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Research Article

Natural Biocides for Mitigation of Sulphate Reducing Bacteria

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This study addresses the potential usage of various herbaceous plants extract including betel leave extract (BLE), green tea (GTE), turmeric (TE), belalai gajah (BGE), garlic extracts (GE), and manjakani extracts (ME) as future biocides of SRB that are natural and nontoxic. Study revealed that retardation of growth was obtained after addition of 5mL of natural biocides to 100 mL of the culture. Reduced biomass growth was observed with most of the tested biocides, dictated by lower biomass contents accepts for ginger and garlic. The planktonic growth was successively suppressed with addition of GTE, TE, and BGE, where the biomass production was decreased by more than 80.0% compared to the control experiments. GRE increased the growth of planktonic bacteria while the GE induced the formation of biofilms, showed by increase in biomass productions with over 23.4% and 77.46% enhancements, respectively. These results suggest that turmeric, green tea, and belalai gajah plants extracts are highly potential biocidal agents for mitigating SRB, thus controlling the effect of MIC on metal surfaces. However, the chemical stability, potential toxicity, and consistent performance of the extracts need further investigation for optimization of its use on a real field scale.

1. Introduction

Corrosion is a daily phenomenon that posed a serious hazard on the mechanical structure of building, transportation, piping, automotive, and many more [1-4]. This natural occurring phenomenon was induced by several folds with the existing microbiologically influenced corrosion (MIC), which is caused by both the aerobic and anaerobic bacteria [5, 6]. MIC has been classified into three categories to distinguish the various mechanisms which are caused by the extracellular electron transfer (EET) and metabolites secreted by microbes and caused by microbial species that secrete enzyme or other corrosive chemical [7]. The biocorrosion that often occurs in the presence of metallic compound (e.g., iron and steel) was impossible to ignore since the use of the material is prominent in various field including transportations, building, and engineering. In the oil and gas industries, the microbial corrosion was mainly due to the activities of sulphate reducing bacteria (SRB) and nitrate reducing bacteria (NRB). SRB was responsible for most instances of accelerated corrosion to ships and offshore steel structures. The SRB, under the anoxic conditions (e.g., in oil and gas pipelines), reacts with sulphur compounds in the

seawater and produces hydrogen sulphide (H₂S), which is corrosive to the metallic structures.

The cause of SRB activities had increased the direct operational expenses due to failure of structure and operational malfunctions. The replacement cost for the pipeline due to biocorrosion was reported to be \$250 million dollar per annum [8]. Besides that, the direct cost for combatting its existence alone causes billions of dollars, where some oil companies allocated minimum millions of dollars for the price of biocides alone [8]. The economic and technical impact, accounted for billions of dollars caused by the MIC, has boosted researchers to find alternatives to reduce or mitigate the process considerably.

The current strategies using chemical biocides to kill the SRB have shown great success [9]. However the use of chemical based materials imposed hazard on the environment, in which any countries have imposed stricter regulation on using biocides in any industries, thus encouraged the search for greener biocides. Despite the presence of numerous studies on producing new type of green biocides, researchers are still far from reaching the main goals to produce environmental-friendly type biocides. Therefore the

current research is focusing on producing 'greener' biocides with greater performance or at par with the existing chemicals biocides. There are several papers reporting on the use of green biocides, either independently or mixed with the existing chemical biocides in certain ratios, as an alternative to reduce the dependency on these harmful chemicals [10-12]. Others reported the use of the natural resources, ranging from waste material to plant extracts, and had shown considerable effects on combatting the activities of the SRB bacteria [13-15]. Chelators such as EDDS (a green chemical) are also investigated as a biocide enhancer to combat SRB biofilm [12]. For instance, cow urine at much lower concentration managed to kill all the existing SRB compared to its chemical counterparts [16]. Besides that, biosurfactant produce by Bacillus licheniformis managed to kill all the SRB after 3 hours of addition at 1% concentration [17]. Other natural resources from herbaceous extract showed significant effects towards SRB activities although they do not completely inhibit the growth. The antimicrobial properties found in Neem extract had reduced the SRB activities by 50% [8]. The antimicrobial effect of tested natural plant extracted has been attributed to the presence of compounds such as flavonols, phenolic acid, terpenes, anthocyanins, stilbenes, and tannis. [18].

