Journal of Chemical and Petroleum Engineering (JChPE)
Print ISSN: 2423-673X Online ISSN: 2423-6721

Desulfurization Capabilities of Indigenous Bacteria Isolates for Enhanced Sulfur Removal

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ARTICLE INFO

Article History:
Received: 03 January 2024
Revised: 08 May 2024
Accepted: 11 May 2024

Article type: Research

Keywords:
Biocatalytic Desulfurization,
Contaminated Soil,
Crude Oil,
Dibenzothiophene,
Microbial Growth

ABSTRACT

Desulfurization is an essential part of refinery operations due to the numerous effects of sulfur on refinery equipment and fuel product quality. Biocatalytic desulfurization methods potentially promote energy savings due to the mild temperature, and low-pressure operating conditions involved. Furthermore, the biological process results in a negligible level of undesirable products and emissions without lowering the calorific value of the fuel. In this study, indigenous microorganisms that thrive in hydrocarbon environments were isolated, characterized, and identified from waste oil dump sites at major garages in the Kumasi Metropolis, Ghana, and used to desulfurize crude oil. Preliminary screening resulted in 132 microbial isolates with 26 most potent isolates selected for the final identification and their use for desulfurization. The isolates identified included Stenotrophomonas maltophilia, pseudomonas aeruginosa, alcaligenes faecalis, enterococcus faecalis, xanthomonas maltophilia, pseudomonas maltophilia, and pseudomonas putida. The highest level of desulfurization was 73.5% at 40 °C by stenotrophomonas maltophilia. An isolate of Enterococcus faecalis, which has not been widely explored for its sulfur removal potential could remove 61% sulfur from crude oil and is worth further research.

Introduction

Sulfur is a naturally occurring compound in crude oil. When fuel is burned, sulfur combines with oxygen to form emissions (SO_x) that contribute to decreased air quality and adverse environmental and health effects [1, 2]. The presence of sulfur in vehicular fuels also increases the release of other environmentally harmful compounds [3]. Humans are exposed to sulfur in its diverse forms through the constant use of sulfur-containing fuels, which equally affect the surroundings.

Desulfurization processes are designed to remove sulfur and its compounds from crude oil. These processes are of significant industrial and environmental relevance as they provide the bulk of sulfur used in industry, sulfur-free compounds that could otherwise not be used in a

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Journal of Chemical and Petroleum Engineering, 2024, 58(2): 289- 309.
Publisher: University of Tehran, College of Engineering *DOI: 10.22059/jchpe.2024.368971.1467*

great number of catalytic processes, and also reduce the release of harmful sulfur compounds into the environment, particularly sulfur dioxide which leads to acid rain [4].

Protecting the environment and its effect on health has been a focus over the years and hence, different sulfur removal techniques have been investigated and applied. Some techniques include hydrodesulfurization, oxidative desulfurization, extractive desulfurization, adsorptive desulfurization, and bio-desulfurization [5-16]. In bio-desulfurization, microorganisms that can utilize sulfur as part of a metabolic pathway are relied on to remove sulfur from crude oil or other petroleum distillates. A great number of these microorganisms have been isolated from waste oil-contaminated soils [17, 18] and crude oil or petroleum distillate storage tanks [19]. The soil is a rich habitat for the diverse taxon of microbes due to its physiological heterogeneity [20]. The soil pH and salinity strongly drive microbial abundance, diversity, composition, and functions [21-23].

Bio-desulfurization has gained a lot of attention in the past decades because of several highlighted benefits compared to the well-used hydrotreatment process. Some of the key benefits associated with the application of bio-desulfurization include its environmental friendliness and ability to get rid of recalcitrant organosulfur compounds [24], the fact that the process requires ambient pressure and temperature conditions indicative of reduced energy requirement [25, 26], the by-products obtained being less toxic as well as the fact that the needed microorganisms can readily be isolated from the environment.

A major setback of bio-desulfurization for large-scale industrial applications is the identified slow kinetics of the process [27] as well as the cost of isolating and applying microorganisms on a large scale [28]. The application of genetic engineering and nanotechnology to modify these microbial systems has been studied extensively to overcome some of these setbacks. Sadare et al. [25] reported the utilization of nano-adsorbents which are found to enhance the transfer of dibenzothiophene (DBT) from bacteria. To manage the cost of obtaining microorganisms, the method of isolating microorganisms from waste oil dump sites, stored fuels as well as human bodies to obtain Indigenous isolates to reduce cost has been explored. In addition, the genes responsible for expressing enzymes in sulfur degradation through particular metabolic pathways in several species of microbes have been identified. Wang et al. [29] and Parveen et al. [30] are among the researchers who successfully explored genetic engineering to modify bacteria for desulfurization. Li et al. [31] observed a 13.8-fold improvement from the recombinant strain (Escherichia coli) compared to the wild strain. The DBT removal mechanism by Rhodococcus rhodochrous (MTCC 3552) [32], through a sulfur-specific pathway is presented in Fig. 1 below.

In this specific 4S pathway, dibenzothiophene is converted to the final product, 2-hydroxybiphenyl through a series of steps catalyzed by the DszA, DszB, and DszC enzymes. In the 4S pathway, the C-S bond is broken without compromising the C-C bond, hence preserving the fuel quality [33].

The current research focuses on isolating different bacteria strains from the waste oil dump sites of different garages within the Kumasi metropolis. The presence of the previously identified genes responsible for sulfur degradation was confirmed in these isolates. The sulfur degrading bacteria which had the potential to thrive in the hydrocarbon environment were used to remove sulfur compounds from model high sulfur crude oil samples at different temperatures.

