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Biodesulfurizing Microbes in the Petroleum Refinery Areas of Saudi Arabia

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Abstract

Gordonia sp., Rhodococcus, Paenibaccilus, Mycobacterium **and many other desulfurizing strains have shown good potential for dibenzothiophene (DBT), 4, 6-Dimethyldibenzothiophene (4-6-Dimethyl dibenzothiophene) and other organosulfur biodesulfurization. These are microbes which have 4S pathway to remove S from remaining calcitarant organosulfur compounds even after deep desulfurization. Sulfur compounds present in crude oils, diesel and petrol when combust in engines they emerge out in the form of elemental Sulfur, which causes environmental and health problems. Therefore, efforts are going to remove this Sulfur compounds by Hydrodesulfurization (HDS) treatment. Some organosulfur compounds remain there even after HDS, which can only remove by highly evolved microbes residing nearby petroleum-contaminated areas in refineries zone. Nature has such adopted and evolved microbes for the bioremediation of such toxic substances. Here we have isolated and characterized highly evolved and adopted Biodesulfurizing microbes present around oil refineries in Kingdom of Saudi Arabia and prepare the culture collection of such highly evolved and adopted biodesulfurization microorganisms for future application of applied Industrial petroleum refineries, which can reduce the Sulfur load in the petroleum products. The several (10 different types) microbes have been reported in these soils to grow in sulfur compounds. Out of these microbes one microbe desulfurizes by 4S pathway. It was identified to be *Rhodococcus erythropolis* type named as *Rhodococcus erythroplis* KAU10. They show good potential for various organosulfur compounds (DBT, 2,4,6-Trimethyl Benzothiophene, Benzothiophene, Dibenzyl sulfide, Benzonaphthothiophene, Dibenzothiophene sulfone, along with crude oil and Petrol and Diesel. Isolated strain *Rhodococcus erythroplis* KAU10 have good potential for Biodesulfurization.**

Keywords: Biodesulfurization, Isolation, Characterization, *Rhodococcus erythroplis* KAU10, 4S Pathway

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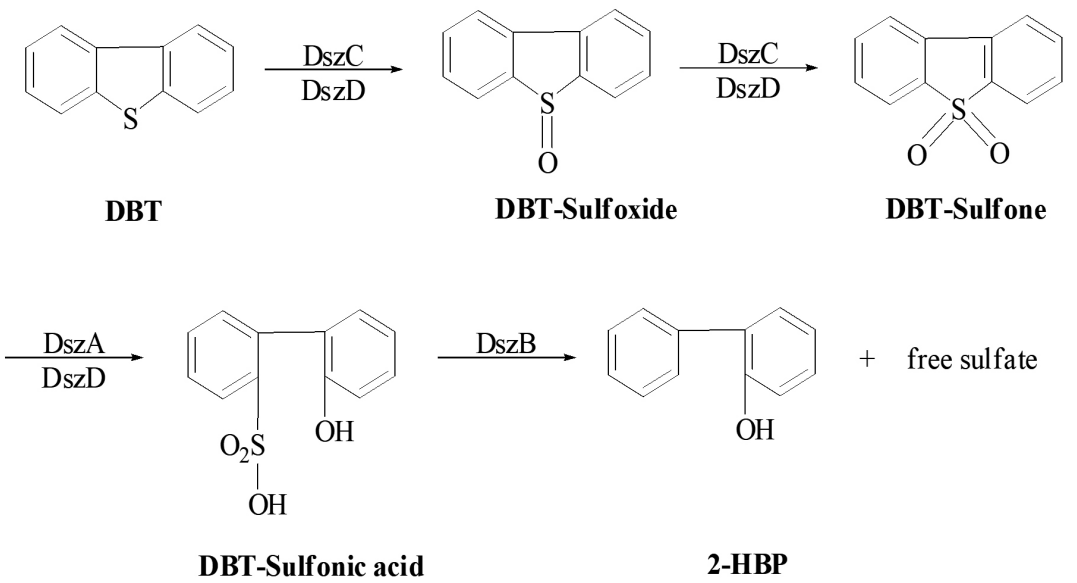
INTRODUCTION

