewater purification through BOD reduction, as displayed in Tables 2 & 3 (He et al., 2017).

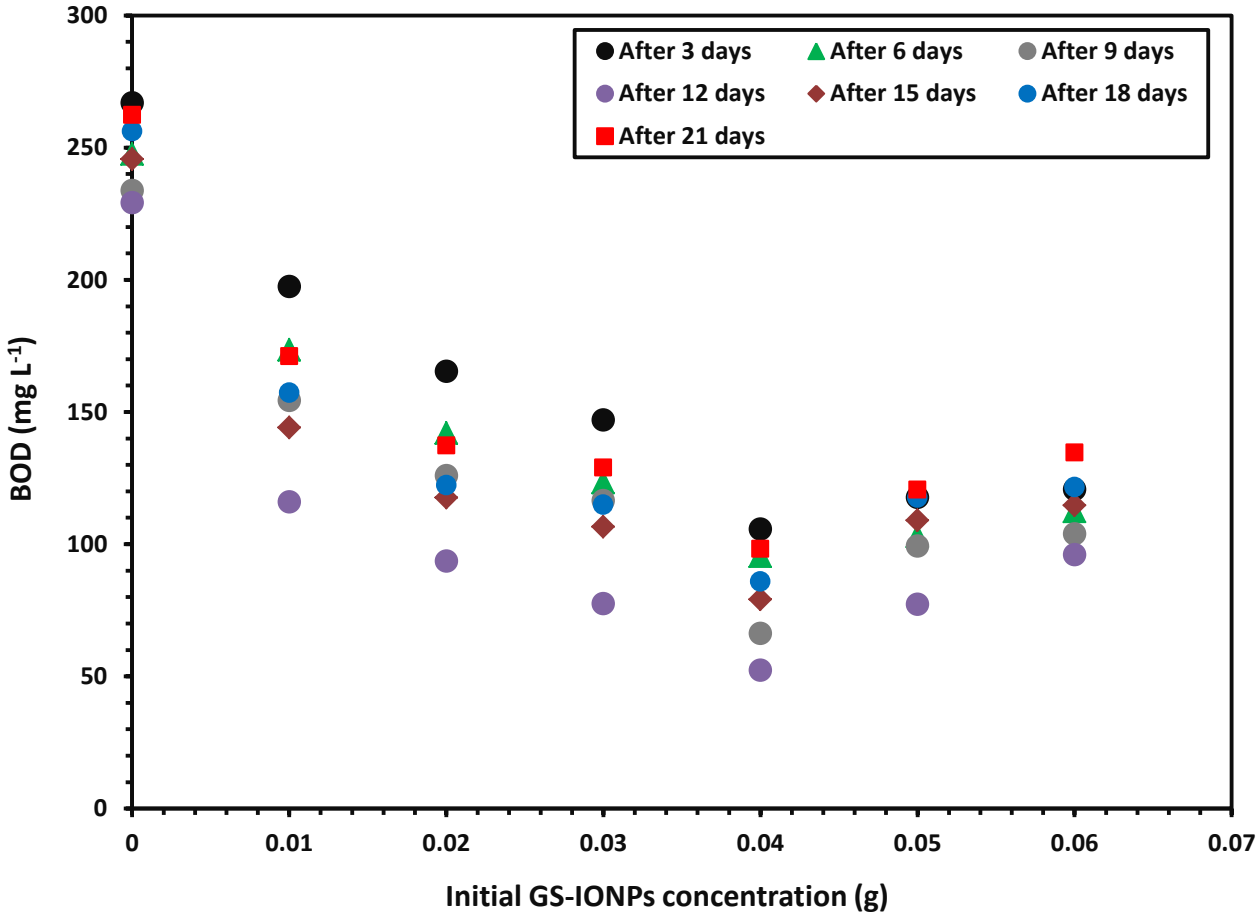

Figure 4. Influence of greenly synthesized GS-IONPs concentration (mg) on BOD remediation pattern.