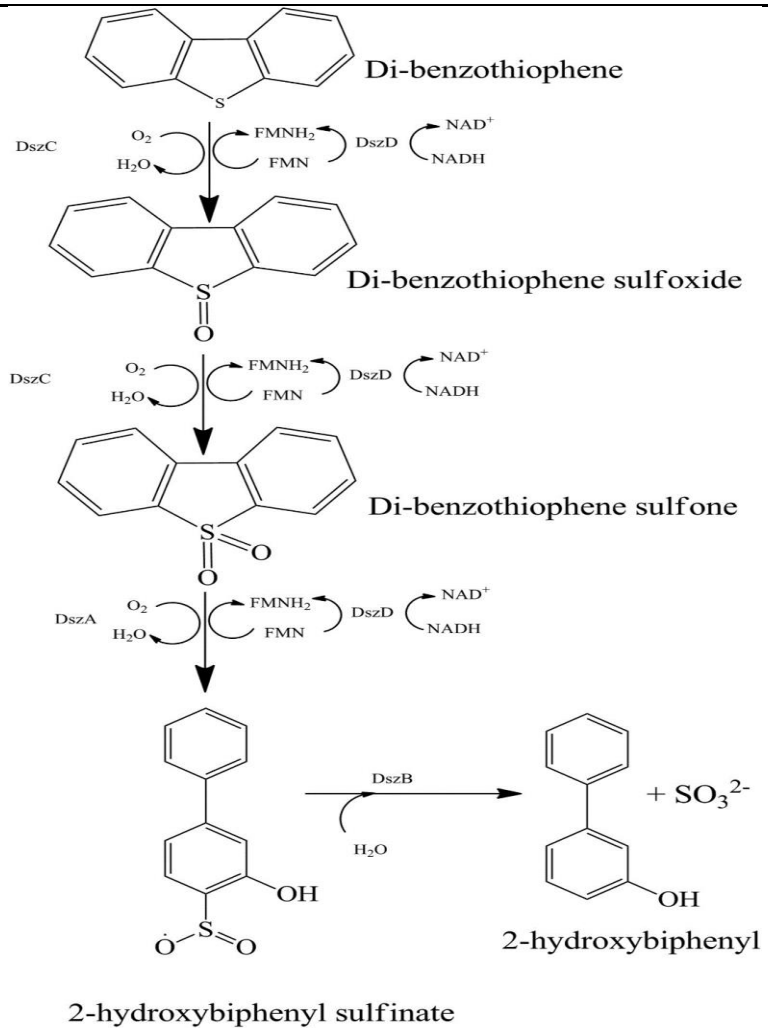


Fig. 1. Bio-desulfurization of DBT by Rhodococcus rhodochrous (MTCC 3552)

Materials and Methods

Materials

Mannitol salt agar, Mueller Hinton agar, nutrient broth, nutrient agar, and Bushnell Has agar (Oxoid Ltd, Basingstoke, UK), Glycerol (Surechem, Ipswich, UK), 0.5 McFarland standard (Hardy Diagnostics, Santa Maria, USA), crystal violet, and hydrogen peroxide (Ernest Chemist, Accra, Ghana), and ASI standard for sulfur (Fisher Scientific, UK).

Methodology

Soil Sampling

All sampling sites were located in the Kumasi metropolis and included solely engine oil-polluted soil sites at selected fitting workshops (garages). The selected type of soil was well-soaked in engine oil, up to a depth of 30 cm.

Fig. 2 below shows the map of Ghana with the research area and the various soil sampling sites. A total of 132 samples were collected. At each sampling site, the soil samples were taken at a depth of 5 cm, 10 cm, and 15 cm. Sterile, small plastic zip-lock bags were used to collect the samples and labelled appropriately. Samples were then placed in a sealed flask and transported to the laboratory for preliminary screening.

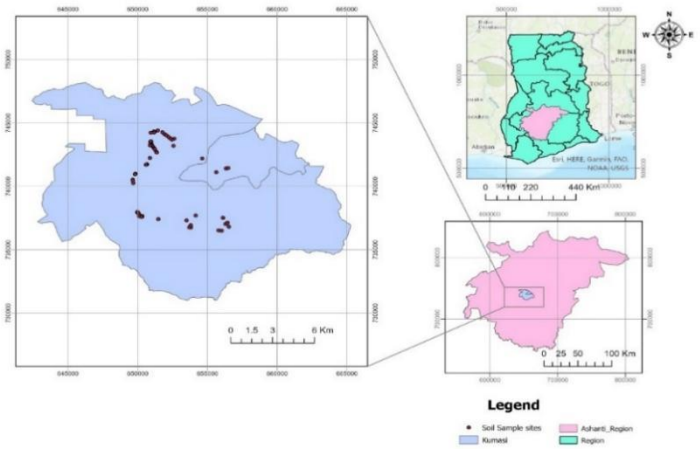


Fig. 2. A map of the sampling sites in the research area (Kumasi Metropolis)

Preparation of Culture Media

Coliform isolation agar, pseudomonas isolation agar, mueller-hinton agar (MHA), nutrient agar, sulfur-indole motility agar, blood agar, and nutrient broth, were prepared according to the manufacturer's protocol.

Preliminary Screening of Samples to Isolate Heterotrophic Microorganisms

To isolate heterotrophic microbes from the samples, the following protocol as previously described by Abeka [34], was followed with some modification.

1 g of each sample was weighed and suspended in 5 mL of sterile distilled water. 20 mL of Tween 80 solution was added. The resulting mixture was vortexed to obtain a uniform suspension. A three-fold 1 in 10 dilution was performed on each sample. 1 mL of the final dilution of each sample was then added to 20 mL sterile nutrient agar, poured into sterile, well-labelled Petri dishes, and incubated at 37 ℃ for 72 hours with daily observation. The resulting individual colonies were carefully picked and used to inoculate 10 mL sterile nutrient broth in appropriately labelled test tubes.

Isolation of Sulfur-Degrading Microbes

To determine the presence of sulfur-degrading microorganisms in the samples, the isolation protocol as described by Farshid et al. [35], was followed with some modification.

The isolates obtained from the preliminary screening were subcultured in a nutrient broth media overnight. 30 g of the sulfur indole motility (SIM) agar was prepared with 1000 mL of sterile water and sterilized in an autoclave. 20 mL portions of the SIM agar were poured into a sterile Petri dish and allowed to set. A loopful of each isolate was streaked in turns aseptically. The plates were then incubated at 37 ℃ with daily observation of growth for 7 days. Sulfur degrading isolates produced a black colour in the medium and were selected.

Organosulfur Utilisation Test

The organosulfur utilization protocol as described by Pokethitiyook et al. [36], was followed with some modifications. The model compound used here was dibenzothiophene (DBT). One milliliter (mL) of each isolate culture was inoculated into a set of test tubes each containing 1mM of DBT. Another set of test tubes containing the MS medium without a sulfur source was

used as a negative control. All the test tubes were incubated on the shaker at 150 rpm at 37 ℃. After 72 hours of incubation, the growth of the selected bacteria was measured by taking the absorbance reading at 610 nm.

Measurement of Emulsion Formation Ability

Some sulfur-degrading microbes can remove sulfur from fuels by forming biosurfactants that make the substrate more readily available [37]. The emulsification index protocol as described by Shahaliyan et al. [38] was followed with some modifications.

The sterile crude oil (150 mL), was added to the sub-cultured isolate (1mL) in a sterile 500 mL bottle. Sterile nutrient broth was then added to make up the volume. The entire height of the mixture was then measured in mm and recorded. The mixture was then incubated at 25 ℃ for 14 days with daily observation. The height of the emulsified layer formed after the 14-day incubation was appropriately measured and the emulsification index, E, was calculated according to Eq. 1 below.

$$E = \frac{\text{height of emulsified layer}}{\text{entire height of solution}} \quad (1)$$

Gibb's Test

To isolate bacteria that can selectively remove sulfur from DBT, Gibb's assay protocol as described by Kayser et al. [39] was used. The presence of 2-hydroxybiphenyl, the end product of DBT desulfurization, was tested for.

