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Antimicrobial and Anticorrosion Effect of Essential oils of Medicinal and Aromatic Plants on Sulphate Reducing Bacteria isolated from Oilfield Produced Water

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ABSTRACT

Medicinal and Aromatic Plants (MAPs) have been known to possess inherent anti-microbial properties. In the present study, locally available essential oils of Lemongrass (LEO) (Cymbopogon flexuosus), Citronella (CEO) (Cymbopogon winterianus) and Patchouli (PEO) (Pogostemon cablin) were analyzed to ascertain their antimicrobial and anticorrosion effects on a native strain of Sulphate Reducing Bacteria (SRB),isolated from produced water sourced from an Oil Collecting Station in India's North-Eastern state of Assam. The Minimum Inhibitory Concentration (MIC) for Lemongrass, Citronella and Patchouli essential oils were determined to be 50 ppm, 100 ppm and 450 ppm respectively, using microdilution susceptibility tests. The minimum concentration of the essential oils that not only inhibit SRB activity, but also kill the target bacterial cells was also determined using Minimum Bactericidal Concentration (MBC) assay. The MBC concentrations of LEO, CEO and PEO were found to be similar to their MIC levels. All the essential oils tested showed SRB biofilm inhibitory effects on glass and N-80 carbon steel coupons. The average rate of biocorrosion calculated based on the weight loss of N-80 steel coupons indicate that the essential oils tested were able to exhibit a significant anticorrosion effect on the treated N-80 steel coupons.

Keywords: Medicinal and Aromatic Plants; Essential oils; Antimicrobial; Anticorrosion; Effect; Sulphate Reducing Bacteria; Oilfield; Produced Water; Assam; India.

INTRODUCTION

In petroleum industries, it is mandatory to control and inhibit sulphate reducing bacteria (SRB), which is usually done by biocide dosage. Regardless of the effectiveness of these biocides, antimicrobial resistance often occurs, particularly in biocide treated biofilms. In addition, the residual concentration, toxicity and persistence of biocides in industrial effluents are of high environmental concern. Hence, alternatives for SRB control are of great interest to the petroleum industry. Essential oils are mixtures of lipophilic and volatile substances, which are known to have components with antibacterial and / or antifungal activity and are potential sources of novel inhibitory substances. The effect of different plant extracts on biofilms has already been demonstrated in the food and medical industry. However studies on the effect of essential oils Medicinal and Aromatic Plants (MAPs) on inhibiting SRB and SRB induced biofilms in the oilfield environment are few. The present study, therefore, aimed to evaluate the efficacy of essential oils of MAPs locally available in Assam, India, in managing SRB population and corrosive behaviour.

EXPERIMENTAL PROCEDURE

ESSENTIAL OILS OF MEDICINAL AND AROMATIC PLANTS (MAPS)

Three Medicinal and Aromatic Plants (MAPs) cultivated widely in Assam, India for essential oil extraction namely Lemongrass (Cymbopogon flexuosus (Steud) Wats.), Citronella (Cymbopogon winterianus Jowitt.) and Patchouli (Pogostemon cablin Benth) were selected for the present study. Authenticated samples of essential oil of Lemongrass, Citronella and Patchouli were procured from The North Eastern Development Finance Corporation Limited (NEDFi) R&D Center for Medicinal and Aromatic Plants located at Khetri, Kamrup District, Assam and used for the present study. The essential oils were stored in small stoppered tubes in a refrigerator at 4°C.

SULFATE REDUCING BACTERIAL STRAIN

The test SRB strain used in this study was isolated from produced water samples collected from the Tank Bottom of a produced water storage tank of an Oil Collecting Station (OCS) of Oil India Limited (OIL), Duliajan, Assam, India. This native strain of SRB was maintained and grown in sodium lactate SRB Medium (NACE, 2004) at 37°C for three successive generations, in anaerobic conditions using sealed serum bottles (10 ml) to obtain a cell density of 105 cells/ml, for use, in subsequent assays. At this cell density i.e. 105 cells/ml, visible blackening of the inoculated serum bottles (10 ml) containing Sodium Lactate SRB Medium was achieved in 72 hours at 37°C.