Current strategies for biocides application combine antimicrobial agent and surfactant and may add green chemical to enhance the efficacies of antimicrobial/biocides. Even with this finding, high concentration of biocides is required (10x or more cont) to eradicate sessile bacteria in biofilm community. Biofilm consists of microorganism, substances including extracellular polymeric substances (EPS), and nutrient entrapped from the water. Cell living in biofilm can employ various defence mechanisms including diffusional limitation, lowered metabolic rate to reduce intake, formation of persistent cells, upregulation of resistance genes, efflux pumps, etc. [16]. These defence mechanisms reduce biocides susceptibility to interact with bacteria. Based on McDonnell and Russell [19], the defence mechanism of bacterial attack by biocides is a sign of evolution and may develop high level of resistance towards biocides. Therefore, green biocides are defined to be substances with highly effective, environmentalfriendly antimicrobial properties with the ability to reduce bacterial chances to develop resistance.

Driving into the biological routes to mitigate Sulphate Reducing Bacterial (SRB) is of great worth in order to allow a better alternative and natural-friendly way in controlling the effect of SRB-induced corrosion. Malaysian-herbs resources have recently been confirmed that a number of natural compounds in plants have an antibiofouling and antibacterial properties [20]. Therefore the present study is aimed at identifying some of the herbaceous plant that may offer the antibacterial properties towards SRB. The benefit of this study is to offer alternative biocides that posed very minimal hazards to the environments but offer similar effectiveness as the chemical biocides.

2. Materials and Methods

2.1. Culture Preservation and Growth. SRB bacteria were isolated from Lutong and Baram Oil Field in Sabah by

Universiti Teknologi Malaysia. The bacteria were preserved in 20% glycerol stock and stored in -80°C for subsequent used [20].

2.1.1. Culture and Growth. Medium was prepared with 4.096 g of MgSO₄, 5.7 g sodium citrate, 1 g of CaSO₄, 1 g of NH₄Cl, 0.5 g of $\rm K_2H_2PO_7$, 1.0 g of yeast extract, and 4.5 ml of sodium lactate. All of the chemicals were dissolved in 1 L distilled water and stirred for 30 minutes before autoclave in 121°C for 20 minutes. 5 mL of ammonium ferum sulphate from stock solution was added to the sterilized Baar's media, prepared by dissolving 6.72 g of ammonium ferum sulphate into 50 mL of distilled water.

The initial pH was adjusted in the range of 6.9 -7.1 with 1M $\rm\,H_2SO_4$ and 1M NaOH. All media components were autoclaved at 121°C for 15 minutes, except for iron(II) sulphate which was filtered sterilized using 0.42 μ m PTFE filter. All the heat/filter sterilized media were mixed in 1L Schott bottle and purged with nitrogen gas for 40 minutes. A loopful of the bacteria from 2-day-old agar culture was transferred to the medium and leave in the 37°C incubator without agitation for 2 days [21].

2.1.2. Efficacies of Growth. SRB were grown in 100 ml anaerobic vials. All procedures involving the transfer of SRB were carried out in anaerobic chamber prior flush with mixture of 90% nitrogen and 10% hydrogen. The procedure for determining the efficacies of biocides to mitigate the growth of sessile SRB was carried out by adding 5 mL/9 mL biocides (1 mg extracts powder dilute in 1 mL of water) into SRB culture on the third day of inoculation.

100 ml of the Baars medium was transferred into each anaerobic vial, followed by addition of 1 mL of two-day-old SRB seed culture, equivalent to $\sim\!\!10^7$ CFU/mL. 5 mL of biocides at concentration of 1000 mg/L was added and mixed vigorously. The serum vials were then sealed and left at 37°C incubator for seven days without agitation. The SRB growth was monitored daily and sample was withdrawn for the measurement of cell count using haemocytometer, conductivity meter, optical density, and biomass.