The pH of the isolated culture was adjusted to 8.0 using 10% (w/v) Na_2CO_3. Then, 50 µL of Gibb's reagent (10 mg of 2,6-dichloroquinone-4chloroimide dissolved in 1 ml of ethanol) was added to each isolate culture and the reaction mixture was incubated at 30 ℃ for 30 minutes. To separate the bacterial cells, the reaction mixture was centrifuged at 10000 rpm, and 4 ℃ for 20 minutes, and the absorbance of the supernatant was measured at 610 nm.

Molecular Isolation and Amplification of Sulfur-Degrading Genes

The following protocol was followed for bacterial DNA extraction, PCR (amplification), and agarose gel electrophoresis for the isolation and amplification of sulfur-degrading microbes, which either bear a hydrocarbon-degrading character or not.

The bacterial DNA extraction (Heat Lysis) method used by Chalini et al. [40] is followed with some modifications. The pure bacteria isolates (1mL sample) were mixed with 0.5 mL PBS and vortexed for 30 seconds. The resulting mixture was then centrifuged at 8000 rpm for 3 minutes. The supernatant was then discarded. 100 µL of nuclease-free water was added to the residue in the Eppendorf tubes and vortexed briefly. The resulting mixture was incubated at 95 ℃ for 5 minutes followed by centrifugation at 14000 rpm for 3 minutes. The supernatant (DNA) was pipetted into a new 1.5 mL Eppendorf tube.

The PCR master mix was formulated according to the following conditions: 2.5 µL of 10X buffer, 2.0 µL of Magnesium chloride. and 0.5 µL of dNTPs were added into a sterile tube. 0.5 µL each of forward and reverse primers (with a concentration of 2.0 nmol/µL) were added and gently mixed. 0.125 µL of Taq polymerase was added, followed by 2.5 µL of DNA template. 16.875 µL of nuclease-free water was added to make the needed volume. The 35-cycle hybridization conditions were set at 94 ℃ for 30 s, 60 ℃ for 55 s, and 72 ℃ for 1 min.

The Agarose Gel Electrophoresis process was as follows: 120 mL of Tris Borate Edetate (TBE) buffer was measured and added to 1.8 g of agarose powder. The mixture was heated for 3 minutes to ensure complete dissolution of the agarose powder. The mixture was then cooled on a thermostatically controlled water bath to 60 ℃. 4 mL of ethidium bromide was then added and poured into the casting tray in the electrophoresis chamber with the gel comb inserted. The

gel in the molten state was left to solidify at room temperature (25 ℃) for 30 to 35 minutes. The gel was then covered in TBE buffer and the amplicons were delivered into the labelled wells. The amplicons were run at 120 V for 30 minutes.

The amplicons were visualized after the electrophoresis process using the infinity gel documentation imaging system.

Carbohydrate Utilization Test

This test focuses on the ability of bacteria to utilize a wide array of sugars. The type of sugar utilized by bacteria helps in predicting the genera they belong to [41].

0.5 g of trypticase soy broth was weighed and dissolved in 4 mL of sterile distilled water in a test tube. 0.2 mL of phenol red was added. The desired carbohydrate (lactose, glucose, and sucrose) was added at a concentration of 0.5 %(w/w) to the tubes separately. Durham tubes were placed inverted in the media to eliminate air bubbles. Sterilisation was done in an autoclave at 115 ℃ for 30 minutes after which the tubes were allowed to cool to a temperature of 45 ℃. The sterilized fermentation broth was then inoculated with each isolate and labelled appropriately. The tubes were further incubated at 37 ℃ for 24 hours. The release of a gas or acid indicated the utilization of specific carbohydrates by each isolate.

Hydrogen Sulfide Production Test

During metabolism, some microorganisms are able to reduce sulfur-containing compounds to sulfides. This helps in biochemically characterising such microorganisms and predicting the genera they belong to. For this test, the protein enrichment media protocol as described by Sonke et al. [42] was followed with some modifications.

Two loops (about 0.02 mL) of isolate suspension were inoculated into 10 mL peptone water. Lead acetate paper was inserted between the cotton wool plug and the tube. The tubes were incubated at 37 ℃ with daily observations for two days. A positive test was denoted by a blackened lead acetate paper. Samples are displayed in Fig. 3.

Fig. 3. Hydrogen sulfide production test

Citrate Utilisation Test

The principle of this test is to detect the ability of a microorganism, which can utilize citrate as the sole carbon source for metabolic procedures with subsequent alkalinity. This is possible for some microbes because**,** they possess a citrate enzyme system, which enables the hydrolysis of citrate to oxaloacetic acid and acetic acid. The citrate utilization protocol as described by Gaurab [43], was followed for this test with some modifications.

Using a straightened wire, the sub-cultured isolates were inoculated into sterile Koser's Citrate medium and incubated at 37 ℃ for 72 hours with daily observation. Citrate utilization was denoted by turbidity and color change of the medium from light green to blue.

Indole Test

This test is performed on bacterial species to detect their ability to convert tryptophan to indole. This type of conversion is possible for some bacteria due to the presence of the tryptophanase enzyme. Tryptophanase catalyses the deamination of tryptophan to indole and ammonium ions [44]. The indole test protocol as described by Marasini et al. [45] was followed with some modifications for this test.

The indole test was performed by growing the isolates in sterile tryptone broth for 24 hours. Following overnight incubation, a few drops of Kovacs' reagent were added to the culture broth, using a sterile pipette. The presence of indole was detected by the appearance of a red layer in the medium while its absence was denoted by a yellow layer.

Catalase Test

Catalase is an enzyme that converts hydrogen peroxide (H_2O_2) to oxygen and water. If bacteria produce this enzyme, in the presence of H_2O_2, the eruption of bubbles is observed, indicating the release of oxygen from the breakdown of H_2O_2 [46].

The isolates were sub-cultured on agar slants at 37 ℃ for 24 hours. A loopful of cells from each agar slant was mixed on a clean microscopic slide with 0.2 ml of 30% hydrogen peroxide. Within 5 seconds to 3 minutes, the appearance of bubbles is indicative of the presence of the catalase enzyme. The appearance of bubbles on the plate could be seen as shown in Fig. 4.

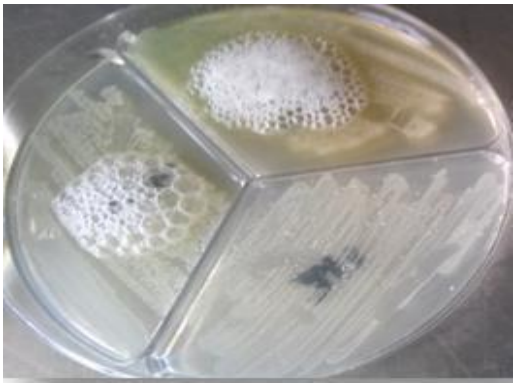


Fig. 4. Picture demonstrating the catalase test for some of the isolates

Utilisation of Selective Media by Isolates

Microbes can easily be characterised based on their ability to selectively grow on certain media. This is due to the presence of some nutrients that are exclusive to their growth [47]. For this test, the isolation protocol as described by Jackson et al. [48], was followed with some modification.