Likewise, the role of the bacterial consortium, on the remediation of COD content (Figure 5). It was inferred that the best COD diminution was attained using 40 mg of GS-IONPs (e.g., temperature 37 °C, and pH 7) after 12 days of incubation. With an increase in GS-IONPs concentrations, the microbial system actively acted well in COD decrement compared with the control specimen (Tables 4 & 5). The removal of COD (mg L^{-1}) increased by 74.76% of that reported using magnetite-free control sample. The findings were in accord with that of BOD results, which hinted that the presence of GS-IONPs (e.g., 40 mg) in cultures efficiently amended the microbial potency for COD mitigation

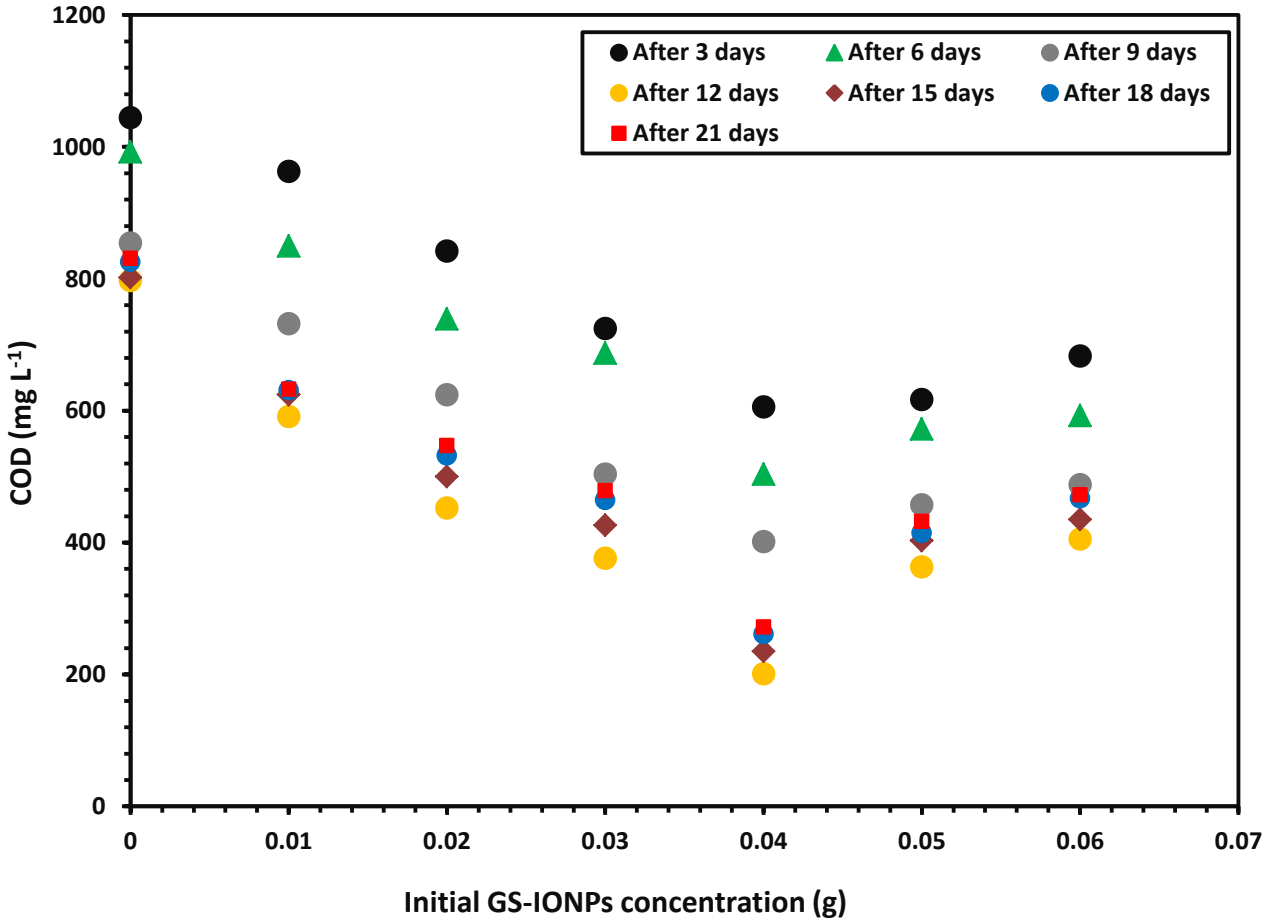

Figure 5. Influence of greenly synthesized GS-IONPs concentration (mg) on COD remediation pattern.

