IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 2, Issue 5 (Jul. – Aug. 2016), PP 46-53
www.iosrjournals.org

Anaerobic Biodesulfurization of Kerosene Part I: Identifying a Capable Microorganism

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Abstract:*Kerosene is the main fuel for domestic use in Nigeria and some other developing countries. It is used in cooking in mostly in poorly ventilated kitchens. The combustion of kerosene produces oxides of nitrogen and sulfur which are precursors to acid rain which is known to destroy buildings, destroy vegetation and is also dangerous to human health. Desulfatiglans anilinicomb, novwas isolated from petroleum products contaminated soil and subsequently evaluated for its ability to desulfurize kerosene. Kerosene obtained from retail outlet in Lagos, Nigeria was provided as the sole source of sulfur in batch cultures and the extent of desulfurization after treatment determined. The desulfurizing bacterium, Desulfatiglans anilini comb, nov., a strict anaerobe, gram negative and an oval to rod shaped bacterium was isolated by enrichment culture and subsequently identified at the Department of Microbiology, University of Lagos, Nigeria was also found to exhibit very high desulfurizing ability towards kerosene at 30^0C and normal atmospheric pressure. The biodesulfurization of kerosene by Desulfatiglans anilini comb, nov.resulted in reduction of sulfur from 48.68 ppm to 12.32 ppm over a period of 72 hours. Gas chromatography analysis with a pulse flow photometric detector (PFPD) revealed that the peaks of Thiophene and 2, 5 – Dimethyl Thiophene significantly decreased after biodesulfurization. This study has been able to establish that Desulfatiglans anilini comb, nov.could effectively desulfurize kerosene and thus may be a promising biocatalyst for practical biodesulfurization of kerosene.*

Keywords:*Anaerobicbiodesulfurization, Desulfatiglans anilini comb, nov., Hydrodesulfurization, Biochemical and Morphological Characteristics.*

I. Introduction

A variety of sulfur-containing heterocyclic organic compounds have been characterized in coal, coal tars and crude oils. Dibenzothiophene (DBT) and substituted derivatives represent important components of these sulfur-containing heterocyclic organic compounds. DBT is representative of a group of heterocyclic compounds which includes thiophene, benzothiophene and other more complex sulfur-containing polyaromatic hydrocarbons. The concentration of sulfur in crude oil is typically between 0.05 and 5.0% (by weight), although values as high as 13.95% have been reported[1]. In general, the distributions of sulfur increase along with the boiling point of the distillate fraction. The combustion of fossil fuels causes serious environmental problems. For instance, carbon dioxide emissions have been implicated in global warming. Nitrogen and sulfur oxides emissions have been shown to be responsible for acid rain, which destroys buildings, kills forests and poison lakes [2].

To reduce sulfur-related air pollution, the level of sulfur in fuels is regulated, and to meet these regulations sulfur must be removed from fuels during the refining process [3]. At the refineries, hydrodesulfurization (HDS) is currently performed to remove sulfur compounds from fossil fuels. This process is done at high temperatures and pressures by metal catalysis and is effective for removing inorganic sulfur and simple organic sulfur compounds. However, it is difficult to remove heterocyclic sulfur compounds using this method. As legislative limits on sulfur emissions have become tighter, the need to remove heterocyclic sulfur compounds from fuel has become more pressing [4]. The high cost and inherent chemical limitations associated with HDS make alternatives to this technology of interest to the petroleum industry. Also, it has been found that increasing the severity of HDS also elicits undesirable effects on fuel quality as other chemical components are reduced at the higher temperatures and pressures needed to achieve low sulfur levels [5]. However, a comparative study of the potentials of some metal oxides on desulfurization of kerosene and diesel oil was carried out by [6], they found out that both PbO_2 and MnO_2 remove over 50% of sulfur in these oils. [7] used a method that combines selective oxidation of highly reactive sulfur compounds and adsorption to remove sulfur in kerosene; their experiment left kerosene whose sulfur content was less than 0.1 ppm. The use of gas phase oxidation to reduce sulfur in diesel was carried out at atmospheric pressure and a temperature range of 90 – 300^0C[8].