MINIMUM INHIBITORY CONCENTRATION (MIC) OF THE ESSENTIAL OILS

In order to establish the minimum concentration of the three essential oils namely Lemongrass Essential Oil (LEO), Citronella Essential Oil (CEO) and Patchouli Essential Oil (PEO) that inhibit the native SRB strain growth, microdilution susceptibility tests were performed [1]. The essential oils were serially diluted in sterile 24-well cell culture plates (Costar® 3526, Corning Inc. New York, USA) to a lowest concentration of 10 ppm in sterile Sodium Lactate SRB Medium to determine the minimum inhibitory concentrations. The indicator native test strain of SRB was grown in Sodium Lactate SRB Medium [2] at 37°C for three successive generations, in anaerobic conditions using sealed serum

bottles (10 ml) to obtain a cell density of 10^5 cells/ml. The cell culture plates containing the different concentrations of the essential oils were incubated after incorporation of the native SRB strain at 37°C for 28 days. All inoculations were carried out in a Whitley-DG 250 Anaerobic workstation (Don Whitley Scientific Ltd., West Yorkshire, UK). Growth of the native test strain of SRB was detected by observing the blackish colour of the medium caused by iron sulfide precipitation in Sodium Lactate SRB Medium. The minimum inhibitory concentration (MIC) was determined as the least amount of antimicrobial substance (essential oil) added that did not result in blackish colour of the medium. Sub-MIC (0.5× MIC) and supra-MIC (2× MIC) of LEO, CEO and PEO were also established.

MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF THE ESSENTIAL OILS

The indicator strain of native SRB was inoculated into the wells of the sterile 24-well cell culture plates (Costar® 3526, Corning Inc., New York, USA) containing sterile sodium lactate SRB medium and incubated at 37°C for 48 hours. LEO, CEO and PEO was then added at their Sub-MIC, MIC and Supra-MIC concentrations. The cell culture plates were then incubated at 37°C for 7 days. All inoculations were carried out in a Whitley-DG 250 Anaerobic workstation (Don Whitley Scientific Ltd., West Yorkshire, UK). Optical density readings were taken in a Shimadzu UV Vis spectrophotometer (UV-2600, Shimadzu Corp., Kyoto, Japan) at a wavelength of 600 nm after one minute of addition of the essential oils to the test system and also after completion of the incubation time period of 7 days to determine the minimum amount of antimicrobial substance (essential oil) required to kill the native SRB in the cell culture wells (MBC). All the tests were done in three replicates.

GLASS COUPONS AND BIOFILM DEVICE

Circular micro glass coverslips of 12 mm diameter and 0.16 mm thickness (Bluestar®, Polar Industrial Corporation, Mumbai, India) were used as the glass coupons for generation of biofilms of the native SRB strains. The device for biofilm formation was a 24-well cell culture plate (Costar® 3526, Corning Inc., New York, USA) with a micro cover glass slip in each well. Biofilms of the native SRB strain used in the study were prepared by inoculating each well of the cell culture plate containing the micro glass coverslips (biofilm device) with a mid-log phase culture (10^5 cells/ml) grown in sodium lactate SRB medium inside a Whitley-DG 250 Anaerobic workstation (Don Whitley Scientific Limited, West Yorkshire, UK). The biofilm device was then incubated at 37°C for a period of 7 days for biofilm generation.

EFFECT OF THE ESSENTIAL OILS ON SRB BIOFILM FORMATION AND STABILITY

LEO, CEO and PEO at sub-MIC, MIC and supra-MIC concentrations were added at the same time in which the native SRB cells were introduced in the biofilm device. All inoculations were carried out in a Whitley-DG 250 Anaerobic workstation (Don Whitley Scientific Ltd., West Yorkshire, UK). The biofilm device was then incubated at 37°C for a period of 7 days for biofilm generation [3]. A positive control with only the SRB cells (without any essential oil) and a media blank (negative control) was also performed in the assay. After the incubation period, unattached or loosely attached cells on the glass surfaces of each well was washed with sterile Sodium Lactate SRB Medium, prior to biofilm cell visualization. SRB cells in the formed biofilms on the glass coupons were stained with 0.1% crystal violet for 10 minutes [4] and visualized using a Olympus BX43 microscope with image analysis software: Q.Capture Pro Version 7.0 (Olympus Corp., Tokyo, Japan).