2.1.3. Biofilm Growth. The carbon steel API-X70 coupon used in this study was obtained from the segment of offshore pipe obtained from local gas pipelines. The pipes were cut into 15 mm x 10 mm, with thickness of 5 mm using metal cutter, and polished with sand paper of 500 and 800 grits. The metal was immersed in ultrasonic bath for 10-15 minutes to remove the dirt and contaminants. It was then autoclaved at 121°C for 15 minutes before being transferred to the 100 mL anaerobic vial and incubated for 2 weeks at 37°C without agitation to promote the biofilms growth. The coupons were then removed and treated with 10% glutaraldehyde in 0.1 M Phosphate Buffer Saline (PBS) buffer and subjected to ethanol washing to preserve the biofilm.

2.2. Extraction of Plant Biocides. Allium sativum (garlic), Zingiber officinale (ginger), Curcuma longa (turmeric), Piper betle (betel leave), Capsicum peppers (chili), oak galls (manjakani), Clinacanthus nutans (belalai gajah), and Camellia

sinensis (green tea) were tested on the SRB culture for its antibacterial activity.

10 g of powdered sample of dried plant parts was extracted with 50% methanol (green tea, turmeric, betel leaves, and garlic), 100% methanol (clove, chili, manjakani, and ginger), and distilled water (belalai gajah leaves) in ratio of 1:5 in a successive manner to produce crude extracts containing wide range of active compounds. The extracts were prepared by maceration of the plant material with the solvents in a shaker for 2 days. The respective extracts were filtered using Whatman No. 1 filter paper and the procedure was repeated for two times followed by drying under reduced pressure at a temperature approximately 45°C in rotary evaporator. The dense residue was collected and stored at 4°C until use. 1 mg/ml of extracts was used for antimicrobial activity.

2.3. Analysis

2.3.1. Scanning Electron Microscopy (SEM). The coupon metal coupons from the anaerobic vials was taken out and immersed in 2.5 wt% glutaraldehyde solution for 8 h and subjected to subsequent ethanol dehydration with series of concentration (30%, 50%, 70%, 100% v/v). The sample was exposed for 10 minutes in each concentration and repeated twice for the final step followed under CO_2 using critical point dryer. Finally, the sample was glued on the stub and coated with Pt alloy prior to SEM observations to increase the resolution signals. The entire surface area of coupon was examined under SEM to locate sessile SRB.

2.3.2. Biomass and Optical Density. 1 mL of sample was withdrawn daily and filtered using 0.45 mm cellulose acetate syringe filter and and washed with distilled water twice. The filter was dried in 60°C oven until consistent weight was achieved, and the biomass was measured using balance to precision. Optical density was measured at 400 nm with UV spectrophotometer (Hitachi) using distilled water as a blank. Values were taken at average from two replications.

3. Results and Discussion

3.1. Preliminary Study on the Efficacies of Biocides on SRB Planktonic Growth. Eight natural biocides extracted from local plants, namely, garlic (GE), turmeric (TE), ginger (GRE), green tea (GTE), betel leaf (BLE), belalai gajah, (BGE), manjakani (ME), and chili (CE), were tested for their inhibitory effects on the SRB growth. The substrates were selected from diversified species for its well-known application among the locals for its antiseptic and antibactericidal effects (REF). These extracts were applied on the third day of incubation and the effects on the SRB growth were monitored on daily basis. 5 mL of 1000 mgmL⁻¹ biocides was added to the bacteria culture and the results were compared with the control experiments. The presence of SRB using the sample from Lutong shows sign of growth after 5-7 hours of cultivations marked by accumulation of dark precipitates due to occurrence of ion Fe²⁺ reductions [8].

