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Desulfurization of Crude Oil and Oil Products by Local Isolated Bacterial Strains

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A B S T R A C T

The presence of organic sulfur-containing oil in the environment is harmful to animals and human health. The combustion of these compounds in fossil fuels tends to release sulfur dioxide in the atmosphere, which leads to acid rain, corrosion, damage to crops, and an array of other problems. The process of biodesulfurization rationally exploits the ability of certain microorganisms in the removal of sulfur prior to fuel burning, without loss of calorific value. In this sense, we hypothesized that bacterial isolates from crude oil and oil products polluted soils can demonstrate the ability to degrade crude oil and oil products as well as dibenzothiophene (DBT), the major sulfur-containing compound present in fuels. The total sulfur bacteria were ranged from $1.6x10^4$- $2.8x10^6$ CFU g $soil^{-1}$ on PCA media and $4.1x10^2$- $2.1x10^6$ CFU g $soil^{-1}$ on basel media (BSM) supplemented with DBT. Those strains which showed great degradation efficiency in case of all investigated hydrocarbons were identified based upon the sequence analysis of their 16S- rRNA. Phenotypic and genotypic examination of the recovered isolates revealed that they belong to the five different genera of *Bacillus*, *Pseudomonas*, *Rodococcus*, *Mycobacterium*, and *Klebsiella*. All isolated bacteria showed to be capable of biodesulfurization of oil or oil products, as they were compared to standard strains (ATCC) and were able to grow in minimal mineral medium supplemented with DBT or 2HBP as the sole sulfur and carbon source. The potential application of these isolates for the biodesulfurization of oil and oil products as well as sulfur-containing compound in fuels prior to combustion was discussed. Furthermore, results indicated that using a microbial consortium might have a promising application in petroleum oil technology and could be potentially used in microbial enhanced oil recovery (MEOR).

Introduction

Sulfur is the most abundant element in petroleum after carbon and hydrogen. The average sulfur content varies from 0.03 to 7.89 mass% in crude oil (Mehran *et al.*, 2007). The sulfur compounds can be found in two forms: inorganic and organic. Inorganic sulfur, such as elemental sulfur, H_2S and pyrite can be present in dissolved or suspended form (Agarwal and Sharma 2010). Organic sulfur compounds such as thiols, sulfides, and thiophenic compounds represent the main source of sulfur found in crude oil. Crude oil and oil products have many components that have to be removed before

they are usable in the marketplace. One of the most toxic elements in the crude products is sulfur. Sulfur forms compounds in oil and oil products, such as hydrogen sulfide, which are very corrosive and extremely toxic. Sulfur in gasoline is not only a source of air pollution, but also plays a significant role in determining the tailpipe emissions of other pollutants, such as nitrogen oxides, carbon monoxides and unburned hydrocarbons. Because of the boiling range, the composition of sulfur compounds in gasoline is unlike that found in diesel oil in which the main sulfur species are dibenzothiophene (DBT) and dibenzothiophenes with substitutions (Monticello, 1998). Mercaptans are a small portion of the gasoline sulfur compounds, whereas thiophene and alkylthiophenes make up the largest portion. Many refineries worldwide are using a variety of methods to reduce the concentration of sulfur in natural gas. Removing sulfur from fuel is becoming a more serious concern as crude oils are getting higher in sulfur content and regulated sulfur limits are becoming lower and lower (Holliger *et al.,* 1997). To date, there is no common method for selective removal of sulfur from oil before its processing, which could be successfully applied on an industrial scale. Biodesulfurization is the application of microbial processes to convert organic sulfur compounds into harmless substances and removing of sulfur. The advantage of the study of biodesulfurization of crude oil is its cost-effectiveness when compared to some physicochemical techniques. Many microorganisms have been reported to use various petroleum hydrocarbons and sulfur compounds, as their sole carbon and energy substrate, despite their extreme insolubility in the aqueous phase. It is possible to desulfurize crude oil directly by selecting appropriate microbial species (Javadli, de Klerk. 2012). Numerous genera of bacteria are known as good hydrocarbon degraders *Rhodococcus, Bacillus, Pseudomonas, Mycobacterium, Klebsiella, Pseudomonas, Actinomycetes, Enterobacter* and *Acinetobacter* (Izumi *et al.,* 1994; Kirimura *et al.,* 2000; Ishii *et al.,* 2005; Al-Zahrani and Idris, 2010; Jamshid *et al.,* 2010; Bhatia, Sharma, 2012; Buzanello *et al.,* 2014); however, reports on the utilization of complex sulfur mixtures like crude oil by isolated microbial species are few. To obtain an efficient desulfurizing bacterial consortium and monocultures, knowledge of the diversity of the microbial community present in sites contaminated with crude oil, their metabolic features and capacity to desulfurize crude oil are of paramount importance. One of the factors that limit biodesulfurization of crude oil is their limited availability to microorganisms. Biodesulfurization has become an alternative way to remedy crude oil and refined products, where the addition of specific microorganism or enhancement of microorganism already present, can improve desulfurizing efficiency (Kvenvolden and Cooper, 2003). In order to develop environmental technologies for crude oil desulfurization, it is necessary to isolate and characterize specific microbial species for evaluation of their efficacy in utilization of sulfur compounds before application to crude oil. Information about efficiency of potential sulfur bacteria of contaminated soil with crude oil or oil refiners in Saudi Arabia is scant.