Additionally, TOC measurement is another helpful tool used to monitor the quality of water. In the same manner, TOC concentrations in the aliquot samples treated with various GS-IONPs obviously dropped with distinguished growth in TOC removal efficacy (e.g., 85.44 %) as an augmentation in the GS-IONPs up to 40 mg, whilst further growing in the GS-IONPs concentrations beyond 40 mg adversely effect on the bacterial growth and activity and raised the TOC content (Figure 6) and (Tables 6 & 7).

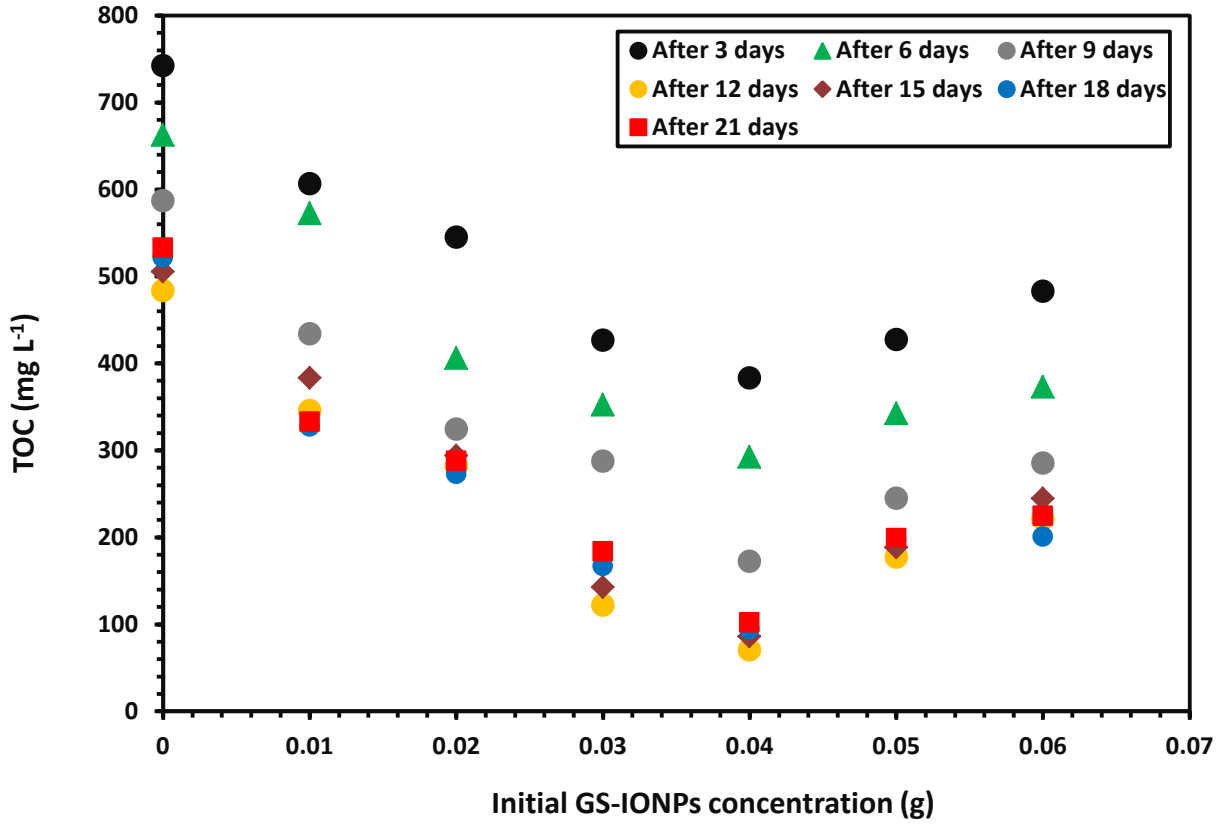

Figure 6. Influence of greenly synthesized GS-IONPs concentration (mg) on TOC remediation pattern.