Biodesulfurization has attracted attention as an alternative process to HDS. The biodesulfurization has many advantages, including lower capital and operating costs coupled with substantially less generation of greenhouse gases [9]. It has been reported that some bacteria utilize DBT as a sole source of sulfur without

breaking its carbon-carbon skeleton. These sulfur-specific pathways are of two types namely the sulfur-specific oxidative pathway also known as the 4S pathway and the sulfur-specific reductive pathway. The sulfur-specific oxidative pathway has been extensively studied [10]. The genes encoding enzymes involved in this pathway have been cloned and sequenced [11]. Bacterial biocatalysts for use in petroleum desulfurization by polyethylene glycol (PEG)–assisted protoplast transformation which produced recombinant *Rhodococcus* with higher ability to biodesulfurize DBT have also been developed [12]. [13]demonstrated an effective way of carrying out effective oxidative desulfurization using air, thus eliminating the need for use, storage and handling of liquid peroxide oxidants, their work produced an ultralow sulfur fuel. Investigation into the efficiency of an ultrasound assisted oxidative desulfurization (USAOD) process in fuel was carried out by [14], their results showed that the sulfur removal in the fuel increased as the dose of the transition metal catalysts increase, their work further revealed that longer sonication time enhances the oxidation process.

There are few reports on the desulfurization activity of sulfur reducing bacteria on DBT and petroleum fractions under well controlled sulfur-reducing anaerobic conditions ([15] and [16]). This is probably due to the expensive nature of reactions under anaerobic condition. The anaerobic route is a potentially attractive biodesulfurization route to apply because of its sulfur specificity. From the pathway, it follows that the calorific value is maintained because C – C bonds are not altered. Furthermore, the reaction pattern is similar to HDS. However, growth under anaerobic conditions proceeds slowly, especially when organic compounds like thiophene and dibenzothiophene are involved in the conversion [17]. From a process point of view, the aerobic route has a major drawback. Diluted sulphate is formed as the end product of the aerobic route that must also be removed, while the H_2S that is formed in anaerobic route can be treated with existing refinery desulfurization plants (as in Claus process). In the aerobic sulfur specific route, oxygen molecules are added to the hydrocarbon skeleton. This is not desirable, because 2-hydroxybiphenyl is involved in the formation of viscous oil sludge (gum) in the fuel. Furthermore, in product inhibition, 2-hydroxybiphenyl formed in the cells will eventually diffuse back to the oil phase, but the phenolic molecule is a well-known biocide [18]. Based on the aforementioned considerations the anaerobic route is chosen in this study.

The objectives of this paper is to achieve desulfurization of kerosene by application of a selected sulfur utilizing bacterium, *Desulfatiglans anilini* comb. nov.as a biocatalyst. For this purpose the microorganism was isolated by the enrichment culture anaerobically, the microorganism was then subjected to various biochemical and morphological tests so as to ascertain its characteristics for the purpose of identification, the identified microorganism was contacted with the kerosene for the biodesulfurization experiment and the extent of biodesulfurization was monitored using a Gas chromatograph to which was attached a Pulse Flow photometric Detector (GC-PFPD).

II. Materials and Methods

The sample site was the petroleum tanker truck park at Orile, a suburb of Lagos, Nigeria. The park is located near the Apapa petroleum products depot. As a result of loading of these trucks with petroleum products, the soil there is highly contaminated with these products. The soil samples were collected in a sterile polythene bags and taken to the laboratory for isolation of microorganisms via enrichment by a method previously described by [19].

The medium for isolation of sulfur reducing bacterium, SRB contained the following substances(g/L): S, 1.000; $Na_2S_2O_3$, 0.500; $(NH_4)_2SO_4$, 0.300; KH_2PO_4, 0.250; $CaCl_2.6H_2O$), 0.200; $FeSO_4.7H_2O$, 0.250; KCl, 0.500, $MgSO_4$, 0.200; $Ca(NO_3)_2$, 0.500; Sodium propionate,1.000; agar-agar(Oxoid), 15.000 and distilled water to 1000ml mark(pH, 7.4). Then, 2.0g from each of the five randomly collected soil samples were weighed out after mixing and transferred aseptically into an Erlenmeyer flask containing 90 ml sterile distilled water to give 10^{-1} ten-fold serial dilution. From this, higher dilutions were made up to 10^{-8}. Then, aliquots(0.1ml) from both low(10^{-2}; 10^{-4}) and high (10^{-6}; 10^{-8})dilutions were plated out on the SRB medium in two replicates by spread plate technique using a sterile hockey stick. The plates were all incubated anaerobically in an anaerobic jar at room temperature ($30^oC\pm$ 2) for 10-14 days. At the end of the incubation, the colonies that developed in the plates were counted; their relative abundance (population density) was estimated by multiplying the plate count by the dilution factor used. Then, pure cultures of the isolates were obtained by streaking technique on SRB medium and stored on agar slants in a refrigerator at 4^oC when not in use.

