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Inhibition Effect of *Rosmarinus officinalis* L. Essential Oil on Environmental Sulfate-Reducing Bacteria induced Corrosion

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Abstract

Sulfate-reducing bacteria (SRB)-induced corrosion plays an important role in environmental and technological fields, causing huge economic and ecological damage. This study was carried out to determine the efficacy of *Rosmarinus officinalis* oil in controlling metal corrosion. Sludge samples were collected from the sulfurous springs in Heet, Anbar governorate, Iraq, to isolate sulfate-reducing bacteria. The samples were enriched using BmA medium under anaerobic conditions. The isolated SRB strain was subjected to biochemical tests and 16S rRNA gene sequencing and identified as *Desulfovibrio desulfuricans*. One set of the microcosm was sampled every week and the effects of *D. desulfuricans* on the metal surface were evaluated, in the presence and absence of the essential oil, by using scanning electron microscope. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of *R. officinalis* oil against *D. desulfuricans* were determined at 20 mg/ml, which was sufficient to kill the bacteria in the enrichment medium. The weight loss of the nails which were incubated in the sediment containing *D. desulfuricans* and *R. officinalis* oil was significantly lower than those incubated in the presence of *D. desulfuricans* alone. Scanning electronic microscopic analysis showed that the oil at 20 mg/ml inhibited the formation of biofilm on the nail's surface. Heavy metal corrosion, cracking and a thick layer of biofilm was observed on the surface of the nail incubated in the sediment without oil treatment. In contrast, addition of *R. officinalis* oil to the sediment with *D. desulfuricans* resulted in a lower rate of corrosion and biofilm layers were thinner.
Keywords: Metal corrosion, SRB, Rosemary oil, Light and scanning electron microscopy.

Резюме

Корозията, предизвикана от сулфат-редуциращи бактерии (SRB), играе важна роля в областта на околната среда и технологиите, причинявайки огромни икономически и екологични щети. Настоящото проучване е проведено, за да се определи ефикасността на масло от *Rosmarinus officinalis* при контролиране на корозията на металите. Събрани са проби от утайки от серните извори в Хит, провинция Анбар, Ирак, с цел изолиране на сулфат-редуциращи бактерии. Пробите са обогатени с помощта на BmA среда при анаеробни условия. Изолираният SRB щам е идентифициран като *Desulfovibrio desulfuricans* чрез биохимични тестове и 16S rRNA генно секвениране. Всяка седмица се взема проба от един комплект от микрокосмоса и се оценяват ефектите на *D. desulfuricans* върху металната повърхност в присъствието и отсъствието на етерично масло, с помощта на сканиращ електронен микроскоп. Минималната инхибираща концентрация и минималната бактерицидна концентрация на маслото от *R. officinalis* срещу *D. desulfuricans* е 20 mg/ml, която е достатъчна за унищожаване на бактериите в обогатяващата среда. Загубата на тегло на пироните, които са инкубирани в утайката, съдържаща *D. desulfuricans* и масло от *R. officinalis* е значително по-ниска от загубата на тегло на пироните, инкубирани в присъствието само на *D. desulfuricans.* Анализът с помощта на сканиращ електронен микроскоп показа, че маслото при концентрация 20 mg/ml инхибира образуването на биофилм върху повърхността на пироните. Наблюдава се корозия на тежките метали, напукване и дебел слой биофилм върху повърхността на пироните, инкубирани в утайката без обработка с масло. Обратно, добавянето на масло от *R. officinalis* към утайката с *D. desulfuricans* води до по-ниска степен на корозия и слоевете на биофилма са по-тънки.

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Introduction

Microbiologically induced corrosion is defined as the deterioration of metals either directly or indirectly by the activity of microorganisms. Microbiologically induced corrosion plays an important role in the natural environmental, economic, and technological fields. More than 75% of corrosion in oil wells and 50% of the corrosion in the wastewater pipeline was due to the sulfate reductive activity of SRB (Martins *et al*., 2009; Tian *et al*., 2017; Lavanya, 2021; Voskuhl *et al*., 2022).

Sulfate-reducing bacteria (SRB) are able to grow in a wide variety of environments such as soils, marine sediments, and mining water content. SRB are unique when compared to other living microorganisms. This is because they utilize sulfate as a terminal electron acceptor, instead of oxygen, and acquire the organic substances to synthesize the carbon source and electron donor. The activities of SRB have led to the reduction of sulfate ion (SO_4^{2-}) to sulfide ion (S^{2-}) and are accompanied by the production of H_2S (Barton, 1995; Groudeva *et al*., 2001; Sheng *et al*., 2007). The generation of H_2S gives rise to a serious odor problem as different sulfide species such as H_2S, HS^- and S^{2-} might coexist in equilibrium in many aquatic systems. In the presence of iron, black ferrous sulfide will form, and undesirable rotten egg smells will be released (Rabus *et al*., 2006).

