

METHOD

Field of invention

- 5 The present invention relates to a mixed microbial culture. The invention also relates to a method for reducing the sulfur content in fossil fuels by using the microbial culture. More particularly, but not exclusively, the invention relates to a method for reducing the sulfur content in bunker oils by using the microbial culture.

10 Background of invention

- Bunker oil is widely used in ships as an important fuel source. A study of total sulfur in 78 crude oils by Ho *et al* (1974) revealed a range of sulfur content in the crude oils between 0.03 to 7.89 wt%. As a result, the combustion of bunker oil has led to a
- 15 significant release of gaseous pollutants (for example, SO_x) and thereby causing air pollution. As such, there are now strict maritime laws that impose a limit of sulfur content in bunker oil. The EU Commission announced on November 22, 2002 that it would impose a limit of sulfur content to 1.5% for all seagoing vessels in the North Sea, English Channel and Baltic region. The Commission also proposed to go for tougher
- 20 international emissions standards through the International Maritime Organization (IMO). This indicates that international maritime business/governmental organizations are very serious about solving the high sulfur content problem in bunker oil. Singapore has a thriving maritime business, with up to about 20 million tons of bunker oil required annually. The maritime activities in Singapore may be affected when more stringent
- 25 regulations on bunker oil's sulfur content are enforced. In Singapore, the present regulation limits sulfur content in bunker oils to 4.5% on a weight basis. The new regulation with a stricter limit of 1.5% sulfur content has been enforced in parts of the world and might be adopted by the rest of the world (including Singapore) in the near future. In order to comply with these environmental standards, it is essential and timely
- 30 to develop effective and economic technologies for desulfurization of bunker oils.

Desulfurization of fossil fuels has always been a focus in the field of fuel quality improvement. Hydrodesulfurization (HDS) is the most widely applied and mature technology in this area. The mechanism of HDS is to reduce the sulfur in the fuel by

hydrogen under high temperature and high pressure, with hydrogen sulfide as the product. However, these conditions have become a disadvantage to HDS, when the regulations on the sulfur content in fossil fuels is to be further tightened, as some of the sulfur existing in the form of organic heterocycles is recalcitrant to HDS. The application
5 of lower sulfur limits might increase the cost of HDS, by requiring higher temperature and pressure and longer reaction time in the process.

As the carbon-sulfur heterocycle compounds make up a high proportion of sulfur in heavy oil, and are recalcitrant to conventional HDS, the biodegradation of these
10 substances are being explored. Biodesulfurization (BDS) has been applied to reduce the amount of sulfur in gasoline and diesel. However, it has not been applied to heavier oils such as bunker oil. A reason of this may be that the environmental standards on the sulfur content in heavy oils, such as bunker oils, were previously less stringent. Another reason is that the physical properties and more complicated sulfur diversities present in
15 heavy bunker oil, for example high viscosity and density, make it difficult to thoroughly mix the oil with the microbial culture used during the biodegradation process. Insufficient mixing can cause a slow mass transfer and low reaction rate, hence a long retention time and big reactor volume. Also, the separation of bacteria from the desulfurized oil phase after the biodegradation can be a problem.

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Therefore, there remains a need for an improved method for reducing the sulfur content in fossil fuels. More particularly, there remains a need for a biodesulfurization method for reducing the sulfur content in bunker oils.

25 **Summary of Invention**

In accordance with a first aspect of the invention, there is provided a method of obtaining a microbial culture for reducing the sulfur content in a fossil fuel, the method comprising the steps of

- 30
- a) obtaining a seed oil sludge from a marine or industrial environment;
 - b) culturing the seed oil sludge under conditions suitable for enrichment of sulfur-biodegrading bacteria; and
 - c) assessing the culture for the ability to reduce the sulfur content in bunker oil.

Advantageously, the microbial culture is highly capable of biodesulfurizing liquid fossil fuels. More particularly, the microbial culture can remove sulfur from sulfuric substances without the destruction of the carbon ring structure.

- 5 Also advantageously, the microbial culture is isolated from natural sources that are economical and easy to cultivate.

In accordance with a second aspect of the invention, there is provided a microbial culture for reducing the sulfur content in a fossil fuel, obtained by the method according
10 to the first aspect of the invention.

In accordance with a third aspect of the invention, there is provided a method for reducing the sulfur content in a fossil fuel, the method comprising: incubating the fuel with a microbial culture according to the second aspect of the invention in a medium
15 under such conditions to reduce the portion of sulfur in the fuel. Preferably, the microbial culture is enriched from an industrial oil sludge.

Preferably, the incubation period is between 25°C to 30°C or higher. More preferably, the incubation period is at about 28°C or higher for about 1 to 8 days.

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Preferably, the fuel and microbial culture volume ratios are between 1:1 to 1:10.

Preferably, the medium is a basal salt medium.

25 Preferably, at least about 40% of the sulfur is removed.

Preferably, the fossil fuel is a liquid or solid at room temperature (20~30°C) and the culture medium is liquid. More preferably, the fossil fuel is bunker oil.

30 Sulfur is removed from fossil fuels containing sulfur by incubation of the fuel with microorganisms that are enriched from an oil sludge. Advantageously, the enriched microbial culture selectively reduces the sulfur without apparently utilizing the fuel as a carbon or energy source.

Biological desulfurization of carbon-sulfur heterocycles, the representatives of which are thiophenic compounds, has two mechanisms: carbon specific and sulfur specific pathways. In the carbon specific pathway, the carbon ring structures of the thiophenic compounds are broken by biological factors, e.g. bacteria, and the compounds are gradually degraded, until the sulfur element is finally released. As this degradation mechanism implies the total destruction of the carbon structure, and hence the loss of the heat value, its application in industry is not desired.

In contrast, the sulfur specific attack on the heterocycles only aims at the sulfur element, and desulfurizes the compounds by turning the covalently bond sulfur to sulfite or sulfate ions, which is water soluble and separable with the oil phase. As there are four sulfur-containing substances along this pathway, it is also called the "4S" pathway. Thiophenic compounds can retain their structural integrity when degraded through the 4S pathway, therefore this mechanism will be the focus of this patent.

Advantageously, the method of the present invention may be carried out under normal pressure and ambient temperature, and the control and operation of the method is less demanding than hydrodesulfurization. In addition, the present method may be used to desulfurize the thiophenic compounds which are recalcitrant to the method of hydrodesulfurization.

By "biodesulfurization", it means to use any biological factors, for example bacteria mixed cultures, pure cultures, enzyme extracts, and the like, to break down the sulfur containing compounds in oils or fossil fuels, and allowing the sulfur to be separable from the oil or fuel.