The isolated microorganism, identified as *Desulfatiglans anilini*comb. novwas suspended in 9 ml of 0.1M sulfur-free phosphate buffer solution (pH 7.0) containing 2% glucose and 1 ml of kerosene for the biodesulfurization experiment in a 100 ml Erlenmeyer flask [19]. The set up was moderately shaken at 180 rpm in a shaker incubator at 30^0C for specific periods. The essence of the shaker incubator is to enhance mass transfer. Also, the growth of the sulfurbacterium,*Desulfatiglansanilini*comb. novin the experimental tube was monitored as described previously by [20]. Thiophene, 2, 5 – dimethylthiophene, benzothiophene and dibenzothiophene were analyzed using Gas Chromatograph 5890 Hewlett Packard, equipped with a Pulsed Flame Photometric Detector (PFPD).

III. Results and Discussions

The microorganism was isolated from oil contaminated soil samples using a sulfur reducing bacteria medium. Colonies obtained at the end of incubation were counted and their population density estimated by multiplying the plate count by the dilution factor used. The pure cultures of the isolate was normally stored on agar slants in a refrigerator at 4^0C when not in use. The isolate was subjected to series of biochemical and morphological tests. The tests have shown the isolate microorganism to be motile, oval to rod like in shape, gram negative, non-spore forming anaerobic microorganism. Table 1 shows the details of the tests and the response to them.

The Gram reaction and cellular morphology was confirmed by optical microscopy. The non-motility of the isolate is an indication that the isolate has no flagellum. Testing negative to the Gram test shows that the isolate has a thin mesh-like cell wall with additional outer membrane that contained lipid separated by periplasmic space. The isolate poorly utilize sodium lactate,the only sodium salt it was not able to utilize very well of all the sodium salts test it under went, and this may be attributed to the inability of the isolate to utilize lactose. It tested positive to hydrogen sulfide test an indication that the isolate is capable of using sulfur-containing amino acid like cysteine. However, it cannot split indole from amino acid. The isolate cannot utilize urea and citrate. The isolate was found to test negatively to the indole, urease and citrate tests. It has been shown to have the capability of utilizing various kinds of sugar such as glucose, xylose and maltose as a source of carbon. However, it could not utilize lactose, this result affirmed the very poor utilization of sodium lactate by the isolate, an indication that the isolate could not utilize lactates as a source of carbon. The non-response of the isolate to the Methyl Red VogesPrausker (MRVP) test is an indication that the isolate would not be active when the pH of the medium lies between 4.4 and 6.2, this feature is peculiar to sulfur utilizing bacteria and this account for the reason why the reaction was carried out at a pH of 7.

Table 1. Cell Morphology and Results of Biochemical Tests

Tests		Observation
Colonial Characteristics on SRB Medium		Smooth edge, raised elevation & cream in color
Shape of cells		Oval to rod shaped
Gram Stain		–
Sodium Lactate Utilization		VP
H_2S Production		+
Indole (Tryptophan utilization)		–
Urease Test		–
Citrate Test		–
Catalase test		–
MRVP(Methyl Red)		–
MRVP(Voges- Proskauer)		–
Motility		–
Glucose		+
Xylose		+
Mannitol		+
Maltose		+
Lactose		–
Gas Production from Glucose		+
Sodium propionate		+
Sodium anilinate		+
Sodium benzoate		+
Sodium butyrate		+
Presumptive Identification of Isolates		*Desulfobacterium anilini*

VP means very poor

Based the biochemical, cultural and morphological characteristics, the isolate was initially presumptively identified to be *Desulfobacterium anilini* [17].Based on phylogenical, phenotypical and chemotaxanomical characteristics, [21]found out that*Desulfobacterium anilini* was clearly a different strains from other species of the genus*Desulfobacterium*. They thus propose a reclassification of*Desulfobacterium anilini* within a new genus, *Desulfatiglans*gen. nov., as *Desulfatiglans anilini*comb. nov.