Since GCC countries are Oil/Fossil, fuel-based countries. The major economy of these countries is based on oil production, refining, and its export to other countries. Nearly 70% of the all-energy oil reserves are from Gulf region. At this time, the confirmed petroleum reserves in the GCC countries account for 30% of the total. At 15.7%, Saudi Arabia is in the lead, accompanied by Kuwait at 6%, the UAE at 5.7%, Qatar at 1.5%, Bahrain at 0.4%, and Oman at 0.3%. In 2022, the GCC nations collectively produced 28.6 million barrels per day, or 32.3% of total global output. Although all GCC nations are impacted by decreasing oil prices, not all are impacted equally. Oman and Bahrain are most impacted by the reduction in oil costs, while Qatar, the United Arab Emirates (UAE), Kuwait and Saudi Arabia (KSA) are not as impacted.[1] As the demand for oil utilization is increasing, the conventional oil well's supplies are decreasing and expected to be exhausted by the end of 2070, so we have to rely on non-convention oil which have more sulfur percentage (Sour crude oil), so it is important to remove sulfur from these oils because they are harmful to the human health, atmosphere, and refinery machines (catalyst used in refining). For sulfur removal, the very old and tradition method is the chemical method where all most all sulfur compounds are removed, but recalcitrant organosulfur compounds remain there even after HDS which after combustion in engines creates environmental problems to human being directly or indirectly. Interesting thing is there that microbes can remove these recalcitrant organosulfur compounds by either reduction method (Kodama pathway) or oxidation method 4S-pathway.[2-6] In Kodama, pathway fuel value is not conserved, while in 4S pathway fuel value of carbon is conserved. 4S pathway is the preferred way of desulfurization because of saving calorific value of organosulfur compounds in industrial perspective. Thermophilic bacteria have also been reported showing the 4S pathways.[7-9] In 4S pathway, phenolic products are formed as the product[10-17] as shown in Scheme 1. As the accumulation of product increases, the activities of this pathway enzyme get decreased/ceased. That is why industrial application of this pathway could not be possible. If we could remove this inhibition by Biotechnological or by Molecular approach, thinks would be easier to apply at industrial scale.[18,19] Here we isolated and characterized Biodesulfurizing microbes from the various oil refineries areas in Saudi Kingdom. Here the measure work was emphasized on potential microbes to be isolated which could fulfil the demand of biodesulfurization of various



Scheme 1. 4S pathway for DBT desulfurization using *DszA, DszB, DszC* and *DszD* enzymes

organosulfur compounds, which remains even after HDS. If the diversity of the microbes is studied and fully characterised, the best quality microbes in terms of biodesulfurization properties, can be made even better by metabolic engineering to remove inhibition by this phenolic product in Saudi Arabia.[20-22] Its industrial scale application can be possible for desulfurization. Crude oil, diesel and petrol may be more precious and valuable for GCC countries, once all the recalcitrant organosulfur compounds are removed or desulfurized[23] by highly evolved microbes from Saudi region. It can boost and enhance the economy of GCC countries by providing sulfur free petroleum products to the Global market. The isolated microorganism identified to be *Rhodococcus erythropolis* KAU10 by morphological, Biochemical, Analytical methods and 16S rRNA gene-based sequencing methods. This strain can grow on various organosulfur compounds and have 4S pathway for biodesulfurization. This strain has potential to be used in Industrial level Biodesulfurization.

METHODOLOGY

Chemicals and materials

All the analytical grade chemicals (DBT, 2-HBP, BT, DBT-sulfone, Gibb's reagents) were obtained from Sigma-Aldrich company. All the chemicals were highly pure (99%) and analytical grade. Media like Luria broth, Agar Powder, Agarose Powder, Gram-staining and bacterial identification kits were purchased from High-Media company. Molecular based chemicals and reagents were purchased from Sigma company. Acetone, Methanol, Ethenol, Ethyl acetate and n-hexane extra pure (99%) were purchased from Merck company.

Overview of Methodologies used

Soils from two different locations were taken for isolation for desulfurzing microbes via 4S pathway. Soil samples were incubated with media containing DBT and incubated for 5 days of each cycles. After 5 weeks of such cycles, bacteria cultures were taken for Gibb's assay by spectrophotometrically. Consequently culture were spread on BSM-Agar media to get the pure colony of doing desulfurization via 4S pathway. Subsequently pure colony were identified by morphologically, biochemically and molecular biologically. Metabolites were identified by GC-MS method and its resting cell activity were compared with *Rhoococcus erythropolis* IGTS culture.