Bacterial communities are difficult to study due to their immense complexity and the potential problems in culture ability of many of the members (Abou-Shanab, 2007). Serological and bacteriological methods are not sensitive enough to differentiate all bacterial isolates (Taghi *et al.,* 2008). Therefore, several molecular approaches now provide powerful adjuncts to the culture-dependent techniques. Now Combination of colonial morphological, physiological, biochemical, serological and molecular markers is essential for successful

identification either to the genus level or more frequently to the species level (Millar *et al.*, 2007).

Bacterial 16S-rRNA is a common target for taxonomic purposes and identification, largely due to the mosaic composition of phylogenetically conserved and variable regions within the gene (Gurtler and Sanisich, 1996, Bayoumi *et al.*, 2010).

This work represents a continuation of our research in the area of petroleum biodegradation technology. The study aims to characterize potential sulfur bacteria isolates from contaminated soil with crude oil or oil refiners. Api profiles as well as 16S-rRNA gene technique were employed for molecular characterization and identification of bacterial isolates. In addition, to describe the ability of selected bacterial strains to desulfurize crude oil and its refined products and to compare local isolated strains with reference commercial strains.

Materials and Methods

Bacterial strains

Local isolates and strains from previous work, laboratory collection and ATCC cultures were used in this research project.

Isolation, identification and culture conditions

Three grams of contaminated soil were added to sterile 250-ml Erlenmeyer flasks containing 50 ml of Bushnell Hass Medium (BHM). The bacterial strain was isolated by repeated enrichment cultures adding crude oil or oil products as the source of carbon and energy. Each crude oil or oil products (crude oil, kerosene, benzene, motor oil, diesel oil and DBT) was supplemented at a final concentration of 200 mg/l. The flasks were incubated in the dark on a rotary shaker at 30°C and 200 rpm for 15 days. At the end of this period, the vials were allowed to settle for 1 hr. The supernatant of each vial was collected and re-suspended in phosphate buffer before being added into new 250-ml Erlenmeyer flasks containing 50 ml BHM and 200 mg/l of substrate compound used. This procedure was repeated five consecutive times totally under the same conditions. Aliquots of every culture were plated on solidified BHM and sprayed with concentrated substrates used to produce solid films on the Petri dishes. The aromatic degrading candidates were identified by the presence of clearing zones around the colonies that indicates substrate utilization. The isolates were identified and named based on morphological, physiological and biochemical characteristics presented in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) and the APi Kit profiling (Api, bioMerieux, France, 2009). Subsequently, bacterial growth is monitored by taking the absorbance at 595 nm.

Cultures and growth rates

Inocula were pregrown in 10 ml nutrient broth medium for 12 h. Cells were grown aerobically in 50 ml Erlenmeyer flasks. Flasks were filled to no more than 20 % capacity. All growth rates were determined with cells growing at 30^{o} C in an incubator shaker at 150 rpm. The absorbency of the culture was measured at approximately 4 h intervals for three days with a spectrophotometer at 595 nm. Cultures were usually harvested at absorbency 0.660. Cell numbers were no longer linear with respect to absorbency above this value. Also, pH of the medium should not change when experiments were terminated at this absorbency. Cells were harvested by centrifugation for 5 min at 3,000 x g at room temperature (Krieg, 1984).

Media were used are LB (Trypton, 10g; yeast extract, 5g; NaCl, 5g; distilled water, 1000

ml) and other strains were grown at 30°C. Basal salt medium (BSM) consisting of: K_2HPO_4 (4 g); Na_2HPO_4 (4 g); NH_4Cl (2 g); $MgCl_2{\cdot}6H_2O$ (0.2 g); $CaCl_2{\cdot}2H_2O$ (0.001 g) and $FeCl_3{\cdot}6H_2O$ (0.001 g) per liter of distilled, deionized water pH 7.0 was used for isolation and growth of the microorganisms under sulfur deficient conditions (Kilbane*et al.,* 1990). Glycerol (20 mM) was used as the carbon source and was omitted when other test compounds were used instead. Soil samples and subsequently isolated strains were inoculated in BSM supplemented with crude oil or oil products (200g/L) as well as 0.5mMdibenzothiophene (DBT) or dibenzothiophenesulfone ($DBTO_2$) or 0.2 mM of $MgSO_4$. The sulfur sources were added to the medium from sterile stock solutions before inoculation (10 mM DBT or $DBTO_2$ in ethanol; 50 mM $MgSO_4$ in deionized water. Media were designated as DBT, $DBTO_2$, or $MgSO_4$ medium, respectively (Wang and Krawiec1994). BSM solidified with 15 g of agar per liter was used for isolating bacterial colonies. All cultures were incubated at 30°C and liquid cultures were shaken at 200 rpm.

