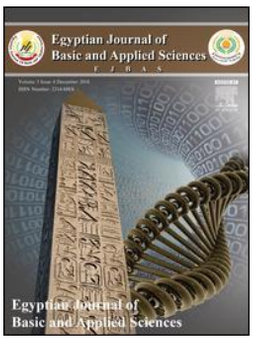

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The purification and functional study of new compounds produced by *Escherichia coli* that influence the growth of sulfate reducing bacteria

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ABSTRACT

The induction and inhibition of sulfate-reducing bacterial (SRB) growth are of academic and biotechnological interests. The purification and functional study of two compounds produced by *Escherichia coli* were reported in this research. One of the compounds induces the growth of SRB and was termed as SRB growth enhancer (SGE) and the other inhibits their growth and was referred to as SRB growth inhibitor (SGI). The *E. coli* was cultured in M9 medium (1:20) and grown at 37°C for 24 h under limited oxygen condition. The cultures were spun and the supernatant was filter-sterilized. The cell-free supernatant was purified by ion-exchange chromatography and size exclusion chromatography. The fractions obtained were tested for inhibitory and enhancing activities of serially diluted *Desulfovibrio indonesiensis, D. vulgaris* and *D. alaskensis*. The effect of SGE and SGI on the morphology of SRB cells and biofilm formation was investigated using scanning electron microscopy (SEM) and atomic force microscopy (AFM). The structural analysis of purified SGE and SGI was carried out using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) spectrometry. SRB cultures in the presence of SGE showed two order of magnitude higher growth**s** than untreated SRB cultures while with SGI they showed growth with two order of magnitude less. The SRB treated with the SGE improved the SRB growth while the inhibitor altered SRB morphology. The MALDI-TOF spectra in linear mode showed the compounds to be an oligomeric series with repeat ~213 m/z unit between each peaks. The data revealed that the molecular mass of SGE is around 1700 Da while that of SGI is estimated as 2400 Da. The result of this study suggests that SGE can be used as a supplement in the media for rapid SRB detection while the SGI can be used for the prevention of SRB colonization.

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SRB; SGE; SGI; *E. coli*; microscopy; MALDI-TOF spectrometry

Introduction

SRB are taxonomically unrelated group of microorganisms that acquire energy for their growth by oxidizing organic substrates and hydrogen. They utilize sulfur compounds, as final electron acceptor during anaerobic growth [1–6]. SRB encompass 60 genera of bacteria, accounting for 220 species [7]. These include proteobacteria, e.g. *Desulfovibrio*, firmicutes, e.g. *Desulfotomaculum* [3,4], archaebacteria, e.g. *Archaeoglobus* [8,9] and thermodesulfobacteria, e.g. *Thermodesulfobacter* [8]. SRB are chemolithotrophic and physiologically distinctive group of anaerobic bacteria. The SRB are widely distributed [9], phylogenetically diverse

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[10] and thrive well in a wide range of environmental conditions [11]. They grow well in anaerobic niches where sulfate reduction is the principal biomineralisation pathway [12].

SRB have been found in soil sediments, storage tanks, water sediments, oil-fields, buried steel piping, shipping industries, ships, nuclear waste containers, water and wastewater plants, marine sediments, hydrocarbon seeps, oil and gas production and storage facilities [6,12–14]. They also use sulfate as a final electron acceptor for the breakdown of the organic nutrients. This results in sulfide production, or if iron is available, as black iron sulfide [7,14]. The ribosomes of SRB are characterized by specific sequence of nucleotides in 16S rRNA (ribosomal ribonucleic acid chains) which helps in their identification. SRB have previously been thought to be strictly anaerobic, although, some genera can tolerate some levels of oxygen [15–18].

SRB have been implicated in biocorrosion, biofouling and hydrocarbon degradation. Early identification of SRB is difficult because they are capable of operating at low cell densities. This implies that rapid or early detection is important to their control [19,20]. Qi et al. [21] supported this statement by emphasizing the difficulty involved in fast enumeration of SRB population in corrosion and environmental studies. Historically, SRB have been detected and enumerated in oil-field systems by the use of culture media that detect only a fraction of the SRB population [22]. Brink et al. [23] emphasized the lack of comprehensive microbiological bioassays methods in oil-fields. Cowan [24] asserted that it takes 28 days to cover the whole SRB detection process and sometimes false reports are obtained. Rapid detection tools available are complex and expensive, e.g., quantitative PCR and SRB Rapid Detection Test Kit using APS reductase as a marker [18,19,25]. Therefore, due to the difficulty in rapid detection of SRB population and the importance of early identification, current studies on SRB detection are directed to using simple and quick approaches especially by supplementing small molecules that can induce microbial growth into microbiological culture media [26].

Another technique central to the control of SRB is the inhibition of their growth or activity. Many approaches have been tried to limit SRB growth in industries so that the production of hydrogen sulfide is prevented. These include the use of nanofiltration, biocompetitive exclusion, chemical inhibitors, biocides and coatings. However, the use of these methods has proved to be expensive, ineffective, or impracticable in the field. Microorganisms and the products of their metabolism are able to degrade most biocides, coatings and chemical inhibitors [27–29]. Also, incidences of equipment failure have been reported with the use of some biocides and most of the biocides are toxic and therefore pose danger to the environment [30,31]. In addition, many of the chemically derived antifouling agents are not environmentally friendly [31]. These account for why urgent research is directed toward the use of microbial metabolites to inhibit the growth of SRB through the production of antifouling enzymes, antimicrobial agents and chemical signals [31].

In nature, SRB have the ability to interact with other microorganisms and the products from these microorganisms [32,33]. When SRB was co-cultured with *E. coli*, it was observed to induce the growth and increase the detection sensitivity of the SRB cultures. Similarly, the addition of cell-free supernatant (CFS) from *E. coli* had a similar effect on the growth/detection of SRB. In order to identify the compounds responsible for the growth induction of *E. coli*, it is important that they be purified and characterized. Therefore, the aim of this study was to purify and identify two bioactive compounds derived from *E. coli* observed to influence the growth of SRB. One of the compounds referred to as SRB growth enhancer (SGE) induces the growth of SRB and the other compound, known as SRB growth inhibitor (SGI), inhibits SRB growth.

Materials and methods

Microbiological techniques

Bacterial strains

The bacterial strains used for this study were *D. indonesiensis* NCIMB 13468 [34,35], *D. alaskensis* NCIMB 13491 [36], *D. vulgaris* NCIMB 8303 and *E. coli*. The bacterial strains were obtained from the Microbiology Research Laboratory, University of Portsmouth, UK.

Media

The growth media used for this study were (i) LB (Luria-Bertani Broth) medium, (ii) MacConkey agar, (iii) M9 medium (100 µl sterile 1 M $CaCl_2$, 200 mL M9 salts 5x (64 g $Na_2HPO_4.7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl, 1 L DH_2O), 2 mL of sterile 1 M $MgSO_4$, 20 mL of 20% glucose and 1 L dH_2O [37]), (iv) vitamin medium (VM) with 500 mg/l of $FeSO_4$ (VMI) and (v) vitamin medium with 50 mgL of $FeSO_4$ (VMR) [38].

The VM medium was composed of 0.5 g KH_2PO_4, 1 g NH_4Cl, 4.5 g Na_2 SO_4, 0.04 g $CaCl_2.2H_2O$, 0.06 g $MgSO_4.7H_2O$, 0.3 g $Na_3C_6H_5O_7.2H_2O$, 25 g NaCl, 2 g casamino acids, 2 g tryptone, 6 g $Na_2C_3H_5O_3$, 1 mL vitamin stock solution, 1 mL trace element stock solution, pH 7.5, and 1 L dH_2O.

The vitamin stock solution contains 0.6 mg vitamin B1 (thiamine), 0.2 mg vitamin B2 (riboflavin), 0.5 mg vitamin B3 (nicotinic acid), 0.6 mg vitamin B5 (pantothenic acid), 0.6 mg vitamin B6 (pyridoxal phosphate), 0.05 mg vitamin B12 (cobalamin), 100 mg vitamin C (L-ascorbic acid), 0.01 mg vitamin H (biotin),1 L dH_2O and the trace elements consist of 1.5 g $C_6H_9NO_6$, 3.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4{\cdot}H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CoSO_4 \cdot 7\ H_2O$, 0.1 g $NiCl_2 \cdot 6H_2O$, 0.1 g $CuCl_2 \cdot 2H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g $KAl(SO_4)_2 \cdot 12H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4 \cdot 2H_2O$, 0.001 g Na_2SeO_3 and 1 L dH_2O.

Standard growth conditions for *E. coli* cells

The *E. coli* culture was refreshed by inoculating a stock culture in freshly prepared M9 or LB medium (1:20) and grown at 37°C for 24 h. The culture was serially diluted ten-fold, plated on MacConkey agar and incubated at 37°C for 24 h to check for purity and for counting of viable bacterial cells expressed as colony forming unit per milliliter (cfu/mL).