Bacteria isolation from DBT-grown cultures

Different crude oil resource locations in Jeddah, Saudi Arabia, were chosen and given the designations 1 and 2 in order to isolate Dibenzothiophene desulfurizing bacteria. 1 was the Jeddah Industrial Area near the refinery at the Jeddah Islamic Sea Port. The area surrounding Jeddah Airport, which has a large oil reserve basin, is where Location 2 (Second Location) was found. Samples of the oil-contaminated soil from these sites were taken in a number of places (10 locations). Each location's sample (50 g) was combined into a single master blend. To isolate DBT-desulfurizing bacteria, 10 mL of the suspension was mixed to 100 mL of basic salt medium (BSM) in a 250 mL flask. As a source of carbon, glucose (5 g/L) and sulfur source from DBT (5 mM/L) were added to this medium. The five-day growth period took place in a spinning shaker at 30 degrees Celsius. When the BSM agar medium had been supplied with glucose and DBT, the culture broth medium was transferred on top of the BSM agar medium after such 5 subcultures.[24] Samples from Workshops were used to isolate all the representative bacterial cultures that were viable and growing primarily.

4S pathway screening by Gibb's assay test

The existence or lack of the 4S pathway in the recovered cultures was discovered by measuring 2-HBP, the pathway's ultimate product, using the Gibb's test. BSM broth containing glucose and DBT was used to cultivate the recovered colonies. After cultivating the bacteria in a DBT-containing medium, the amount of 2-HBP they produced was measured. When Gibb's reagent is combined with aromatic hydroxyl groups, like 2-hydroxy phenyl, a blue complex is generated. Each bacterial culture broth had its pH adjusted to 8.0 with 10% sodium bicarbonate or sodium carbonate, and then 100 µL of each broth was combined with Gibb's reagent (10 mg of 2,6-dichlorquinone-4-chlorimide generated in 1 mL of ethanol) for a 30-minute incubation. The findings of the Gibb's test are displayed through

the production of a blue color complex (indicating a positive result) or a brown hue (indicating a negative result). It was expected that Gibb's assay-positive cells would produce 2-HBP from DBT via the 4S pathway.[25]

Analytical analysis of DBT-based metabolites from bacterial isolates (GC-MS)

After centrifuging the culture broth, the resulting supernatant was adjusted to a pH of 2.0 using 6N hydrochloric acid as the solution. Samples were extracted using the acidified supernatant and ethyl acetate in equal volumes before being analyzed by GC-MS. The Pegasus HT TOFMS was coupled with the GC in the Agilent 7890A GC-MS system. The GC column has the following dimensions: 29.8 m x 0.2 m x 320 m. The sample temperatures ranged from 60 to 320 degrees Celsius throughout the duration of the examination. The temperature increased at a rate of 15 degrees Celsius per minute while the sample was being analyzed. Throughout the entire sample, the temperature remained stable at 280 degrees Celsius. 1µL of DBT culture broth was analyzed in which 1.2 mL/min of helium is used as the carrier gas.

Identification of the 4S pathway's *dsz* operon genes

The desulfurization gene A (*dszA*) gene was amplified using the forward primer 5'-GCGCGGCAAGTTCGATCTGT-3' and the reverse primer 5'- TCCCGCAGGATGTCCTTGATC-3'. In order to amplify the desulfurization gene B (*dszB*) gene, we used the forward primer 5'-ATCGAACTCGACGTCCTCAG-3' and the reverse primer 5'-TCAGGACCACAGCTACAAG3'. Primers 5'- CTGTTCGGATACCACCTCAC-3' and 5'- GTGCCTGAAGGTGTTGCA-3' were used for amplification of the desulfurization C (*dszC*) gene, respectively.[26,27] Primers 5'-GCGCGGCAAGTTCGATCTGT-3' and 5'-GTGCCTGAAGGTGTTGCA-3' were used to amplify the desulfurization (*dsz*) *ABC* operon gene.

Bacterial isolate identification

Colony features

On DBT-containing basal salt media, bacterial isolates that use the 4S route for desulfurization grew in distinctive colonies. Each colony's layout, hue, margin, height, and size were meticulously recorded.