Within the oil well, SRB is associated with the release of H_2S, which lead to the souring of gas and oil reservoirs (Voskuhl *et al*., 2022). By the way, SRB is associated with the corrosion and souring of the oil downstream piping and carrying line (Lavanya, 2021). The activity of SRB will cause surface modifications, creating a local anoxic condition that lacks oxygen. The presence of SRB will decrease the efficiency of secondary oil recovery. Thus, SRB activity reduces the commercial value of crude oil (Tian *et al*., 2017; Deng *et al*., 2018).

Rosmarinus officinalis (Lamiaceae, Rosemary) is a widely cultivated aromatic plant. The essential oil of the plant contains a large number of powerful phytochemicals such as α-pinene, cineol, camphor, limonene, linalool, and camphene. Over the years, rosemary oil has become well known for its antioxidative, antitumoral, and antimicrobial values (Hussain *et al*., 2010; Hudaib *et al*., 2015; Atala and Aldabagh, 2017; de Macedo *et al*., 2020).

To our knowledge, no studies on the effect of *Rosmarinus officinalis* against SRB causing metal corrosion to have been previously published. This prompted us to further investigate the inhibition activity of rosemary oil against SRB-induced corrosion which may help in preventing corrosion.

Materials and Methods

Sample collection

Sediment samples were collected from the sulfurous springs in Heet, Anbar governorate, Iraq. All samples were transferred quickly into a sterile and airtight universal bottle. The bottles must be completely filled with samples in order to create an anaerobic environment. This process should do as fast as possible to ensure less exposure to air. This is because long-term exposure to air will inhibit or kill the SRB. Finally, the samples were brought to the laboratory for the enrichment of SRB as soon as possible, preferably within 24 hours.

Enrichment and isolation of SRB

Postgate B medium (BmA medium) was prepared to enumerate SRB at 30°C for 7 days, in anaerobic conditions (Postgate 1984; Sheng *et al*., 2007). The medium consisted of [g/L]: K_2HPO_4 0.5, NH_4Cl 1.0, $CaSO_4.2H_2O$ 1.0, $MgSO_4.7H_2O$ 2.0, Sodium acetate 3.5, Yeast extracts 1.0, Ascorbic acid 0.1, Thioglycolic acid 0.1, $FeSO_4.7H_2O$ 0.5. Five ml of sediment suspension was inoculated into the McCartney bottles that contained BmA medium in triplicates. The bottles were filled with BmA medium until full before capped. At the same time, a control bottle was prepared by filling up the McCartney bottle with BmA medium without inoculation of suspension. All these bottles were incubated at 30°C for 7 days. Growth of SRB was indicated by the blackening of the medium.

Identification of SRB

Biochemical tests were done to identify isolated SRB according to Postgate and Campbell (1966). Isolation and purification of genomic DNA were carried out using Geneaid kit (Korea) depending on the manufacturer's instruction, followed by amplification of the 16srRNA gene using a universal primer (F5-AGA GTT TGA TCC TGG CTC AG-3) and (R5- AAG GAG GTG ATC CAG CC-3) (Weisburg *et al*., 1991). The amplified product was sequenced in Macrogen company and analyzed using NCBI blast (nlm.nih.gov/Blast.cgi).

Plant sample collection and extraction

Leaves of rosemary (*Rosmarinus officinalis* L.) were collected from the Biology department garden of the College of Science, Baghdad University, Iraq. The plant samples were identified by Prof. Dr. Ali Fadam Al-Mohammadi from the Center of Desert Studies, University of Anbar. Plant samples

were washed with distilled water and left to air-dry.

Rosemary essential oil was extracted by hydrodistillation using a Clevenger apparatus continuously for 4 h, and the extracted oil was kept at 4°C before the test (Fadil *et al*., 2015; Hudaib *et al*., 2015).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of R. officinalis essential oil against SRB

The minimum inhibitory concentration (MIC) was determined using the broth tube dilution method. Serial dilution of the rosemary oil was prepared in a liquid medium which was then inoculated with a standardized volume of SRB and incubated for a given period. The final concentrations of the oil used in this study were 2.5, 5, 10, 20, 40, and 80 mg/ml. Different concentrations of the oil were added to the bottles before SRB was added. Then, a constant 5 ml of SRB was inoculated into the bottles. Each bottle was assayed in triplicates. All the bottles were closed tightly and incubated at 30°C for 7 days. The growth was assessed after the incubation period and the MIC value was read. Blackening of the medium indicated growth of SRB whereas no blackening of the medium showed the activity of SRB was inhibited. Therefore, the lowest concentration of the oil that prevented the blackening of the medium was taken as the MIC value.

MBC was conducted immediately after the results of MIC were recorded. Based on the MIC test result, it was not known whether SRB was inhibited or killed. Therefore, MBC test was performed to determine the lowest concentration of *R. officinalis* oil capable of killing SRB.

SRB from the MIC test was used for the MBC test. Five ml of SRB from each bottle of the MIC test with and without any visible SRB growth was transferred into a new set of McCartney bottles. These bottles were filled with fresh BmA medium. Then, all the bottles were closed tightly and sealed with parafilm.