Microbial and biochemical techniques were employed in this project. The effects of pH, temperature degrees on crude oil and oil products biodesulfurization and growth rates of some isolates were determined. The growth rates of cultures in exponential phase were determine from linear regressions of $\log_{10}$ absorbency vs. time, calculating a least squares fit of data from the exponential growth phase, and determining the slope of this line. The instantaneous growth rate (μ) will be determined from the slope of this line x $\ln_{10}$; μ had the dimensions/h (Koch, 1984).

Optical density and biomass measurements

The turbidity of the cultures was determined by measuring the Optical Density (OD) at a wavelength of 595 nm in 2 ml cuvettes using a spectrophotometer (Biophotometer plus, Eppendorf). The net dry weight for the biomass was determined simultaneously. A 1 mL of culture was centrifuged at 1500 rpm for 10 min, washed twice with distilled water, poured into a pre-weighed container, dried overnight at 90 °C to constant weight and cooled for reweighing. The linear relation between OD_{595} and dry mass was obtained.

Effect of crude oil and oil products concentrations on sulfur bacteria growth activity

Growth of the isolated bacterial strains on different concentrations of crude oil, kerosene, benzene, motor oil, diesel oil and DBT was evaluated by measuring culture optical density (OD) at 595 nm.

Procedure for sulfur removal

The bacteria were used to desulfurize crude oil and/or oil products under three conditions. These include different time duration, temperature and different pH degrees. The desulfurized crude oil and oil products were subjected to ultra violet visible spectrophotometric analysis.

Quantification of sulfur

Biodesulfurized crude oil or oil refiners sample (2ml) was weighed in a conical flask and added to10ml of concentrated HCl contained in Kjedahl digestion flask. Distilled water (20ml) was then added. The contents were shaken to hydrolyze and then allowed to stay for 3 hours. The content was filtered with No.1 Whatman filter paper. The filtrate was kept for analysis. The filtrate (5ml) was poured into a test tube. Distilled water (15ml) and 2ml of conditioning reagent was then added. The test tube was covered and allowed to stand for few hours. A Spatula full of $BaCl_2$ was then added. The turbidity was read

with ultra violet visible spectrophotometer. The other compounds were analyzed using gas chromatograph (GC) equipped with a pulsed flame photoatomic detector (PFPD) according to Aribike *et al.,* (2009).

Molecular genetics analysis

DNA extraction

The cell pellets form all isolates were used to extract genomic DNA using (Jena Bioscience, Germany) extraction kit according to manufacturer's instructions.

PCR amplification of 16S-rRNA gene

Primer sequences used to amplify the 16S-rRNA gene fragment were: U1 [5CCA GCA GCC GCG GTA ATA CG3] and U2 [5ATC GG(C/T) TAC CTT GTT ACG ACT TC3] according to Kumara *et al.,* (2006).The PCR master mix contained10Pmol of each primer and 12.5 µl of 2xSuperHot PCR Master Mix (Bioron, Ludwigshafen, Germany) mixed with 50 to 100 ng of DNA template. Sterile d.H2O was added to a final volume of 25 µl. Thermal cycler (Uno II, Biometra, Germany) program was 94 °C for 4 min., 94 °C for 1 min., 55 °C for 1 min., 72 °C for 1.5 min, the number of cycles was 35 cycle and the post PCR reaction time was 72°C for 5 min.

Analysis of the PCR products

The PCR reaction products were electrophoresed with 100 bp ladder marker (Fermentas, Germany) on 10 x 14 cm 1.5%-agarose gel (Bioshop, Canada) for 30 min using Tris-borate- EDTA Buffer. The gels were stained with 0.5 ug /ml of ethidium bromide, visualized under the UV light (Watanabe et al., 2001)and documented using a GeneSnap 4.00- Gene Genius Bio Imaging System (Syngene, Frederick, Maryland, USA).

Sequencing of 16S-rRNA gene

The 990bp PCR-products of each isolate were purified from excess primers and nucleotides by the use of AxyPrep PCR Clean-up kit (AXYGEN Biosciences, Union City, California, USA) and directly sequenced using the same primers as described for the amplification process. The products were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI Applied Biosystems, Foster City, California, USA) on a 3130XL Genetic Analyzer (Applied Biosystems). The bacterial 16S-rDNA sequences obtained were then aligned with known 16S-rDNA sequences in Genbank using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information, and percent homology scores were generated to identify bacteria (Maniatis *et al.,* 1982).