Growth procedure for SRB

The 1 mL stock culture of each SRB strain (*D. indonesiensis, D. alaskensis* and *D. vulgaris*) was inoculated into 9 mL of VMI medium at 1:10 (v/v) ratio using a syringe and needle to prevent the introduction of oxygen into the vial. The SRB initial inoculum size was determined using a hemocytometer. The cultures were incubated at 37°C for 7 days. The presence of SRB was revealed by the change of the growth medium from light yellow to black color at the base of the vial as a result of formation of insoluble iron sulfide (FeS) due to reaction of hydrogen sulfide (H_2S) produced by metabolizing cells with iron sulfate in the medium.

Procedure for obtaining cell-free supernatant

An overnight culture of *E. coli* (5 mL) was inoculated into 95 mL of M9 medium at 1:20 (v/v) (18 x 100 mL vials) and incubated at 37°C for 6, 12, 18, 24, 48 and 72 h in limited oxygen conditions. After incubation, 0.1 mL each of the 100 mL *E. coli* culture was serially diluted ten-fold and inoculated on MacConkey agar to check for purity of the culture and determine the viable counts. Each of the 100 mL cultures was spun in SORVALL RC 5 C Plus at 18,000rpm (38,828 g) for 35 min using rotor SS-34, 4°C. The supernatant obtained was filter-sterilized *via* a 0.22 µm Millipore filter unit (Stericup) *in vacuo* to obtain a cell-free supernatant (CFS) and stored at 4°C for further purification.

Purification of cell-free supernatant by ion-exchange chromatography

The first stage for the purification of the CFS was ion-exchange chromatography. This procedure was carried out using 5 mL of Q Sepharose Fast Flow™ resin in 1.0 × 10 cm Econo-Column®

(Bio-Rad). The column was equilibrated with 10 volumes of 50 mM NaCl in 10 mM Tris-HCl, pH 8.0 using 1 mL/min flow rate. The CFS was diluted 1 in 3 with 10 mM Tris-HCl, pH 8.0. The column was connected to Bio-Rad Biologic Low Pressure (LP) system and a computer system installed with Bio-Rad Biologic LP system software. The column was washed with 10 volumes of 50 mM NaCl in 10 mM Tris-HCl, pH 8.0 and the bound materials in the column were eluted with step gradients of 125, 250 and 500 mM NaCl in 10 mM Tris-HCl, pH 8.0. The partially purified SGE fractions were tested for activity on the growth of *D. indonesiensis* in VMR medium.

Large-scale purification of SGE using ion-exchange chromatography

The *E. coli* was inoculated in 100 × 100 mL (10 L) of M9 medium using 1:20 dilution of cells and incubated at 37°C for 18 h in limited oxygen condition. The *E. coli* cultures were spun at 13,000 rpm (22278 g) for 35 min at 4°C in SORVALL RC 5 C Plus centrifuge using rotor SLA-1000. The supernatant was sterilized using a 0.22 µm Millipore filter unit (Stericup) to obtain a CFS and was stored at 4°C for further purification. To purify the 10 L CFS, ion exchange chromatography was used and elution was by step gradient (Figure 1). The column (5.0 × 10 cm Econo-Column® (Bio-Rad)) contains 100 mL of Q Sepharose Fast Flow™ resin (GE Healthcare). The column was equilibrated with 20 volumes of 50 mM NaCl, 10 mM Tris-HCl, pH 8.0 using 10 mL/min flow to ensure washing of the unbound materials. Prior to loading, the CFS was diluted 1 in 3 with 10 mM Tris-HCl, pH 8.0 and applied by pumping onto the column. The column was washed with 10 volumes of 50 mM NaCl, 10 mM Tris-HCl, pH 8.0 and the bound materials in the column were eluted with step gradients of 125, 250 and 500 mM NaCl in 10 mM Tris-HCl, pH 8.0 using Bio-Rad Biologic LP system. The chromatographic fractions were also collected and stored at 4°C for testing and for further purification.

Purification of SGE using size exclusion chromatography

The step 3 fraction (100 mL) obtained from large-scale purification of SGE was concentrated to 3 mL by ion-exchange chromatography (Figure 2). This is because, in order to use gel filtration efficiently, the volume of loading fraction for this size of column (2.5 x 75 cm Econo-Column® (Bio-Rad) with cross-sectional area of 4.91 cm^2) should not be more than 3 mL. The column used contains Toyopearl (HW-40S) (TOSOH Bioscience LLC) with a fractionation range of 100–7000 Da. The column was prepared with 200 mL of HW-40S resin and was equilibrated with 10 volumes of 50 mM NaCl in 10 mM Tris-HCl, pH 8.0 using 0.5 mL/min flow rate. In all the purifications by size exclusion, 50 mM NaCl in 10 mM Tris-HCl, pH 8 was used as the mobile phase.

The concentrated step 3 fraction was applied to the gel filtration column using 0.5 mL/min flow rate. The fractions without the void volume were then pooled and again concentrated to 1 mL using Q Sepharose Fast Flow resin and eluted by one step gradient of 700 mM NaCl in 10 mM Tris-HCl, pH 8 (Figure 2). The HW-40S column was washed with 10 volumes of 50 mM NaCl in 10 mM Tris-HCl, pH 8 after which the concentrated fraction was applied to the column using 0.5 mL/min flow rate. During gel filtration, two distinct peaks were detected (Figure 3). These peaks subsequently named as SGI (Peak 1) and SGE (Peak 2) were pooled separately and sterilized by filtration into a sterile vial (Fisher Scientific) using Minisart 0.22 µm syringe filter (Fisher Scientific) and de-oxygenated for 30 min by sterile nitrogen gas. It was immediately covered with a sterile injection stopper (Fisher Scientific) and sealed using an aluminum crimp seal (Fisher Scientific). Both fractions were tested for functional activities.

Procedure for testing SGI and SGE on SRB growth

Prior to testing, the fractions were filter-sterilized using a syringe driven Millex^{R} sterile filter unit with 0.22 µm MF-Millipore^{TM} membrane and de-oxygenated by sterile nitrogen for 30 min. The test organisms were grown in VMI medium at 1:10 (v/v) ratio in anaerobic condition at 37°C for 7 days. The fractions were tested by serially diluting *D. indonesiensis*, *D. vulgaris* and *D. alaskensis* in VMI up to 10^{-5} dilution (test for inhibition) and VMR (test for enhancement) and incubated at 37°C. Fractions were introduced into each of the dilutions at 1:20 (v/v) ratio. Controls were set up without fractions. Growth was examined and recorded daily by observing the presence of insoluble black iron sulfide at the base of the vials. All the tests were done under anaerobic conditions.

SEM analysis on SGI and SGE treated SRB

The effect of SGI and SGE on the morphology of SRB cells and biofilm formation was investigated using an SEM, JEOL 6060LV. Glass coupon (7 x 10 mm) was placed inside 9 mL of VMR medium in a 10 mL vial. The medium was de-oxygenated using nitrogen gas for 30 min and sterilized by autoclaving for 15 min. An aliquot of the 7 days old culture of *D. indonesiensis* was added into the medium using 1:10 (v/v) ratio and SGI was added into the medium at 1:20 (v/v) ratio. The culture was incubated at 37°C for 1, 4 and 7 days. All the above procedures were carried out using aseptic technique. Two controls were set up one with SGI only and another comprising a culture of *D. indonesiensis* only. This procedure was repeated for SGE. Glass coupons containing biofilms were fixed with Gluteraldehyde (4 %) in phosphate buffer (0.1 M) overnight at 20 (±2)°C and rinsed in phosphate buffer (0.1 M) twice at 10-min interval. The glass coupons were then fixed with 3 drops of osmium tetroxide (1 %) in sodium cacodylate buffer (0.1 M) at 20 (±2)°C for 1 h and mixed gently using B7925 rotator to ensure uniform distribution of the solvent. The coupons were rinsed twice in 0.1 M phosphate buffer at 10-min interval. This was followed by 10 min sequential exposures to 30, 50, 70, 90 % (v/v) aqueous solutions of absolute ethanol (reagent grade) and final immersion for 10 min in 100 % acetone (reagent grade). Following dehydration, two drops of hexamethyldisilazane were placed on each coupon to cover the slides and the specimens were incubated overnight at 20 (±2)°C. The glass coupons were placed on aluminum stubs and sputter-coated with gold using Q150 R ES for 5 min. The Au-coated specimens were viewed at 15 kV accelerating voltage value.