Apart from that, one bottle filled with BmA medium and SRB was used as a positive control bottle whereas another bottle containing chloramphenicol and SRB acted as a negative control. The bottles were incubated at 30°C for 7 days. SRB grew in fresh BmA medium if only they were inhibited but not killed. MBC endpoint was identified as the minimum concentration of *R. officinalis* essential oil capable of killing SRB inoculated into the medium.

Preparation of laboratory microcosm

To study the effect of *R. officinalis* oil in controlling SRB-induced metal corrosion, a laboratory-scale microcosm method was used. Based on the MBC determination, the MBC value of *R. officinalis* oil (20mg/ml) was used. Nails that were dirt free were used in this test. The initial and final mass of nails was measured to determine any weight loss.

The sediment sample used in this experiment was collected from the same sulfurous springs. Meanwhile, six liters of soil samples were used in this experiment. Sodium acetate and sodium sulfates such as Na_2SO_4 and $MgSO_4.7H_2O$ were added to the soil. Twenty-one g of sodium acetate, 6 g of Na_2SO_4, and 12 g of $MgSO_4.7H_2O$ were measured. Once these chemical compounds were prepared, they were mixed with the sediment in a big bowl. Each microcosm was a plastic cup and was initially half-filled with gently packed sediment. For each microcosm, three pieces of nails were inserted into the sediment. Once this was done, sediment was then added to occupy ¾ of the volume of the cup. Finally, the cup was topped up with soil and the top of the cup of each microcosm was sealed with cellophane tape to create an anaerobic environment. There were 6 sets of microcosms, i.e., Set A with sediment, SRB, rosemary oil, nails, and soil topping, and another set (Set B) without rosemary oil. All the microcosms were then incubated at 30°C in the dark for 6 weeks. Every week, one microcosm from Set A and another from Set B were sacrificed for the determination of the weight of the nails and SRB determination while after 4 and 6 weeks, Scanning Electron Microscopic (SEM) analysis was also performed. The nails were washed with distilled water and then dried in the oven at 55°C for 24 hours. After 24 hours, the nails were observed under a scanning electron microscope (SEM). At the same time, a small quantity of the sediment (15 g) from the area where the nails were embedded was enriched in BmA medium for the presence of SRB. Blackening of the medium indicated that SRB was present in those areas. Finally, the mass of the nails was determined using an electronic weighing machine and the results were recorded. Each experiment was done in triplicates.

Observation of the biofilm under a light microscope and by scanning electron microscope

A nail from Set A (microcosm containing rosemary oil) and Set B (microcosm without rosemary oil) was removed from the microcosm and placed in a petri dish. The nails were rinsed with distilled water with much care without destroying

the surface of the nail, dried in the air, and observed under the light microscope.

Another nail from each set of microcosms was also removed at the end of the incubation period. The biofilm that formed on the surface of the nail was observed under SEM after being prepared using the standard procedure. A scanning electron microscope (TESCAN VEGA 3, USA) was used to observe and study the morphology of the SRB and biofilm.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows, version 26, SPSS Inc., Chicago, IL). Data summary was done with descriptive statistics such as the mean and standard deviation of the mean. Statistical significance was determined by One-Way Analysis of Variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test. A *p*-value of less than 0.05 was considered to be a significant difference in all statistical analyses.

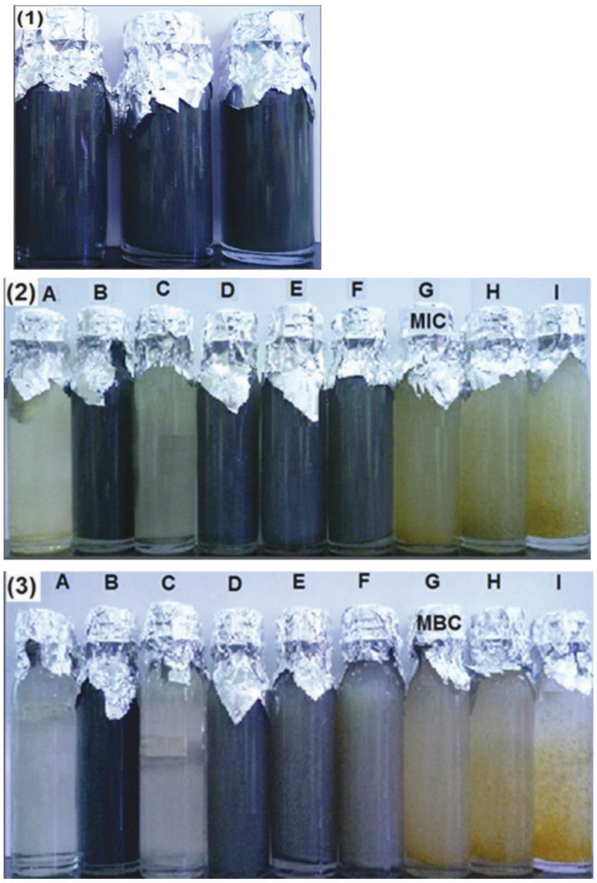

Fig. 1. (1) Growth of SRB in BmA medium containing iron. (2) Determination of MIC value of *R. officinalis* oil on *D. desulfuricans*. A: Medium control, B: Inoculum control, C: Chloramphenicol control, D: 2.5 mg/ml, 5 mg/ml, F10 mg/ml, G: 20 mg/ml (MIC), H: 40 mg/ml, I80 mg/ml. (3) Determination of MBC value of *R. officinalis* oil on *D. desulfuricans*. A Medium control, B: Inoculum control, C: Chloramphenicol control (with SRB inoculums), D: 2.5 mg/ml, E: 5 mg/ml, F:10 mg/ml, G: 20 mg/ml (MBC), H: 40 mg/ml, I 80 mg/ml.