In the biodesulfurization experiment, the organism was suspended in a sulfur free phosphate medium containing 2% glucose and the kerosene in the ratio 9:1. It is worthy of mention that sulfur-containing organic compounds, namely; thiophene, 2, 5 – dimethyl thiophene, benzothiophene and dibenzothiophene were run as standards and used to identify the peaks in the kerosene samples for both the control and experimental designs. However, thiophene and 2, 5 – dimethyl thiophene were the only ones found kerosene. It is easier for the organism to utilize carbon in glucose which is in aqueous state in which the organism is also suspended if available than kerosene which is oil. This is to provide alternative source of energy to the organism apart from

the fuel thus ensuring that the biodesulfurization of thiophene and 2, 5 – dimethyl thiophene took place via a sulfur-specific degradation pathway. Further, the cells of*Desulfatiglans anilini* comb. novwere observed at the interface of the aqueous and oil phase when the reaction broth was centrifuged. The implication of this is that if the cells were at the bottom of the aqueous phase, then the *Desulfatiglans anilini* comb. novmust have secreted emulsifiers to overcome the poor solubility of the fuel. This can be achieved by breaking down the fuel into smaller droplets. When this is done, then the structure of the fuel must have been distorted thus reducing its calorific value. However, this did not happen, rather the reaction was possible by the microorganism increasing the cell surface hydrophobicity [22].

The initial value of the optical density measurement of the cells of the microorganism prior to the start of the experiment was found to be 0.930 at a wavelength, λ of 510 nm. The colorimeter used for the measurement is the Coulomb 1998 version. At the end of the experiment (72 hours), the absorbance has increased to 0.936. The value of the absorbance was found to be equivalent to standard plate counts of 6.40 x 10^6 cfu/ml for initial value, and a final value, 6.44 x 10^6 cfu/ml. This slight increment in population growth is considered insignificant, hence, the organism was assumed to have used the sulfur compounds for sustenance only.

The Gas Chromatography as shown in Figure1revealed that the kerosene contained 6.955 mg/l of thiophene and 41.724 mg/l of 2, 5 – dimethyl thiophene. No benzothiophene and dibenzothiophene were found in the kerosene. Figure 1 shows the GC-PFPD peaks for all of the sulfur compounds in the kerosene (approximately 48.679 mg/l sulfur initially) before the biodesulfurization by *Desulfatiglans anilini* comb. nov. After treatment of the kerosene with the microorganism for 72 hours, all of the peaks were found to have significantly decreased as shown in Figure 2.