CONDITIONING N-80 STEEL COUPONS WITH ESSENTIAL OILS

N-80 steel coupons (20 mm × 10 mm × 2 mm) were cleaned in 18% HCl, which was then neutralized by immersion in a saturated sodium bicarbonate solution. Finally, the coupons were washed with distilled water, rinsed in acetone, and dried in an air stream [3]. Conditioned surfaces were obtained by immersion of N-80 steel coupons in LEO, CEO and PEO at 25 and 50 ppm solutions for 7 days at 25°C. The remaining amount of LEO, CEO and PEO that was not absorbed to the metal surface was then removed by rinsing with deionised water and dried with sterile air to obtain clean uniformly surface layered (conditioned) coupons.

ANTI-CORROSION EFFECT OF ESSENTIAL OILS ON SRB INDUCED CORROSION

Two assays were used to test the anti-corrosion effect of the essential oils under study, on SRB induced corrosion on N-80 steel coupons: Assay 1: Conditioned N-80 steel coupons with LEO, CEO and PEO at 25 and 50 ppm concentrations, were placed in serum bottles containing Sodium Lactate SRB Medium inoculated with 10^5 cells/ml of the native test strain of SRB and incubated for 28 days at 37°C in a Jeio Tech IL-21A incubator (Jeio Tech Co, Ltd, Seoul, Korea). Assay 2: Cleaned N-80 steel coupons (without surface layering conditioning) were placed in serum bottles containing Sodium Lactate SRB Medium and the essential oils (LEO, CEO and PEO) at 25 and 50 ppm concentrations. These serum bottles were then inoculated with 10^5 cells/ml of the native test strain of SRB and incubated for 28 days at 37°C in a Jeio Tech IL-21A incubator (Jeio Tech Co, Ltd, Seoul, Korea).

After the incubation period the coupons from both the assays were retrieved and coupon surfaces were cleaned (washed in acid, neutralized with sodium bicarbonate, rinsed in water and acetone, and dried in an air stream) [3]. The coupon weight loss was determined by measuring the weight of the coupon using an analytical balance (Sartorius AG model BSA 224S-CW, Goettingen, Germany) before and after completion of the assays. Weight of the coupons were measured in grams and the weight loss recorded in the coupon in the negative control (media blank) was subtracted from the weight loss values obtained for the treated samples and the positive control (inoculated with 10^5 cells of native SRB strain and without any antimicrobial substance i.e. essential oil).The corrosion rate (CR) of N-80 steel coupons was then calculated as per NACE standard SP0775-2013 (NACE,2013) and is expressed in millimeters per year (mm/y) , using 7.86 g cm^{-3} as the density of N-80 carbon steel. Scanning Electron micrographs of the surface features of the untreated N-80 steel coupons were also captured using SEM S-3600N (Hitachi High-Technologies Corp., Japan).

RESULTS

LEO, CEO and PEO were tested against a native SRB strain. MIC of LEO, CEO and PEO were determined by visualizing the inhibition of the physiological activity of the native SRB strain to produce the blackish colour in the Sodium Lactate SRB medium by iron sulfide precipitation. The MIC of LEO, CEO and PEO was established as 50 ppm, 100 ppm and 450 ppm respectively based on cell culture plates assays containing the essential oils in varying concentrations starting from a lowest concentration of 10 ppm to 600 ppm. A representative image of one such cell culture plate assay taken after 28 days of incubation at 37°C is shown in Figure 1. The Sub-MIC (0.5× MIC) and supra-MIC (2× MIC) of LEO, CEO and PEO were also derived and are presented in Table 1.

LEO used in the present study exhibited the lowest MIC against the native test SRB strain at 50 ppm whereas the highest value was recorded for PEO at 450 ppm (Table 1). The value of MIC observed for CEO was 100 ppm. The inhibitory effect of LEO, CEO and PEO in inhibiting the physiological activity of SRB under the assay conditions can be attributed to the sole or synergistic effect of known naturally occurring anti-microbial substances as their major constituents. These anti-microbial substances that are amongst the major components of the essential oils are namely Citral, Geraniol and Geranyl acetate in the case of LEO [5,6]. The major anti-microbial components of CEO are Geraniol, Citronellol, Citronellol, Citronellal [7]while the anti-bacterial property of PEO can be attributed to its constituents Pogostone and Patchoulol [8].Reports on similar antimicrobial studies with medicinal plant essential oils specifically on Sulphate Reducing Bacteria (SRB) encountered in the Oil and Gas Industry could not be found. However, Korenblum and her co-workers in 2013, while studying the anti-microbial action of Cymbopogon citratus essential oil collected from the medicinal plant garden of the Federal University of Sergipe in Brazil against SRB isolated from a soured oil reservoir in Purdu Bay, Alaska, reported the MIC of LEO to be 0.17 mg/ml (170 ppm) [3]. They further observed that there was no difference in the inhibition action of Lemongrass essential oil and its major anti-microbial component (Citral). They therefore, concluded that the MIC values for LEO and highly pure commercial Citral (>96% purity) procured from Sigma-Aldrich, Australia, were same [3]. In comparison the results obtained in the present study with the Lemongrass species grown widely in the state of Assam, India i.e. Cymbopogon flexuosus (Lemongrass) and Cymbopogon winterianus (Citronella) have shown better inhibition of the native SRB test strain with an MIC value of 50 ppm and 100 ppm, respectively (Table 1).