Combining biochemical analysis with microscopy

The DBT desulfurizing bacteria's morphological form, gram and spore stains were performed. The biochemical tests, which were conducted in accordance with predetermined protocols, included hydrogen sulfide production, the hydrolysis of starch, methyl red, casein, sucrose, catalase, indole, oxidase, Voges-Proskauer, lipid activity, nitrate reduction, , urea hydrolysis and citrate utilizationtests.[28-30]

16S rRNA sequencing bacterial isolates

The bacterial isolates that tested positive for DBT desulfurization underwent 16s rRNA sequencing and phylogenetic analysis at the central facilities of our university. The primers 5'-GCAATAACAGGTCTGTGATGCCC-3' (forward) and 5' GCATCACAGACCTGTTATTGC-3' (reverse) were used to amplify the 16S rRNA gene.[31]

Chromosomal DNA isolation

Separate pure bacterial culture transfers were made in 100 mL of BSM broth mixed with carbon source of glucose and sulfur source as dibenzothiophene in 500 mL flask in capacity, where they were shaken at 30°C for 4 days at 180 rpm. The culture broths underwent a ten-minute centrifugation at 10,000 rpm following the incubation time. Then, liquid nitrogen was used to crush 0.1 grams of the mycelium from each isolate on a spotless porcelain dish. Each isolate's crushed mycelium was then put into a tube with 500 µL of TE buffer and lysozyme enzyme, and it was cultured there for 30 minutes at 37°C. The tube was then treated for 30 minutes at 55°C with 20 µL of proteinase K and 10 µL of SDS. The mixture was chilled shortly after the incubation period, spun for five minutes at 10,000 revolutions per minute, and then processed with a phenol-chloroform (1:1) mixture. The aqueous part of the combination had been moved to a sterile tube, and 90% of an ethyl alcohol solution was employed to produce a DNA precipitate at -20°C. The pellet of DNA was subsequently retrieved after 15 minutes of centrifuging at 8,000 rpm. Final DNA pellet was mixed in Tris-EDTA buffer, 20 µL of RNase enzyme was placed in it, and mixture was incubated for a

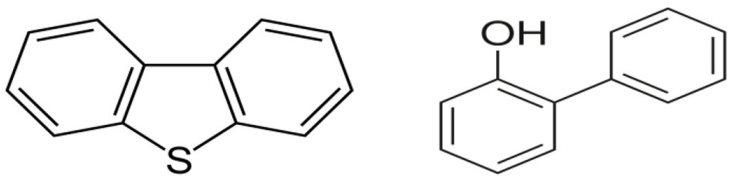
single hour at 37 degrees Celsius to produce pure DNA witch is free from RNA contamination. The genetic material DNA was precipitated once again using ethyl alcohol (90%) at -20°C. After spinning (for 10 minutes at 10,000 rpm),[32] a pure DNA pellet was produced, and the DNA's purity was assessed using a Ultra-visible (UV) spectrophotometer.

Gene/s characterization

The Polymerase chain reaction (PCR) was done using 50 ul of buffer, 10% dimethyl sulfoxide, 6 mM magnesium chloride, 2.5 units of Taq Pol, and 30 pmol of each primer. The DNA input taken was 100 ng. Initial temperature was set at 94°C for 1 minute to denature the mixture, then it was lowered to 57°C for 60 seconds to anneal the mixture, then it was raised to 72°C for 1 minute to extend the hybridized primers, and finally it was raised to 72°C for 5 minutes to complete the amplification in 35 cycles. After that, an agarose gel electrophoresis was performed on the PCR reaction mixture using a 1 kb DNA ladder as a size marker. The sequencing of rRNA genes was done using the dideoxy chain termination method.[33] The 16S rRNA gene sequences of the KAU10 isolate were uploaded to NCBI Genbank.

Reactions of resting cells

The isolates were cultured for 48 hours at 30°C in 500 mL flasks with 250 mL AVSG medium containing 100 mg DBT/L. After collecting the cells by centrifugation at 9000 g for 30 minutes at 4°C, they were washed twice in 0.1 m potassium phosphate buffer (pH 7.0) and kept at 20°C. 1 mL



(A) (B)