Results

Enrichment, isolation, and identification of SRB

The enrichment cultures of SRB by using BmA medium under anaerobic conditions showed that the bacteria were presented in the collected samples. Growth of SRB was indicated by blackening of the BmA medium, which contained iron, and turbidity of the BmA medium without iron.

The BmA medium with iron turned black after 1 to 2 days while the BmA medium without iron turned turbid after 3 to 5 days. The BmA medium in the presence of iron which was originally light yellow turned black and wall growth could be observed around the wall of the bottles. This blackening of the medium was due to the formation of ferrous sulphide, formed from the reaction between sulfide and iron. Sulfide was formed from the dissimilatory reduction of sulfate by SRB under anaerobic conditions. However, the BmA medium without iron turned turbid as there was no formation of ferrous sulfide. Figure 1A shows the blackening of SRB in the BmA medium with iron.

The isolated SRB were subjected to biochemical tests and 16S rRNA gene sequencing and identified as *D. desulfuricans* (Table 1).

Table 1. Biochemical test results of *D. desulfuricans* isolated from sediment samples

Test	Result
Motility	+
Catalase	-
Urea	+
Indol	+
Nitrate	+
Sulfur, Indole, Motility (SIM) test	+

Comparative sequence analysis showed the identity of the DNA sequences which encode for the 16S rRNA gene of the studied sulfate-reducing bacteria strain with similar sequences as those from the GenBank database. The nucleotide sequence of the isolated SRB had 99% homology with sequences *D. desulfuricans* strain Essex 6 (Accession number 104990) deposited in the GenBank.

Effects of R. officinalis essential oil on the growth of D. desulfuricans

The final concentrations of the oil used in this study were 2.5, 5, 10, 20, 40, and 80 mg/ml. Once these concentrations of the oil were inoculated into

the bottles, *D. desulfuricans* was added and followed by incubation at 30°C for 7 days. The growth of *D. desulfuricans* was detected by the blackening of the BmA medium. The lowest concentration that did not cause blackening of the medium was considered as the MIC value. In this study, it was observed that the MIC value for *R. officinalis* oil against *D. desulfuricans* was 20 mg/ml (Fig. 1B).

Based on the results, the bottles containing oil concentrations of 2.5 mg/ml, 5 mg/ml and 10 mg/ml showed blackening of the medium. Therefore, *D. desulfuricans* was not killed at these concentrations. However, when the MIC culture containing 20 mg/ml of oil concentration was subcultured into the new medium, no blackening of the medium could be observed, suggesting the MIC value at 20 mg/ml was effective in killing *D. desulfuricans*. Therefore, the MIC and MBC value for *R. officinalis* oil was the same at 20 mg/ml. Figure 1C shows the MBC value of *R. officinalis* oil.

Effects of R. officinalis essential oil in controlling metal corrosion caused by D. desulfuricans

Reduction in the mass of nails buried in the sediment without *R. officinalis* oil was significantly higher than the loss of mass of nails buried in the sediment with oil (Table 2). A thick black layer was formed on the surface of the nails buried in sediment without oil, but such layer was not present on nails buried in sediment with *R. officinalis* oil.

Based on the results, the reduction in the mass of the nails increased with the incubation period. The highest mass loss was observed in week 6 while the least mass loss of the nails was observed after just 1 week of incubation. On the other hand, a small quantity of the sediment (15 g) from the area where the nails were embedded was subcultured in fresh BmA medium. This step was to ensure that SRB was active and present in areas near the nail. Based on growth in BmA medium, SRB was present in the area where the nails were embedded. However, the growth of SRB in the area without the addition of oil was much stronger as compared to that in the presence of oil (Fig. 2), suggesting that *D. desulfuricans* was inhibited by *R. officinalis* oil. In some cases, wall growth could be observed in the bottles without oil treatment, but it was absent in the bottles inoculated with sediment and oil. Therefore, it can be concluded that *R. officinalis* oil can reduce the metal corrosion induced by *D. desulfuricans*.

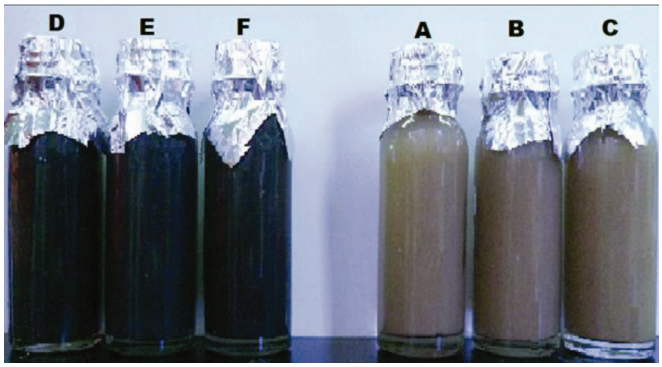

Fig. 2. The growth of *D. desulfuricans* around the nail after 6 weeks incubation; A, B and C: Growth of *D. desulfuricans* in sediment around the nail in Set A microcosm with oil. D, E and F: Growth of *D. desulfuricans* in sediment around the nail in Set B microcosm without oil.