Minimum Bactericidal Concentration assays (Figure 2) for Lemongrass essential oil (LEO), Citronella essential oil (CEO) and Patchouli essential oil (PEO) was established by adding each of the essential oils at their Sub-MIC, MIC and Supra-MIC (Table 1) concentrations to sterile sodium lactate SRB medium in cell culture plates that were inoculated with the native test strain of SRB. Optical density readings taken from the wells containing LEO, CEO and PEO at their Sub-MIC, MIC and Supra-MIC concentrations after one minute of addition of the essential oils to the test system and also after completion of the incubation time period of 7 days is shown in Table 2. The MBC of LEO, CEO and PEO was established as the same value as their MIC, as no cell growth was evident from the optical density readings from any of the three replicate wells, in case of each of the essential oils tested.

The MBC of LEO, CEO and PEO in the present study was established as the same value as their MIC, as no cell growth was evident from the optical density readings in the MBC assay (Table 2). This result is in agreement to the results obtained by Korenblum and her co-workers in 2013, with regards to the MIC and MBC values of the essential oil of *Cymbopogon citratus* against the non-native test SRB strain *Desulfovibrio alaskensis* NCIMB 13491[3]. The results indicate that the test essential oils namely LEO, CEO and PEO showed a bactericidal effect against the native SRB strain. The optical density readings taken from the wells containing LEO, CEO and PEO at their Sub-MIC, MIC and Supra-MIC concentrations after one minute of addition of the essential oils to the test system and also after completion of the incubation time period of 7 days showed that there was an immediate bactericidal effect of the essential oils upon contact with SRB cells (Table 2).

Reduction in cell density and growth inhibition of the biofilms of the native test strain of SRB at MIC and Supra-MIC concentrations of LEO,CEO and PEO was visualized in the micrographs obtained using a Olympus BX43 Microscope (Figure 3). Microscopic visualization of the SRB biofilms indicate that at their MIC and supra-MIC concentrations LEO, CEO and PEO were able to reduce cell density of the native SRB strain and subsequently arrest biofilm formation. Sub-MIC levels for LEO showed relatively higher reduction in the number of SRB cells in comparison to sub-MIC levels of CEO and PEO. Untreated SRB cells (positive control) formed dense biofilms on glass surfaces (Figure 3 J).

Results obtained from the study of SRB biofilms on glass coupons indicate that the essential oils used in the present study not only inhibited biofilm formation but also reduced biofilm cell densities in comparison to the untreated control. LEO, CEO and PEO were able to reduce SRB cell numbers and prevent further biofilm development at their MIC and supra-MIC levels. Only LEO was able to reduce bacterial numbers in its sub-MIC concentration of 25 ppm whereas minimal reduction was seen in the case of CEO and PEO at their sub-MIC levels (Figure 3).

The inhibition of SRB biofilm formation was observed on N-80 carbon steel coupons conditioned with LEO, CEO and PEO (Figure 4). Dense black corrosion precipitates could be observed in the positive control N-80 steel coupons which were devoid of any antimicrobial treatment, while the treated coupons showed inhibited biofilm development (Figure 4). The average corrosion rates determined in the case of Assay 1 and Assay 2 are shown in Table 3.There was a slight corrosion detected on the blank media coupons (negative control), which was considered as chemical corrosion and as such this was subtracted from the weight loss values of the treated coupons and untreated positive control (Table 3). The average corrosion rates calculated for the coupons are also presented in Table 3. SEM micrographs of the untreated coupons, before and after completion of the assays, demonstrate the nature and extent of biocorrosion on the N-80 steel coupons by the test native strain of SRB under *in-vitro* conditions (Figure 5).