The selective media employed here included, pseudomonas isolation medium and coliform selective medium. About 13 g of each selective media was weighed into a 500 mL infusion bottle and re-suspended in 450 mL distilled water. The preparation was then sterilized at 115 ℃ for 30 minutes. It was then stabilized at 45 ℃ for 15 minutes. The solution was then transferred into labelled sterile Petri dishes in 10 mL portions. Two loops of the isolates were spotted on the agar plate in turns. The plates were incubated at 37 ℃ for 24 hours. The procedure was performed in triplicates for each sample.

Gram Staining Reaction

The culture (0.1 mL) was heat-fixed on a slide. 5 drops of ammonium oxalate crystal violet solution were added and washed off with sterile water after 20 seconds. About 5 drops of Lugol's iodine solution were then added and allowed to stand for 30 seconds. A few drops of 95% ethanol were added and washed off with sterile water after 5 seconds. The slide was then counterstained with drops of 0.5% safranin solution which was washed off with sterile water after 30 seconds. The slide was then viewed under the microscope for its reaction to Gram's stain as outlined by Moyes et al. [49]. The appearance of purple colonies indicates the presence of gram-positive bacteria whereas the appearance of pink colonies, indicate the presence of gram-negative bacteria.

Morphological Characterisation

This test focuses on characterizing the isolates based on their shape, size, color and pattern of growth on an agar plate. The morphological characterization protocol as described by van Teeseling et al. [50] was followed with some modifications. For this study, 20 mL nutrient agar was melted and stabilized at 45 ℃. It was then poured into labelled sterile Petri dishes and allowed to set after which a loopful of the culture was spotted on the agar surface. The plates were then incubated at 37 ℃ for 72 hours with daily observation. Fig. 5 below shows a petri dish with an identified isolate. The shape of the isolates was noted when observed under the microscope to aid in the identification.

Fig. 5. A picture depicting petri dish morphology for *Pseudomonas putida* strain

Matrix-Assisted Laser Desorption/Ionisation-Time of Flight (MALDI-TOF) Test

The target plate was inoculated with 1 uL loopful of the isolate to form a thin film. One microliter (1 µL) of the matrix solution was added and the plate was left to dry. The plate was exposed to a beam of laser for a short period and was placed in the bio-typer. The spectra were obtained and analysed using the GeenaR software and compared with standard microbial databases [51].

Quantitative Measurement of Sulfur-Reduction Ability

The organisms were investigated for their ability to remove sulfur from crude oil using the protocol described by Shahaliyan et al. [38], with some modifications.

Sterile crude oil (100 mL), was added to the sub-cultured isolate (1 mL) in a sterile 250 mL bottle. Sterile nutrient broth was then added to make up to a volume of 200mL. The mixture was then incubated at 25 ℃ for 72 hours with daily observation. The residual sulfur content for each mixture was then determined using a sulfur analyser. This procedure was repeated at a temperature of 40 ℃ and 55 ℃ to assess the effect of temperature on sulfur removal.

Results and Discussion

Preliminary Screening of Samples to Isolate Heterotrophic Microorganisms

The routine media, nutrient agar used, allowed a vast array of microorganisms present in the sample to grow. A total of 132 isolates were obtained from the preliminary screening in nutrient agar. Table 1 shows all the isolates that were obtained. Ra et al. [52] reported on isolation of 6.0×10^2 bacteria plate counts from a petroleum contaminated soil and 4.0×10^3 bacteria plate count from a different petroleum contaminated soil. This indicates that a wide range of bacteria isolates can be obtained from petroleum-contaminated soils.

Isolation of Sulfur-Degrading Microbes

The isolates that can utilize sulfur as a substrate for growth were the focus of this study. All the isolates from the preliminary screening were further screened on sulfur indole motility (SIM) medium, to assess their growth in a sulfur environment. 64 isolates showed growth which was shown by positive signs and were selected for subsequent tests. The results are shown in Table 1.

Table 1. Utilization of sulfur for growth for all isolates

Isolates	SIM	Isolates	SIM	Isolates	SIM
$7MZ^a_1$	+	$2OF^c_3$	+	$4AS^a_1$	-
$6MZ^a_3$	+	$1OF^a_2$	+	$2AS^a_1$	-
$1MZ^a_3$	+	$3OF^a_2$	+	$1AS^a_2$	-
$6MZ^a_1$	+	$3OF^c_2$	+	$1AY^a_1$	-
$1MZ^a_3$	+	$2OF^a_3$	+	$1AY^b_1$	-
$6MZ^a_2$	+	$1OF^a_1$	+	$2AY^a_1$	-
$1MZ^a_1$	+	$1OF^b_1$	+	$2AY^b_1$	-
$7MZ^a_2$	+	$1OF^c_1$	+	$3KR^a_1$	-
$1MZ^b_2$	-	$4OF^a_3$	+	$2KR^a_1$	-
$1MZ^b_3$	-	$1OF^b_2$	+	$2KR^b_1$	-
$7MZ^a_2$	+	$1OF^c_2$	+	$1KR^a_2$	-
$7MZ^a_3$	+	$3OF^a_3$	+	$4P^a_1$	-
$7MZ^b_1$	+	$3OF^b_1$	+	$2P^a_1$	-
$7MZ^b_2$	+	$3OF^C_3$	+	$3P^a_1$	-
$7MZ^c_1$	+	$1OF^b_3$	+	$3P^a_2$	-
$7MZ^c_2$	-	$4S^a_3$	-	$3P^b_2$	-
$7MZ^b_3$	-	$4S^b_3$	+	$1AM^a_1$	-
$7MZ^c_3$	+	$4S^c_3$	+	$1AM^b_1$	-
$6MZ^b_1$	+	$4S^a_2$	+	$1AM^a_2$	-
$6MZ^b_2$	+	$4S^b_2$	+	$1AH^a_1$	-
$6MZ^c_1$	+	$4S^a_1$	-	$1AH^b_1$	-
$4MZ^a_1$	+	$4S^a_2$	-	$2AH^a_1$	-
$4MZ^b_1$	+	$1S^a_2$	-	$2AH^b_1$	-
$4MZ^c_1$	-	$1S^b_2$	-	$2AH^a_2$	-
$4MZ^a_2$	+	$1S^a_1$	+	$2AH^b_2$	-
$4MZ^b_2$	+	$1S^b_1$	+	$3AH^a_1$	-
$4MZ^c_2$	+	$1S^a_3$	-	$3AH^a_2$	-
$4MZ^a_3$	-	$5S^a_1$	+	$3AH^b_2$	-
$4MZ^b_3$	-	$5S^b_1$	+	$3AH^b_1$	-
$4MZ^c_3$	-	$5S^b_2$	-	$4AS^a_1$	-
$3MZ^a_1$	-	$1AB^a_1$	+	$2AS^a_1$	-
$3MZ^b_1$	-	$1AB^b_1$	+	$1AS^a_2$	-