AFM analysis on SGI and SGE treated SRB

The effect of SGI and SGE on the morphology of SRB cells and biofilm formation was also carried out using Multi-Mode/NanoScope IV scanning probe microscope, Bruker, Santa Barbara, CA, USA. Glass coupon (7 x 10 mm) was placed inside a 9 mL of VMR medium in a 10 mL vial. The medium was de-oxygenated using nitrogen gas for 30 min and sterilized by autoclaving for 15 min. An aliquot of the 7 days old culture of *D. indonesiensis* was added into the medium using 1:10 (v/v) ratio and SGI was added into the medium at 1:20 (v/v) ratio. The culture was incubated at 37°C for 7 days. All the above procedures were carried out aseptically. A control was untreated culture of *D. indonesiensis*. This procedure was repeated for SGE. The glass coupon was removed from the medium by using forceps and placed on a surface of freshly cleaved muscovite mica (1 cm^2; Agar Scientific, Stansted, Essex, UK), left for 2 min, rinsed with dH_2O and dried in an N_2 stream. The surface was then attached to a nickel disk mounting assembly (1 cm^2) using double-sided adhesive tape and placed on top of the AFM scanner. AFM studies were performed in air under ambient conditions (T = 23°C, RH = 21%) using the J-scanner (max. xy = 200 µm). Scanning was performed in contact mode using a V-shaped, silicon nitride cantilever with an integrated tip

(lever D: t = 0.6 µm, l = 205 µm, w = 25 µm, k = 0.06 N m-1; model: NP-10, Bruker, France). The images were subsequently processed and dimensions measured using NanoScope Analysis software (V 1.4, Bruker).

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis on SGI and SGE

Samples preparation for MALDI experiments involved preparation of 50 mM of triethylammonium bicarbonate (TEAB) buffer (SIGMA-ALDRICH) and dissolving lyophilized SGI and SGE fractions in 50 µL of TEAB buffer. The 50 µL of TEAB buffer was placed in a spin cartridge and 100 µl of TEAB was added. This was spun six times at 13000 rpm for 3.5 min at room temperature using Galaxy 14 D centrifuge (VWR). This is to ensure complete buffer exchange to TEAB buffer to improve resolution. Mass spectrometry plate reader was prepared by washing with isopropanol, water and methanol and was allowed to dry. The 3 µL of SGI samples was mixed with 3 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix, spotted on the microplate slide and allowed to dry. The MALDI micro MXTM (Micromass MS Technologies) was calibrated using a mixture of des-ArgBradykinin, Angiotensin I, Glu-Firbopeptide B and Neurotensin (Applied Biosystems). The screening of SGI and SGE fractions was made for mass-charge-ratio (m/z) range of 500–6000 Da in positive modes. Control used was 10 mM Tris-HCl, pH 8, matrix (α-cyano-4- hydroxycinnamic acid (CHCA)), TEAB buffer, mixture of TEAB buffer and matrix.

Results

The representation of 25 different elution profiles results of the first stage of purification, carried out by ion-exchange chromatography is shown in Figure 1. The step gradients of 125 and 250 mM NaCl were used to remove the main quantity of the high molecular weight compounds such as DNA, protein, polysaccharides and glycoproteins present in the CFS. Fraction of step 3 gradient (~100 mL) was used for further purification by gel filtration. The chromatogram for obtaining concentrated fraction is presented in Figure 2.

The chromatogram obtained when concentrated fraction obtained in Figure 2 was applied to the HW-40 S column is shown in Figure 3. During gel filtration, two distinct peaks were detected. At least 75 repeat experiments were done and generated the same results. The molecular mass of the peak 1 and 2 compounds is higher than 1355 Da estimated from the molecular size of the compounds in these peaks, the position of vitamin B12 (1355 Da) and Blue Dextran (2,000,000 Da).

Results of testing fractions from gel filtration on SRB growth

The results of testing fractions from gel filtration on SRB growth showed that compounds present in peak 2 had inducing effect, while peak 1 exhibit slight inhibitory effect on the growth of SRB strains (Table 1, Figure 4). When serially diluted cultures of each SRB strains (*D. indonensiensis, D. vulgaris* and *D. alaskensis*) in VMR medium were tested with peak 2 fraction, growth was observed up to 10^{-4} dilution after 2 days of incubation (Table 1), whereas untreated SRB culture had growth only up to 10^{-2} dilutions (Table 1, Figure 4). This is a two order of magnitude difference between growth of untreated SRB culture and SRB treated with peak 2 fraction.

To optimize bioactivity test of peak 1 fraction for inhibitory effects on SRB growth, VMI medium was used. The VMI medium contains 500 mg/L of $FeSO_4.7H_2O$, which is optimum for SRB growth. The idea here was to check if the medium will show a better inhibition of SRB growth by peak 1 fraction. The results of testing peak 1 fraction on SRB growth are shown in Table 2 and Figure 5. It revealed

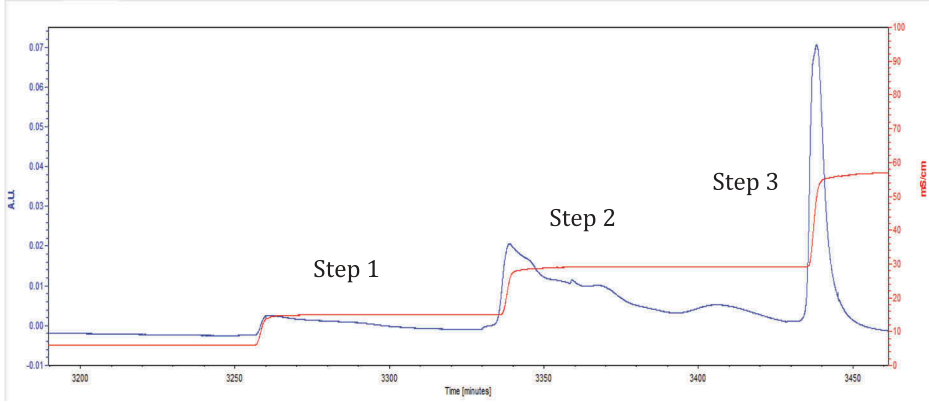

Figure 1. Chromatogram of elution of CFS from *E. coli* using step gradients.

Step 1: 125 mM NaCl in 10 M Tris-HCl, pH 8, Step 2: 250 mM NaCl in 10 mM Tris-HCl, pH 8, step 3: 500 mM NaCl in 10 mM Tris-HCl, pH 8.0. The blue line is UV (A.U.) absorbance measured at 280 nm while the red line indicates conductivity (mS/cm).

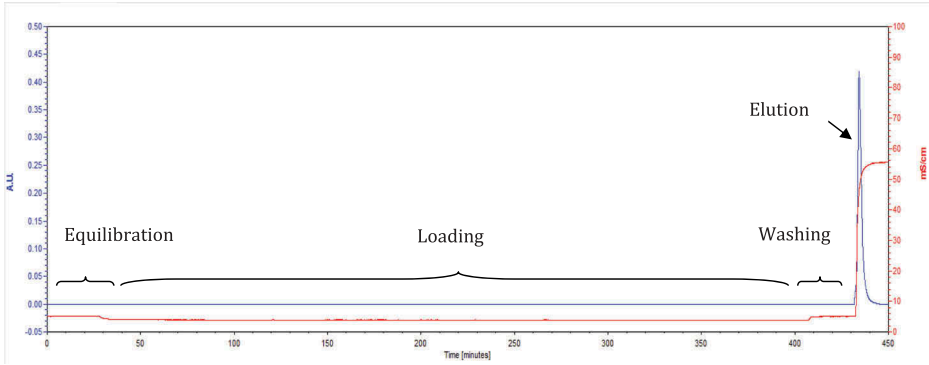

Figure 2. Chromatogram of elution of step 3 fraction.

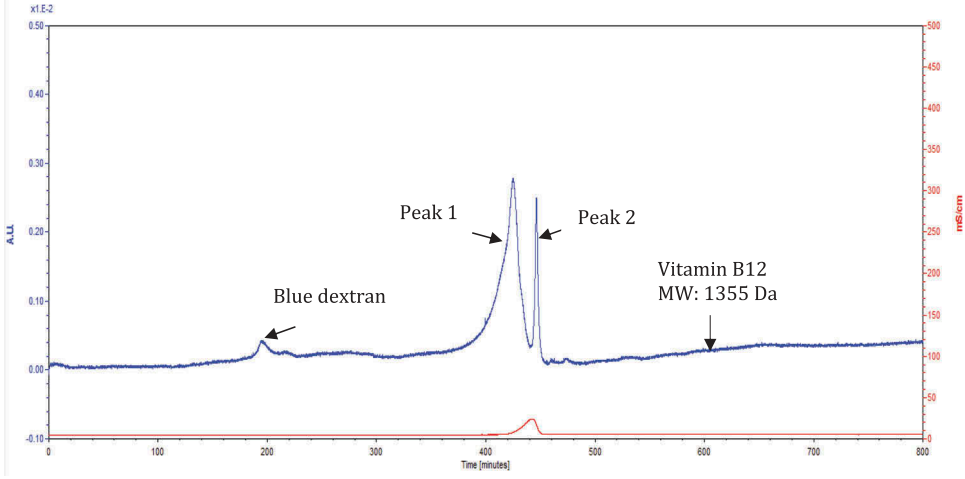

Figure 3. Chromatogram of concentrated step 3 fraction on HW-40 S column.

Peak 1 is subsequently referred to SGI and peak 2, SGE. The blue line on the chromatogram is UV absorbance measured at 280 nm, the red line indicates conductivity (mS/cm). Multiple experiments were done.

Table 1. SRB growth in VMR medium in the presence and absence of peak 2 fraction.