Figure 1. Stucture of Dibenzothiphene (DBT)-A and 2-Hydroxy biphenyl-(2-HBP)-B

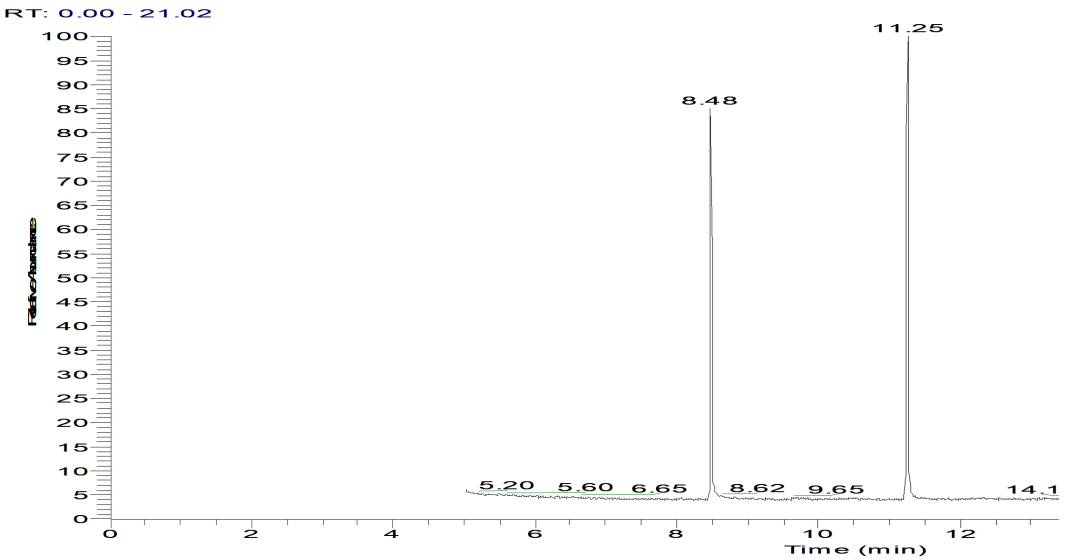


Figure 2. GC-MS Chromatogram obtained for the bacterial isolate KAU10 from broth extract showing peaks of parent compound, DBT at 11.25 min. and metabolites formed, 2-HBP at 8.48 min

of the cell suspension was added to 33mL of 30% (v/v) glycerol and 10 mL of 1% (v/v) DBT in ethanol in a test tube until the final concentration was 6.60 g/L. The resting cell reaction was allowed to continue for 1 hour at 30°C with 150 rev/min of reciprocal shaking.

RESULTS AND DISCUSSION

DBT-supporting microorganisms

10 bacterial representative types that were predominantly growing on BSM medium added with DBT were taken from two different oil-contaminated sites for the current study. The Gibbs test was used to check each of isolates either having 4S pathway metabolic activity or not, and one bacterium (designated as KAU10) isolated from the mix soil of sites 1 and 2 was discovered to be positive for the 4S pathway. The Gibb's test, which revealed the development of blue color due to produced 2-HBP, the ultimate byproduct of the 4S pathway, validated this.[34] The Gibb's assay was previously used by a number of researchers to assess the desulfurization activity of bacteria via the 4S pathway.[35-37] Because of their extensive culture in basal salt medium (BSM) supplemented with glucose as a carbon source and DBT as the only sulfur source, the other isolates in our analysis that tested negative for the Gibb's assay may be using the Kodama pathway and Van Afferden system to metabolize DBT.

GC-MS to detect 4S pathway's intermediates and final product

To find the metabolites of the 4S pathway, the Gibb's test positive bacterial isolate (KAU10) underwent GC-MS analysis. The chemicals with retention times of 11.2 and 8.48 minutes in the KAU10 isolation culture broth were characterized as DBT (parent compound) and the 2-HBP (formed final product), respectively, by GC-MS (Figure 1 and 2). Li et al. used GC-MS to examine DBT cultured media from Mycobacterium sp. X7B and found DBT sulfone and 2-HBP. Using the newly isolated Gordona strain CYKS1 and the GC chromatogram of DBT grown broth, Rhee et al. discovered 2-HBP during DBT desulfurizing research. The significant fragmentation ions at m/z 139 were seen in the DBT mass spectra of the KAU10 isolate, along with a molecular [M+] ion peak at m/z 184. The molecular weight of DBT is represented by this peak. Major metabolite of KAU10 showed a molecular [M+] ion peaks at the molecular mass of (m/z) 141, 139, 115, 102, 89,70, 63 51,39, 27) obtained from the library of GC-MS machine, confirms the metabolite to be 2-HBP which is the end product of 4S pathway from DBT as a parent organosulfur compound. Each molecule has its distinctive breaking patterns by them they are identified. This finding provides strong support for several previous publications.[38-40] as seen in Figure 3. However, when Akhtar et al. used GC-MS to examine the mass spectra of DBT culture liquid media of *Rhodococcus* species, they found that the molecular ion peaks for DBT sulfone and 2-HBP