Figure 1 shows the effect of the biocides on the biomass growth of planktonic SRB on the day seven. All the natural

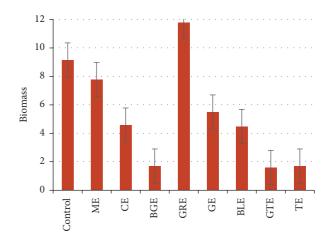


FIGURE 1: Effects of biocides on the growth of SRB bacteria, on the 7^{th} day of cultivation.

plants extracts show reductions in growth, except for ginger. The strongest growth retardation was observed with BGE, GTE, and TE, where the biomasses obtained were 81.4% (1.7 $\rm gL^{-1}$), 82.5% (1.6 $\rm gL^{-1}$), and 81.4% (1.7 $\rm gL^{-1}$) 7 lower than the control experiments (9.15 $\rm gL^{-1}$), respectively. However, the GRE (11.8 $\rm gL^{-1}$), on the other hand, promotes the growth of planktonic biomass with 29.0% enhancements over the control experiments. Moderate inhibitions were achieved with CE, ME, BGE, and GE, where the planktonic biomasses were decreased by 10 -50% of control experiment.

The concentration of biocides was fixed at 50 mgmL⁻¹, added on the 3rd day of cultivation. Addition of sodium lactates to the medium served as carbon source for both catabolism and metabolism processes [21].

A few methods were used to determine that the growth of the SRB was determined using two methods, qualitatively and quantitatively. Visual observation was carried out daily to observe the thickness and culture condition. The bacteria harbour and stick on the glass creating a condensed layer of thick residue that contained bacteria, metal ores, and medium. The layer that sticks on the glass was considered as biofilm growth and was noted as excessive biofilm (+++) to less (+) or none biofilm (0) formed. Other than that, the turbidity of culture was measured using UV spectrometer to evaluate the growth of the planktonic SRB. Table 1 shows the result from the visual observation.

3.2. Effect of Concentration on the Retardation of the Planktonic Growth. The effect of concentrations on the planktonic growth was carried out by dispensing and filtering 1 mL of sample, washing subsequently with distilled water, and drying in 60°C oven until constant weight was achieved. This procedure was carried out on daily basis after the addition of biocides. It was observed that, at 50 mgmL⁻¹, the retardation of planktonic growth was recorded with all types of biocides as opposed to the control, dictated by the declining of biomass weight. Figure 2 presents the profile of the planktonic biomass weight. Rapid decline of the planktonic growth was observed after the introductory of biocides in day three, while the

Sample	Blackening	Turbidity	Viscosity	Biofilm
Control	++++	+++	+++	+++
Garlic	++++	+++	++++	++++
Green Tea	++	+++	+	Nd
Turmeric	++	++	+	Nd
Belalai Gajah	+++	++	++	++
Manjakani	+++	++	++	++
Ginger	++++	+++	++	+
Betel Leave	+++	++	+	+

TABLE 1: Visual observation of the culture characteristic after treatment with biocides.

Note: ++++: excess; +: less; Nd: no detected.

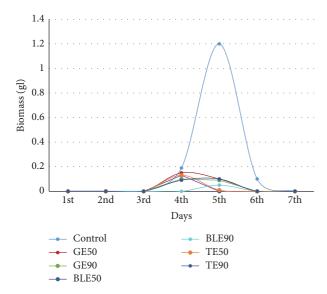


FIGURE 2: Effects of biocides concentration on the planktonic biomass. The culture was grown for day seven, and the biocides were injected on day three.

control continued to grow and achieve maximum in day five. The declining trend in the control experiment after day 6th might be caused due to depleting of the essential nutrient. On the other hand, the growth of SRB after the biocides addition was repressed immediately, and the declining effect took place in the 4th day of incubations.

Referring to Figure 3, the highest decline of planktonic growth was observed with tea extracts (77.46 %), followed by garlic (64.79%) and betel leave (18.31%). However, after increasing the biocides concentration from 50 mgmL⁻¹ to 90 mgmL⁻¹, the growth exhibited variety of responses. Reduction of growth, as compared to the growth at 50 mgmL⁻¹, biocides were further enhanced by ~23.40% and 77.78% for BLE and GTE, respectively (Figure 3). On the other hand, the garlic extracts resulted in 52.80% enhancement, which is least expected. However, further observation was carried out to measure the total growth (planktonic + biofilm-wall growth) because some of the biocides triggered excess wall growth (Table 1) before withdrawing conclusive remarks. The result is presented in Figure 5.