Observation of nail surface and formation of biofilm on the surface of the nail under light microscope

The surface of the nails and the formation of biofilm were also observed under the light microscope. Before incubation, the nail's surface was smooth with no corrosion or biofilm formation (Fig. 3A). The nails that were incubated in the sediment with *D. desulfuricans* but without *R. officinalis* oil

Table 2. The average loss of mass of nails (g) embedded in the sediment containing *D. desulfuricans* with oil and without oil over 6 weeks incubation

Week	Average loss of mass of nails (g) embedded in sediment + *D. desulfuricans* + nails	
	Set A (with *R. officinalis* oil)	Set B (without *R. officinalis* oil)
1	^{a}0.0002 ± 0.00005	^{a}0.0004 ± 0.0002
2	^{a}0.0006 ± 0.0001	^{b}0.0019 ± 0.0004
3	^{a,b}0.0011 ± 0.0003	^{e}0.0115 ± 0.0003
4	^{b}0.0018 ± 0.0001	^{f}0.0311 ± 0.0002
5	^{c}0.0032 ± 0.0003	^{g}0.0607 ± 0.0007
6	^{d}0.0044 ± 0.0003	^{h}0.0812 ± 0.0006

Values are mean ± standard deviation of three replicates. Values with different superscript letters are significantly different ($p < 0.05$) based on one-way ANOVA and Tukey HSD test

for 4 and 6 weeks showed the formation of a sticky and rough layer on the surface (Fig. 3A1 and 2). Such layers known as biofilm are formed when inhibitory substances such as *R. officinalis* oil are not present to inhibit *D. desulfuricans*. The biofilm formation in week 6 was much higher than the biofilm, which was formed in week 4 because *D. desulfuricans* grew and increased in members with time to cause a higher corrosion rate. The nails which were incubated in sediment with *D. desulfuricans* plus *R. officinalis* oil after 4 and 6 weeks showed biofilm formation but it was only a fine layer compared to the nails which were incubated in the absence of *R. officinalis* oil (Fig. B1 and 2). The biofilm did not colonize the whole surface of the nail. This observation proved that the nails coated with *R. officinalis* were prevented from SRB-induced corrosion, suggesting that *R. officinalis* was inhibitory towards *D. desulfuricans*.

new population of bacteria formed on the surface of the nail incubated in the sediment with *D. desulfuricans* without oil (Fig. 4-5). On the other hand, the surface of the nail incubated in the sediment with *D. desulfuricans* and *R. officinalis* oil showed limited biofilm formation (Fig. 4-2 and Fig. 4-4). A compact structure could be observed on the surface of the nail incubated in the sediment containing *D. desulfuricans* alone when compared to the nail incubated in sediment with *D. desulfuricans* and *R. officinalis* oil. Differences in the condition of the surfaces of those nails indicated that *R. officinalis* oil exhibited anti-SRB activity in the laboratory microcosm.

Discussion

Basically, SRB are often involved in microbial corrosion, especially when they are in close contact with metal surfaces and lead to the formation of biofilm. A thick and slimy biofilm is usually