The inhibition of SRB biofilm formation was also observed on N-80 carbon steel coupons with LEO, CEO and PEO at 25 and 50 ppm concentrations (Figure 4). Although inhibition of SRB biofilm and reduction in bio-corrosion rates was seen in the treated coupons in both the assays, the average corrosion rates calculated for the coupons (Table 4) indicate that the surface layering of the coupons (Assay 1) was less effective than the direct incorporation of the antimicrobial substance in the assay system (Assay 2).

Based on the classification of average corrosion rates [9] the essential oils of Lemongrass, Citronella and Patchouli reduced the severity of the SRB induced bio-corrosion in the N-80 steel coupons to low from the high corrosion rate observed in case of the untreated control (positive control) (Table 4). The performance of LEO was the best followed by CEO, with the least anti-corrosion activity being shown by PEO in both the assay conditions (Table 4). SEM micrographs of the untreated coupons demonstrate high SRB biocorrosion on the N-80 steel coupons under in-vitro conditions. Pitting corrosion was also observed in the micrographs in addition to surface corrosion with serration and wave like undulations (Figure 5).

Table 1: Minimum Inhibitory Concentrations (MIC) of the essential oils

LEO: Lemongrass essential oil (Species: *Cymbopogon flexuosus)*; CEO: Citronella essential oil

Medicinal and Aromatic Plant Essential Oil	**Minimum Inhibitory Concentration (MIC)** (ppm)	**Sub- Minimum Inhibitory Concentration (Sub- MIC)** (ppm)	**Supra- Minimum Inhibitory Concentration (Supra-MIC)** (ppm)
LEO	50	25	100
CEO	100	50	200
PEO	450	225	900

(Species: *Cymbopogon winterianus*); PEO: Patchouli essential oil (Species: *Pogostemon cablin*)

Table 2: Spectrophotometric readings for the MBC Assay of the essential oils

Essential Oil / Test Condition	Optical Density (O.D.) readings at 600 nm after 1 minute of addition of test essential oil in the assay system			Optical Density (O.D.) readings at 600 nm after 7 days of addition of test essential oil in the assay system		
	Sub- MIC	MIC	Supra-MIC	Sub- MIC	MIC	Supra-MIC
LEO	0.433	0.122	0.120	0.401	0.125	0.115
CEO	0.515	0.145	0.145	0.563	0.147	0.145
PEO	0.727	0.145	0.146	0.756	0.149	0.146
Positive Control (Untreated)		0.939			1.215	
Negative Control (Media blank)		0.062			0.062	

LEO: Lemongrass essential oil (Species: *Cymbopogon flexuosus)*; CEO: Citronella essential oil (Species: *Cymbopogon winterianus*); PEO: Patchouli essential oil (Species: *Pogostemon cablin*). Values are mean of three replicates.

Table 3: Effect of the essential oils on SRB induced corrosion on N-80 steel coupons

Assay 1: Essential oil surface layered coupons					Assay 2: Essential oil added in culture media with coupons				
Anti-corrosion Treatment	**Initial Weight (g)**	**Final Weight (g)**	**Mass loss (g)**	**Average Corrosion Rate (mm/y)**	**Anti-corrosion Treatment**	**Initial Weight (g)**	**Final Weight (g)**	**Mass loss (g)**	**Average Corrosion Rate (mm/y)**
LEO(50 ppm)	3.6377	3.6354	0.0023	0.0191	LEO(50 ppm)	3.9151	3.9138	0.0013	0.0108
LEO(25 ppm)	3.8856	3.8815	0.0041	0.0340	LEO(25 ppm)	3.8790	3.8776	0.0014	0.0116
CEO(50 ppm)	3.8602	3.8569	0.0033	0.0274	CEO(50 ppm)	3.6655	3.6635	0.002	0.0166
CEO(25 ppm)	3.6966	3.6883	0.0083	0.0688	CEO(25 ppm)	4.0480	4.0456	0.0024	0.0199
PEO(50 ppm)	3.9115	3.9046	0.0069	0.0572	PEO(50 ppm)	3.3622	3.3608	0.0014	0.0116
PEO(25 ppm)	3.9767	3.9678	0.0089	0.0738	PEO(25 ppm)	4.0028	3.9998	0.003	0.0249
Positive Control (Untreated)	3.9161	3.8920	0.0241	0.1998	Positive Control (Untreated)	3.9161	3.8920	0.0241	0.1998

Values are mean of three replicates. Final weight of coupons calculated after subtracting 0.0004 grams of weight loss observed on the coupon of Negative Control (Media Blank) from the values obtained by weighing the coupons in analytical balance. Positive control used, was same for both the assays. The average corrosion rate of N-80 steel coupons was calculated as per NACE standard SP0775-2013. LEO: Lemongrass essential oil; CEO: Citronella essential oil; PEO: Patchouli essential oil.