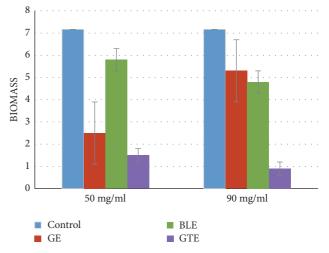


FIGURE 3: Effect of biocides concentration on the growth of planktonic SRB in 100 mL vials at 37°C in anaerobic condition. The biomass concentration was evaluated at the day 6th.

According to Farjana et al. [14], tea extracts at certain concentration prevent the adhesion of bacteria to the abiotic surfaces caused by the active biomolecules called proanthocyanidin that had been reported to interact with bacterial outer membrane that prevents the adhesion. Failure to attach either reversibly or irreversibly limits the survivability of these SRB, which caused the growth to reduce dramatically.

Second evaluation was carried out for determination of overall biomass productions (planktonic and sessile growth) by filtering the biomass content and dried in oven until constant weight was achieved. The biomass concentration was evaluated for the treatment using 3 types of biocides concentration at 90 mgmL⁻¹. The result in Figure 5 shows that the overall biomass content after introduction of GE was boosted by 6-fold compared to control. Meanwhile, the planktonic growth only shows less than 2-fold increase (Figure 3). GE results in increased viscosity of the medium, which secured the anaerobic condition for the metabolic activities inside the vial. This condition is preferable for the growth of SRB; thus excess formation of biofilm and wall growth was observed (Figure 4). The first speculation on the use of garlic extract (50 mgmL⁻¹) provides reduction of planktonic growth compared

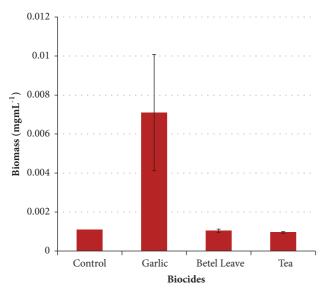
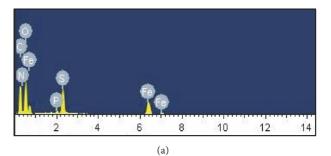
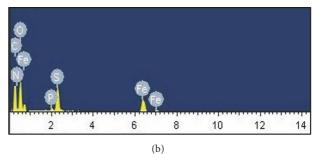


FIGURE 4: Effects of biocides (garlic, betel leave and tea) at concentration of 90 mgmL $^{-1}$ on the overall biomass weight collected after $7^{\rm th}$ days of incubation.

to control experiments, shown by the reduction in biomass harvested. Through secondary observation, these extracts also promoted strong growth on the vials wall boosted to several folds (Figure 4). It is speculated that the planktonic SRB moves towards the wall and created layers of cells, and if continued further it would form permanent biofilms. The migration of cells from the suspension to the vials wall reduced the concentration of planktonic cells and increased the thickness biofilm. It is expected that the quorum sensing of the cells is induced with existence of certain chemicals in the garlic extracts that promotes the communication between cells and greatly enhanced the formation of biofilm. Furthermore after increasing the biocides concentration from 50 mgmL⁻¹ to 90 mgmL⁻¹, the amount of planktonic biomass also increased by half (compare to 50 mgmL⁻¹) due to excess growth on the biofilm, where some of the loosely bound cells were released back to the suspension. On the other, the garlic, for example, encourages the formation of biofilm (wall growth) or the aggregation of cells. It is speculated that the extract of garlic was oily and thus might have altered the viscosity of the medium, reduced the solubility of oxygen, and thus contributed to highly anaerobic conditions in the growth bottle. The increased viscosity with highly anaerobic conditions might have boosted the growth on the wall (biofilm) and thus results in less reading of planktonic biomass (Figure 1) in comparison to control.