By "fossil fuel", it means any hydrocarbon product derived from petroleum, coal, shale, oil, including crude oil, lignite, synthetic fuels derived therefrom, and mixtures thereof.

By "enrichment", it means any process that uses selective culture media and incubation conditions to isolate microorganisms from the natural environment. The enriched mixed microbial culture of the present invention is obtained from such a process.

Examples

The following examples are presented to further illustrate and explain the present invention and should not be taken as limiting in any regard.

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All references herein mentioned are hereby incorporated by reference.

Example 1

- 10 This example shows the biodesulfurization of three model thiophenic compounds by a microbial culture according to the present invention. The microbial culture was enriched from the oil sludge.

The three compounds consisted of thiophene (TH), benzothiophene (BTH), and DBT.
15 These three sulfur species are all carbon-sulfur heterocycles with increasing molecular weights. The reason for such a choice is that the alkylated derivatives of BTH / DBT are abundant in heavy oils, and the metabolism pathways for BTH / DBT and BTH / DBT derivatives were quite similar (MacPherson et al. 1998). Dibenzothiophene (DBT) represents a broad range of sulfur heterocycles found in petroleum. In diesel fuels, up to
20 70% of the sulfur content is made up of DBT and substituted DBT. The ratio is estimated to be even higher in heavy oils, such as bunker oils (Yap et al., 2003). An aerobic process was chosen for the Example considering the efficiencies of biodesulfurization, the easiness of parameter control and the original environment of the collected oil sludge, from which bacterial seed was detached and enriched. The efficiencies of desulfurization
25 (dsz) bacteria in DBT metabolism were evaluated and the roles of non-desulfurization (non-dsz) bacteria in the microbial community were evaluated and discussed. Biodegradation intermediates were monitored using GC-MS and sulfur distribution in different phases (inorganic/organic compounds in water and biomass) was analyzed in detail.

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Chemicals and culture medium

Thiophene (TH), benzothiophene (BTH), and dibenzothiophene (DBT) were purchased from Sigma Aldrich (USA). All chemicals used in the experiment were of analytical grade, commercially available and used without further purification. The culture medium was a sulfur-free basal salt medium (BSM) consisting of (in g L⁻¹) KH₂PO₄ 2.44, Na₂HPO₄ 5.57, NH₄Cl 2.00, MgCl₂•6H₂O 0.36, FeCl₃•6H₂O 0.001, MnCl₂•4H₂O 0.004 and glycerol 1.84. The final medium pH was 7.0. Organosulfur solution (OS) consisting of TH 1.68 g, BTH 2.68 g, and DBT 3.68 g per liter of pure ethanol was provided as sole sulfur source. Ethanol was used to dissolve the sulfur species as they are non water-soluble, but it would not be used in the next example with actual bunker oils. In the experiment, the volume ratio of added OS and operation capacity was 1:40. The final concentrations of TH, BTH and DBT were all initially 0.5 mM in the reactor. The medium for bacterial plate counting had the same composition as the culture medium described above. 15 g of noble agar were added per liter for solidification.

15 Seed source and screening approach

The mixed microbial culture was detached from the oil sludge with OS solution as the sole sulfur source, and was incubated under aerobic condition for 3 to 4 weeks. The result was a mixed microbial culture that is more abundant in the biodesulfurizing population, compared to the original seed sludge.

The seed for biodesulfurization was enriched from the oil sludge collected from the island Pulau Sebarok (Singapore). Such an environment was selected as the source of seed sludge, as it might increase the abundance of desulfurizing bacteria in the seed. During bacterial culturing, besides the basic nutrient media only three model sulfur species were fed to the biosystem as food sources, targeting the further enrichment of BDS bacteria in the mixed culture. For detachment, the oil sludge was mixed with BSM at a volume ratio of 1:5 and reciprocally shaken for 4 days (150 rpm, room temperature). Afterwards, 50 mL of the yellowish aqueous phase was transferred to a 1-L flask containing 250 mL of BSM supplemented with OS (2.5% v/v) and the mixture was reciprocally shaken for 7 days (150 rpm, room temperature). This enrichment process was repeated three times before the microbial culture was applied in the reactor.

Reactors set-up

The biodesulfurization process was carried out in a batch-mode reactor for 8 days. 300 mL of the seed sludge was transferred into a reactor, replenished with 100 mL of 5 times concentrated BSM and 12.5 mL of OS. The mixture was then topped up to 500 mL with MilliQ water. Another identical reactor was prepared as the control with the same composition except that the 300 mL of seed sludge was replaced by 300 mL of MilliQ water. A small pinch of HgCl_2 was used to ensure the control reactor bacteria-free. Headspace aeration was chosen for oxygen supply. The inlet air was filtered through a 0.22- μm pore sized filter to eliminate possible contamination by circulation, and the air diffuser was fixed at about 5 cm above the liquid surface. Both reactors were reciprocally shaken (150 rpm, room temperature).

Analytical methods

Gas chromatography - mass selective detector (GC-MSD) analysis

Model thiophenic compounds and their metabolites in the reactors were monitored by GC (Agilent 6890N, USA) equipped with MSD (Agilent 5973N) and J&W DB-5ms column after being recovered by liquid/liquid extraction in n-hexane from the culture medium. The extraction procedure was: 2 mL of the sample was mixed with 1 mL of n-hexane and the mixture was shaken manually, kept still for 5 min and the upper layer was removed to a scaled glass bottle. The above operation was repeated totally four times. The extract was pooled and supplemented with n-hexane to a final volume of 4 mL before GC-MS analysis. The GC oven temperature program was: initial 40 °C and held for 3 min, 45 °C/min to 150 °C and held for 1 min, 25 °C/min to 300 °C and held for 2 min. The injector temperature was 300 °C in splitless mode; and the interface temperature was 300 °C.

Ion chromatography (IC) analysis

IC analysis with the Shimadzu system (Japan), which was composed of SIL-10Ai auto injector, LC-10Ai liquid chromatography, DGU-12A degasser, CTO-10A column oven, CDD-10 conductivity detector, and SCL-10A system controller, was chosen to monitor

the soluble inorganic ions in samples. The operation conditions were: isocratic mode with a flowrate of 1 mL min⁻¹; oven temperature 40°C; sampling frequency 2Hz; and runtime 15 minutes. The mobile phase was composed of (g L⁻¹) Bis-tris, 0.76 and 4-hydroxybenzoic acid, 1.105, and was filtered (0.2µm, polyamide membrane) before use.