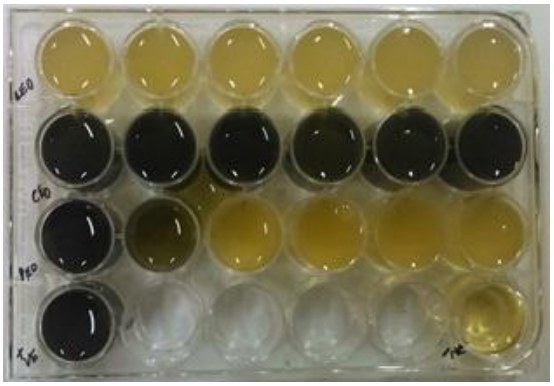

Figure 1: Minimum Inhibitory Concentration assay for the MAP essential oils

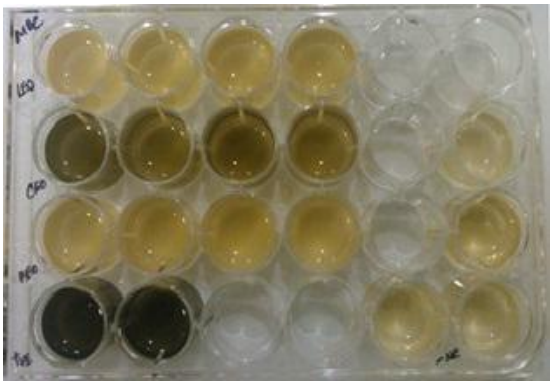

Figure 2: Minimum Bactericidal Concentration assay of the MAP essential oils

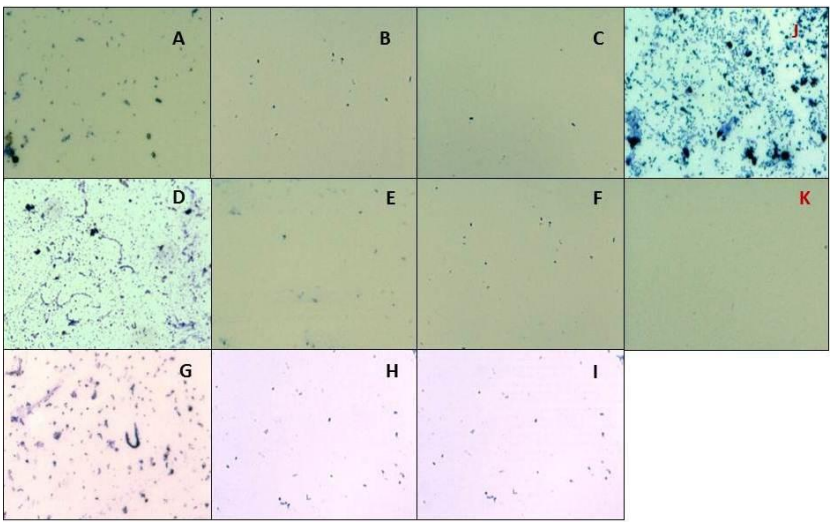

Figure 3: Light Micrographs of stained SRB biofilm on Glass coupon after 7 days.
A, B and C: LEO at sub-MIC, MIC and supra-MIC levels; D, E and F: CEO at sub-MIC, MIC and supra-MIC levels; G, H and I: PEO at sub-MIC, MIC and supra-MIC levels. LEO: Lemongrass essential oil; CEO: Citronella essential oil; PEO: Patchouli essential oil. J: Positive control (Untreated SRB biofilm), K: Negative control (culture media blank). Images captured in 40X magnification in Olympus BX43 microscope.