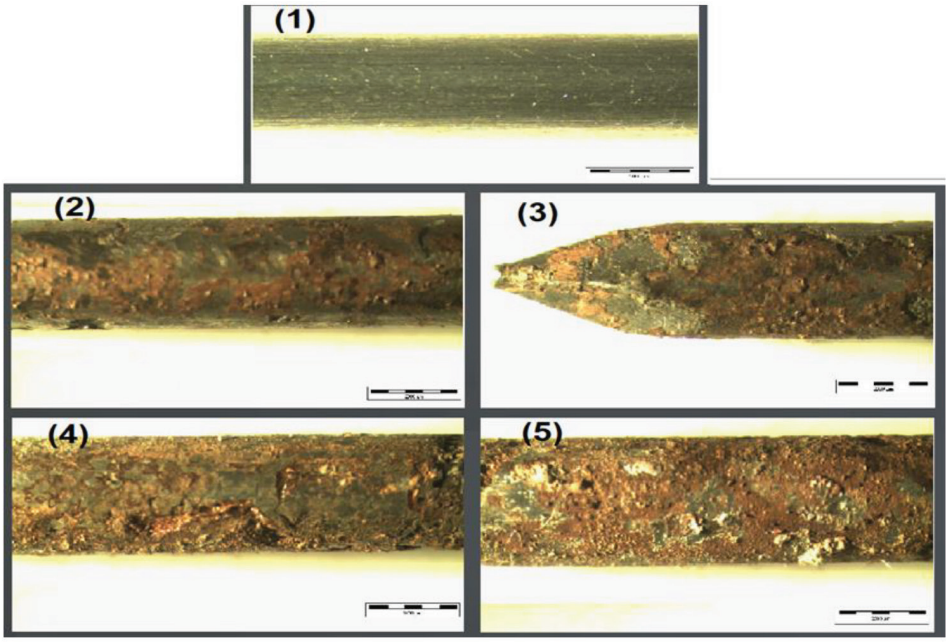

Fig. 3. Observation on nail surface under light microscope (Magnification:40X); (1) before incubation in sediment containing *D. desulfuricans*, (2) after 4 weeks incubation in sediment containing *D. desulfuricans* in the presence of *R. officinalis* oil (Set A), (3) after 4 weeks incubation in sediment containing *D. desulfuricans* in the absence of *R. officinalis* oil (Set B), (4) after 6 weeks incubation in sediment containing *D. desulfuricans* in the presence of *R. officinalis* oil (Set A), and (5) after 6 weeks incubation in sediment containing *D. desulfuricans* in the absence of *R. officinalis* oil (Set B).

Observation on the biofilm via scanning electron microscope

The surface of the nail which contained *D. desulfuricans* biofilm was also observed under a scanning electron microscope. Before incubation, the surface of the nail was clear with no biofilm formation (Fig. 4-1). However, the surface of the nail which was incubated in the sediment with *D. desulfuricans* for 4 weeks showed the formation of thick biofilm (Fig. 4-3). After 6 weeks of incubation, the

formed on the surface of corroded metals (Sheng *et al*., 2007). Therefore, the protection of structures against corrosion has become a critical step at this moment.

Although there have been many reports on the antimicrobial, antioxidant, and antitumoral activities of *R. officinalis* essential oil (Hussain *et al*., 2010; Hudaib *et al*., 2015; Atala and Aldabagh, 2017; de Macedo *et al*., 2020), our study is of considerable significance as SRB-induced corrosion

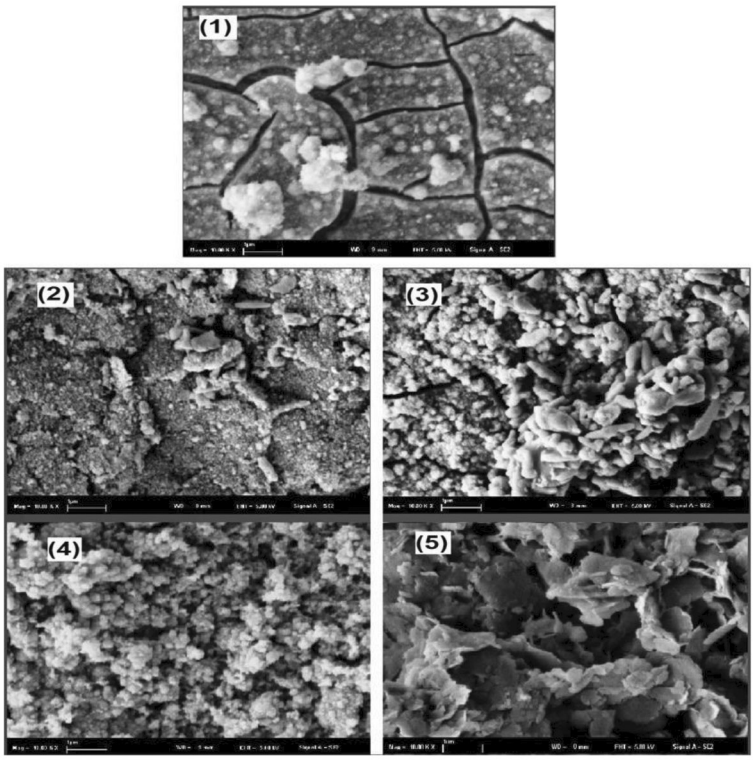

Fig. 4. Scanning electron microscopy micrograph of the nail surface **(1)** before incubating in the sediment, (2) incubated in the sediment containing *D*. *desulfuricans* and *R*. *officinalis* oil in week 4 (Set A), (3) incubated in the sediment containing *D*. *desulfuricans* without *R*. *officinalis* oil in week 4 (Set B), (4) incubated in the sediment containing *D*. *desulfuricans* and *R*. *officinalis* oil in week 6 (Set A), and (5) incubated in the sediment with *D*. *desulfuricans* without *R*. *officinalis* oil in week 6 (Set B).

plays an important role in environmental and technological fields, causing huge economic and ecological damage worldwide. This fact necessitated a search for more potent, low-cost, and environmentally friendly agents from natural resources including plants.

SRB was successfully isolated from the sulfurous springs in Heet, Anbar governorate, Iraq. In particular, Postgate Medium B was used to enrich SRB. This medium is known as enrichment medium. where extra nutritional supplements are added. The growth of SRB could be observed after 1- and 2-day incubation. The blackening of the BmA medium was due to the dissimilatory reduction of sulfate by SRB under anaerobic conditions. Sulfide will react with iron to produce ferrous sulfide. However, the light-yellow color of the medium changed to turbid but not black when SRB was re-inoculated in BmA medium without iron. This condition occurred because no iron was provided to react with the sulfide produced. Therefore, it can be concluded that there was no formation of ferrous sulfite. Overall, this observation confirmed that the blackening of the medium was due to SRB. The isolated SRB strain was subjected to biochemical tests and 16S rRNA gene sequencing and identified as *D. desulfuricans*.