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Plate counting

The bacteria concentration in the seed reactor was monitored by colony counting on specific agar plates (with OS as the sole sulfur source). The original sample was serially
10 diluted with phosphate buffer saline (PBS) solution. 50 µL of the diluted samples at 10⁻⁴, 10⁻⁵ and 10⁻⁶ levels were chosen for inoculation. After two days' incubation at 37°C, colonies on the agar surface were counted by visual inspection. Plates with colony numbers between 30 and 300 were used to calculate the colony forming units (CFU) of bacteria in the inoculation. All plates were double checked at the end of experiment.

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Biomass concentration and sulfur content

Biomass concentration was monitored according to APHA 1998 standard method (APHA 1998). For each sample, 5 mL was taken and filtered through a glass-fiber filter paper (preheated under 105°C for 24 hrs). The biomass, i.e. suspended solids (SS), were
20 collected by scratching deposits on glass-fiber filter papers and later sent to CHNS elemental analysis (PerkinElmer 2400, US) for its organic carbon, hydrogen, nitrogen and sulfur content.

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Results

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Desulfurization bacteria counting and biomass generation

Colonies appearing on the agar plate mainly consisted of three morphologies based on visual observation: (1) round, shiny, light orange, opaque and relatively large; (2) round, shiny, white, opaque and tiny; and (3) creamy, shiny, undulate and medium-sized. The counting result was based on total colony numbers without further classification (Fig. 2).

- 5 From day 0 to day 1, a slight decrease in bacteria CFU was observed (from 2.7×10^8 to $8.2 \times 10^7 \text{ mL}^{-1}$). From day 1 to day 4, a steady increase in bacteria CFU followed and the value reached $2.2 \times 10^9 \text{ mL}^{-1}$ on day 4. Furthermore, Figure 3 depicts the time profile of biomass concentration in the seed reactor. The SS concentration increased from 3.5 g L^{-1} to 4.4 g L^{-1} during the 8 days of operation.

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Time profiles of thiophene, benzothiophene and DBT

- Time courses of DBT concentrations in the seed and control reactors were depicted and compared in Figure 4. For the seed reactor, the DBT concentration was on a continuous decline since day 0 and was reduced to below detection limit from day 6 onwards. For
15 the control reactor, the DBT concentration stayed relatively constant and was close to the calculated value of the addition (0.25 mM). The concentrations of TH and BTH dropped below the detection limit (0.005 mM) within the first day of operation in both the sample and control reactors (data not shown here). Therefore the seed sludge used was
20 able to efficiently desulfurize DBT, and the reaction was due to biological function instead of physical or chemical one. In contrast, the concentrations of thiophene and benzothiophene decreased below 0.005 M after one day of reactor operation in both the sample and control reactors, due to most likely the effect of air stripping.

25 *Metabolites in the desulfurization pathway of DBT*

- The GC chromatograms of the n-hexane extract from the seed and control reactors were analyzed and compared. On day 0, the total ion chromatographs (TIC) for two reactors had similar patterns, with all three thiophenic compounds well detected and few other
30 peaks. Through quantification, the concentration levels of TH, BTH, and DBT in both reactors were close to theoretically calculated values of the TH, BTH and DBT. On day 6, the TIC for control and seed reactors became more complicated and presented totally different patterns (as shown in Figures 5 and 6). The retention time and probable

identities of major peaks in Figures 5 and 6 are listed in Tables 1 and 2 with the help of mass spectrometry (MS) analysis.

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Table 1. Identities of the main organic compounds in seed reactor on day 6 (extracted by n-hexane)

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No.	Retention Time (min)	Name	Probability (%)
1	3.107	Cyclohexane	73.8
2	3.414	Furan, tetrahydro-2,5-dimethyl-	67.4
3	3.522	Furan, tetrahydro-2,5-dimethyl-	63.2
4	4.515	Cyclotrisiloxane, hexamethyl-	96.4
5	5.614	cyclotetrasiloxane, octamethyl-	93.8
6	7.217	benzene, 1,3-bis(1,1-dimethylethyl)-	77.8
7	8.861	phenol, 2,4-bis(1,1-dimethylethyl)-	53.9
8	9.036	p-Hydroxybiphenyl	77.0
9	12.466	dibenzothiophene sulfone	96.9

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Table 2. Identities of the main organic compounds in control reactor on day 6 (extracted by n-hexane)

No.	Retention Time (min)	Name	Probability (%)
1	3.128	Cyclohexane	76.5
2	3.424	tetrahydro-2,5-dimethyl furan	66.1
3	3.532	tetrahydro-2,5-dimethyl furan	66.6
4	4.516	hexamethyl cyclotrisiloxane	96.2
5	5.610	octamethyl cyclotetrasiloxane	94.9
6	6.402	p-Trimethylsilyloxyphenyl-bis(trimethylsilyloxy)ethane	20.6
7	7.217	benzene, 1,3-bis(1,1-dimethylethyl)-	76.4
8	8.860	phenol, 2,4-bis(1,1-dimethylethyl)-	53.2
9	10.603	Dibenzothiophene	97.8

5 In the seed reactor, DBT could not be detected on day 6, while DBT-sulfone and HBP were found with relatively high abundance at 12.466 min and 9.036 min, respectively. However, in the control reactor, DBT was still at a high concentration level and no DBT-sulfone or HBP was detected.

10 The time profiles of DBT, DBT-sulfone, and HBP in the seed reactor are concluded in Figure 7. Two more curves were added in addition to the DBT degradation curve as shown in Figure 4: the DBT-sulfone and hydroxybiphenyl (HBP) curves. These two compounds are the important intermediates in the 4S pathway of DBT biodesulfurization. It could be seen that their abundances increased with the decrease DBT concentration, and obvious accumulation appeared towards the end of the experiment. Therefore the DBT desulfurization was conducted in this mixed culture through the sulfur-specific 4S pathway, which was much desirable. With the loss of DBT, DBT-sulfone and HBP were produced and accumulated. The DBT-sulfone concentration experienced a steady

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increase during the first 3 days, and declined slightly from day 3 to day 4, after which a fast accumulation occurred until day 6 and a sharp decrease followed to the end of the experiment. The concentration of HBP was relatively stable compared with DBT-sulfone. A steady accumulation of HBP occurred from day 0 to day 2, after which no obvious increase or decrease was observed in HBP concentration.

Sulfur Balance

The total amount of organic sulfur in the reactor was initially 24 mg (in sulfur) through calculation. Figure 8 depicts the time profile of total sulfur content in biomass in the seed reactor. In Figure 8, two series of data were presented: "original" series represent results originally obtained from the CHNS elemental analysis; "calibrated" series represent data calibrated under certain assumptions that would be discussed in the next section. In Figure 8, the initial total sulfur amount in biomass was 1.8 mg in the original series and 6.7 mg in the calibrated series, while the final total sulfur amount in biomass increased to 24.6 mg in the original series and 26.4 mg in the calibrated series. Thus, the difference between the final and initial sulfur amount was 22.8 mg in the original series and 19.7 mg in the calibrated series, both of which are comparable to the total sulfur amount added (24 mg).