3.3. Observation with SEM and EDX. The SEM micrographs and EDX spectra for the control specimen and sample tested with GTE and TE are shown in Figures 5 and 6. The EDX analysis was employed to gather information on the surface composition in the absence and presence of biocides of the SRB culture. For this test, the biocides were added at the beginning of the cultivation and the samples were analysed after two weeks. The carbon steel coupon was





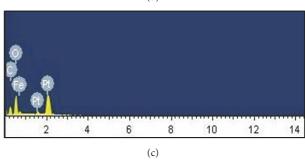


FIGURE 5: EDX spectra of carbon steel coupons: (a) control; (b) after addition of 90 mg/mL GTE; and (c) after addition of 90 mg/L of TE.

treated according to Section 2.3.1 and analysed with EDX machine. Considerable amount of ferrous ion, sulphurous compound, phosphorus, carbon, nitrogen, and oxygen was present on the surface (Figures 5(a) and 5(b)). Addiction of GTE (Figure 5(b)) suppressed the peak of the ferrous ion as opposed to the control (Figure 5(a)), which was an indicator of reduced corrosion. Interestingly, the peak of ferrous ion at 6-8 keV (Figure 5(c)) was totally absent in the spectra which suggested that the addition of TE had successfully suppressed the corrosion considerably. Besides that, elimination of strong sulphide peak on the metal surface (Figure 5(c)) provides additional hypothesis that the turmeric extracts (TE) had successfully stopped the corrosion after its addition. The absence of nitrogen, phosphorus, and sulphide peaks was obvious in the sample treated with TE (Figure 5(c)).

The SEM analysis was carried out for visual observation of the intact surface. The analysis shows that the carbon steel coupon was covered with the black clumpy deposits, products from the SRB corrosion. The SEM micrograph for the control specimens showed uneven surface with very black and thick lumps. This could be an indicator of highly corroded surface. However the condition of the coupon surface was less clumpy with even blackish tone being observed with GTE and TE treatment (Figures 6(b) and 6(c)). The black deposit was a

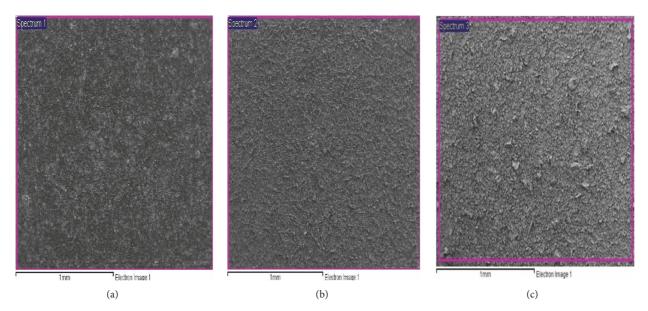


FIGURE 6: Surface evaluation by SEM micrograph. (a) Control, (b) treatment (GTE); and (c) treatment with turmeric (TE) with green tea.

result from the reactions of sulphides generated by SRB with ferrous iron in the medium.

In actual practice, the existence of SRB can cause dealloying of the metallic surface which caused permanent damage and altered its morphological structure. The result from the SEM and EDX spectra supports the results from this study that turmeric extracts may serve as good inhibitor for the SRB growth, thus reducing the corrosion activities.

4. Conclusions

Early observation of the works revealed the potential of natural biocides to be manipulated for the mitigation of sulphate reducing bacteria. Different biocides exhibited variety of response towards the growth of SRB. Some biocides enhanced the growth of planktonic SRB, while some induced the formation of biofilm. Extracts from green tea leave, belalai gajah, and turmeric show promising results as they not only prevented the formation of biofilm/wall growth, but also retarded the overall SRB's growth. These biocides repressed the planktonic growth by more than 50% compared to the untreated sample. The decline will subsequently contribute to the reduction of the SRB-induced corrosion, although the actual rate of corrosion was not measured in this work. The tea, turmeric, and betel leaves extracts show potential to be explored as they provide strong effects towards multiplication of the SRB due to reducing planktonic growth and absence of biofilm. The existence of certain chemicals in these biocides that may either damage the cellular structure of the SRB or simply prevent the quorum sensing and limits the cellto-cell communications provides new insights into highly potential biocides to be explored and thus commercialized. More works are needed to define the exact mechanism of these biocides and their influences on reduction of SRB growth.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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