	Day 1			Day 2				Day 3			
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
SRB	+	+	-	+	+	-	-	+	+	-	-
+ Peak 2 fraction	+	+	+	+	+	+	+	+	+	+	+
+ Peak 1 fraction	+	-	-	+	-	-	-	+	±	-	-

(+) SRB growth, (-) no SRB growth, (VMR) vitamin medium with 50 mg/l of $FeSO_4$. Several independent experiments was done in triplicate. SRB: *D. indonensiensis, D. vulgaris* and *D. alaskensis*. SRB initial inoculum size: 1.0–2.5 × 10^7 cfu/mL.

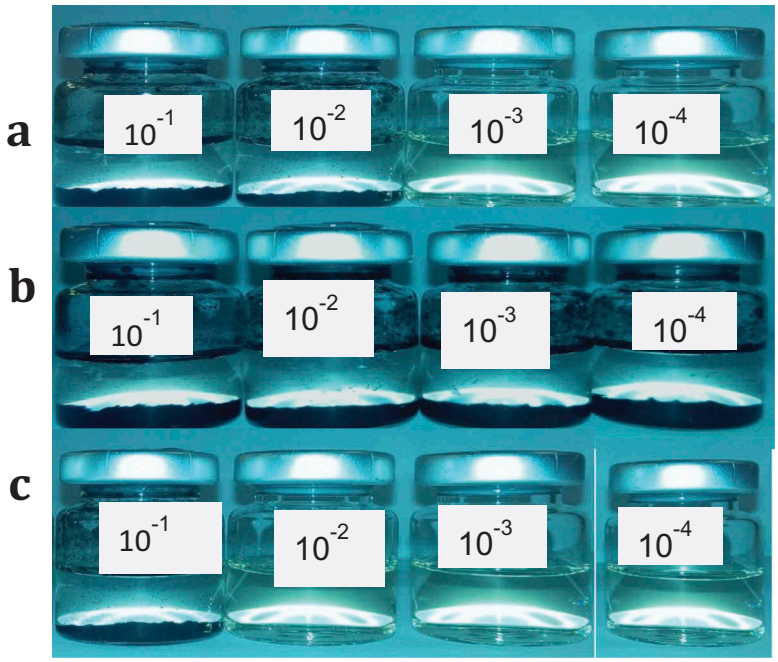

Figure 4. 2 days growth of SRB in the presence and absence of peak 2 fraction.

(a) Growth of control SRB, (b) Growth of SRB with peak 2 fraction, (c) Growth of SRB with peak 1 fraction. The black precipitate at the base of the vials indicates SRB growth.

that SRB strains tested with peak 1 fraction had growth up to 10^{-2} dilution after 3 days incubation (Table 2, Figure 5) when compared with untreated SRB that had growth up to 10^{-4} (Table 2, Figure 5). This shows a two order of magnitude growth inhibition of each SRB strain by the peak 1 fraction when compared with untreated SRB.

Thus, the peak 2 fraction induces the growth of the SRB while the peak 1 fraction inhibits their growth and is referred to as SGE and SRB growth inhibitor (SGI) respectively.

Microscopic characteristics of SRB growth in the presence of SGE and SGI

The representation of images obtained when cultures of *D. indonensiensis* in VMR medium was supplemented with SGI and viewed using SEM is shown in Figure 6. SGI fraction with no addition of bacterial cells is in Figure 6 a. There were no major changes observed between the morphology of cells, and biofilm formation of untreated (Figure 6 b) and SGI-treated SRB cells (Figure 6 c) after 1 day of growth. After 4 days of growth, the SGI-treated cells showed deformity

Table 2. SRB growth in VMI medium in the presence and absence of peak 1 fraction.

	Day 1			Day 2				Day 3			
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
SRB	+	+	-	+	+	+	-	+	+	+	+
+ SGI	+	+	-	+	+	-	-	+	+	-	-

(+) SRB growth, (-) no SRB growth, (SGI) SRB growth inhibitor, (VMI) vitamin medium (VM) with 500 mg/l of $FeSO_4$. Several independent experiments were done in triplicate. SRB: *D. indonensiensis, D. vulgaris* and *D. alaskensis*. SRB initial inoculum size: 1.0–3.5 × 10^7 cfu/mL.

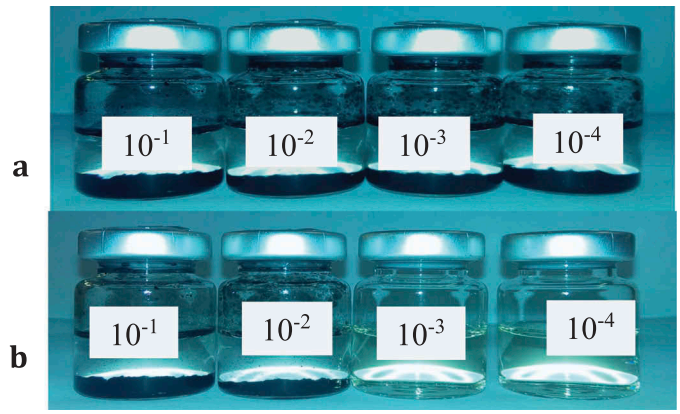

Figure 5. 3 days growth of SRB in VMI medium in the presence and absence of SGI.
(a) Growth of control SRB, (b) Growth of SRB with SGI. The black precipitate at the base of the vials indicates SRB growth.

in morphology and stunted growth due to their interaction with SGI (Figure 6 e) but untreated cells appeared normal (Figure 6 d). This suggests that the SGI had a negative impact on the morphology and integrity of the SRB cultures that may eventually lead to the impairment of their cellular functions resulting in cell death. On the seventh day of incubation, the untreated *D. indonensiensis* cells appeared normal (Figure 6 f) but the SGI-treated cells showed a pronounced deformity, making the negative impact of SGI on *D. indonensiensis* cultures more obvious (Figure 6 g). In this case, the integrity of the cell was compromised and the cell content discharged due to the interaction of SRB with the inhibitor.

The representation of scanning electron micrographs obtained when cultures of *D. indonensiensis* in VMR medium was supplemented with SGE is shown in Figure 7. The micrograph of SGE fraction incubated without SRB is presented in Figure 7 a. *D. indonensiensis* supplemented with SGE showed increased cell numbers and enhanced growth at each incubation period due to the presence of SGE (Figure 7 c, e and g) when compared to the control (Figure 7 b, d and f). Thus, the SGE induced the growth rate of the SRB cultures perhaps due to a reduction in time for their cell division. After 4 days of incubation, there was a notable interaction between the SGE fractions and the *D. indonensiensis* culture, which resulted in the growth induction of the SRB (Figure 7 e) unlike the untreated cultures (Figure 7 d). After 7 days incubation of *D. indonensiensis* with SGE in Figure 7 g, the EPS matrix encapsulates the SRB cells making them appear much healthier compared to the control cultures (7 f).