Discussion

The viable count of bacteria and biomass concentration in the sample reactor revealed that both dsz and non-dsz bacteria experienced an increase in abundance. With the three thiophenic compounds as the sole sulfur source in agar plates, the result of viable count could represent the concentration variation of dsz bacteria, while the SS was considered as a good indicator of total biomass in the reactor. For the viable count, data of days 5, 6 and 7 were not included in Figure 2 because the dilution levels (10^{-4} , 10^{-5} , 10^{-6}) were not high enough and the colonies on agar surface were too many to be accurately counted. Still, it was estimated that the exponential growth of dsz bacteria started after a short lag phase from day 0 to day 1 and went on until day 6. The DBT concentration level dropped below the detection limit since day 6 in the seed reactor

(referring to Figure 4) and it might have repressed the bacteria's further growth with insufficient sulfur source.

Comparison of the two time profiles in Figure 4 proved that the loss of DBT in the seed reactor was mainly attributed to biological degradation rather than physical or chemical processes. Moreover, it was confirmed that the mixed bacterial seed enriched from the oil sludge was able to degrade DBT efficiently. In the control reactor, a slight ascending was observed in DBT concentration during the first 6 days. It was probably caused by the better emulsion in the reactor after longer time of mixing, since initially the added DBT floated on the liquid surface in a particulate form and was difficult to be homogenously sampled. In evaluating the time cause of TH and BTH, rapid loss of two compounds was observed in both reactors. Therefore the decrease of concentration was most likely caused by abiotic factors (e.g. instable compound structures, air stripping). No further study has been carried out on this aspect. In the GC chromatograms of day 6, the major components (other than DBT, DBT-sulfone and HBP) eluting from 3.0 min to 15.0 min were quite similar for both the control and the seed reactors (Table 1 and Table 2). Their possible sources might be the impurities introduced by extraction solvent, organic sulfur compounds, medium components or other experimental procedures. As they appeared in both the sample reactor and the abiotic control with similar abundance, they were not likely to affect the results in this study.

In the '4S' pathway of DBT metabolism, the last step of transformation catalyzed by the desulfinate was likely to be the rate-limiting step (Gray et al. 1996), and it might be the cause of the accumulation of DBT-sulfone and slow production of HBP (Figure 7) in this study. Other factors, e.g. insufficient introduction of specific enzymes, repression of certain dsz genes by the accumulation of HBP, or impact of non-dsz bacteria on the metabolites, might also be responsible for the observed profiles of DBT-sulfone and HBP. Considering the impact of non-desulfurization bacteria, the first concern was whether these bacteria utilized the metabolites for carbon source. Based on the full screening of GC-MS data for all extract samples, no organosulfur compounds other than DBT / DBT-sulfone were identified, indicating that the aromatic structure in thiophenic compounds was not degraded for carbon source. However, the produced HBP might become bioavailable for certain bacteria during the exponential growth phase and was

partly degraded as carbon source, which might account for its relatively stable concentration shown in Figure 7.

A sulfur balance was attempted in this example for a better evaluation of the system performance. Totally 24 mg of organic sulfur was added to the sample reactor as the sole sulfur source. As the biodesulfurization went on, five partitions became potential sinks to the added sulfur: (1) the non-degraded thiophenic compounds; (2) the organic metabolites (i.e., DBT sulfone, DBT sulfone etc.); (3) the soluble inorganic ions (i.e., sulfite, sulfate and thiosulfate); (4) the cellular material in bacteria; and (5) the surrounding atmosphere. At the end of operation, all three thiophenic compounds (TH, BTH, and DBT) were below detection limit in GC-MS analysis. Thus, the contribution of partition (1) as a sulfur sink could be neglected. For partition (2), the detected metabolite, DBT-sulfone, was one sink for total sulfur. However, without calibration standards, the amount of sulfur in DBT-sulfone was not quantified. Another organic metabolite in the '4S' pathway, 2-(2'-hydroxyphenyl) benzene sulfinate (HPBS) also contains sulfur element. Since HPBS is more hydrophilic than DBT-sulfone and HBP (Watkins et al., 2003), it might resist the extraction with n-hexane. Therefore, the organic compounds in the aqueous phase were monitored as well. Full screening and identification was done for the aqueous samples with GC-MS and no soluble sulfur-containing compounds were detected. To verify partition (3) mentioned above, inorganic ions in the aqueous phase were also investigated. Ion chromatography was applied to detect three targeted ions (sulfite, sulfate and thiosulfate). However, none of them was found with a significant level (above 2 mg L^{-1}) in samples. The most probable reason for such a result is that sulfite (if there were any) would be oxidized to sulfate immediately after formation in an aerobic environment, and sulfate is a bioavailable sulfur source for most bacteria. Thus, sulfate (if formed) was likely to be fast assimilated by both dsz and non-dsz bacteria in the mixed microbial community in a sulfur-limiting medium. Thus, the most likely sink for sulfur was partitions (4) and (5), the biomass and surrounding atmosphere. As the accumulation of sulfite / sulfate could strongly repress the dsz genes in dsz bacteria, the assimilation of inorganic sulfur into the biomass (especially by non-dsz bacteria) was beneficial to a continuous biodesulfurization with high efficiency.

In evaluating the sulfur content in biomass, results from CHNS elemental analysis were calibrated. The reason for calibrating original data was because some glass-fiber debris

might mix in the biomass samples when the suspended solids was collected by scratching filter papers after filtration. Although glass-fiber filter paper was not combustible, it might 'dilute' biomass and cause negative error in weight and thus the final results of C, H, N, and S content in biomass. To eliminate the potential error, the original carbon content of all samples was adjusted to 53.3%, which equals to the value of carbon content in the commonly used biomass formula $\text{CH}_{1.78}\text{N}_{0.24}\text{O}_{0.33}$, and the corresponding sulfur content was adjusted proportionally. The total sulfur content in biomass was calculated by the following formula:

$$\text{Total sulfur content (mg)} = \text{Sulfur content per unit suspended solids (\%)} \times \text{Suspended solids concentration in the sample (g L}^{-1}\text{)} \times \text{Operation volume of the sample reactor (500 mL)} \times 0.001 (\text{L mL}^{-1}) \times 1000 (\text{mg g}^{-1})$$

The result of total sulfur content in biomass (Figure 7) indicated that after biodesulfurization, most organic sulfur added in the bioreactor ended up as part of biomass and a small portion was transformed to intermediate organosulfur (eg. DBT-sulfone) in the culture medium or was lost through abiotic process. Thus, a preliminary sulfur balance was achieved.