Data analysis

Data collected were statistically analyzed by using SPSS program package. Tests of significance were done using least square difference test according to Steel and Torrie (1977). All experiments were repeated at least three times.

Results and Discussions

Prevalence of bacteria in polluted soils

Sulfur desulfurizing bacteria were estimated in contaminated soil with oil or oil products (Table 1). Sulfur desulfurizing bacteria enumerated on 2 different media shown in table 1. Moisture contents were ranged from 77.3- 85.1% in all collected samples. The total sulfur bacteria were ranged from$1.6x10^4$-$2.8x10^6$ CFU $gsoil^{-1}$ on PCA media and $4.1x10^2$ - $2.1x10^6$CFU g $soil^{-1}$on basal media supplemented with DBT. The highest numbers were obtained from Kerosene

($2.1x10^6$ CFU g $soil^{-1}$) and 2HBP ($2.8x10^6$ CFU g $soil^{-1}$) treated samples on PCA media and $2.1x10^6$CFU g $soil^{-1}$from DBT supplemented media. The lowest numbers were obtained from motor oil ($0.9X10^4$ CFU g $soil^{-1}$) and crude oil ($1.6x10^4$) on PCA media and kerosene ($4.1x10^2$ CFU g $soil^{-1}$) on DBT supplemented media. Furthermore, numbers of growing desulfurizing bacteria was higher on PCA media than basic media plus DBT. This could be explained by nature of oil or substrate added to contaminated soil. In general, the presence and numbers of sulfur desulfurizing bacteria were various among soil samples (Table 1). No growth was observed on both media for other soil samples contaminated with other oil products heavy crude oils, 3.96 % sulfur (bitumen), and gasoline (Data not shown).

All bacteria showed to be capable of biodesulfurization of oil or oil products, as they were able to grow in PCA media and minimal mineral medium supplemented with DBTor 2HBPas the sole sulfur and carbon source. Therefore, all wiled local bacterial flora grow on both media showed broad specificity for sulfur removal from oil and oil refiners. These growing sulfur desulfurizing bacteria showed broad specificity for sulfur removal whether crude oil, oil products or substrates i.e. DBT or HBP as sole sources of sulfur.

Dibenzothiophene DBT (in hexadecane) was used as model oil to carry out a stable continuous desulfurization (Castorena *et al.*, 2002; Youssef and El-Abyad 2015; Amin, 2011). Almost all of the bacteria reported could degrade DBT to 2-HBP or its derivatives through a sulfur-specific pathway (Castorena *et al.*, 2002; Amin, 2011; Bhatia and Sharma, 2012). These bacteria can be used to lower sulfur levels in oil products. Therefore, isolates showed broad specificity for sulfur removal.

Enrichment and isolation of desulfurizing bacteria

All twelve isolates under study (labeled from an 'TU- S' series as TU-S1, −S2, −S3, −S4, −S5, −S6, −S7, −S8, -S9, -S10, -S11, and -S12) (Table 2) showed to be capable of biodesulfurization of oils, as they were able to grow in minimal mineral medium (BSM) supplemented with DBT as the sole sulfur and carbon source. Isolates from various polluted soils were isolated by enrichment culture technique and deposited in our microbial bank at Taif University, Saudi Arabia in our laboratory. The isolates were identified on the basis of their cultural, physiological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (9^{th} edition) (Holt *et al.,* 1994) and Api kit profiles (ApiBioMerieuxsa, 2009). Phenotypic examination of the recovered isolates revealed that they belong to the five different genera of *Bacillus, Pseudomonas, Rodococcus, Mycobacterium,* and *Klebsiella* (Table 2). Furthermore, more than five isolates were isolated from free contaminated soil with crude oil or oil products. All selected strains showed optimal growth at 35^oC but grows in two different media. Strains were local wild isolates isolated by enrichment culture technique from oil refinery at Jeddah, and some gas stations at Taif, KSA. Many investigators have been isolated and studied sulfur biodesulfurizing bacteria around the world from oil and oil products of contaminated soil (Anderson and Lovley 2000; López-Cortés *et al.,* 2006; Melnyk *et al.,* 2011; Pfeffer *et al.,* 2012; Srujana Kathi and Khan, 2013).

Identification, morphological, and biochemical characterization of isolate

Isolates from various polluted soils were isolated by enrichment culture technique. Further support to the assignment of these isolates was given by positive results for the

Gram test, as well as by cells morphology under light microscopy. The twelve isolates were identified on the basis of their cultural, physiological, biochemical characteristics (API profiles) and 16S-rRNA gene sequencing (Table 3). Table (3) showed five isolated biodesulfurizing genera.