The effects of SGI and SGE on SRB growth using AFM

The representation of atomic force micrographs of *D. indonensiensis* grown for

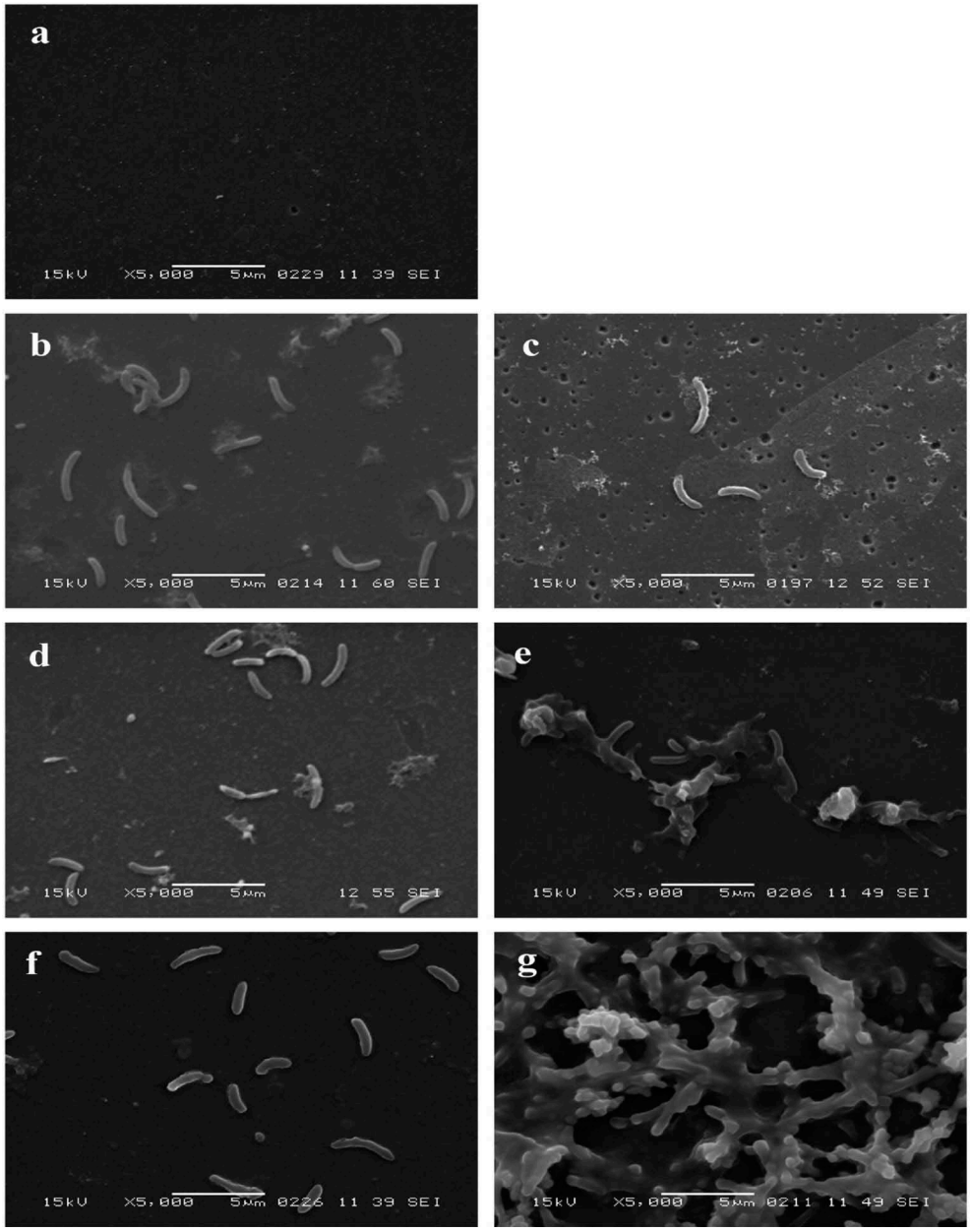

Figure 6. Scanning electron micrographs of SRB treated with SGI.

(a) SGI fraction only, (b) untreated *D. indonensiensis* after 1 day of growth, (c) *D. indonensiensis* treated with SGI after 1 day of growth, (d) untreated *D. indonensiensis* after 4 days of growth, (e) *D. indonensiensis* treated with SGI after 4 days of growth, (f.) untreated *D. indonensiensis* after 7 days of growth, (g) *D. indonensiensis* treated with SGI after 7 days of growth. The same SRB inoculum size of 2.5×10^7 cfu/mL was used for the experiment.

7 days with SGI and SGE are presented in Figure 8. *D. indonensiensis* treated with SGI had deformed cells (Figure 8 b). In contrast, *D. indonensiensis* treated with SGE had increased number of bacterial cells and enhanced growth (Figure 8 c) while the growth of untreated *D. indonensiensis* appeared normal (Figure 8 a).

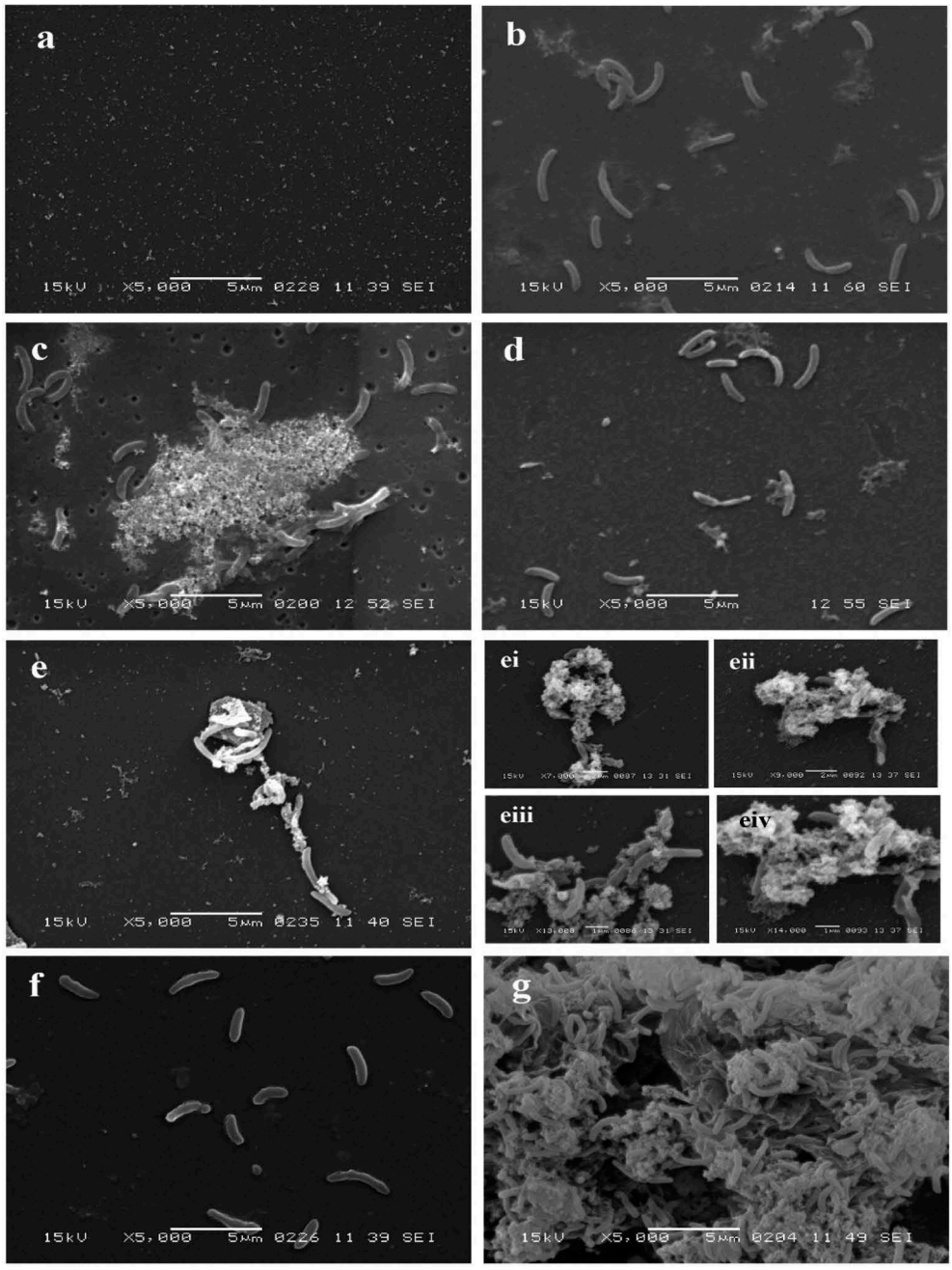

Figure 7. Scanning electron micrographs of SRB treated with SGE.

(a) SGE fraction only, (b) untreated *D. indonensiensis* after 1 day of growth, (c) *D. indonensiensis* treated with SGE after 1 day of growth (d) untreated *D. indonensiensis* after 4 days of growth (e) *D. indonensiensis* treated with SGE after 4 days of growth; (ei) x7000, (eii) x9000, (eiii) x13000, (eiv) x14000, (f.) Untreated *D. indonensiensis* after 7 days of growth, (g) *D. indonensiensis* treated with SGE after 7 days of growth. The same SRB inoculum size of 2.5×10^7 cfu/mL was used for the experiment.

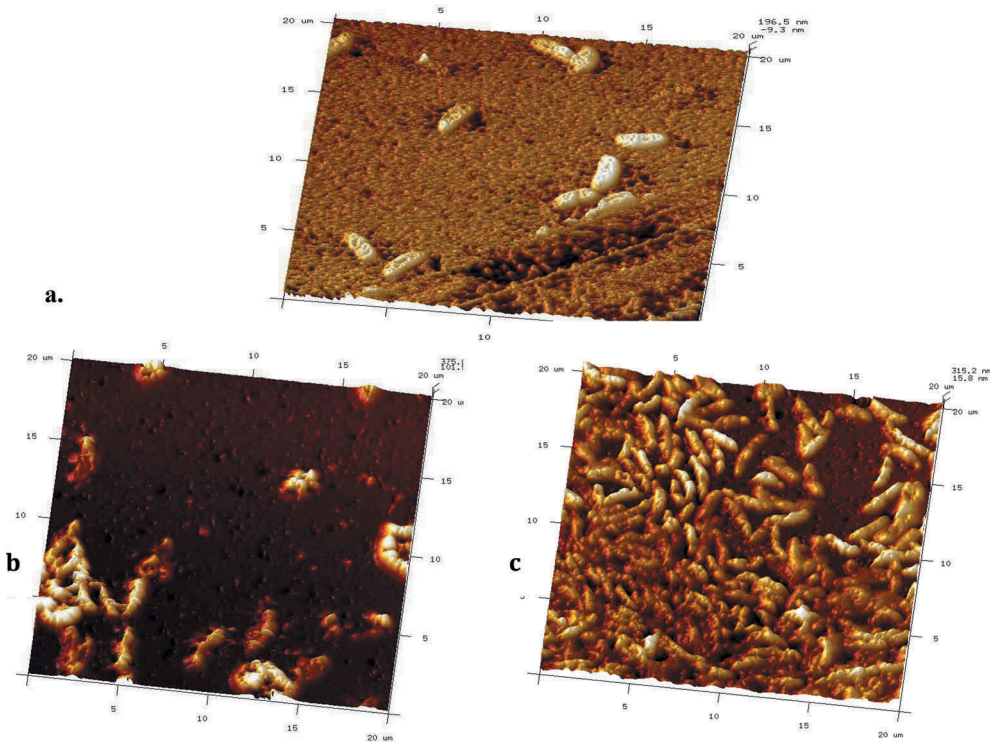

Figure 8. The atomic force micrographs of treated and untreated SRB.