$3MZ^{c}_{1}$	-	$1AB^{a}_{2}$	+	$1AY^{a}_{1}$	-
$3MZ^{a}_{2}$	-	$1AB^{b}_{2}$	+	$1AY^{b}_{1}$	-
$3MZ^{b}_{2}$	+	$1AB^{C}_{2}$	+	$2AY^{a}_{1}$	-
$3MZ^{c}_{2}$	+	$2AB^{a}_{1}$	-	$2AY^{b}_{1}$	-
$3MZ^{a}_{3}$	+	$2AB^{b}_{2}$	+	$3KR^{a}_{1}$	-
$2MZ^{a}_{1}$	+	$2AB^{a}_{2}$	+	$2KR^{a}_{1}$	-
$2MZ^{b}_{2}$	+	$3AB^{a}_{1}$	+	$2KR^{b}_{1}$	-
$5MZ^{a}_{2}$	-	$3AB^{a}_{2}$	+	$1KR^{a}_{2}$	-
$5MZ^{b}_{3}$	-	$4AB^{a}_{2}$	+	$4P^{a}_{1}$	-
$5MZ^{a}_{1}$	-	$4AB^{b}_{2}$	+	$2P^{a}_{1}$	-
$2OF^{a}_{1}$	+	$4AB^{a}_{1}$	+	$3P^{a}_{1}$	-
$2OF^{b}_{2}$	+	$4AB^{b}_{1}$	-	$3P^{a}_{2}$	-

Organosulfur Utilization Test

Sulfur is predominant in the organic form in fuels. The effectiveness of a sulfur-degrading microbe in the bioremediation of sulfur fuels comprises its ability to reduce sulfur in its organic form. With this test, forty-nine isolates showed growth in the DBT-enriched MS media which is of interest. The results of these tests are displayed in Table 2. The ability of bacteria isolates to show growth in the DBT enriched media indicates the ability of these isolates to utilize DBT which is the target sulfur compound, desired to be removed. Similar results were also obtained by Pokethitiyook et al. [36], and Rath et al. [53].

Table 2. Isolates that utilized DBT for growth

Isolates	$\lambda_{max610nm}$	**isolates**	$\lambda_{max610nm}$	**isolates**	$\lambda_{max610nm}$
$7MZ^{a}_{1}$	0.823	$4S^{b}_{3}$	0.023	$4MZ^{b}_{2}$	0.021
$6MZ^{a}_{3}$	0.444	$4S^{c}_{3}$	0.033	$4MZ^{c}_{2}$	0.355
$1MZ^{a}_{3}$	0.058	$4S^{a}_{2}$	0.231	$3MZ^{b}_{2}$	0.214
$6MZ^{a}_{1}$	0.122	$4S^{b}_{2}$	0.516	$3MZ^{c}_{2}$	0.098
$1MZ^{a}_{3}$	0.322	$1S^{a}_{1}$	0.024	$3MZ^{a}_{3}$	0.369
$6MZ^{a}_{2}$	0.864	$1S^{b}_{1}$	0.064	$2MZ^{a}_{1}$	0.024
$1MZ^{a}_{1}$	0.336	$5S^{a}_{1}$	0.311	$2MZ^{b}_{2}$	0.063
$7MZ^{a}_{2}$	0.786	$5S^{b}_{1}$	0.159	$2OF^{a}_{1}$	0.099
$7MZ^{a}_{2}$	0.655	$1AB^{a}_{1}$	0.487	$2OF^{b}_{2}$	0.288
$7MZ^{a}_{3}$	0.632	$1AB^{b}_{1}$	0.223		
$7MZ^{b}_{1}$	0.612	$1AB^{a}_{2}$	0.455		
$7MZ^{b}_{2}$	0.131	$1AB^{b}_{2}$	0.132		
$7MZ^{c}_{1}$	0.456	$1AB^{C}_{2}$	0.368		
$7MZ^{c}_{3}$	0.621	$2AB^{b}_{2}$	0.091		
$6MZ^{b}_{1}$	0.412	$2AB^{a}_{2}$	0.134		
$6MZ^{b}_{2}$	0.523	$3AB^{a}_{1}$	0.067		
$6MZ^{c}_{1}$	0.552	$3AB^{a}_{2}$	0.032		
$4MZ^{a}_{1}$	0.134	$4AB^{a}_{2}$	0.0211		
$4MZ^{b}_{1}$	0.633	$4AB^{b}_{2}$	0.095		
$4MZ^{a}_{2}$	0.122	$4AB^{a}_{1}$	0.133		

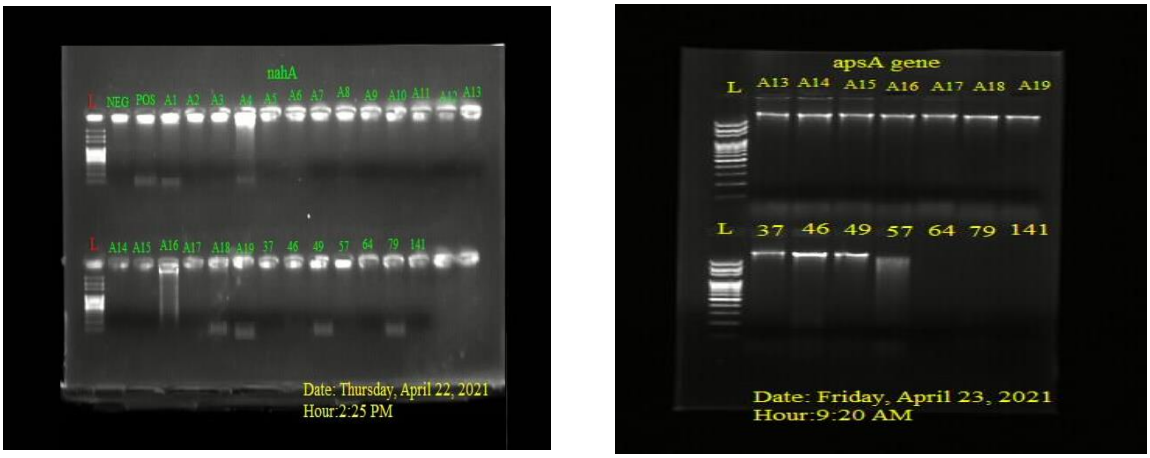
26 isolates that showed an absorbance reading of 0.2 and above were used in the subsequent tests. Usually, an isolate that shows an absorbance (optical density) of 0.2 and above indicates a higher possibility of growth rate. The closer the optical density to 1, the better the growth rate [18].