These isolates were identified on the basis of their cultural and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (9^{th} edition) (Holt et al., 1994) and Api kit profiles (ApiBioMerieuxsa, 2009). The examination of the recovered isolates revealed that they belong to five different genera: *Bacillus, Pseudomonas, Rodococcus, Mycobacterium,* and *Klebsiella*.

The data of 16S-rDNA sequence analysis showed that 16S-rDNA sequence of isolates S1-S12 were 98% identical to that of *Bacillus pumlius*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Bacillus subtilis*, *Bacillus pumlius*, *Rodococcus erythropolis*, *Rodococcus ruber*, *Mycobacterium pheli*, *Mycobacterium pheli*, *Klebsiella oxytoca*, *Mycobacterium goodie*, *Bacillus subtilis*, respectively.

All selected strains showed optimal growth at 35^{o}C but grows in different media amended with DTB, oil or oil products. Isolates showed to be capable of biodesulfurization of oils or oil products, as they were able to grow in minimal mineral medium supplemented with DBT as the sole sulfur and carbon source.

The natural environment, such as polluted soil or oil field, usually provides the best niches to source microorganisms with potential for BDS activities. As these microorganisms are cultivated and isolated in the laboratory for the purpose of BDS, they display different potentials arising from their different genetic make-ups and conditions that they were previously acclimatized to. For BDS reactions, whole cells or cell extracts can be used. In the case of whole cells, these can be resting cells as well as growing ones (Nuhu 2013). Previously, only Gram bacteria were harnessed for these desulfurization activities (Gunametal, 2006). Elsewhere, the resting cells of *Rhodococcus erythropolis* SHT87 isolated from oil- contaminated soil in Tehran was found to contain three sulfur-metabolizing genes, namely dszA, dszB and dszC (Davoodi-Dehaghani *et al.*, 2010).

A newly identified *Microbacterium* sp. NISOC-06 was employed to achieve close to 95% desulfurization of 1 mmol/L DBT during a 2-week incubation period (Papizadeh etal.2010). Apart from BTH, *Mycobacterium phlei* WU-0103 can also utilize another heterocyclic sulfur-containing compound, naphtha [2, 1-b] TH, and 52% reduction in sulfur content of a 12-fold diluted crude straight-run light gas oil fraction was accomplished (Ishii *et al.*, 2005).

While equal percent reduction in TSC of Liaoning Crude oil (from 3,600 to 1,478mg/L) was achieved in a longer period of time (72h), 99%reduction in total sulfur level of DBT in tetradecane was accomplished, under controlled pH and temperature, by the thermiphilic bacterium, *Mycobacterium goodie* X7B (Li *et al.*, 2007). Other, biodesulfurizing bacteria were isolated and identified *P. stutzeri* (Dinamarca *et al.*, 2010), *P. putida* (Alcon *et al.*, 2005), *R. erythropolis* (Ansari *et al.*, 2009), *Mycobacterium* sp. (Chen, 2008), *Bacillus subtilis* (Kirimura *et al.*, 2001; Ohshiro *et al.*, 2005; Al-Bahry *et al.*, 2016) and *Klebsiella* sp. 13T (Bhatia and Sharma, 2012).

Table.1 Enumeration of desulfurizing bacteria in different contaminated soil samples polluted with oil and oil products

Sample[+]	Media		
	Moisture %	PCA[*]	BSM+DBT[**]
Crude oil	79.3	$1.6x10^4$	$1.6x10^4$
Diesel oil	85.1	$2.31x10^5$	$3.9x10^3$
Kerosene oil	81.2	$2.1x10^6$	$4.1x10^2$
Benzene oil	80.1	$1.1X10^5$	$1.1X10^6$
Motor oil	77.3	$0.9X10^4$	$1.5X10^5$
DBT treated	82.5	$1.7x10^5$	$2.1x10^6$
2HBP treated	83.2	$2.8x10^6$	$1.4x10^4$

+, Each sample is an average of 3 mixed samples. *, Stilinovi andHrenovic(2009); **, Kilbane *et al.*, (1990)

Table.2 Morphology, physiology, and growth of five selected biodesulfurizing bacteria

Identification Code	TU-S2	TU-S5	TU-S7	TU-S10	TU-S12
Proposed Name	*Pseudomonas putida*	*Bacillus pumils*	*Rodococcus erythropolis*	*Klebsiella oxytoca*	*Bacillus subtilis*
Colony color	Yellowish	Creamy	white	Clear	Creamy
Morphology	Short rods	*Bacilli*	Filaments	Short rods	*Bacilli*
Gram Stain	-	+	+	-	+
Motility	+	+	-	+	+
Oxidase reaction	+	+	+	-	+
Catalase reaction	+	+	+	+	+