Table 1. Colony characteristics of the bacterial isolates

Colony Character	KAU10 Isolate
Configuration	Round
Color	White
Elevation	Convex
Size	4.8 mm
Margin	Ciliate

Table 2. Morphological, Gram staining, Spore shape and Biochemical tests

Characteristics	Bacterial Isolate KAU10
Microscopic observation	
Morphological shape	Filamentous
Spore shape	Oval to circular oval
Gram staining	Gram postive
Biochemical Tests	(+/-) / Positive/ Negative
Sucrose Test	+
Starch Hydrolysis	+
Catalase test	+
Casein Hydrolysis	+
Oxidase test	+
Citrate utilization test	+
H2S production test	+
Indole test	-
Lipid Activity	+
Voges-Proskauer test	-
Methyl red test	+
Nitrate reduction test	+
Urea Hydrolysis	+

were quite similar. DBT-sulfone was also detected with our culture isolate (data not shown).

DBT desulfurizing bacteria's amplified dsz operon genes

The presence of the dsz ABC operon genes was confirmed by the increased expression of these genes in the bacterial strain KAU10 (Figure 4). It is noteworthy that both isolates contained the dsz operon on their genomic DNA, which is consistent with a previous finding. Shavandi et al. found the dsz operon genes on the chromosome of Gordonia alkanivorans RIPI90A, which are necessary for DBT desulfurization. However, the dsz operon was discovered to be present on plasmid in the *Rhodococcus erythropolis* IGTS8

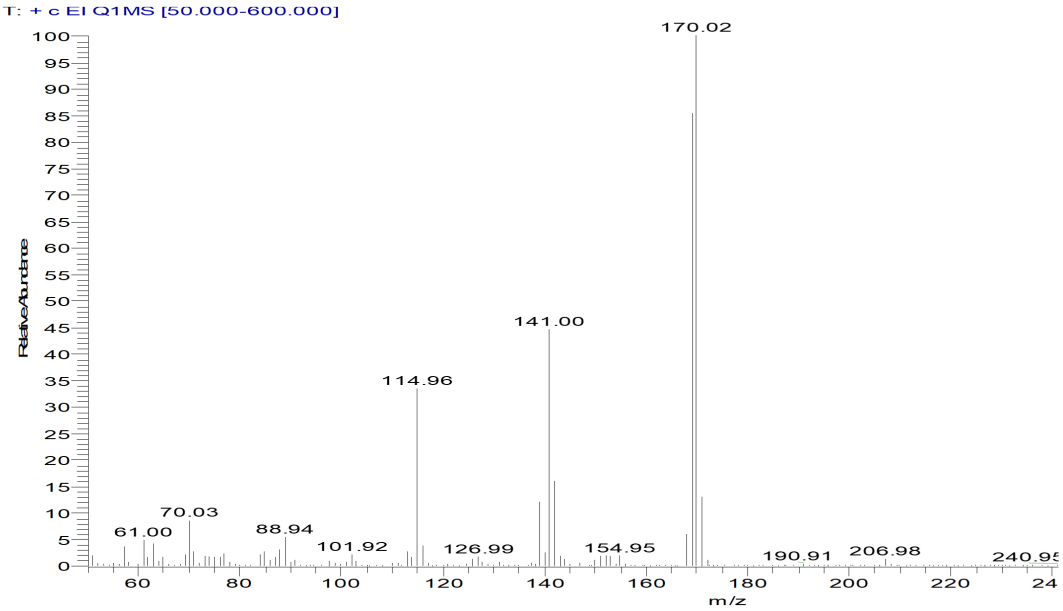


Figure 3. Shows the mass spectrum of 2-HBP metabolite obtained from bacterial isolate KAU10