Measurement of Emulsion Formation Ability

The ability of the isolates to form emulsion as part of their sulfur degradative mechanism was investigated. None of the isolates produced a stable emulsion in crude oil under 48 hours. A stable emulsion (biosurfactant) formation property is a major characteristic of good hydrocarbon degraders [5-55], which is not desired in this case. The isolates are expected to degrade sulfur without degrading the hydrocarbon bonds. Lyu et al. [37] indicated that the presence of the biosurfactant aids in the emulsion formation for improvement in the desulfurization, however, without biosurfactants, desulfurization is still possible.

Molecular Isolation and Amplification of Sulfur-Degrading Gene

The apsA gene has been proposed as a highly useful phylogenetic marker for sulfur-degrading microbes [56]. 26 isolates were investigated for the presence of the sulfur-degrading gene, apsA. 21 isolates out of the 26 also showed DNA bands for the nahA gene (which is the hydrocarbon-degrading gene) together with the sulfur-degrading gene (apsA). This indicates that these isolates can survive in the hydrocarbon environment and may degrade hydrocarbons in the absence of other substrates such as sulfur. Fig. 6a shows the results for the nahA gene identification and Fig. 6b shows some of the isolates with some of the ApsA gene.



A B

Fig. 6. a) Gel picture showing nahA gene, and b) gel picture showing apsA gene

Gibbs Test

The results are displayed in Table 3 below. Two of the isolates, $6MZ^{a}_{3}$ and $1AB^{a}_{2}$, were positive for the 4S sulfur-degradation metabolic pathway. The blue color is indicative of the formation of 2-hydroxybiphenyl (2-HBP) compound, the end product of the 4S pathway [57]. All the other isolates in this study may have been using alternate pathways for sulfur degradation. The brown coloration indicates a complete utilization of sulfur, whereas the purple color change indicates that the sulfur compound has been metabolized into ferrous sulfide (FeS) [58].

Table 3. Gibbs Test Results at 610 nm absorbance wavelength

Isolates	Colour Change	Inference	Absorbance
$7MZ^{a}_{1}$	Dark blue	-	0.898
$6MZ^{a}_{3}$	Transient blue to dark brown	+	0.746
$1AB^{a}_{2}$	Transient blue to dark brown	+	0.535
$1AB^{a}_{1}$	Brown	-	0.156
$1MZ^{a}_{3}$	Brown	-	0.981
$6MZ^{a}_{2}$	Brown	-	0.167
$1MZ^{a}_{1}$	Brown	-	0.122
$7MZ^{a}_{2}$	Brown	-	0.156
$7MZ^{a}_{2}$	Brown	-	0.115
$7MZ^{a}_{3}$	Purple	-	0.462
$7MZ^{b}_{1}$	No colour change	-	0.042
$5S^{a}_{1}$	No colour change	-	0.092
$7MZ^{c}_{1}$	No colour change	-	0.122
$7MZ^{c}_{3}$	Purple	-	0.068
$6MZ^{b}_{1}$	No colour change	-	0.059
$6MZ^{b}_{2}$	No colour change	-	0.054
$6MZ^{c}_{1}$	No colour change	-	0.045
$4S^{b}_{2}$	Purple	-	0.105
$4MZ^{b}_{1}$	No colour change	-	0.099
$4S^{a}_{2}$	No colour change	-	0.129
$2OF^{b}_{2}$	No colour change	-	0.072
$1AB^{b}_{1}$	Purple	-	0.505
$1AB^{C}_{2}$	Purple	-	0.622
$4MZ^{c}_{2}$	Purple	-	0.898
$3MZ^{b}_{2}$	Purple	-	0.646
$3MZ^{a}_{3}$	Purple	-	0.531

Biochemical Reactions

A total of 26 isolates were screened for this test and the results are shown in Table 4. The tests involved the ability of the organisms to utilize an array of selected sugars. Almost all the isolates fermented sugar satisfactorily. Their unique ability to break down some compounds was also exploited here. Selective media such as the pseudomonas isolation agar and coliform selective media were also observed to be utilized by some of the isolates. Biochemical tests are conventional and relatively inexpensive tests that help in the identification of bacteria. The utilization of these sugars indicates the type of enzymes produced by the bacteria [59]. All these contribute to microbial characteristic description and identification. These biochemical reactions aid in the identification of various isolates (up to the species level) based on the biochemical activities (including nutritional and metabolic capabilities).

Table 4. Isolate Biochemical Reactions

S	CAT	IN	MRVP	CIA	PIA	H_2S	Lac	CIT	Gluc	Mal
$7MZ^{a}_{1}$	+	-	MR-VP-	+	+	+	-	+	+	+
$6MZ^{a}_{3}$	+	-	MR-VP-	+	+	+	-	+	+	+
$1AB^{a}_{2}$	+	-	MR-VP-	-	-	-	-	+	+	+
$1AB^{a}_{1}$	+	-	MR-VP-	+	+	+	-	+	-	+
$1MZ^{a}_{3}$	+	-	MR-VP-	-	-	-	-	+	-	+
$6MZ^{a}_{2}$	+	-	MR-VP-	+	+	+	-	+	-	+
$1MZ^{a}_{1}$	+	-	MR-VP-	-	-	-	-	+	+	+
$7MZ^{a}_{2}$	+	-	MR-VP-	+	+	+	-	+	-	+
$7MZ^{a}_{2}$	+	-	MR-VP-	+	+	+	-	+	-	+
$7MZ^{a}_{3}$	+	-	MR-VP-	-	-	-	-	+	-	+
$7MZ^{b}_{1}$	+	-	MR-VP-	+	+	+	-	+	+	+
$5S^{a}_{1}$	+	-	MR-VP-	+	+	+	-	+	+	+

$7MZ^{c}_{1}$	+	-	MR-VP-	+	+	+	-	+	+	+
$7MZ^{c}_{3}$	+	-	MR-VP-	+	+	+	-	+	+	+
$6MZ^{b}_{1}$	+	-	MR-VP-	+	+	+	-	+	-	+
$6MZ^{b}_{2}$	+	-	MR-VP-	-	-	-	-	+	-	+
$6MZ^{c}_{1}$	+	-	MR-VP-	+	+	+	-	+	-	+
$4S^{b}_{2}$	+	-	MR-VP-	-	-	-	-	+	+	+
$4MZ^{b}_{1}$	+	-	MR-VP-	+	+	+	-	+	+	+
$4S^{a}_{2}$	+	-	MR-VP-	-	-	-	-	+	+	+
$1AB^{C}_{2}$	+	-	MR-VP-	-	-	-	-	+	+	+
$4MZ^{c}_{2}$	+	-	MR-VP-	-	-	-	-	+	+	+
$3MZ^{b}_{2}$	+	-	MR-VP-	-	-	-	-	+	+	+
$3MZ^{a}_{3}$	+	-	MR-VP-	+	+	+	-	+	-	+
$2OF^{b}_{2}$	+	-	MR-VP-	-	-	-	-	+	-	+
$1AB^{b}_{1}$	+	-	MR-VP-	+	+	+	-	+	+	+

Key: CAT: catalase test, IN: indole test, MRVP: Methyl red Voges-Proskauer test, CIA: coliform isolation agar, PIA: Pseudomonas isolation agar, H_2S: Hydrogen sulfide test, CIT: Citrate test, Gluc: glucose test, mal: maltose test, lac: lactose test, Iso: isolates.