Example 2

Effect of microbial culture on sulfur species in bunker oil

Bacterial cultures enriched in Example 1 were further screened for desulfurization of bunker oil in this example.

Bunker oils (two types, #1 and #2) having different sulfur contents were used in this example to test the desulfurization capability of the microbial culture, which were previously enriched from oil sludge and had been applied in biodesulfurization of three model sulfur species in Example 1. Detailed information on the physical properties of bunker #1 (Marine Fuel Oil cst380) and #2 (cst619) are provided in Appendix I, and the main organic constituents were identified by GC-MSD and listed in Appendix II. The bunker oil #1 has slightly higher sulfur content than bunker oil # 2.

50 mL of the seed sludge was taken from the sample reactor from Example 1 into a 50-mL centrifuge tube and centrifuged at 3300 rpm for 15 minutes. After that, the supernatant was removed and the settled solids were replenished with 30 mL of fresh BSM and 0.5 mL of glycerol, which provides an environment rich in growth nutrients except sulfur for bacteria. The mixture was reciprocally shaken for 24 hrs under room temperature (around 28 °C) before being applied to the bunker oils. In this example, every 20 mL of the adjusted seed sludge was mixed with 5 mL of the bunker fuel. The mixture was gently stirred and incubated for 72 hrs under room temperature. The CHNS elemental analyzer was applied to determine the combustible elements contents of each sample.

The oil-bacteria mixtures were prepared in triplicate. Before and after 72 h of incubation, the oil samples and biomass samples (both the untreated blank and treated) were characterized of their elemental composition CHNS elemental analyzer. The results of the combustible element contents are listed in Table 3.

Table 3. The CHNS content in the untreated and treated bunker oil under the oil:water ratio of 1:4

Sample ID	Carbon (%)	Hydrogen (%)	Sulfur (%)	Nitrogen (%)	Carbon/sulfur
bunker #1 BT1	75.19	17.49	3.64	2.92	20.66
bunker #1 BT2	78.48	19	4.21	2.13	18.64
bunker #1 BT3	83.19	21.66	4.9	1.6	16.98
bunker #2 BT1	87.23	24	5.22	1.69	16.71
bunker #2 BT2	72.16	19.44	4.14	1.36	17.43
bunker #2 BT3	95.79	25.8	5.48	1.63	17.48
bunker #1 AT1	47.72	20.17	3.09	1.19	15.44
bunker #1 AT2	50.25	16.5	2.97	0.84	16.92
bunker #1 AT3	66.39	22.01	3.95	0.83	16.81
bunker #2 AT1	46.26	21.31	3.02	0.58	15.32
bunker #2 AT2	33.89	22.41	2.65	0.18	12.79
bunker #2 AT3	24.95	16.31	1.87	-0.09	13.34

BT= before treatment; AT= after treatment

The sulfur contents of each bunker sample before and after treatment were compared in Figures 9 and 10. It is indicated that the sulfur content apparently declined after the treatment with the seed sludge. The average sulfur content decreases for bunker fuel #1 and bunker fuel #2 were respectively 21.4% and 49.3% (Figure 9). The difference in reduction extent for bunker fuel #1 and #2 might be caused by their different viscosity and chemical compositions. The detailed comparison of the sulfur content in each triplicate for bunkers #1 and #2 were presented in Figure 10.

Except for the sulfur content, the carbon/sulfur ratio was also evaluated in Figure 11 and Figure 12. The average C/S ratio in the treated bunker fuel was decreased by 12.6% in bunker #1 and decreased by 19.7% in bunker #2 (Figure 11). The detailed comparison of the C/S ratio in each triplicate for bunker #1 and #2 were presented in Figure 12. It seems carbon structures were also affected while sulfur content declined with the function of bacteria.

Biodesulfurization with microbial culture: Focus on Oil/Water Mixing Ratio

Based on the results obtained in Example 1 and the evaluation of the microbial culture effect on sulfur species in bunker oils, the biodegradations of organosulfur species present in bunker oil were further studied. This example sets out the optimal conditions for the biodesulfurization of bunker oils by varying volumetric ratios of bunker oil and culture medium. The oil-water volumetric ratios of 1:3 and 1:10 were chosen based on the results shown in literature (Labana *et al*, 2005). Two different bunker oil samples (Bunker Oil #1 and Bunker Oil #2) were characterized on the basis of organosulfur species distribution.

Biodegradations of organosulfur species present in the reactor were monitored during different time periods by suitable analytical methods including GC-MS, Elemental Analyzer and inductively coupled plasma-optical emission spectrometry (ICP-OES). The change in the organosulfur content of bunker oil was observed for 0, 48, 72 and 96 hrs. The activity of the desulfurizing bacteria was expected to vary with the time due to changes in its environment and also the extent of degradation of a different organosulfur species could be different. Bacteria also required time to adapt to changes in reactor conditions. Therefore, it was important to monitor the performance of the bioreactor for

different time periods in order to design the bioreactor based on optimal reaction conditions. The performance evaluation of the bioreactor was carried out and the mechanism of the bioprocess was studied. The following model diagram (Figure 13) illustrates the overall approach followed in this example.

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Materials and methods

The same information as provided in Example 1 will not be repeated here, including chemicals and culture medium, seed source and bacteria screening, and some analytical methods (elemental analysis for total organic sulfur in bunker, IC for soluble sulfur in aqueous medium, organic sulfur and carbon analysis of biomass) etc. Some additional information is iterated hereafter.

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Bioreactor selection and set up

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The biodegradation of bunker oil was carried out in a 50 mL centrifuge tube with an operational volume of approximately 27 mL. Separation of the three phases in the bioreactor namely, bunker oil, biomass and aqueous phase determined the choice of the bioreactor. When centrifuge tubes were used for the bioprocess, the separation of individual phases was possible. Two different bunker oil and culture medium volume ratios were experimented, namely 1:3 and 1:10. The effect of time on biodegradation of bunker oil by desulfurizers was observed. The time periods (hrs): 0, 48, 72 and 96 were used to study the change in bunker oil's organosulfur content.