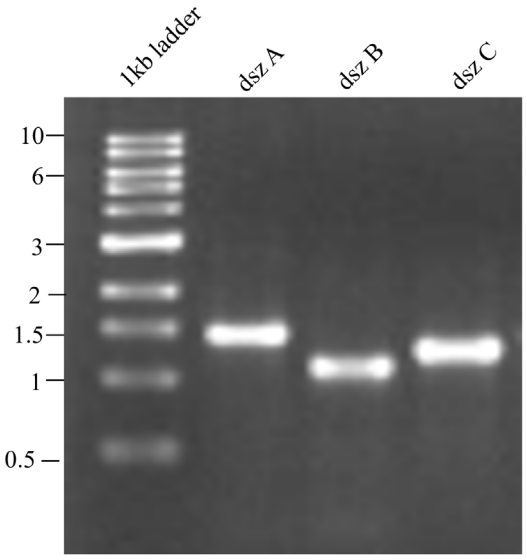


Figure 4. Shows the dsz operon genes of the KAU10 bacterial strain as they appeared on a PCR gel

model strain used in biodesulfurization studies.

The desulfurizing bacteria are Gram positive, filamentous, and provisionally identified as Rhodococcus based on colony characteristics (Table 1 and 2), morphological (shape), clear zone assay, staining (Gram's and Spore) (Figure 5), and biochemical (citrate utilization test, urea hydrolysis, hydrogen sulfide production, indole test, methyl red, lipid activity, Voges-Proskauer test, and nitrate reduction test) studies. Comparable morphological and biochemical testing allowed Taddei et al. to conclude that *Rhodococcus* species were the soil bacteria under study. Both bacterial isolates' 16S rRNA genes were sequenced after being amplified using the common primers. KAU10, a bacterial isolate, has incomplete rRNA gene sequences that were 1,393 bp long. KAU10 and *Rhodococcus erythropolis* were related to one another, according to the phylogenetic study.

Using NCBI's BLASTn program, it was possible to calculate the species' percentage of identity. *Rhodococcus erythropolis* IGTS8 was the source of 99.91% of the identities. Given that the rRNA gene is the oldest, most conserved, and most dispersed portion of the microorganisms' genomes, it has shown to be a universal tool for phylogenetic analysis and the study of relationships among organisms. Despite the fact that prokaryotes have three distinct ribosomal RNAs-5S, 16S, and 23S only the 16S rRNA sequence is employed since its nucleotides are both evenly sized and simple to sequence. In order to create a phylogenetic tree, the distance-matrix approach is utilized with the rRNA sequence. By logging variations in two or more organisms' sequences and analysing them using software, the evolutionary distance is calculated. Since there is a chance that the genome has undergone changes that would bring the sequence back to its original state, a statistical correction factor is used. The phylogenetic tree is built following the evolutionary distance measurement. The length of the branches separating the two microorganisms is directly inversely correlated with the differing evolutionary distances of the two species. Phylogenetic trees can be constructed in a variety of ways based on the software/computer application and the number of microorganisms.[41]

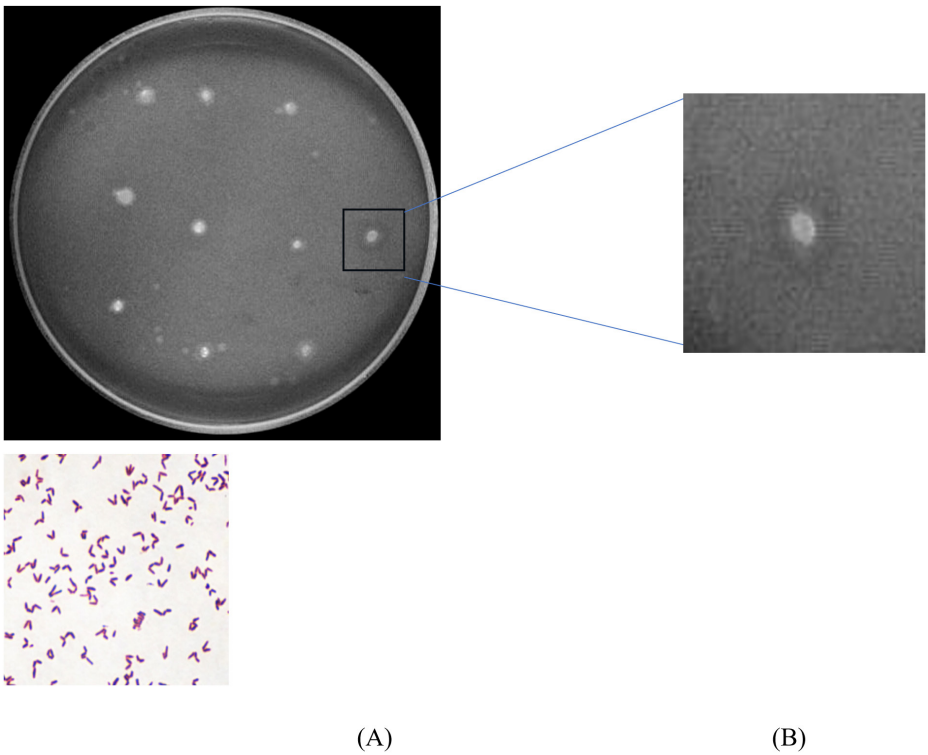
Identification of bacteria via the 4S route that have been found to be DBT desulfurization positive