Table.3 Identification of selected local wild isolated bacteria and designated codes

Number	Identification	Lab code
1	*Bacillus pumilus*	TU-S1
2	*Pseudomonas putida*	TU-S2
3	*Pseudomonas stutzeri*	TU-S3
4	*Bacillus subtilis*	TU-S4
5	*Bacillus pumilus*	TU-S5
6	*Rodococcus erythropolis*	TU-S6
7	*Rodococcus ruber*	TU-S7
8	*Mycobacterium pheli*	TU-S8
9	*Mycobacterium pheli*	TU-S9
10	*Klebsiella oxytoca*	TU-S10
11	*Mycobacterium goodii*	TU-S11
12	*Bacillus subtilis*	TU-S12

Table.4 Specific growth rate and optical density of selected isolated strains on oil and oil refineries (benzene, kerosene and diesel)

Isolates	Growth rate (h^{-1})				O.D.			
	Oil	Diesel	Kerosene	Benzene	Oil	Diesel	Kerosene	Benzene
TU-S2	0.0342	0.0273	0.0142	0.0042	49	37	39	46
TU –S5	0.0437	0.1365	0.0232	0.0342	63	56	41	55
TU –S6	0.0351	0.2430	0.0311	0.0352	70	46	46	37
TU –S9	0.0241	0.0362	0.0351	0.0441	57	43	56	46
TU -S 10	0.0231	0.1621	0.0342	0.0243	46	48	53	39
TU –S12	0.0343	0.0461	0.0243	0.0342	67	55	47	43

Table.5 Performance of selected local isolates of desulfurization of oil and oil products[a]

Isolate	Sulfur removed%[b]				
	Crude oil	Diesel	Kerosene	Benzene	Motor oil
TU-S2	31.0	21.1	33.2	21.2	21.6
TU-S5	25.0	19.3	31.0	25.0	29.0
TU-S6	26.1	20.1	29.0	27.0	29.0
TU-S9	23.2	15.4	32.0	26.1	**30.3**
TU-S10	24.3	21.0	19.0	19.3	18.4
TU-S12	19.3	20.0	22.0	17.2	21.2

[a] = All experiments were carried out according to the details in Materials and Methods section, b = Each value represents the average value obtained from triplicate flasks.

Table.6 Comparative performance of selected local isolates and commercial strains of desulfurization of oil and oil products [a]

Strain*	**Sulfur removed%[b]**					
	Crude oil	**Diesel**	**Kerosene**	**Benzene**	**Motor oil**	**DBT**
Local						
***P. putida*TU-S2**	**31.0**	**21.1**	**33.2**	**21.2**	**21.6**	**37.6**
***B. pumilus*TU-S5**	**25.0**	**19.3**	**31.0**	**25.0**	**29.0**	**33.2**
***R. erythropolis*TU-S7**	**26.1**	**20.1**	**29.0**	**27.0**	**29.0**	**34.5**
Commercial						
R. erythropolis	**23.2**	**15.4**	**32.0**	**26.1**	**30.3**	**29.4**
Desulfobacteriumanaline	**24.3**	**21.0**	**19.0**	**19.3**	**18.4**	**30.1**
Thiobacillusthiooxidances	**19.3**	**20.0**	**22.0**	**17.2**	**21.2**	**23.2**

Table 3 shows morphological, physiological and biochemical characteristics of selected isolates. Strains were local isolates isolated by enrichment technique. Colony morphology on nutrient agar plate, S12showed creamy, big spreading, finely wrinkled and slimy. In S2showed yellowish, small, opaque irregular colonies with earthy odors, S7 was medium white colony with gray center, and S10 was small clear colony (Table 3). In blood agar plates showed the hemolysis. Phenotypic examination of the recovered microorganisms revealed that they belong to the genera of *Bacillus, Rodococcus, Klebsiella* and *Pseudomonas* (Table 3). Five isolates *Pseudomonas putida* S2, *Bacillus subtilis* S12, *Bacillus pumils* S5, *Rodococcus ruber* S7, *Klebsiella oxytoca* S10 showed good growth on Bushnell- Haas medium amended with crude oil as a sole carbon source and were selected based on the growth and degradation ability. All selected strains showed optimal growth at 35^{o}C.

Growth kinetics

All selected isolates grow on petroleum oil and oil products (Table 4). Isolate S5, S6 and S9 showed best growth on crude oil, diesel, kerosene and benzene, respectively, except S9 showed best growth on both kerosene and benzene only (Table 4). Isolate S10 and S12 showed the lowest growth rate on examined oil and oil products. Also, Isolate S6 showed highest optical density on crude oil, and isolate S5 showed good optical density on both diesel and benzene. Isolate S5 and S9 showed best optical density (56) on diesel and kerosene, respectively.