Bacteria may degrade hydrocarbons and use them as carbon sources sole. Besides, the NPs can be crucial in the bacterial remediation of hydrocarbons pollutants by enhancing microbial growth (Alabresm, 2020; Kumari and Singh, 2016). Therefore, the combination between NPs and hydrocarbon-degrading bacteria could produce an effective alternative to conventional wastewater treatment (Pete et al., 2021). Cerqueira et al. (2011) (Cerqueira et al., 2011) demonstrated that the bacterial consortium exhibited remarkable hydrocarbon degradation capabilities, by decreasing the aromatic and aliphatic fractions by 51.8 % and 90.7 %, respectively. Moreover, the bacterial performance during the production of biosurfactants has resulted in a 39.4% decrease in surface tension in the culture medium and a 55.1% increase in emulsifying activity. The findings indicated that the bacterial consortium has the potential to be employed in the bioremediation of petroleum hydrocarbons-laden wastewater, simultaneously promoting the decontamination of aquatic systems and upgrading industrial productivity (Cerqueira et al., 2011).

Energy plays a significant role in a microbe's capability to degrade hydrocarbon pollutants. Magnetite NPs could facilitate bacterial bioactivity and metabolite because of their physicochemical properties (e.g., surface area and quantum size effects). This enhancement effect may be explained by the fact that iron is slowly released from the iron source, which keeps the proper iron concentration and inhibits high iron concentration, which could be toxic for bacteria.

Proper iron could promote bacteria growth (Bestawy et al., 2020). Moreover, biosurfactants are surface-active biomolecules produced by microorganisms and have diverse applications petroleum biotechnology and environmental bioremediation (Fenibo et al., 2019; Jimoh and Lin, 2019).

Numerous articles have demonstrated the significance of hydrocarbon-degrading bacteria in biosurfactants production. Fazaeli et al. (2020) showed that *Staphylococcus hominis* could produce a sufficient quantity of the biosurfactant (Fazaeli et al., 2020). Parthipan et al. (2017) (Parthipan et al., 2017) reported that the strain *Bacillus subtilis* was shown to be more resilient than other reported biosurfactant generating bacteria in the efficiency of crude oil degradation due to their characteristic capacity for enzymes production. As a result, they may be employed to eliminate the hydrocarbon pollutants from a polluted environment (Parthipan et al., 2017). Kalaimurugan et al. (2022) (Kalaimurugan et al., 2022) demonstarted that *Bacillus safensis* can produce biosurfactants with possible benefits in wastewater remediation and can be used for large-scale processing in biosurfactant industries (Kalaimurugan et al., 2022). Keskin et al. (2015) and Eddouaouda et al. (2011) (Eddouaouda et al., 2012; San Keskin et al., 2015) documented that the green surfactants production was recorded in *Staphylococcus Sp. (Staphylococcus xylosus; Staphylococcus epidermidis)* with the ability to emulsify different hydrocarbon substrates. Lemaire et al. (2020) (Lemaire et al., 2020) displayed that the *Shewanella genus* is essential for many biotechnological applications, mainly the bioremediation of hydrocarbon contaminants. Sharma et al. (2019) (Sharma et al., 2019) study results demonstrated that the biodegradation of hydrocarbon by *Agrobacterium fabrum* in the glucose (co-substrate) presence under the best running conditions (e.g., pH 6.0, and 30.0 $^{\circ}$C). They revealed that the strain produced 5.77 ± 0.3 g L^{-1} of biosurfactant and yeast extract (C: N = 2:1) with high emulsification activity of $65 \pm 0.5\%$ (Sharma et al., 2019).

The magnetite NPs improvement in the production of biosurfactants by microorganisms for hydrocarbon-contaminated environments leads to enhancements at all levels, including performance, cost-effectiveness, and environmental compatibility (Dhanya, 2021; Liu et al., 2013). Iron NPs enhanced the expression of genes involved in the synthesis of biosurfactants in *Bacillus Sp*. (e.g., *Bacillus subtilis*) by increasing the cell membranes permeability, resulting in a more efficient surfactin secretion (Yang et al., 2020).