This research confirms that the *Rhodococcus erythropolis* KAU10 species is an aerobic bacterium that uses the aerobic DBT desulfurization (4S) pathway. Comparing them to microbial strains that display anaerobic biodesulfurization routes, they will be more

Table 3. Specific activity comparison

Strain Cell	Conc. (g/l)	Activity (U)
KAU10	6.6	12.50
R. erythropolis IGTS8	6.6	4.36

1 U = 1 mol 2-HBP/g-cell/h.



(A) (B)

Figure 5. Colony morphology and DBT clear zone assay (A) and gram stain of KAU10 (B)

significant economically and commercially. By processing the oil in an aqueous phase, the water-soluble sulfite that is formed by microorganisms with an oxidative DBT desulfurization route can be easily disposed of. In contrast, the reductive process of anaerobic desulfurization involves the removal of sulfur from DBT as hydrogen sulfide that ultimately leaves sulfur atoms. Desulfurization is possible with anaerobic strains, but the process is laborious, costly, and time consuming to maintain. Additionally, unfavourable compounds can arise from the organic fuel components.[35] It is also possible to genetically alter these *Rhodococcus erythropolis* KAU10 for increased DBT biodesulfurization activity. These genetically enhanced biodesulfurizing *Rhodococcus erythropolis* KAU10 strains have the potential to be commercially significant and effective when used to desulfurize fuels throughout the procedure used in refineries to make fuels free of sulfur.

Activity of Resting Cells

To ascertain their relative activity, the one strains were put through an identical experiment with the control strain, IGTS8, and the outcomes are displayed in Table 3. *R. erythropolis* IGTS8 had the second-highest activity level, with 4.36 units, whereas strain KAU10 had a 12.50-unit output. Under the assessed experimental circumstances, all three strains outperformed the control strain. This suggests that the new strains have a good chance of being used as bio-catalysts for oil desulfurization. The almost three fold increase activity is good indication for commercial purposes. This increased activity might be due to better sulfur utilization found in the petroleum contaminated soils in high temperature and high humidity in the area of refinery in Jeddah, Saudi Arabian regions. As soils are highly contaminated with sulfur products. This may be because of better adaptation by KAU10 in such a harsh condition of high temperature and high sulfur contents in the soil.

CONCLUSION

One potential DBT desulfurizing *Rhodococcus erythropolis* KAU10 species was isolated from oil-contaminated soils near refineries for the current study. The commercial significance of these desulfurizing species is based on their ability to desulfurize DBT found in fuels without destroying the main ring structure of DBT or decreasing the energy content of fuel (mileage), chemical used as a study model for biocatalytic desulfurization via the 4S route. It has been discovered that the dsz operon (A, B, and C) that controls the 4S pathway is present in the *Rhodococcus erythropolis* KAU10 species. In the biodesulfurization of fuels, this DBT desulfurizing organisms become increasingly important from an ecological and commercial standpoint. This strain is capable of growing on a variety of organosulfur compounds, demonstrating a high and broad range of sulfur compound desulfurization, which is required by the present industrial level of desulfurization. This isolated microbes have more potential for desulfurization than reported previously, hence could be a good option for alternate microbe based sulfur removal from the petroleum products. It has good and promising scope for desulfurization. Its furthur industrial level optimizations are needed to make its use in hydrodesulfurization process and petroleum refineries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AA conceptualized the project and performed the experiments. AA, OAB, MSN and VA wrote, reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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