In general, microorganisms produce biosurfactants to increase their interfacial area for contact to give improved uptake of hydrophobic substrates. However, it has been observed that the exopolymers synthesized by these strains in media with glucose as carbon and energy source, had a remarkable capacity of emulsifying hydrocarbon compounds (Martinez-Checa *et al.,* 2002).

Biodesulfurization of oil and oil products by bacteria: The results obtained with crude oil and oil products removal by 6 selected isolated strains (Table 5) indicated that the concentration of sulfur decreases after 4 days of incubation in all treatment by different isolates. Isolate S2 (*P. putida*) removed the highest amount of kerosene (33.2%) followed by crude oil (31%). Also, strain S9 (*M. phlei*) removed (32%) of kerosene (Table 4). Isolate S10 (*Klebsiella oxytoca*) and S12 (*B. subtilis)* removed the lowest amount of oil or oil products tested (Table 4). Furthermore, isolate S9 (*M. pheli*) and S12 (*B. subtilis*) removed the lowest amount of diesel oil (15.4%) and benzene (17.2%), respectively (Table 4). Also, the results obtained with DBT removal (0.3 mM DBT) by S2, S6 and S9 indicated that the concentration decreases after 4 days of incubation (data not shown). Our experiment showed a removal of 100% of sulfur after 8 days of incubation with 0.3 mM DBT concentrations.

Local isolated bacteria had the potential to desulfurize crude oil or oil refiners but with different rates using it as sole sulfur source. Microorganisms, particularly *Rhodococcus* (Izumi *et al.*, 1994), *Bacillus* (Kirimura *et al.*, 2000; Buzanello *et al.*, 2014), *Pseudomonas* (Al-Zahrani and Idris, 2010, Jamshid *et al.*, 2010), *Mycobacterium* (Ishii *et al.*, 2005), *Klebsiella* (Bhatia, Sharma, 2012)species have been found to metabolize crude oil and oil products as well as DBT as a source of sulfur by cleaving the C–S bond of sulfur compound in crude oil products or DBT via a sulfur-specific pathway (4 S pathway) without affecting the carbon skeleton. Tong *et al.*, (2005) reported that *Rodococcus* spp desulfurizing organic sulfur of diesel oil by resting cells. *Rhodococcus* sp. FS-1, which

can specially break the C-S bond of dibenzothiophene (DBT) and convert DBT into 2-hydrobenzophene by "4S" pathway, is used to decrease the sulfur content in diesel oil and it was strongly high. Also, other results indicated that *M. phlei* WU-0103 may have a good potential as a biocatalyst for practical biodesulfurization of diesel oil. Other microorganisms *i.e. Stachybotrys bisbyi* TUSb1 formed a compound free of sulfur (biphenyl). The desulfurization and formation of biphenyl was determined by the continuity of culture from 4 to 10 days at 35°C in the concentration of 0.3 mM DBT. HPLC results suggest that the final metabolite of dibenzothiophene by *Stachybotrys bisbyi* TUSb1 is the biphenyl. The final product biphenyl suggests that the metabolic pathway used by *Stachybotrys bisbyi* TUSb1 in the biodesulfurization process with 0.3 mM of DBT, indicated the specific via of the 4 S (Gherbawy *et al.,* 2016). The desulfurization of DBT and formation of 2HBP have been detected by many bacteria (Omori *et al.*, 1992).Bacteria tested metabolized a broad range of organic sulfur compounds in crude oils and oil products, suggesting its potential application to the desulfurization of petroleum oil and oil products.

Comparative experiments were carried out to provide information relevant to the biodesulfurizing trait of 3 reference strains and 3 local isolates (Table 6). In general, the rates of removing sulfur from oil or oil refiners by selected local isolates were higher than references strains (ATCC) cultures being the highest was S2 (*P. putida*) 33.2 % on kerosene and 37.6 % on DBT after 24 hr only of incubation at 35^{o}C (Table 5).

However, the relative biodesulfurization rates for commercial strains *R. erythropolis*, *D. analine* and *T. thiooxidances* showed the highest amount of removing sulfur of kerosene 32% and 30.3% of motor oil, as well as 30.1% and 23.2% of DBT when used as sole sulfur sources, respectively (Table 5). The experiment showed a removal of 100% after 10 days of incubation with crude oil or oil products and pH of the culture medium was measured after 7 days (data not shown).