Diverse commercial biosurfactants and laboratory-formed samples have been examined as stabilizers for the metallic nanoparticles. It was observed that biosurfactants produced by microorganisms could play an incredibly significant role in nanoparticles stabilization. Therefore, Biosurfactant use has emerged as a green alternative for enhancing nanoparticles stabilization. The mode of action is through adsorbing onto metallic nanoparticles, surface stabilizing the nanoparticles, and avoiding consequent aggregation. Treated oily wastewater with iron NPs was feasible for reusing in other processes or reinjection in the empty fields (Mohammed and Al-Zuheri, 2020).

Table 2. Average (±SD) of replicates tests of bacterial growth media on biochemical oxygen demand (BOD) content, using different concentrations of GS-IONPs (mg) at different time intervals.

Time (days)	**GS-IONPs primary concentration (mg)**						
	Control	**10**	**20**	**30**	**40**	**50**	**60**
3.0	267.02 ± 0.58	197.52±1.81	165.39± 0.77	147.08± 2.00	105.80± 1.92	117.77± 2.29	120.81±0.61
6.0	247.63±1.68	173.50±2.79	142.05±1.53	123.28±1.35	95.50±2.95	102.97±2.84	112.44±2.58
9.0	233.80± 1.37	154.36± 1.77	125.91± 1.53	116. 50± 2.25	66.36± 1.46	99.33± 1.97	104.00±2.65
12.0	229.21± 0.71	116.00± 3.61	93.67± 1.53	77.50± 2.18	52.33± 2.08	77.33± 2.08	96.07±2.49
15.0	245.65±2.15	144.11±1.02	117.59±1.23	106.55±1.39	79.09±0.54	109.00±1.00	114.67±3.06
18.0	256.24±1.04	157.33±2.08	122.33±2.08	115.00±1.00	85.96±1.07	117.67±1.53	121.67±1.53
21.0	262.38±1.98	171.15±1.23	137.33±2.08	129.00±1.00	98.31±1.07	120.67±1.53	134.67±2.52

Table 3. GLM test for variation in bacterial growth media on biochemical oxygen demand (BOD) content, using different concentrations of GS-IONPs (mg) with different time intervals.

Growth media	**Source**	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F-value**	**P-value**
Bacterial growth media biochemical oxygen demand + different concentrations GS-IONPs	Conc. of NPs (mg)	6	38291	38291	6382	105.15	0.000
	Time (days)	6	373587	373587	62264	1025.93	0.000
	Error	134	8133	8133	61	-	-
	Total	146	420010	-	-	-	-

Table 4. Average (±SD) of replicates tests of bacterial growth media on chemical oxygen demand (COD) content, using different concentrations of GS-IONPs at different time intervals.

Time (days)	GS-IONPs concentration (mg)						
	Control	**10**	**20**	**30**	**40**	**50**	**60**
3.0	1044.79 ± 3.27	963.19±2.63	842.39± 1.71	725.08± 1.88	606.13± 2.03	617.11± 1.81	683.15±1.95
6.0	992.58±2.01	850.16±1.26	739.39±1.57	687.28±2.26	503.90±2.05	512.30±1.91	592.78±2.15
9.0	854.25± 4.02	732.36± 1.82	624.25± 1.53	504. 16± 2.25	401.69± 1.09	457.33± 1.10	488.22±1.11
12.0	797.56± 2.26	591.34± 0.90	452.51± 2.50	376.61± 1.77	201.28± 1.02	363.55± 3.07	405.48±0.46
15.0	802.18±1.59	624.30±0.72	500.01±1.24	426.56±2.28	235.33±2.52	403.00±3.00	435.00±2.65
18.0	825.68±2.03	631.41±1.01	532.25±1.64	495.00±1.00	261.28±1.12	414.67±4.16	467.11±1.02
21.0	830.99±1.30	632.78±1.57	547.45±1.88	478.97±0.95	272.19±1.06	432.12±1.18	472.44±2.69

Table 5. GLM test for variation in bacterial growth media on chemical oxygen demand (COD) content, using different concentrations of GS-IONPs (mg) with different time intervals.