MIC is defined as the lowest possible concentration of an antimicrobial agent required to inhibit the visible growth of microorganisms. MBC on the other hand is the lowest concentration of an antimicrobial that can completely kill or lyse the microorganisms (Madigan *et al*., 2009).

In this study, the MIC and MBC values of rosemary oil against SRB were 20 mg/ml. Therefore, MIC and MBC were equal also against SRB. However, the bottles with oil concentrations of 2.5, 5, and 10 mg/ml showed blackening of the medium. Therefore, it revealed that SRB was not killed at these concentrations.

Based on the MBC determination, the MBC value of rosemary oil was 20 mg/ml. This concentration of oil was used to study the effects of rosemary oil on SRB-induced metal corrosion. It was not known whether this concentration of oil was sufficient to control the corrosion of nails or a higher concentration of oil functions. Laboratory-scale microcosm was conducted using sediment with the bacteria plus 20 mg/ml of rosemary oil.

The results indicated that the reduction in the mass of the nails buried in the sediment with *D. desulfuricans* but without *R. officinalis* oil was significantly higher than the loss of mass of nails buried in the sediment with the bacteria and rosemary oil.

The reduction of mass was due to the corrosion rate induced by *D. desulfuricans*. Upon extended incubation, the corrosion reaction caused the formation of biofilm on the surface of the nails. Biofilm formation usually began with the initial attachment of bacteria to the surface. Extracellular polymeric substances are the content that is present in the biofilm. Extracellular polymeric substances consist of polysaccharides, proteins, and nucleic acid (Beech and Sunner, 2004), and they could increase the rate of metal corrosion. On the other hand, the loss of mass of nails buried in the sediment with *D. desulfuricans* and rosemary oil could still be observed but to a lower value when compared to those buried in the sediment with *D. desulfuricans* alone. This was because the biocorrosion rate was reduced by *R. officinalis* oil.

Based on the results, the reduction in the mass of the nails increased as the incubation period increased. It can be concluded that rosemary oil did not inhibit all corrosion rates of the metal but there was an indication that it could reduce SRB-induced metal corrosion. The observation of *D. desulfuricans* cells within the biofilm could sometimes be a tedious job or they could not be observed clearly at all. This might be due to the nature of SRB, which embeds itself within the EPS matrix (Antony *et al*., 2007; Deng *et al*., 2018; Li *et al*., 2021).

A scanning electron microscope was used in order to obtain a clearer image of the biofilm structures. The results indicated that *D. desulfuricans* grew at different rates in the sediment containing *D. desulfuricans* with and without oil. The growth of *D. desulfuricans* caused continuous biofilm formation and the bacteria were attached to the surface of the nail (Al-Darbi *et al*., 2002; Lavanya, 2021)

The effectiveness of the inhibitory activities of *R. officinalis* essential oil is primarily influenced by their powerful phytochemical compositions. The main components in rosemary oil are α-pinene, cineol, camphor, limonene, linalool, and camphene (Hussain *et al*., 2010; Hudaib *et al*., 2015; Atala and Aldabagh, 2017; de Macedo *et al*., 2020). Essential oils have lipophilic features that allow them to pass across the bacterial cell wall, which leads to bacterial cell disruption due to its effect on the plasma membrane permeabilization, loss of ions, and collapse of proton pumps (Md Zain *et al*., 2018; Vaithiyanathan *et al*., 2018; Dong *et al*., 2022; Annemer *et al*., 2022).

In conclusion, the present study indicated that the essential oil of rosemary has the potency to reduce bio-corrosion. This finding gives the possibility for the use of this oil as a source of corrosion protective agent.

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References

Al-Darbi, M. M, Z. M. Muntasser, M. Tango, M. R. Islam (2002). Control of microbial corrosion using coatings and natural additives. *Energ. Source Part A.* **24**: 1009-1018.

Annemer, S., A. Farah, H. Stambouli, A. Assouguem, M. H. Almutairi, A. A. Sayed, I. Peluso, T. Bouayoun, N. A. Talaat, A. L. El Ouali, Y. Ezoubi (2022). Chemometric investigation and antimicrobial activity of *Salvia rosmarinus* spenn essential oils. *Molecules* **27**: 2914.

Antony, P. J., S. Chongdar, P. Kumar, R. Raman (2007). Corrosion of 2205 duplex stainless steel in chloride medium containing sulphate-reducing bacteria. *Electrochim. Acta* **52**: 3985-3994.

Atala, M. L., M. A. Aldabagh (2017). Antibacterial activity of *Rosmarinus officinalis* and *Dodonaea viscosa* leaves extracts against *Escherichia coli* and *Staphylococcus aureus*. *Iraqi J. Sci.* **58**: 1393-1397.