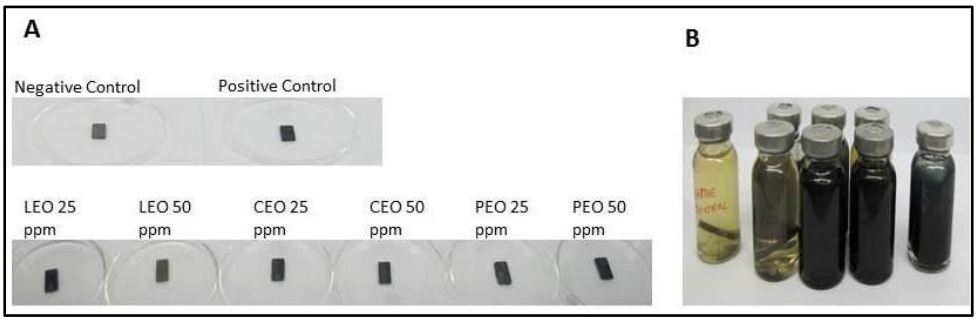

Figure 4. SRB biofilm formation on N80 steel coupons.
A: N-80 Coupons at 25 and 50 ppm of LEO, CEO and PEO. B: Experimental setup for biocorrosion study using serum bottles. LEO: Lemongrass essential oil; CEO: Citronella essential oil; PEO: Patchouli essential oil

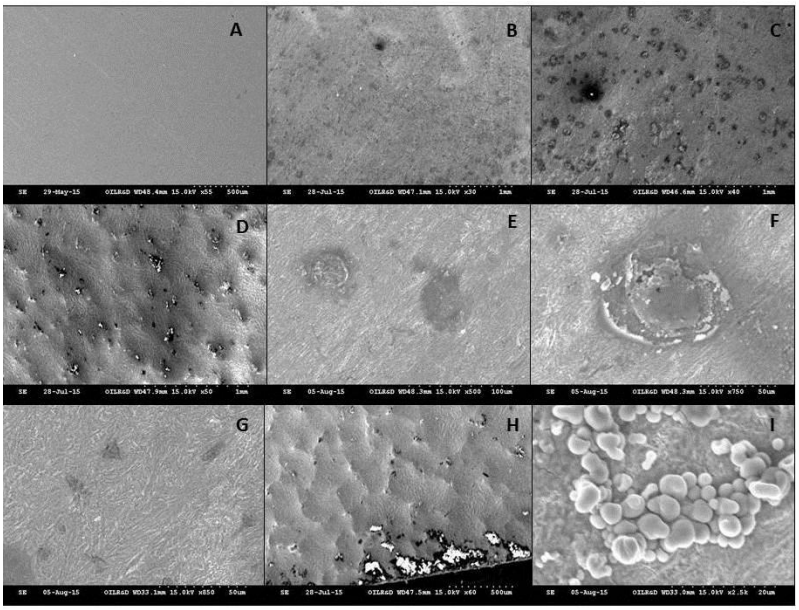

Figure 5. Nature and extent of bio-corrosion induced by the native test strain of SRB on the (untreated control) N80 steel coupons.

A: Surface before start of the assays; B: Surface after end of 28 days incubation period; C: Coupon surface showing pitting corrosion; D: Surface showing wave like undulations with pit formation; E and F: Surface morphology of the pits; G: Corroded surface showing serration; H: Wave like surface undulation with pits near the edges containing bacterial biomass; I: Bacterial cells attached to the surface.

CONCLUSIONS

The present study clearly establishes the antimicrobial and anticorrosion activity of the selected (MAPs essential oils against SRB induced biocorrosion under laboratory conditions. To the best of our knowledge the research work embodied in this manuscript is the first effort in scientifically demonstrating the antimicrobial and anticorrosion effects of Lemongrass (Cymbopogon flexuosus), Citronella (Cymbopogon winterianus) and Patchouli (Pogostemon cablin) of Assam, India against SRB isolated from oil field water of Duliajan, Assam, India. Lemongrass Essential Oil and Citronella Essential Oil used in the present study exhibited relatively low MIC value of 50 ppm and 100 ppm, respectively in comparison to Patchouli Essential Oils which exhibit a relatively high MIC value of 450 ppm. Significant antimicrobial and anticorrosion activity was also observed for these essential oils, in the biofilm inhibition assays performed on glass and N-80 steel coupons. Lemongrass and Citronella essential oils have been found to show promising results and future efforts may be aimed at successfully managing SRB population and SRB induced biocorrosion in the Oil Field installations using these naturally occurring, less toxic, eco-friendly and sustainable alternatives to the chemical biocides, currently in usage. Sourcing of these widely grown MAPs essential oils for future field application in the oilfield installations of Assam, , India is also expected to provide a boost to cultivation of these plant species thereby generating an income source for the farmers and leading to overall economic growth of the bio-resource rich region.

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