(a) Untreated *D. indonensiensis* after 7 days of growth, (b) *D. indonensiensis* treated with SGI after 7 days of growth, (c) *D. indonensiensis* treated with SGE after 7 days of growth. The same SRB inoculum size of 3.5×10^7 cfu/mL was used for the experiment.

Characteristics of SGI and SGE by Maldi-Tof spectrometry

The spectra of SGI and SGE in positive mode are shown in Figures 9 and 10 respectively. The spectra suggest the compounds to be an oligomeric series with repeat unit of ~213 m/z. In both spectra, there is a gradual increase in the relative abundance as the m/z decreases. The dominant peaks in Figure 9 spectrum are observed at m/z 674.3, 888.3, 1100.3, 1313.3, 1526.3 and 1739.3 with the least m/z value giving the highest relative abundance. However, in Figure 8.13 spectrum, dominant peaks are observed in m/z 673.1, 886.3, 1099.7, 1312.7, 1525.7, 1738.7, 1951.7, 2164.7 and 2400. According to Pavia et al. [39], the heaviest significant m/z value often represents the molecular ion in mass spectrum, therefore, m/z value of 2400 in SGI indicates the molecular mass of SGI to be around 2400 Da (Figure 9). Similarly, the m/z value of 1700 in SGE indicates the molecular mass of SGE to be around 1700 Da (Figure 10).

Discussion

Based on the combined results from gel filtration chromatography, and MALDI-TOF spectrometry, the molecular weight of the two compounds is estimated to be around 1700 Da for SGE and SGI, 2400 Da. This shows that these compounds have low molecular weight properties. Their low molecular weight property suggests that the compounds being siderophore cannot be ruled out. Brandel et al. [40], Zheng and Nolan [41]; Saha et al. [42] and Ahmed and Holmström [43] described siderophores as metabolites that have low-molecular-mass, have strong affinity for ferric ion (Fe^{3+}) and are often excreted and exported by microorganisms during low iron

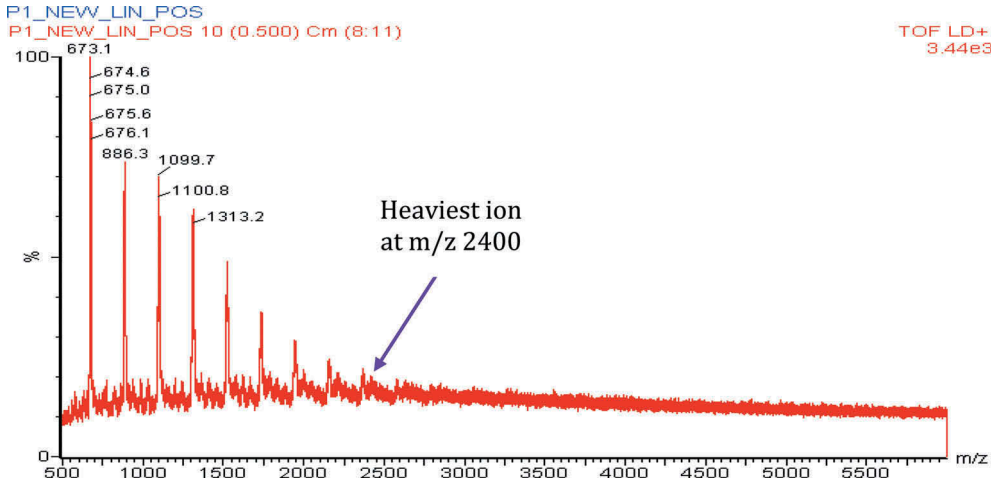

Figure 9. SGI in positive mode.

The spectrum contains fragments with a repeating unit of ~213 m/z between each peak. The heaviest significant m/z value of 2400 indicates the molecular weight to be around 2400 Da.

conditions. In this study, M9 medium was utilized for the production of SGE and SGI from *E. coli*, but M9 medium is devoid of iron.

However, according to Neilands [44], iron is one of the most essential elements required for the growth of microorganisms. Similarly, when there is none/low iron availability in a medium, most bacteria devise a means to scavenge iron from the environment using siderophores which have a very high affinity for Fe^{3+}, thereby making iron available to the microbial cell [44–47]. Mohandass [45] reported that siderophores are mostly novel compounds and contain many modified amino acids that are naturally not found elsewhere. Also, Martinez et al. [46], Ali and Vidhale [47] and Ahmed and Holmström [43] added that siderophores have several biotechnological, agricultural, environmental and

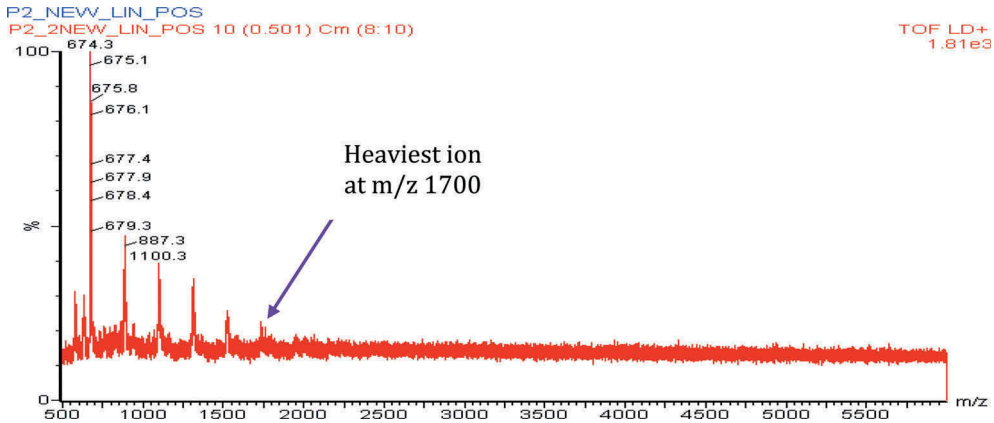

Figure 10. SGE in positive mode.

The spectrum contains fragments with a repeating unit of ~213 m/z. The heaviest significant m/z value of 1700 indicates the molecular weight to be around 1700 Da.

medicinal applications. According to Martinez et al. [46], most bacteria synthesize one or more types of siderophores, which are often secreted into the growth medium. For example, in iron-deficient medium *Hafnia alvei* produces ferrioxamine G (672 Da) and ferrioxamine E (653.53 Da) that are capable of inducing the growth of self and other bacteria [48,49], *Ustilago sphaerogena* produces ferrichrome (687.70 Da) [50,51], a marine bacterium *Alteromonas haloplanktis* synthesizes bisucaberin with molecular weight of 400.47 Da [52,53], acinetobactin (346.34 Da) is produced by *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa* produces two siderophores, namely, pyoverdin (1365.42 Da) and pseudobactin (1039.82 Da) [45,54–56].

The other possibility that the compounds are not siderophore is related to the size. The results obtained do not suggest that SGE is a siderophore produced by *E. coli* because the molecular weight is greater than the molecular weight of enterobactin, a siderophore produced by *E. coli* with a molecular weight of 669.55 Da [57-58]. Similarly, the autoinducer purified from *E. coli* has a molecular weight of 500 Da [59]. In addition, the SGI has not been documented in literature as a compound produced by *E. coli*. Thus, the SGE and SGI to our knowledge have not been previously described. Although iron can be acquired by bacterial cells using other means aside from siderophore. Messenger and Barclay [60] argued that iron can be acquired by the cell *via* the binding of iron to less specific cell wall components, for example Gram-negative bacteria uses lipopolysaccharides (LPS) as the most important nonspecific cation-binding element. Lipopolysaccharides are the principal component of Gram-negative bacteria and they give them their endotoxic and antigenic properties. The outer membrane also contains a proteinporins that serves as a substrate transport channel and helps in the semi-permeability of the small molecules [61]. The results obtained from the count of *E. coli* and the activities of SGE indicate that there is a correlation between the total viable bacterial counts and the production of the SGE. Similarly, Carvalho et al. [62] reported the optimum production of bioactive compounds from *Bacillus subtilis* R14 during stationary phase under oxygen limitation. In the same way, Rattanachuay et al. [63] grew *Pseudomonas* sp. W3 for 18 h to produce extracellular compounds that have inhibitory effects on pathogenic vibrios.

The SGE can be used as a rapid detection tool for SRB. If SGE is incorporated to commonly used media for detecting and isolating SRB, it can help to detect SRB with low cell densities, it can also enhance SRB growth and detection. This is similar to the argument of Bertrand et al. [26] who stated that microbiological culture media can be supplemented with small molecules that can induce or inhibit microbial growth. This detection sensitivity is comparable with complex rapid techniques, for example SRB Rapid Detection Test Kit using APS reductase as a marker [19]. However, the advantages of this approach are that this method (i) allows the use of culture method often used for SRB enumeration and detection, (ii) rapid SRB growth and detection sensitivity, (iii) living and metabolically active SRBs can be seen, (iv) SRB which cannot be recovered using standard culture procedures and therefore, often wrongly thought as dead can be reactivated.