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From the 1 L enrichment reactor, 20 mL of acclimated seed sludge was transferred into the 50 mL centrifuge tube and centrifuged at the speed of 12000 rpm for 8 minutes. The supernatant liquid was transferred back into the enrichment reactor. For the bunker oil and culture medium ratio of 1:3 (v/v), the centrifuge tube was topped up with 20 mL of BSM (with no sulfur source added). Approximately 7 mL of Bunker Oil #1 was also added and biomass attached to the bottom of the centrifuge tube was re-suspended. For the bunker oil and culture medium ratio of 1:10 (v/v), the centrifuge tube was topped up with 25 mL of BSM (with no SS added). Approximately 2.5 mL of Bunker Oil #1 was also added and biomass attached to the bottom of the centrifuge tube was re-suspended. All tests were conducted in duplicates. Similar procedure was followed for the preparation of

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samples for Bunker Oil #2. Since the effects of time and bunker oil and water ratio (v/v) on the bioprocess were to be studied, a total of 24 samples were prepared in identical 50 mL centrifuges. The centrifuge tubes (bioreactors) were reciprocally shaken at the speed of 150 rpm under room temperature. The picture of the bioreactors setup is shown in Figure 14.

Sampling procedure and sample pretreatment

The centrifuge tubes were taken from the incubator after specific time periods (48 hrs, 72 hrs and 96 hrs). In order to analyze the sulfur content in bunker oil, they were centrifuged for 15 minutes at the speed of 10000 rpm, and then the biomass and aqueous medium were separated. After centrifuging, the biomass was collected at the bottom of the centrifuge tube, while the bunker oil was obtained as a separate layer above the aqueous medium. Few drops of bunker oil were collected into 5 mL glass vials for further analysis. The remaining portion of bunker oil was discarded in such a way that the aqueous medium could be collected easily. The aqueous medium was transferred using pipettes into a 2 mL centrifuge tube and was centrifuged for 8 minutes at the speed of 11000 rpm. The supernatant was filtered using 0.22 μ m PTFE filter into a 5 mL glass vial for sulfur analysis. Finally, the biomass (if any) left at the bottom of the 50 mL centrifuge tube was collected in 2 mL centrifuge tubes and allowed to dry overnight in an oven at 60°C. The dried samples of biomass were then used for analysis of its sulfur content.

Analytical methods

GCMS for organosulfur species in bunker oil

The sample for GC-MS analysis was prepared by diluting the bunker oil using hexane as the solvent. Table 4 shows the weight [g] of bunker oil diluted using 5 mL of hexane. 1 μ L of the sample was taken in a syringe and injected into GC-MS for organosulfur analysis.

Table 4. Mass of bunker oil in 5 mL of hexane

Bunker Oil type/ Oil-water ratio	Weight [g]			
	0 [hr]	48 [hrs]	72 [hrs]	96 [hrs]
BunkerOil#1; 1:3[v/v]	0.0144	0.0198	0.0275	0.04
BunkerOil#1; 1:10[v/v]	0.0144	0.0075	0.0608	0.0665
BunkerOil#2; 1:3[v/v]	0.0134	0.0143	0.029	0.0465
BunkerOil#2; 1:10[v/v]	0.0134	0.0066	0.0482	0.0614

HP series 6890 gas chromatograph, equipped with mass selective detector and J&W DB-5ms column was used with helium carrier gas flow rate of 7.5 mL min^{-1} . The injector temperature was 300°C , in splitless mode. The oven temperature program was set as follows. 40°C held for 3 min, $30^\circ\text{C min}^{-1}$ to 250°C and held for 3 min, $10^\circ\text{C min}^{-1}$ to 310°C and held for 1.5 mins.

ICP-OES for total dissolved sulfur in aqueous medium

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Due to the presence of chloride ions in high concentration ($> 100 \text{ mg L}^{-1}$), ion chromatography (IC) technique is not suitable to determine the sulfur species in the aqueous medium. The total dissolved sulfur present in the aqueous medium was then determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) method. The apparatus used was Perkin Elmer Optima 2000DV Optical Emission Spectrometer.

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The entire optical system was purged for three hours with Nitrogen gas to eliminate the effects of oxygen adsorption bands in the wavelength range of 170-200 nm. The calibration curve for sulfate was prepared using 10 mg L^{-1} and 100 mg L^{-1} sulfate standards at wavelengths of 180.669 nm and 181.975 nm. 5 mL liquid samples were prepared in 15 mL centrifuge tubes and the liquid samples were analyzed using ICP-OES technique. The liquid flow rate of 1.5 mL min^{-1} and a delay time of 60 seconds between consecutive sampling were set. MilliQ water was used as the blank for the instrument.

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Results and discussion

GC-MS analysis of bunker oil samples

- 5 Using GC-MS, seven organosulfur compounds were detected by matching the MS spectra with the available database of organic compounds. Figures 15 and 16, show the Total Ion Chromatogram (TIC) of Bunker Oil #1 and #2 samples at 0 hr.

10 In order to detect a target compound like dibenzothiophene (DBT), the selective ion chromatogram corresponding to DBT was extracted by inputting its molecular weight. The extracted ion chromatogram and the MS spectra of DBT are shown in Figures 17 and 18.

15 The intensity of the compound was calculated by integrating the area under the ion chromatogram peaks corresponding to their retention time. For example, the retention time for DBT was approximately 9.7 min and its intensity was calculated upon integration of the peak at 9.7 minutes from the TIC (original). Further, the intensity for DBT was also calculated by integration of the peak at 9.7 minutes from the extracted ion chromatogram (extracted).

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The typical MS spectra of the seven identified organo sulfur species (excluding DBT) are shown in Appendix III. For several compounds, the intensity could not be evaluated using TIC (original). Therefore, the extracted ion chromatograms were used to calculate the intensity of the compound in area units. For dimethyl DBT the main MS peaks were
25 observed over a range of retention time, which might be attributed to its isomers. As a result, several intensities could be obtained for the retention time range upon integration of the extracted chromatogram peaks. In order to compare the intensity of dimethyl DBT with other compounds, the intensities of dimethyl DBT obtained for the retention time range were summed up.

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GC-MS was also used to characterize the two bunker oil types on the basis of the intensity of the identified organosulfur species. It can be seen in Table 2, that the weights of bunker oil samples used for the GC-MS analysis were different. Therefore the

intensity of the compound was reported in the unit of area /g of bunker oil (specific area units) by dividing the original area by the weight of the oil.

Comparison of organosulfur species' intensities in bunker oils

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The intensities of different organosulfur species were calculated in the units of area/g of bunker oil (specific area). Figure 19 compares the distribution of the organosulfur species on the basis of specific area. After full screening of the chromatogram, both of bunker oils were found to contain at least 7 kinds of sulfur heterocycles (not including the isomers), including 4 methylated benzothiophenes, dibenzothiophene, DBT and 2 methylated DBT. No organo-sulfur species of other structures were found, nor were thiophenic compounds with higher molecular weight. Therefore the main organo-sulfur species in the bunker oils were proved to be methylated thiophenic compounds. As the degradation mechanism of methylated thiophenes is similar to that of their mother compounds, BTH and DBT, the results obtained with model sulfur species were useful and important. The specific area calculated is dependent on the sensitivity of the compound to the detector. For example, dimethyl DBT has the highest response during the analysis. However, dimethyl DBT may not be the most abundant form of organosulfur present in bunker oil. The quantitative analysis of specific compound was not determined because the standard substances for all were not available. Comparing the specific area values of the different organosulfur compounds, it is evident that Bunker Oil #1 has relatively higher values compared to Bunker Oil #2. This can be due to the difference in the physical characteristics of both bunker oils.