Gram Staining

Gram-positive bacteria are microorganisms that can retain the primary dye (crystal violet) even after washing during the gram stain reaction. This is so because the cell wall of gram-positive microbes has a higher concentration of starch granules. This is not true for the gram-negative bacteria. Their granules are membrane-bound and very hard to stain. Sulfur-reducing bacteria are mostly gram-negative according to Rana et al. [60]. However, some gram-positive bacteria have been isolated and applied for desulfurization, such as *Rhodococcus* sp. [30]. Out of the 26 isolates, 25 were found to be gram-negative with sulfur-degrading capabilities. However, one isolate ($7MZ^{a}_{3}$) was observed to be gram-positive and its sulfur-degrading capabilities will be worth exploring further.

Morphological Characterisation

The morphology of the 26 isolates observed under the microscope is presented in Table 5. The morphology of the isolates is part of the process of identification and classification of bacteria [61, 62] and the morphology gives an idea of the fitness and environmental adaptive characteristics of these bacteria isolates [63]. The morphology is said to have a direct impact on biological functions that include but are not limited to motility, acquisition of nutrients, resistance to stress, and interaction with other microorganisms [50].

Table 5. Morphology of isolates

Isolates	Morphology	Possible genera
$7MZ^{a}_{1}$	Mucoid appearance of light cream colony clusters	Stenotrophomonas, or Pseudomonas
$6MZ^{a}_{3}$	Slightly pigmented cream colony clusters	Pseudomonas, or Stenotrophomonas
$1AB^{a}_{2}$	Mucoid appearance of light cream colony clusters	Stenotrophomonas, or Pseudomonas
$1AB^{a}_{1}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$1MZ^{a}_{3}$	Cream colony clusters with thin layered feathery edges	Alcaligenes, Pseudomonas or Achromobacter
$6MZ^{a}_{2}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$1MZ^{a}_{1}$	Cream colony clusters with brownish zones	Pseudomonas, Xanthomonas or Stenotrophomonas
$7MZ^{a}_{2}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$7MZ^{b}_{2}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$7MZ^{a}_{3}$	White colony clusters with pink zones	Staphylococcus, Bacillus or Rhodococcus
$7MZ^{b}_{1}$	White colony clusters with greenish zones	Pseudomonas or Stenotrophomonas

$5S^{a}_{1}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$7MZ^{c}_{1}$	Small circular clusters with yellow zones	Xanthomonas or Achromobacter
$7MZ^{c}_{3}$	Cream colony clusters with brown zones	Pseudomonas, Xanthomonas or Stenotrophomonas
$6MZ^{b}_{1}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$6MZ^{b}_{2}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$6MZ^{c}_{1}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$4S^{b}_{2}$	Mucoid appearance of light cream colony clusters	Stenotrophomonas, or Pseudomonas
$4MZ^{b}_{1}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$4S^{a}_{2}$	Mucoid appearance of light cream colony clusters	Stenotrophomonas, or Pseudomonas
$1AB^{C}_{2}$	Small circular clusters with yellow zones	Xanthomonas or Achromobacter
$4MZ^{c}_{2}$	White colony clusters with greenish zones	Pseudomonas or Stenotrophomonas
$3MZ^{b}_{2}$	Mucoid appearance of light cream colony clusters	Stenotrophomonas, or Pseudomonas
$3MZ^{a}_{3}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$2OF^{b}_{2}$	White colony clusters with irregular edges	Alcaligenes, Pseudomonas or Achromobacter
$1AB^{b}_{1}$	Mucoid appearance of light cream colony clusters	Stenotrophomonas, or Pseudomonas

Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Test

MALDI-TOF is an analytical technique of mass spectrometry, which allows a very sensitive, relatively high speed as well as excellent sensitivity in identifying microorganisms [64]. The method has been used to identify isolates up to the species level and applied widely in different areas, ranging from clinical diagnosis to environmental microbiology [65-70]. The identity of the isolates to the species level has been displayed in Table 6. Most of the identified bacteria have been previously isolated from different regions and used in different studies on desulfurization of different feedstock. *Enterococcus faecalis*, however, has not been explored for bio-desulfurization. It is a gram-positive bacteria that is usually found in the gastrointestinal tracts of humans and animals and is also present in soils, water, and sewage. It can usually thrive with or without oxygen [71]. It is described as a mesophile, but has been reported to survive for about 30 minutes at temperatures around 60 °C [72].

Table 6. MALDITOF test results

Isolates	Identity	Isolates	Identity
$7MZ^{a}_{1}$	*Stenotrophomonas maltophilia*	$7MZ^{c}_{3}$	*Pseudomonas maltophilia*
$6MZ^{a}_{3}$	*Pseudomonas aeruginosa*	$6MZ^{b}_{1}$	*Alcaligenes faecalis*
$1AB^{a}_{2}$	*Stenotrophomonas maltophilia*	$6MZ^{b}_{2}$	*Alcaligenes faecalis*
$1AB^{a}_{1}$	*Alcaligenes faecalis*	$6MZ^{c}_{1}$	*Alcaligenes faecalis*
$1MZ^{a}_{3}$	*Alcaligenes faecalis*	$4S^{b}_{2}$	*Pseudomonas putida*
$6MZ^{a}_{2}$	*Alcaligenes faecalis*	$4MZ^{b}_{1}$	*Alcaligenes faecalis*
$7MZ^{a}_{1}$	*Stenotrophomonas maltophilia*	$7MZ^{c}_{3}$	*Pseudomonas maltophilia*
$6MZ^{a}_{3}$	*Pseudomonas aeruginosa*	$6MZ^{b}_{1}$	*Alcaligenes faecalis*
$1AB^{a}_{2}$	*Stenotrophomonas maltophilia*	$6MZ^{b}_{2}$	*Alcaligenes faecalis*
$1AB^{a}_{1}$	*Alcaligenes faecalis*	$6MZ^{c}_{1}$	*Alcaligenes faecalis*
$1MZ^{a}_{3}$	*Alcaligenes faecalis*	$4S^{b}_{2}$	*Pseudomonas putida*
$6MZ^{a}_{2}$	*Alcaligenes faecalis*	$4MZ^{b}_{1}$	*Alcaligenes faecalis*
$7MZ^{a}_{1}$	*Stenotrophomonas maltophilia*	$7MZ^{c}_{3}$	*Pseudomonas maltophilia*

Effect of Temperature on Sulfur Removal Ability

The results displayed in Fig. 7 represent sulfur removal efficiencies of all the isolates at various temperatures.