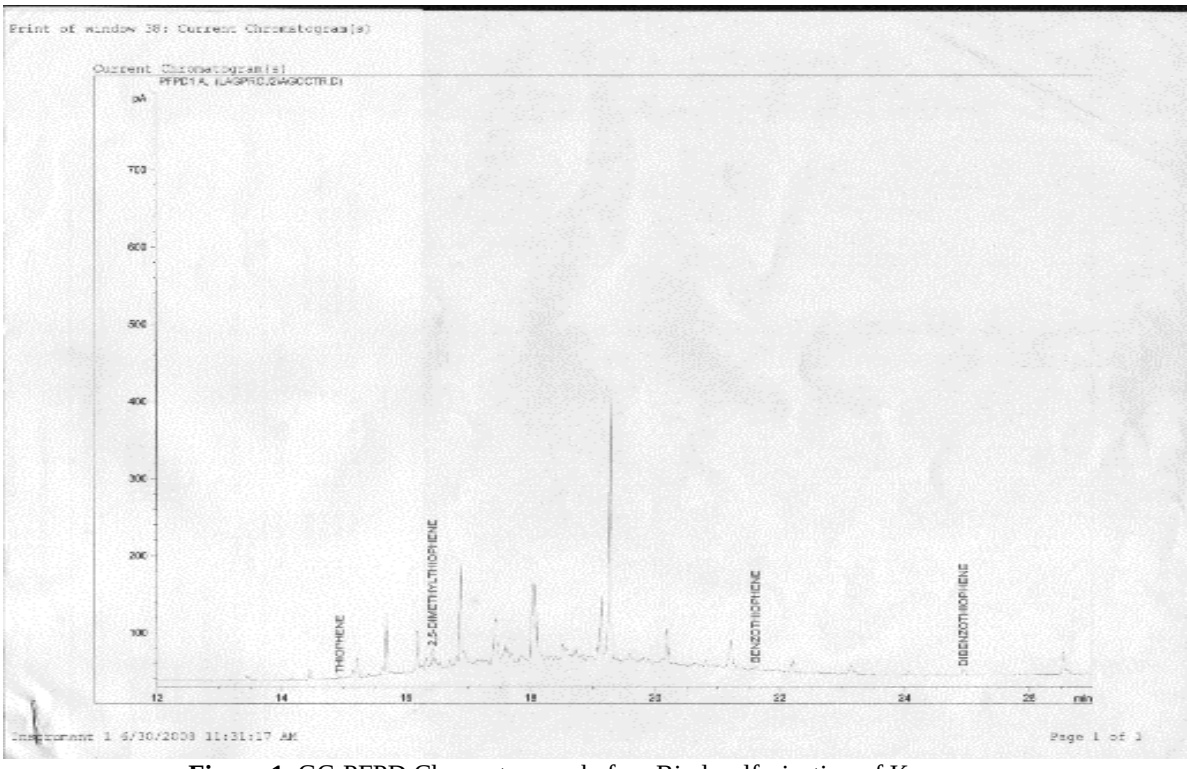

Figure 1. GC-PFPD Chromatogram before Biodesulfurization of Kerosene

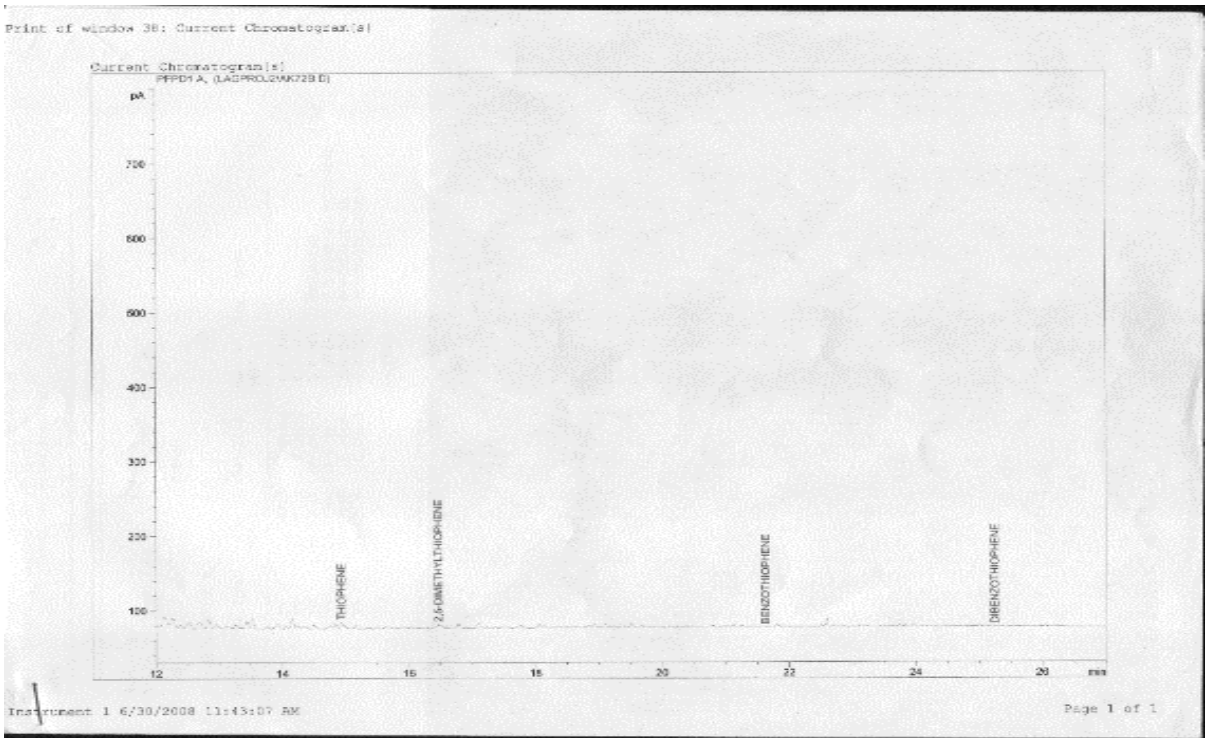

Figure 2. GC-PFPD Chromatogram 72 Hours after Biodesulfurization of Kerosene

It is important to note that the sulfur compounds with retention times longer than 5 minutes nearly disappeared. Such characteristics of desulfurization by cells of *Desulfatiglansanilini*comb. novare opposite or complimentary to those of hydrodesulfurization, in which sulfurcompounds with a shorter residence time are more easily desulfurized [23]. Based on these results, cells of *Desulfatiglans anilini* comb. nov are considered to have a sufficiently broad substrate specificity to desulfurize major organic sulfur compounds contained in kerosene.

The pattern of kerosene biodesulfurization by the cells of *Desulfatiglans anilini* comb. nov.is shown in Figures 3, 4 and 5.It can be seen that thiophene with an initial concentration of 6.955 mg/L was reduced to 0.992 mg/L at the end of 72 hours.

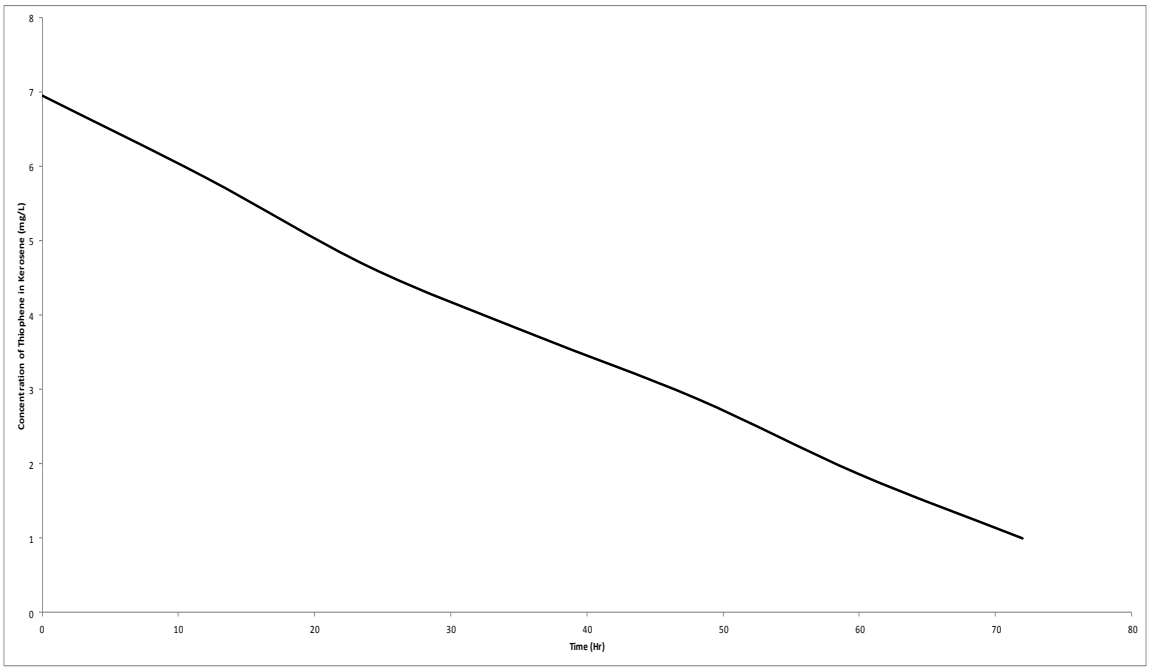

Figure 3: The Concentration Time Profile of ThiopheneBiodesulfurization in Kerosene

This represents 85% biodesulfurization.The profile as shown in Figure 3 is linear. When this happens, the kinetics of the biodesulfurization process is defined as zero order meaning that the thiophene consumption is independent of its concentration. This implies a high affinity for the thiophene by the microorganism. The possibility of all of it being consumed should the contact be allowed to exceed 72 hours is high. It is worthy of mention that thiophene is an aromatic compound and as such its stability is enhanced due to the ability of the electrons in the π orbitals to delocalize thereby providing it with high resonance energy. The electron pair on its sulfur are significantly delocalized in theπ electron system. Theoretical calculations have suggested that its degree of aromaticity is less than that of benzene [24]. Consequent of its aromaticityis its stability or recalcitrance to hydrodesulfurization and non-exhibition of properties seen in thioethers.