25 *GC-MS analysis: Bunker Oil #1 for oil/water ratio 1:3 [v/v]*

The changes in the intensity of the organosulfur compounds were monitored for time periods namely, 0, 48, 72 and 96 hours. Figure 20 shows the changes in specific area values for the organosulfur species for Bunker Oil #1 with the volumetric oil/water ratio of 1:3. There has been a consistent decrease in the intensities of BTH derivatives and confirms their biodegradation over the period of time except for diethyl BTH where no much change in its intensity was found for the observed time periods.

In the case of DBT and its derivatives, the intensity of methyl DBT demonstrated a continuous decrease in the working period. The biodegradation of dimethyl DBT started after an initial lag time and it has been observed that the biodegradation of dimethyl DBT was significant after 48 hrs. The possible reason could be that, the desulfurizers require
5 some time to get adapted and for activation of enzymes specific to the biodegradation of dimethyl DBT. The intensity of DBT was surprisingly increased first in the first 48 hours and followed by a decrease to below its original level, with unknown reasons.

GC-MS analysis: Bunker Oil #1 for oil/water ratio 1:10 [v/v]

10 Figure 21 shows the changes in specific area values for the organosulfur species for Bunker Oil #1 with the volumetric oil/water ratio of 1:10. Again, there has been significant decrease in the intensities of methyl BTH and dimethyl BTH between 0 and 48 hrs. It was also observed that their intensities increased during the time period between 48 and
15 72 hrs and the reason for this phenomenon is unclear. Overall, the figure shows the importance of the environmental conditions on the bioprocess. A similar trend was also observed with respect to methyl DBT.

20 However the measured results for DBT, dimethyl DBT, trimethyl BTH and diethyl BTH may not be accurate due to possible operational errors as their intensities were found to be higher than the initial values at specific times after the onset of the bioprocess, if otherwise the biodegradation didn't function well. In general, it can be understood that the activity of the desulfurizers in the bioreactor were affected by the oil/water ratio. The microbial community had higher degrading abilities for bunker #1 when the oil/water ratio
25 was 1:3 compared to 1:10. The possible reason could be that with an oil/water ratio of 1:10, the amount of water could have been high enough to reduce the contact of the bacteria with bunker oil.

GC-MS analysis: Bunker Oil #2 for oil/water ratio 1:3 [v/v]

30 Figure 22 shows the changes in specific area values for the organosulfur species for Bunker Oil #2 with the volumetric oil/water ratio of 1:3. It can be inferred that there has been a decrease in the intensities of all the organosulfur compounds except for DBT, during the time period 0 and 48 hours. After 48 hours it has been observed that their

intensities increased. This may be due to the decrease in the activities of bacteria after 48 hours as the conditions in the bioreactor could have been less favorable for them to survive during time periods beyond 48 hours but there are no other data available supporting these. However, selective desulfurizers were able to degrade DBT during the course of bioprocess, with a slight decrease of DBT intensity observed from 70 hrs onwards.

Overall, the desulfurizers in the bioreactor were capable of degrading the organosulfur species until 48 hours and after 48 hours their growth seems to have been affected due to possible changes in the conditions within the reactor, resulting in the decline of biodegradation of organosulfur species.

GC-MS analysis: Bunker Oil #2 for oil/water ratio 1:10 [v/v]

Figure 23 shows the changes in specific area values for the organosulfur species for Bunker Oil #2 with the volumetric oil/water ratio of 1:10. It can be inferred that there has been significant decrease in the intensities for all the seven organosulfur compounds between 0 and 96 hrs. For the cases of DBT, diethyl BTH and trimethyl BTH, the biodegradation started after an initial lag time. It has been observed that the biodegradation of these compounds was significant after 48 hrs. The possible reason could be that, the desulfurizers require some time to get adapted and also for the activation of enzymes specific to the biodegradation of dimethyl DBT.

In general, it can be understood that the activity of Bunker Oil #2 desulfurizers in the bioreactor were also affected by the oil/water ratio. The bacteria had higher degrading abilities for bunker #2 when the oil/water ratio was 1:10 compared to 1:3. This may be possibly due to the reason that under higher oil content, the conditions were less favorable for the growth of bacteria. Therefore, with oil/water ratio of 1:3, the amount of water could be lower than the minimum water requirement. The different favorable oil/water ratio of bunker oils #1 and #2 might be attributed to their different chemical natures: Bunker Oil #2 was even heavier compared to Bunker Oil #1 and also has a higher viscosity.

Elemental analysis of Bunker Oil #1

In order to determine the total combustible (organic) carbon and sulfur in bunker oil samples, elemental analysis was done. Figures 24a, 24b, and 24c show the sulfur content (wt %), carbon content (wt %) and carbon/sulfur ratio (C/S) for the two ratios of oil/water when bunker oil #1 was used. The graphs show the average values of duplicated tests for each sample. The y-axis error bar denotes the maximum and minimum deviation from the average. The horizontal line in each figure represents the initial value at 0 hrs.

It can be observed from Figure 24a that the sulfur content decreased in the Bunker Oil #1 samples with time after 48 hrs when the oil/water volumetric ratio was maintained as 1:3. This result is also generally consistent with the GC-MS results for Bunker Oil #1 for 1:3 oil/water ratios. When oil/water ratio was 1:10, no decrease in the sulfur content was observed in the bunker oil #1 samples for the different time periods. This may be due to the reason that the water content was present in excess for the desulfurizers to effectively degrade the organosulfur species in bunker oil #1. As explained earlier, when bunker oil #1 was used, the oil/water ratio of 1:10 might have provided more water than necessary for the bacteria. As a result, the contact between the bacteria and bunker oil #1 could have been affected.

Figure 24b shows that for oil/water ratio of 1:3, the carbon content also decreased with time when bunker oil #1 was used whilst that for bunker #1 at 1:10 ratio of oil/water behaved differently. Figure 24c also demonstrates the decrease in C/S ratio over time for Bunker Oil #1 for both oil/water ratios (except for bunker #1, 1:10 ratio at 96 hrs), which indicated that there might be bacteria in the mixed culture actively degrading the carbon components in the bunker oil.