Barton, L. A. (1995). Sulphate Reducing Bacteria. In: Atkinson, T. and R. F. Sherwood (Eds), Biotechnology Handbooks, Springer Science+Business Media, LLC, New York.

Beech, I. B, J. Sunner (2004). Biocorrosion: towards understanding interactions between biofilms and metals. *Curr. Opin. Biotechnol.* **15**: 181-186.

de Macedo, L. M., É. M. D. Santos, L. Militão, L. L. Tundisi, J. A. Ataide, E. B. Souto, P. G. Mazzola (2020). Rosemary (*Rosmarinus officinalis* L., syn *Salvia rosmarinus* Spenn.) and its topical applications: A review. *Plants* **9**: 651.

Deng, Q., X. Wu, Y. Wang, M. Liu (2018). Activity characteristics of sulfate reducing bacteria and formation mechanism of hydrogen sulfide. *Appl. Ecol. Environ. Res.* **16**: 6369-6383.

Dong, Y., S. Guang-Ling, Z. Zheng (2022). Naturally effective inhibition of microbial corrosion by bacterium-alga symbiosis on 304 stainless steel, *J. Clean. Prod.* **356**; 131823.

Fadil, M., A. Farah, B. Ihssane, T. Haloui, S. Rachiq (2015). Optimization of parameters influencing the hydrodistillation of *Rosmarinus officinalis* L. by response surface methodology, *J. Mater. Environ. Sci.* **6**: 2328-2336.

Groudeva, V., L. Gazso, S. Groudev, R. Dimkov, A. Kujumdzieva, T. Nedeva, T. Rasheva (2001). Environment protection and biotechnology. National bank for industrial microorganisms and cell cultures, Sofia.

Hudaib, M. M., K. A. Tawaha, H. S. Hudaib, A. H. Battah (2015). Chemical composition of volatile oil from the aerial parts of *Rosmarinus officinalis* L. grown in Jordan. *J. Essent. Oil-Bear. Plants.* **18**: 1282-1286.

Hussain, A. I., F. Anwar, S. A. S. Chatha, A. Jabbar, S. Mahboob, P. Singh-Nee Nigam (2010). *Rosmarinus officinalis*

essential oil: antiproliferative, antioxidant and antibacterial activities. *Braz. J. Microbiol.* **41**: 1070-1078.

Lavanya, M. A (2021). Brief insight into microbial corrosion and its mitigation with eco-friendly inhibitors. *J. Bio. Tribo. Corros.* **7**:125.

Li, Z., J. Zhou, X. Yuan, Y. Xu, D. Xu, D. Zhang, D. Feng, F. Wang (2021). Marine biofilms with significant corrosion inhibition performance by secreting extracellular polymeric substances. *ACS Appl. Mater. Interfaces* **13**: 47272-47282.

Madigan, M. T., J. M. Martinko (2009). Brock Biology of Microorganisms, 12th Edition. Pearson Prentice Hall. Southern Illinois University Carbondale, USA.

Martins, M., M. L. Faleiro, R. J. Barros, A. R. Veríssimo, M. A. Barreiros, M. C. Costa (2009). Characterization and activity studies of highly heavy metal resistant sulphate-reducing bacteria to be used in acid mine drainage decontamination. *J. Hazard. Mater.* **166**: 706-713.

Postgate, J. R. (1984). The sulphate-reducing bacteria. 2nd edition., Cambridge University Press, London, UK.

Postgate, J. R., L. L. Campbell (1966). Classification of *Desulfovibrio* species, the nonsporulating sulphate-reducing bacteria. *Bacteriol. Rev.* **30**: 732-738.

Rabus, R., T. A. Hansen, F. Widdel (2006). Dissimilatory sulphate and sulphur-reducing prokaryotes. *Prokaryotes* **1**: 659-768.

Sheng, X., Y. P. Ting, S. O. Pehkonen (2007). The influence of sulphate-reducing bacteria biofilm on the corrosion of stainless steel AISI 316. *Corros. Sci.* **49**: 2159-2176.

Tian, H., P. Gao, Z. Chen, Y. Li, Y. Li, Y. Wang, J. Zhou, G. Li, T. Ma (2017). Compositions and abundances of sulfate-reducing and sulfur-oxidizing microorganisms in water-flooded petroleum reservoirs with different temperatures in China. *Front. Microbiol.* **2**:143.

Vaithiyanathan, S., K. Chandrasekaran, R. C. Barik (2018). Green biocide for mitigating sulfate-reducing bacteria influenced microbial corrosion. *3 Biotech.* **8**: 495.

Voskuhl, L., D. Brusilova, V. S. Brauer, R. U. Meckenstock (2022). Inhibition of sulfate-reducing bacteria with formate, *FEMS Microbiol. Ecol.* **98**: 1-10.

Weisburg, W. G., S. M. Barns, D. A. Pelletier, D. J. Lane (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**: 697-703.

Zain, W. Md. W. S., N. I. H. Salleh, A. Abdullah (2018). Natural biocides for mitigation of sulphate reducing bacteria. *Int. J. Corros*. **2018**: 3567569.