Figure 4 shows the profile of 2, 5 – Dimethylthiophenebiodesulfurization in kerosene by the cells of *Desulfatiglans anilini* comb. nov.The substrate, 2, 5 – Dimethylthiophene was reduced to11.323 mg/L from an initial concentration of 41.715 mg/L, this represents a 73% extent of biodesulfurization.

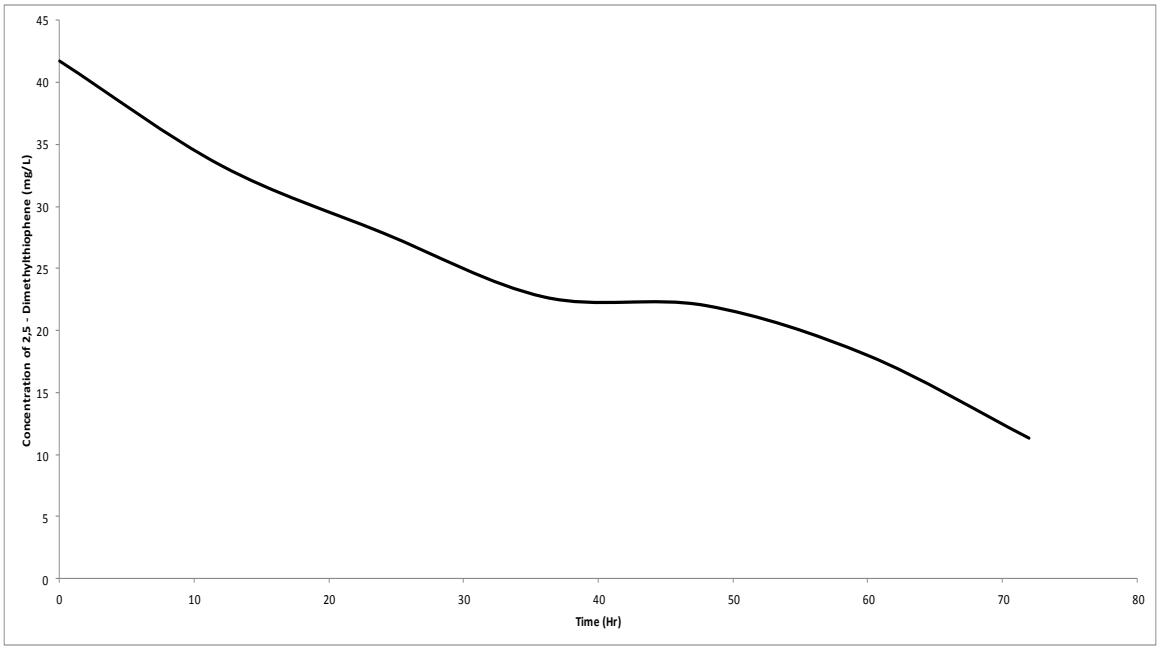

Figure 4: The Concentration Time Profile of 2, 5 - DimethylthiopheneBiodesulfurization in Kerosene

Like thiophene, it is also aromatic, the results showed that it is more difficult to desulfurize than the thiophene. The reason may not be farfetched, the position of the methyl substituents are symmetry, this position on its own confer some level of stability on the substrate. The methyl groups also provide steric hindrances to the sulfur which is the target of the organism. The presence of other sources of sulfur such as thiophene and others not reported with easier accessibility may also be responsible for the relatively low level of extent of biodesulfurization. The level may be higher if the 2, 5 – Dimethylthiophene was the only source of sulfur.

The superimposition of Figures 3 and 4 gives rise to Figure 5 which shows the concentration – time profile of kerosene biodesulfurization within the space of polyaromaticsulfur hydrocarbon standards. It showed that *Desulfatiglans anilini* comb. nov.steadily desulfurized the kerosene decreasing its sulfur concentration from 48.67 to 12.30 mg/l at the end of 72 hours, this represents 75% biodesulfurization.