Growth media	Source	DF	Seq SS	Adj SS	Adj MS	F-value	P-value
Bacterial growth media chemical oxygen demand + different concentrations of GS-IONPs	Conc. of NPs (mg)	6	1852489	1852489	308748	444.34	0.000
	Time (days)	6	3804482	3804482	634080	912.56	0.000
	Error	134	93109	93109	695	-	-
	Total	146	5750080	-	-	-	-

Table 6. Average (±SD) of replicates tests of bacterial growth media on total organic carbon (TOC) content, using different concentrations of GS-IONPs (mg) at different time intervals.

Time (days)	GS-IONPs concentration (mg)						
	Control	**10**	**20**	**30**	**40**	**50**	**60**
3.0	494.89 ± 0.83	406.59±2.80	345.19± 0.59	326.81± 2.09	283.17± 2.42	327.54± 2.17	346.35±3.22
6.0	441.82±2.15	372.43±2.53	300.82±1.99	252.48±2.50	202.41±2.46	242.40±2.59	272.89±2.41
9.0	391.52± 0.84	333.96± 2.06	224.35± 3.53	187.80± 1.08	122.32± 2.29	185.08± 2.04	220.44±1.57
12.0	322.60± 2.49	245.66± 1.71	182.22± 2.17	122.01± 1.61	70.44± 0.49	97.33± 2.08	121.33±1.06
15.0	363.57±1.42	283.19±2.50	227.61±1.22	172.78±2.51	95.89±1.01	118.40±1.21	164.77±3.23
18.0	381.48±1.49	307.33±2.08	242.95±2.54	195.33±0.58	121.56±1.45	167.82±1.65	191.86±1.67
21.0	410.59±1.38	352.83±1.66	298.00±1.00	234.52±1.96	171.28±1.04	199.03±0.93	244.83±2.64

Table 7. GLM test for variation in bacterial growth media on total organic acid (TOC) content, using different concentrations of GS-IONPs (mg) with different time intervals.

Growth media	Source	DF	Seq SS	Adj SS	Adj MS	F-value	P-value
Bacterial growth media total organic acid + different concentrations of GS-IONPs	Conc. of NPs (mg)	6	1256797	1256797	209466	12.84	0.000
	Time (days)	6	2159917	2159917	359986	22.07	0.000
	Error	134	2185324	2185324	16308	-	-
	Total	146	5602038	-	-	-	-

4. Conclusion

In summary, the present study provides momentous insights into the introduction of greenly synthesized iron oxide nanoparticles (GS-IONPs) as growth-enhancing material for some bacterial species isolated from wastewater treatment unit located in natural gas facility, Port Said, Egypt. The progress in the bacterial microbiome community was analyzed spectrophotometrically at different interval times. Moreover, the designed mixture of GS-IONPs and grown bacterial microbiome have been extensively employed in the remediation of crude oil. The findings revealed that the removal % of COD, BOD, and TOC was interestingly promoted by 74.76, 77.17, and 85.44%, respectively using 0.04 g of GS-IONPs, compared to the non-treated GS-IONPs sample. To sum up, the present study proposed a momentous scenario for the potency of nanomaterials in bioremediation.

-Credit Authorship

Abeer M. Salama: Conceptualization, Methodology, Investigation, and Writing original draft.**Emanne Rashad:** Writing original draft, and Editing.**Ahmed M. Elgarahy:** Writing original draft, Reviewing, and Editing. **Khalid Z. Elwakeel:** Reviewing, and Editing.

Declarations

-Ethical Approval. Not applicable

-Consent to Participate. Not applicable

-Consent to Publish. Not applicable

-**Competing Interests.** The authors declare that they have no competing interests.

Availability of data. All data generated or analyzed during this study were included in the submitted article. In addition, the datasets used or analyzed during the current study were available from the corresponding author on reasonable request.

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