All biodesulfurizing bacteria whether local isolates or standard strains in our experiments were able to desulfurize all crude oil or oil refiners but with different rates using it as sole sulfur source. Strains from the bacterial genus *Rhodococcus* were most often reported, such as *R. erythropolis*. In literature, a number of other microorganisms, particularly *Rhodococcus* (Izumi *et al.,* 1994), *Bacillus* (Kirimura *et al.,* 2000), Buzanello *et al.,* 2014, *Pseudomonas* (Al-Zahrani and Idris, 2010, Jamshid *et al.,* 2010), *Mycobacterium* (Ishii *et al.,* 2005), *Klebsiella* (Bhatia, Sharma. 2012), *Arthrobacter* (Seo *et al.,* 2006) and *Gordonia* (Li *et al.,* 2006) species have been found to metabolize crude oil and oil products as well as DBT as a source of sulfur by cleaving the C–S bond of sulfur compound in crude oil products or DBT via a sulfur-specific pathway (4S pathway) without affecting the carbon skeleton. These results suggested that hydrodesulfurization (HDS) of crude or oil products through microbial activities has been shown to be a potential alternative to HDS, since HDS cannot remove the heterocyclic organo-sulfur compounds such as dibenzothiophene (DBT) (Sumedha and Sharma, 2010) which represent about 70% of the sulfur in crude oils. Thermophilic microorganisms are more appropriate to be used for BDS applications following HDS (Bhatia and Sharma, 2012). Further, using local isolates is more reliable and better than imported strains which are more adapted to our environment.

In addition, using a consortium of 3 local isolates, *P. putida* TU-S2, *B. pumlius* TU-S5, and *R. erythropolis* TU-S7 Removed 90% of sulfur of crude oil, and refined petroleum products: kerosene, benzene, diesel as well as

the model compound DBT after 3 days only (data not shown).

The overall low levels of remaining DBT after only 48 h in culture with the ~100 times higher starting level of DBT in the media (0.5 mM), it seems clear that a fast-consumption metabolism is occurring. In this circumstance, it would be reasonable to expect that stoichiometric high levels of HBP would be found in culture from the use of DBT at the beginning of the 4S pathway. However, the relatively low overall levels (<0.07 mM) of HBP detected and the lack of a pattern for its accumulation in the tested media (data not shown) suggest that most of the HBP produced may have been somehow consumed or degraded after its synthesis. Furthermore, during its growth, high levels of DBT were removed in the first 24 hours, and a rapid DBT degradation within the first hour of incubation was observed. Detection of 2-hydroxybiphenyl (HBP), a marker for the 4S pathway, suggests strains have metabolical capability for DBT desulfurization. The presence of $MgSO_4$ in growth medium as an additional sulfur source has interfered with DBT degradation. Although the HBP detection data would also indicate that genes related to this metabolism (such as *dsz*C and *dsz*B) should be operational, homologous sequences for these genes were lacking, which suggest the existence of a specific mechanism and genes not yet described for DBT consumption/HBP formation in the *B. pumilus* and other isolates (Buzanello *et al.*, 2014). To account for the lack of stoichiometric relationship between DBT and HBP, the existence of another DBT-consumption metabolism that includes the destruction of the molecule ring cannot be discarded. Further research involving biochemical/molecular tests and full-genome sequencing is currently underway to address this question in more details.

It is concluded to the best of our knowledge, this is might be the first report of the identification of five different genera as a sulfur removing bacteria in Saudi Arabia. Most organisms cannot efficiently desulfurize crude oil, oil products and both BTs and DBTs which are present in hydrodesulfurized petroleum oil. In our study, all isolates were employed as catalyst for the biodesulfurization of hydrodesulfurized oil products. Bacteria isolated and commercial strains could break the C–S bond of DBT and converting it into 2-hydrobenzophene (2-HBP) by the ‘‘4S’’ pathway. The desulfurization results indicated that the mixed bacteria could efficiently desulfurize most of the heterocyclic sulfur compounds in the hydrodesulfurized oil refiners (data not shown). Therefore, our strains showed broad specificity for sulfur removal and a further investigation is highly recommended. The results revealed the possibility to use these bacteria as for the reduction of sulfur content in petroleum oil where they cause pollution problems. Furthermore, results indicated that using a microbial consortium might have a promising application in petroleum oil technology and could be potentially used in microbial enhanced oil recovery (MEOR).

Recommendations

In order for biodesulfurization to realize commercial success, a variety of process considerations must be addressed including reaction rate, emulsion formation and breakage, biocatalyst recovery, and both gas and liquid mass transport. Biodesulfurization offers an attractive alternative to conventional hydrodesulfurization due to the mild operating conditions and reaction specificity afforded by the biocatalyst. Worldwide, it has been realized that pollution prevention is more economical and socially responsible than clean up, and inevitably the demand for waste site remediation technologies will

abate. Before such perceived advances can be realized, however, a fundamental knowledge database of sulfur bacteria performance under complex environmental parameters must be attained. This can only be achieved through careful studies and a comparative life cycle assessment that balances risk with biotechnological benefit.

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