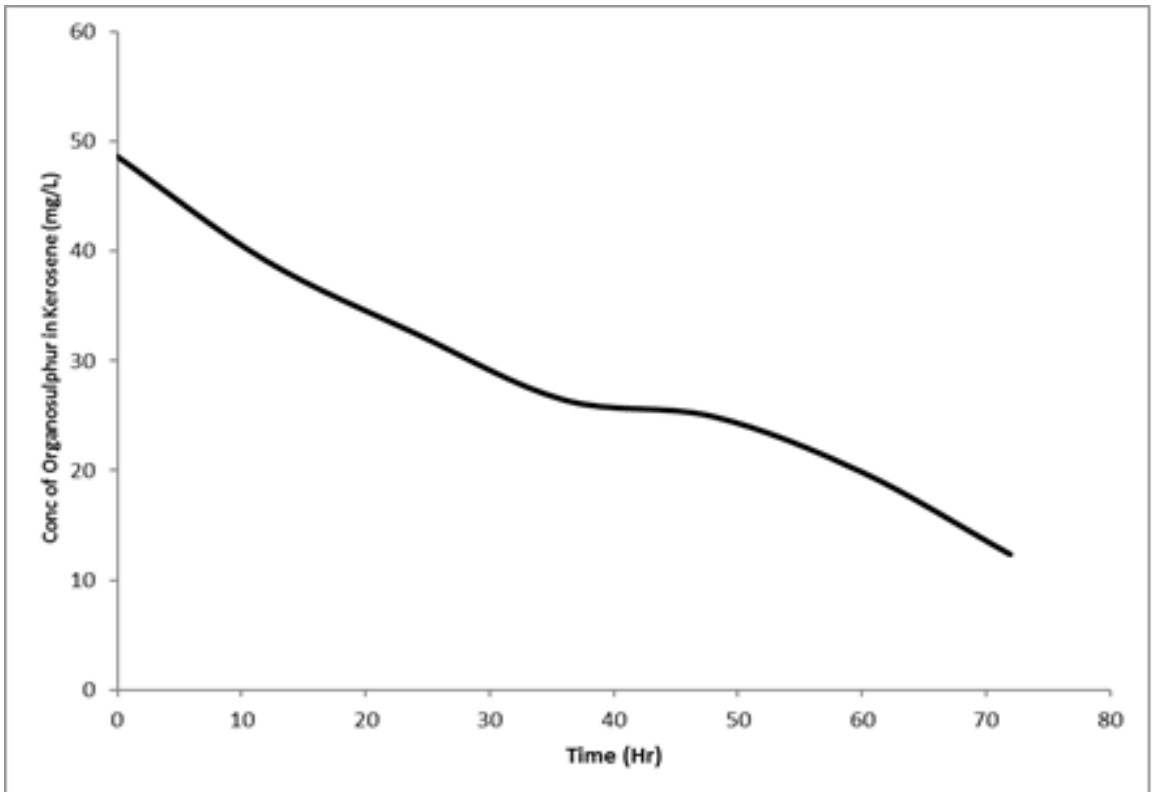

Figure 5: The Concentration – Time Profile of Kerosene Biodesulfurization

This is a remarkable feat at a reaction temperature of only 30^0C and normal atmospheric pressure, extremes of reaction conditions would have been required in hydrodesulfurization to attain the same level of desulfurization. The ability of *Desulfatiglans anilini* comb. nov to desulfurize both the thiophene and 2, 5 – Dimethylthiophene is a demonstration of the broad substrate specificity of the organism to sulfur-containing organic compounds contained in kerosene. From the viewpoint of a practical process, biodesulfurization at ambient temperature and pressure of kerosene containing various types of thiophene derivative is advantageous, since cooling treatment of the oil to ambient temperature would be unnecessary.

In conclusion, *Desulfatiglans anilini* comb. nov, a strict anaerobe could effectively desulfurize organosulfur compounds in kerosene. It could achieve the selective cleavage of C-S bonds at ambient temperature and pressure conditions by a sulfur-specific reductive desulfurization pathway. The biodesulfurization pattern of thiophene follows a zero order kinetics. Furthermore *Desulfatiglans anilini* comb. nov may be a useful desulfurizing biocatalyst possessing broad substrate specificity toward organosulphur compounds in fuel.

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