According to Liu et al. [64], the cell membranes of bacteria are semi-permeable and this may allow the inflow of the growth promoter thereby enriching the cells and encouraging biofilm formation. The more abundant viable cells observed with SGE correlate to the more rapid growth observed in the liquid medium of the bioassay result. During purification of SGE, the inhibitor of SRB growth (SGI) was discovered. The development of biofilms follows three stages and includes irreversible attachment of single bacteria colonies on biotic or abiotic surfaces using structures such as pili, extracellular polymeric substances (EPS) and fimbriae. The next stage is growth. Once bacterial colonies are attached to surfaces, the bacteria replicate actively to form a complex structure referred to

as glycocalyx [65] and the cycle continues following dispersion of planktonic cells from the biofilm matrix [66,67]. However, if the initiation of SRB colonization on surfaces is prevented or if their growth is impeded, this will inhibit biofilm formation and eventually lead to the limitation of damage such as biocorrosion, equipment failure, biofouling, hydrocarbon degradation and reservoir souring facilitated by SRB. Meanwhile, the cell deformity, bulge and elliptical changes observed in SGI-treated SRB cells are similar to reports obtained when Mn^{2+} and Cd^{2+} were incubated with cultures of SRB strain *Salmonella daqing*, isolated from Daqing oil-field [64]. Similarly, Mishra and Malik [68] reported changes in the physiology and morphology of bacterial cells when exposed to heavy metal.

Several studies have described some inhibitors of SRB growth that are derived from bacteria; for example, Jayaraman et al. [69] and Zuo [29] reported that indolicidin, bactenecin, and polymyxin produced by *Paenibacillus polymyxa* are capable of inhibiting SRB growth. *Bacillus brevis* produces a compound referred to as gramicidin-S that inhibits the growth of *Desulfovibrio orientis, D. vulgaris* and *D. gigas* [29,31,70] and thereby reduced corrosion caused by the SRB. In addition, *Bacillus licheniformis* secretes γ-polyglutamate and polyaspartate that reduce SRB growth [29,71,72]. The mechanism of SRB growth prevention by these organisms has been suggested and include either the production of antimicrobial agents [29,73] or attack on the adenosine 5′-phosphosulphate (APS) and bisulfate reductase (DSR) responsible for hydrogen sulfide production in SRBs [14]. Similarly, the SGE may function in SRB induction by increasing their growth rate while the SGI may function by causing damage in the cells as observed in this study. The MALDI-TOF spectra showed the presence of low molecular weight compounds in the range of 1700 Da for SGE and 2400 Da for SGI. The spectra showed equal and repeating units of ~213 m/z between the peaks. According to Wallace and Guttman [74], the equal and repeating units are characteristic spectra of condensation homopolymers. MALDI-TOF spectra revealed that the compounds are small molecular weight biomolecules and that the two molecules are very closely related.

Conclusions

Two low molecular weight compounds have been isolated and purified from *E. coli* and are referred to as SRB growth enhancer (SGE) and SRB growth inhibitor (SGI). The biomolecules possess at least one negatively charged group. Functional analysis showed inhibitory effects of the inhibitor on the growth of SRB and SGE revealed their growth induction. The heaviest ion of SGI was at m/z 2400 and indicates the molecular weight of SGI to be around 2400 Da, while the heaviest ion of SGE was at m/z 1700 and indicates the molecular weight of SGE to be around 1700 Da.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] Guan S. Synergistic protection against microbiologically influenced corrosion using a 100 % solid polyurethane incorporated with antimicrobial agents. 490 Geanchie Drive Milton Ontario, Canada: A report of the Madison chemical industrial Inc.; 2000. p. 1–14.

[2] Zhang C, Wen F, Cao Y. Progress research in corrosion and protection by sulfate reducing bacteria, 2011 3rd international conference on Environmental Science and Information Application Technology (ESIAT 2011). Procedia Environ Sci. 2011;10:1177–1182.

[3] Keller KL, Wall JD, Chhabra S. Methods for engineering sulfate reducing bacteria of the genus. Desulfovibrio Methods Enzymol. 2011;497:503–517.

[4] Kakooei S, Ismail MC, Ariwahjoedi B. Mechanisms of microbiologically influenced corrosion: a review. World Appl Sci J. 2012;17 (4):524–531.
[5] Fichtel K, Mathes F, Könneke M, et al. Isolation of sulfate-reducing bacteria from sediments above the deep-subseafloor aquifer. Front Microbiol. 2012;3:65.
[6] AlAbbas FM, Bhola R, Spear JR, et al. Electrochemical characterization of microbiologically influenced corrosion on line pipe steel exposed to facultative anaerobic *Desulfovibrio* sp. Int J Electrochem Sci. 2013;8:859–871.
[7] Barton LL, Fauque GD. Biochemistry, physiology and biotechnology of sulfate-reducing bacteria. Adv Appl Microbiol. 2009;68:41–98.
[8] Sherry A, Gray ND, Ditchfield AK, et al. Anaerobic biodegradation of crude oil under sulfate-reducing conditions leads to only modest enrichment of recognized sulfate reducing taxa. Int Biodeterior Biodegrad. 2013;1–9. DOI:10.1016/j.ibiod.2012.04.009
[9] Plugge CM, Zhang W, Sholten JCM, et al. Metabolic flexibility of sulfate reducing bacteria. Front Microbiol. 2011;2(81):1–8.
[10] Castro HF, Williams NH, Ogram A. Phylogeny of sulfate-reducing bacteria. FEMS Microbiol Ecol. 2000;31:1–9.
[11] Grein F, Ramos AR, Venceslau SS, et al. Unifying concepts in anaerobic respiration: insights from dissimilatory sulfur metabolism. Biochim Biophys Acta. 2013;1827:145–160.
[12] Dolla A, Fournier M, Dermoun Z. Oxygen defence in sulfate-reducing bacteria. J Biotechnol. 2006;126:87–100.
[13] Jack TR. *Biological corrosion failures*, ASM international ASM handbook volume 11: failure analysis and prevention. American Society for Microbiology, Washington DC, United States; 2002. p. 882–890.
[14] Muyzer G, Stams AJ. The ecology and biotechnology of sulfate -reducing bacteria. Nature Rev Microbiol. 2008;6:441–454.
[15] Hardy JA, Hamilton WA. The oxygen tolerance of sulfate reducing bacteria isolated from the North Sea waters. Curr Microbiol. 1981;6:259–262.
[16] Wargin A, Olańczuk-Neyman KM, Skucha M. Sulfate reducing bacteria, their properties and methods of elimination from Groundwater. Polish J Environ Study. 2007;16(4):639–644.
[17] Lodowska J, Wolny D, Jaworska-Kik M, et al. The chemical composition of endotoxin isolated from intestinal strain of *Desulfovibrio desulfuricans*. Sci World J. 2012. Article ID 647352. DOI:10.1100/2012/647352
[18] Zhang-Sun W, Augusto LA, Zhao L, et al. *Desulfovibrio desulfuricans* isolates from the gut of a single individual: structural and biological lipid A characterization. FEBS Lett. 2015;589:165–171.
[19] Horacek GL. Field experience with an SRB rapid detection test kit. SPE Drilling Engineering Texas, United States; 1992. p. 275–278.
[20] Kondo R, Shigematsu K, Butani J. Rapid enumeration of sulfate reducing bacteria from aquatic environments using real-time PCR. Plankton Benthos Res. 2008;3(3):180–183.
[21] Qi P, Zhang D, Wan Y. A novel sulfate-reducing bacteria detection method based on inhibition of cysteine protease activity. Talanta. 2014;129:270–275.
[22] Ingvorsen K, Vester F. Improved most-probable-number method to detect sulfate-reducing bacteria with natural media and a radiotracer. Appl Environ Microbiol. 1998;64 (5):1700–1707.
[23] Brink DE, Vance I, White DC. Detection of *Desulfobacter* in oil field environments by non-radioactive DNA probes. Appl Microbiol Biotechnol. 1994;42:469–475.
[24] Cowan JK (2005). *Rapid enumeration of sulfate reducing bacteria*. Proceedings of CORROSION/ 2005, Paper No. 05485, NACE International Houston, TX, p. 16. https://www.onepetro.org/conference-paper/NACE-05485.
[25] Eden B, Laycock PJ, Fielder M (1993). *Oilfield reservoir souring, Health and Safety* –Offshore Technology Report.
[26] Bertrand S, Bohni N, Schnee S, et al. Metabolite induction *via* microorganism co-culture: A potential way to enhance chemical diversity for drug discovery. Biotechnol Adv. 2014;32:1180–1204.
[27] Muthukumar N, Maruthamuthu S, Mohanan S, et al. Oil soluble corrosion inhibitor on microbiologically influenced corrosion in diesel transporting pipeline. Portugaliae Electrochim Acta. 2007;25:319–334.
[28] Little BJ, Lee JS. *Microbiologically Influenced Corrosion*. Kirk-Othmer encyclopedia of chemical technology. John Wiley & Sons, Inc. New Jersey, United States; 2009. DOI:10.1002/0471238961.micrlitt.a01.
[29] Zuo R. Biofilms: strategies for metal corrosion inhibition employing microorganisms. Appl Microbiol Biotechnol. 2007;76(6):1245–1253.