Elemental analysis of Bunker Oil #2

Figures 25a, 25b, and 25c show the sulfur content (wt %), carbon content (wt %) and Carbon/Sulfur ratio (C/S) for the two ratios of oil/water when bunker oil #2 was used. Similarly, the horizontal line in each figure represents the initial value at 0 hr. The graphs show the average values of two replicates used for each sample. The y-axis error bar denotes the maximum and minimum deviation from the average.

It can be observed from Figure 25a, that the sulfur content decreased in the Bunker Oil #2 samples more significantly when the oil/water volumetric ratio was maintained as 1:10. This result is also generally consistent with the GC-MS results for Bunker Oil #2 for 1:10 oil/water ratio. When oil/water ratio was 1:3, the water content in the bioreactor may be lesser than the minimum requirement for the bacteria to survive. Therefore no decrease in the sulfur content was observed in the bunker oil #2 samples for the different time periods with oil/water ratio of 1:3. The increase in the sulfur content for the oil samples between 0 and 48 hours may be due to the inefficient separation of the bunker oil and biomass, thus the sulfur in biomass occurring in oil might affect the measurement.

Figure 25b shows that for oil/water ratio of 1:10, the carbon content also decreased with time when bunker oil #2 was used. Figure 25c, demonstrates the decrease in C/S ratio between 0 and 96 hrs for Bunker Oil #2 for both oil/water ratios. In the case when oil/water ratio was 1:10, a significant decrease in both sulfur and carbon contents was observed between 48 and 72 hours and an increase between 72 and 96 hours. This demonstrates the variability in the activities of bacteria due to changes in conditions within the bioreactor over various time periods.

Sulfur balance

In order to understand the sulfur balance within the bioreactor system, the sulfur analysis was done in the biomass and aqueous medium phases. The separation of the individual phases namely bunker oil, biomass and aqueous medium was achieved by centrifuging all the samples at a high speed. Separation of biomass was carried out at the speed of 10000 rpm for 15 minutes. Centrifugation at higher speeds may cause the biomass to breakup.

An example is shown when bunker oil #2 was used and the oil/water ratio was 1:10. Figure 26 shows the change in sulfur content in the biomass as weight percentage for different time periods of the bioprocess. It can be observed that there was an accumulation of sulfur content in the biomass from a very low initial value (approximately

0%) to about 1.5% at 48 hours. After 48 hrs, the sulfur content in the biomass decreased steadily.

Conclusions

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The following conclusions may be made in this example:

1. Organosulfur species in bunker oil can be biodegraded with the enriched bacteria. The highest removal efficiency obtained from the batch study is 49.3% of sulfur
10 in bunker.
2. Bunker Oil #1 and #2 were characterized on the basis of the intensities of the target organosulfur species using GC-MS method. Several organosulfur compounds like BTH, DBT and their derivatives were identified. The intensities of these compounds were
15 also calculated and compared over various conditions, to monitor the changes in distribution of organosulfur compounds.
3. Both the elemental analysis and GC-MS analysis of the bunker oil samples have shown the biodesulfurization of bunker oil by bacteria are dependent on several
20 parameters such as bunker oil/culture medium ratios, degradation time, and bunker oil types.
4. Bacteria desulfurized bunker oil #1 more efficiently when the volumetric oil/water ratio was maintained as 1:3 on the basis of elemental and GC-MS analysis. The
25 oil/water ratio of 1:10 was not suitable for the biodegradation of organosulfur species in bunker oil #1.
5. Bacteria desulfurized bunker oil #2 more efficiently when the volumetric oil/water ratio was maintained as 1:10 on the basis of elemental and GC-MS analysis. The
30 oil/water ratio of 1:3 was not suitable for the biodegradation of organosulfur species in bunker oil #1. The different favorable oil/water ratio of bunker oils #1 and #2 might be attributed to their different chemical natures: Bunker Oil #2 was even heavier compared to Bunker Oil #1 and also has a higher viscosity.

6. The carbon content in the bunker oil was affected in the process of biodegradation, as the C/S ration decreased over time for both fuel types and oil/water ratios. Therefore, it will be explored in further studies to enhance the activity of the 4S pathway bacteria in the mixed culture, and to reduce the carbon degradation in the biodesulfurization process.

The mixed culture obtained from oil sludge showed remarkable ability in BDS of thiophenic compounds and their derivatives, both as pure substance (model sulfur species) and as mixtures in real bunker oils. The important parameters in this process were determined by our experiments to be: bacteria abundance and diversity, reactor design, reactor operational parameters (mixing, duration, sulfur species type and abundance, oil water ratio, etc.), separation after test, and last but not least, temperature.

The invention has high potential to be used by oil refineries and oil suppliers etc., to improve the oil quality thus to sell at a much higher price. As evidenced in the lab data, a preliminary investigation indicated the feasibility of bunker oil BDS with a significant decrease of sulfur content (~50%) in bunker oil. Considering a further study to optimize the operation conditions, it is believed that the biodesulfurization of bunker oil technology has a wide prospective future in application.

Whilst there has been described in the foregoing description preferred embodiments of the present invention, it will be understood by those skilled in the technology concerned that many variations or modifications in details of design or construction may be made without departing from the present invention.

CLAIMS

1. A method of obtaining a microbial culture for reducing the sulfur content in a fossil fuel, the method comprising the steps of
 - 5 a) obtaining a seed oil sludge from a marine or industrial environment;
 - b) culturing the seed oil sludge under conditions suitable for enrichment of sulfur-biodegrading bacteria; and
 - c) assessing the culture for ability to reduce the sulfur content of bunker oil.
- 10 2. A microbial culture for reducing the sulfur content in a fossil fuel, obtained by the method of claim 1.
3. A method for reducing the sulfur content in a fossil fuel, the method comprising: incubating the fuel with a microbial culture according to claim 2 in a medium under such
15 conditions to reduce the portion of sulfur in the fuel.
4. A method according to claim 2 or 3, wherein the microbial culture is enriched from an industrial oil sludge.
- 20 5. A method according to claims 3 or 4, wherein the incubation period is at about 28°C or higher for about 1 to 8 days.
6. A method according to any one of claims 3 to 5, wherein the fuel and microbial culture volume ratios are between 1:1 to 1:10.
- 25 7. A method according to any one of claims 3 to 6, wherein the medium is a basal salt medium.
8. A method according to any one of claims 3 to 7, wherein at least about 40% of
30 the sulfur is removed.
9. A method according to any one of claims 3 to 8, wherein the fossil fuel is a liquid or solid at room temperature (20~30°C) and the culture medium is liquid.

10. A method according to claim 9, wherein the fossil fuel is bunker oil.