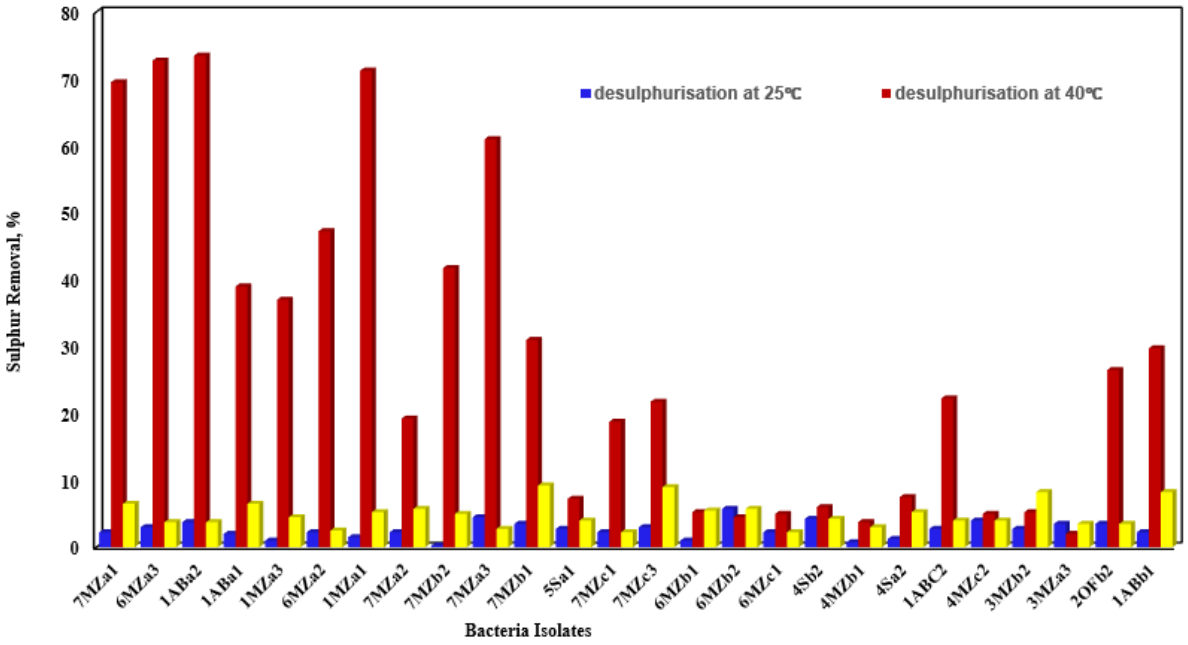


Fig. 7. Comparison of microbial desulfurization at different temperatures

From the results, it can be seen that temperature has a profound effect on the level of sulfur removal from crude oil by all the isolates. The highest removal rate for most of the identified isolates in this research was found to be 40 ℃, indicating their possible mesophilic characteristics. The growth temperature for all the isolates (identified in this research) ranges from a minimum of 10-20 ℃ and a maximum of 40-45 ℃ with the most optimum temperature around 37 ℃ [73, 74]. From the results above, five isolates could remove more than 60% of the sulfur that was present with the highest removal of 73.5% at 40 ℃. According to Nassar *et al* [6], using *paenibacillus glucanolyticus* for desulfurization resulted in about 94% sulfur removal at 33.5 ℃ within 120 hr. Shahaby and Essam-El-din [75], using *Pseudomonas putida* for desulfurization of crude oil resulted in sulfur removal of 31% at 30 ℃. Also, using *Rhodococcus erythropolis* resulted in 26.1% sulfur removal from crude oil at 30 ℃. A sulfur removal of 25% was also achieved from crude oil by *Bacillus pumilus*, also at 30 ℃. Yu et al. [76] also achieved a sulfur removal of 62.3% from Fushun crude oil and 47.2% from Sudanese crude oil at 30 ℃ within 72 hr by using *Rhodococcus erythropolis*. The observed higher removal rates at 40 ℃ could be due to the effect of temperature on the chemical properties and reaction rates. These reactions are catalysed by various enzymes including the DszA, DszB, and DszC enzymes that are known to have an optimal temperature of 37 ˚C in the desulfurization process [77]. (At the elevated temperature of 50 ℃, these enzymes and others are probably deactivated and the isolates are unable to survive and multiply. A detailed study of the optimum growth requirements for selected isolates will be conducted in another research.

Conclusion

Bio-desulfurization is a very promising method and hence the isolation and utilization of indigenous microorganisms, which have the potential to remove sulfur is the right step in the right direction. 26 most potent desulfurization isolates were successfully obtained from waste engine oil dump sites in the local environment. After 72 hours, a desulfurization efficiency of

5.75% was achieved at 25 ℃, in comparison with 73.5% efficiency at 40 ℃ and 9.25% at 55 °C. Out of the twenty-six most potent isolates, five removed more than 60% of sulfur which were present in the crude oil at a temperature of 40 ℃. The five isolates included two strains of Strenotrophomonas maltophilia, two strains of Pseudomonas aeruginosa, and a strain of Enterococcus feacalia. The isolation and utilization of these indigenous bacteria from waste oil dump sites capable of sulfur removal was fruitful and could be a source of a less expensive source of microbes, compared to the commercial ones. Enterococcus feacalia which is among the best five isolates has not been widely explored for bio-desulfurization and this could be worth researching further. The optimum recommended temperature of about 40 ℃ should be employed for effective desulfurization using these isolates. Future research could focus on genetic modification of the isolates to improve desulfurization efficiency and rate, the pH, and the absence or presence of some nutrients in enhancing the sulfur removal.

Declaration

The authors declare that there is no conflict of interest about in manuscript. Funding for the research was from the authors with support from the management of KNUST Engineering Education Project (KEEP), the management of West Coast Gas Ghana (WCGG) especially Mr. Emmanuel Amonoo Johnson (MD), and Mr. Courage Dey, Elsco Company Limited (MD). This manuscript is part of a Ph.D. research and all data are duly presented in the manuscript.

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How to cite: Boateng E.K, Danquah K.O, Ankudey E.G, Darkwah L, Boateng D.K. Desulfurization Capabilities of Indigenous Bacteria Isolates for Enhanced Sulfur Removal. Journal of Chemical and Petroleum Engineering 2024; 58(2): 289-309.