[30] Gittens JE, Smith TJ, Suleiman R, et al. Current and emerging environmentally-friendly systems for fouling control in the marine environment. Biotechnol Adv. 2013;31:1738–1753.
[31] Zarasvand KA, Rai VR. Microorganisms: induction and inhibition of corrosion in metals. Int Biodeterior Biodegrad. 2014;87:66–74.
[32] Oh YJ, Jo W, Yang Y, et al. Influence of culture conditions on *Escherichia coli* O157: h7biofilm formation by atomic force microscopy. Ultramicroscopy. 2007;107(10–11):869–874.
[33] Wikieł AJ, Datsenko I, Vera M, et al. Impact of *Desulfovibrio alaskensis* biofilms on corrosion behaviour of carbon steel in marine environment. Bioelectrochemistry. 2014;97:52–60.
[34] Feio MJ, Beech IB, Carepo M, et al. Isolation and characterization of a novel sulfate-reducing bacterium of the *Desulfovibrio* genus. Anaerobe. 1998;4(2):117–130.
[35] Feio MJ, Beech IB, Carepo M, et al. Validation list no. 75. Int J Syst Evol Microbiol. 2000;50:1415–1417.
[36] Feio MJ, Zinkevich V, Beech IB, et al. *Desulfovibrio alaskensis* sp. nov., a sulfate-reducing bacterium from a soured oil reservoir. Int J Syst Evol Microbiol. 2004;54 (5):1747–1752.
[37] Miller JH. Experiments in molecular genetics. New York: Cold Spring Harbor Laboratory; 1972.
[38] Zinkevich V, Beech IB. Screening of sulfate-reducing bacteria in colonoscopy samples from healthy and colitic human gut mucosa. FEMS Microbiol Ecol. 2000;34:147–155.
[39] Pavia DL, Lampman GM, Kriz GS, et al. Introduction to Spectrosocpy (5th edition). Cengage Learning, USA; 2013.
[40] Brandel J, Humbert N, Elhabiri M, et al. Pyochelin, a siderophore of *Pseudomonas aeruginosa*: physicochemical characterization of the iron (III), copper (II) and zinc (II) complexes. Dalton Trans. 2012;41:2820.
[41] Zheng T, Nolan EM. Siderophore-based detection of Fe (III) and microbial pathogens. Metallomics. 2012;4(9):866–880.
[42] Saha R, Saha N, Donofrio RS, et al. Microbial siderophores: a mini review. J Basic Microbiol. 2013;53(4):303–317.
[43] Ahmed E, Holmström SJM. Siderophores in environmental research: roles and applications. Microb Biotechnol. 2014;7:196–208.
[44] Neilands JB. Siderophores: structure and function of microbial iron transport compounds. J Biol Chem. 1995;270(45):26723–26726.
[45] Mohandass C. Bacterial siderophores and their biotechnological applications. In: Ramaia N, editor. Marine microbiology: facets & opportunities. India: National Institute of Oceanography; 2004. p. 169–174.
[46] Martinez JS, Carter-Franklin JN, Mann EL, et al. Structure and membrane affinity of a suite of amphiphilic siderophores produced by a marine bacterium. Proc Nat Acad Sci. 2003;100 (7):3754–3759.
[47] Ali SS, Vidhale NN. Bacterial siderophore and their application: a review. Int J Curr Microbiol App Sci. 2013;2(12):303–312.
[48] Reissbrodt R, Rabsch W, Chapeuaurouge A, et al. Isolation and identification of ferrioxamine G and E in *Hafnia alvei*. Biol Metals. 1990;3:54–60.
[49] Gledhill M, Buck KN. The organic complexation of iron in the marine environment: a review. Front Microbiol. 2012;3(69):1–17.
[50] Young M, Edited by. Vol. 3, New York: Pergamon press; 1985. p. 275–295.
[51] Andrews SC, Robinson AK, Rodríguez-Quiñones F. Bacterial iron homeostasis. FEMS Microbiol Rev. 2003;27(2–3):215–237.
[52] Takahashi A, Nakamura H, Kameyama T, et al. Bisucaberin, a new siderophore sensitizing tumor cells to macrophage mediated cytolysis. II. physico-chemical properties and structure determination. J Antibiot. 1987;40(12):1671–1676.
[53] Mansson M, Gram L, Larsen TO. Production of bioactive secondary metabolites by marine vibrionaceae. Mar Drugs. 2011;9(9):1440–1468.
[54] Faraldo-Gómez JD, Mark SP, Sansom MSP. Acquisition of siderophores in gram-negative bacteria. Nat Rev Mol Cell Biol. 2003;4:105–116.
[55] Vandenende CS, Vlasschaert M, Stephen YK, et al. Functional characterization of an aminotransferase required for pyoverdine siderophore biosynthesis in Pseudomonas aeruginosa PAO1. J Bacteriol. 2004;186(17):5596–5602.
[56] Balado M, Souto A, Vences A, et al. Two catechol siderophores, acinetobactin and amonabactin, are simultaneously produced by Aeromonas salmonicida subsp. Salmonicida. Sharing Biosynthetic Pathway ACS Chem Biol. 2015;10:2850–2860.
[57] Neilands JB. Siderophores. Arch Biochem Biophys. 1993;302(1):1–3.
[58] Albrecht M, Osetska O, Abel T, et al. An enantiomerically pure siderophore type ligand for the diastereoselective 1: 1 complexation of

lanthanide (III) ions. Beilstein J Org Chem. 2009;5(78):1–8.
[59] Freestone PPE, Williams PH, Lyte M, et al. (2003). Method for inducing bacterial growth using autoinducers. http://www.google.co.uk/patents/US6649398. Assessed on 15 September, 2015.
[60] Messenger AJM, Barclay R. Bacteria, iron and pathogenicity. Biochem Educ. 1983;11 (2):54–63.
[61] Banoub JH, El Aneed A, Cohen AM, et al. Structural investigation of bacterial lipopolysaccharides by mass spectrometry and tandem mass spectrometry. Mass Spectrom Rev. 2010;29:606–650.
[62] Carvalho ALU, Oliveira FHPC, Mariano RLR, et al. Growth, sporulation and production of bioactive compounds by Bacillus subtilis R14. Braz Arch Biol Technol. 2010;53 (3):643–652.
[63] Rattanachuay P, Kantachote D, Tantirungkij M, et al. Inhibition of shrimp pathogenic vibrios by extracellular compounds from a proteolytic bacterium Pseudomonas sp. W3. Electron J Biotechnol. 2010;13(1):1–11
[64] Liu Z, Yang S, Bai Y, et al. The alteration of cell membrane of sulfate reducing bacteria in the presence of Mn(II) and Cd(II). Miner Eng. 2011;24:839–844.
[65] Dunne WM. Bacterial adhesion: seen any good biofilms lately? Clin Microbiol Rev. 2002;15:155–166.
[66] Oyewole OA. The Relationship of biofilms and physicochemical properties of soil samples with corrosion of water pipelines in Minna, Niger State, Nigeria. Cont J Microbiol. 2011;5(2):1–10.
[67] Langer S, Schropp D, Bengelsdorf FR, et al. Dynamics of biofilm formation during anaerobic digestion of organic waste. Anaerobe. 2014;29:44–51.
[68] Mishra A, Malik A. Recent advances in microbial metal bioaccumulation. Crit Rev Environ Sci Technol. 2013;43:1162–1222.
[69] Jayaraman A, Mansfeld FB, Wood TK. Inhibiting sulfate- reducing bacteria in biofilms by expressing the antimicrobial peptides indolicidin and bactenecin. J Ind Microbiol Biotechnol. 1999;22:167–175.
[70] Morikawa M. Beneficial biofilm formation by industrial bacteria Bacillus subtilis and related species. J Biosci Bioeng. 2006;101:1–8.
[71] Mansfeld F, Hsu H, Ornek D, et al. Corrosion control using regenerative biofilms on aluminum 2024 and brass in different media. J Electrochem Soc. 2002;149:B130–B138.
[72] Ornek D, Jayaraman A, Syrett BC, et al. Pitting corrosion inhibition of aluminum 2024 by Bacillus biofilms secreting polyaspartate or g-polyglutamate. Appl Microbiol Biotechnol. 2002;58:651–657.
[73] Tsygankova LE, Vigdorovich VI, Esina MN, et al. Inhibitory and bactericidal action of the biocorrosion agents «INCORGAS» and «AMDOR». Bioelectrochemistry. 2014;97:154–161.
[74] Wallace WE, Guttman CM. Data analysis methods for synthetic polymer mass spectrometry: autocorrelation. J Res Natl Inst Stand Technol. 2002;107:1–17.